Using PCR Technque In Comparison With Other Tests In The Diagnosis Of Pulmonary TB Associated With Mycotic Infections In AL-Qadisiya Province.

Adnan H. AL-Hamadani* , Jaafar k. AL-Mousawiand Orass M.Al-Taee*****

الخلاصة

أجريت هذه الدراسة في محافظتي الديوانية والنجف لغرض تشخيص حالات التدرن الرئوي باستخدام تقنية تفاعل السلسلة المتبلمرة (PCR) ومقارنتها مع بعض الفحوصات الروتينية والحديثة. جمعت 250 عينة قشع ودم ومن كلا الجنسين وبأعمار مختلفة لمرضى التدرن الرئوي الذين راجعوا مركز الأمراض الصدرية والتدرن في محافظتي الديوانية والنجف للمدة من كانون الأول 2006 إلى حزيران 2007.أظهرت النتائج أن النسبة المئوية للإصابات بالتدرن هي 40% أما الإصابات الفطرية المرافقة للتدرن فكانت نسبتها 30% بينما نسبة الإصابات الفطرية الرئوية فكانت نسبتها 30. بينت الدراسة أن نسبة حساسية(Sensitivity) وخصوصية(Specificity) فحص PCR في تشخيص حالات التدرن الرئوي هي(94.2% ، 100%) على التوالي وعند مقارنة هذه النتائج مع الفحوصات الروتينية الأخرى مثل الفحص المباشر والزرع ألمختبري فقد لوحظ أن نسبة حساسية وخصوصية هذه الفحوصات كانت (51.4% , 86.6%) للفحص المباشر و (100% ، 88.2%) للزرع ألمختبري وعلى التوالي.

نستنتج من ذلك بان الزرع ألمختبري يعد الأكثر دقة في التشخيص إلا أن وجود بعض السلبيات والصعوبات(drawbacks) تجعل منه اقل استعمالاً في تشخيص المرض وخاصة في الوقت الحاضر أهمها فترة الحضانة التي تستغرقها البكتريا من 4-8 أسابيع ، يليه فحص PCR ويمتاز بسرعة التشخيص

Abstract

Background: **Tuberculosis (TB) remains an important public health problem world wide. It is one of the leading infectious diseases in the world and is responsible for more than 3 million deaths and 8 million new cases annually.The report of World Health Organization (WHO) estimated, for the year 2002 a global incidence of 8.8 million new cases, including 3.9 million smear-positive subjects.**

***Aim of the study*: We aimed to evaluate the effectiveness of available rapid diagnostic tests to identify TB infection.**

*Professor, College of Medicine AL- Qadissiya University.

**Assistant Professor,College of Medicine, Kufa university.

***Assistant Teacher,College of Medicine AL- Qadissiya University.

(البحث مستل من رسالة الماجستير)

The main goal of this study was to use the PCR technique for the rapid detection of tuberculosis using sputum sampling and body fluid specimens other than respiratory secretions.

***Methods*: During the period from December 2006 to May 2007, A total of 50 samples (sputum) were selected from 250 outpatients.The study regard's the culture of sputum as the gold standard test to other methods.**

***Results:*All of the examined patients with TB, the cause was *M. tuberculosis*.Clinical and laboratory diagnosis of the suspected patients revealed that 30 patients were suffering from PMIs , 15 of them had mixed infection.Twenty patients appeared to have pulmonary tuberculosis without mycotic infection. The sensitivity, specificity, PPV, NPV and Accuracy rate of PCR test were (94.2 %, 100%, 100%, 88.2% and 96 %), respectively.**

***Conclusions*: The AFB stain was easy but not sufficient to diagnosis the pulmonary TB alone, whereas the use of LÖwenstein-Jensen medium sensitive enough for diagnosis of pulmonary TB, but need along time to get the results. The polymerase chain reaction (PCR) test gave a high sensitivity and specificity in comparison with other done test, with its advantages of greater speed and effectiveness than conventional detection methods. It was successfully to identify the *M. tuberculosis*, particularly when the staining for acid- fast bacilli is negative and there was a lack of growth on culture or when fresh material has not been collected for culture.**

Introduction

Tuberculosis is a systemic infection manifested only by the evidence of an immune response in most exposed individuals. In some infected persons the disease either progresses or, more commonly, reactivates after asymptomatic period (years) (1). The most common reactivation form is chronic pneumonia with fever, cough, bloody sputum, and weight loss. Spread outside of the lung also occurs and is particularly devastating when it reaches the central nervous system. The natural history follows a course of chronic wasting to death aptly called "consumption' in the past (1).During the past years, systemic mycoses have been globally emerging as a problem of increasing importance in infectious diseases. This is to the growing population of immunocompromised patients due to outbreak of some factors such as: the use of antibiotic and corticosteroid, radiotherapy and chemotherapy, malignant diseases, acquired immune deficiency syndrome (AIDS), diabetes mellitus and use of immunosuppressive drugs in recipients' organ transplantation. All

of these were considered as a predisposing factor to fungal infection and due to acute and chronic diseases (2). Mycotic infections are common and their incidence is ever increasing (3).They usually represent a diagnostic challenge to physicians as their real difficulty in distinguishing them from other infections particularly tuberculosis, but there are numerous clinical clues that can help in suggesting the presence of systemic mycosis. However the diagnosis of mycotic infections still remains a serious problem. The clinical picture lacks specific manifestations; usually it does not differ substantially from bacterial infections (4). The rapid detection and identification of clinically important Mycobacterium spp, is essential for patient management and infection control (5). We know that 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, for example in children. On the other hand, microscopic identification and culture of mycobacteria in sputum are the most common methods for diagnosis of pulmonary disease, but the detection of extra pulmonary TB is often more difficult (6). In microbiology, DNA amplification using Polymerase chain reaction (PCR) has allowed great progress to be made in the rapid and accurate diagnosis of infections 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 (7).

Methods

The samples (sputum) were selected from outpatients who attended to Al-Qadisyia Centre of Tuberculosis and Chest Diseases, Al-Najaf Centre of Tuberculosis and Chest Diseases, and private clinics, where AFB smear microscopy is available but mycobacterial cultures are not routinely performed.

AFB SMEAR MICROSCOPY

The AFB smear examination was performed on uncentrifuged specimens using Ziehl-Neelsen (ZN). staining. After direct smear examination, the remainder of the sputum specimen was transferred to the plane tube, where culture facilities were available, and was digested using sputolysin/sodium hydroxide (4%) processing, and then mycobacterial culture was performed.Also Lacto phenol cotton blue dye which used for the staining of fungal elements in microscopic examination(8).

MYCOBACTERIAL CULTURE MEDIA FOR SPUTUM SPECIMENS

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).The sediment was divided in to two portions. One portion was inoculated in to Lowenstein-Jensen (LJ) slants were prepared for each specimen, incubated at 35°C to 37°C for up to eight weeks, and inspected weekly for growth. A specimen was considered *M tuberculosis* culture–positive if the culture media (LJ slants) grew *M tuberculosis*; negative if the culture media indicated no growth and none grew *M tuberculosis*. The second portion of the sediment was used in DNA extraction process. The remainder of the sediment was transferred to an Eppendorf tube and stored at - 20°C if not immediately processed. Other culture media was Sabouraud's dextrose agar (SDA-HiMedia India) with Streptomycin and penicillin, It was used for the isolation of molds for clinical specimens (10).

PCR detection Method

I. Method of DND extraction and purification *(*Cinnagen Inc- Iran)

Homogenized sputum for the detection of *M.Tuberculosis*.

One hundred µl of homogenized sputum sample was taken and putted in a 95 ^{o}C water bath for 20 minutes and then followed the protocol .In samples with high protein content, 5ul protease were added to homogenized sputum and then placed in a 55 ^{o}C, 3 hr, then placed in a 95 ^{o}C water bath for 20 min, then follow the protocol of Cinnagen Inc.

II. Method of PCR detection (Cinnagen Inc – Iran)

The polymerase chain reaction amplification was performed using assay based on a repetitive sequence IS 6110 of MTB and by the application of two oligonucleotide primers IS1 and IS2 for the detection of a 163-bp DNA fragment.

III. PCR results analysis

The results of the PCR were performed in post amplification area. 10µl from amplification samples was directly loaded in a 2 % agarose gel containing 0.5mg/ml ethidium bromide without adding loading buffer in electrophoresis and the products were visualized by UV transillumination.

IV. Agarose gel electrophoresis.

Two concentrations of agarose gel were prepared (1% and 2%) as needed. The concentration of 1% agarose was used in electrophoresis after DNA purification process, while 2% agarose was used after PCR detection.

Statistical Analysis

All results were performed by Chi square test at the level of significant when P-value < 0.01.The specificity,sensitivity and diagnostic accuracy of the results were also calculated by applying the following equations: a / a + b = sensitivity, d / d + c = specificity. (a = the total number of positive cases, b = false positive those bearing positive reading from negative samples, d =total number of true negatives, c=those with negative reading from positive cases , positive and negative predictive values also calculated . Diagnostic accuracy = specificity + sensitivity / 2 (11).

Results

The laboratory diagnosis depends on the conventional methods; the patients were divided into three groups according to the test applied. These groups were: 1st group included all the clinically suspected patients (250) who were diagnosed by ZN stain for the detection of tuberculous bacilli in their sputum, 180 (72%) out of 250 examined patients were negative and 70 (28%), were positive. The 2nd group included 50 out of 250 clinically suspected patients who were diagnosed by others tests such as, LJ culture media and PCR test. The 3rd group was 10 cases of non specific chest disease included in this study, who were also diagnosed by two tests.

Pulmonary infections according to etiologic agents

All of the examined patients with TB, the cause was M. tuberculosis. Clinical and laboratory diagnosis of the suspected patients revealed that 30 patients were suffering from PMIs , 15 of them had mixed infection. Twenty patients appeared to have pulmonary tuberculosis without mycotic infection (Table 1).

Table -1: Pulmonary infection according to etiologic agents and their percentage.

Type of infection	No. of cases	%
TB	20	40
Mycosis & TB	15	30
Mycosis	15	30
Total	50	100%

The fungal etiological agents of pulmonary infection that associated with *M. tuberculosis* throughout the study were represented by 9 species (Table 2). Four *Aspergillus* species was represented by 16 isolates obtained from 16 patients. One *Candida* species was represented by 4 isolates obtained from 4 patients. Tow *Cryptococcus neoformans* isolates from 2 patients.Two *Rhizopus oryzae* isolates from 2 patients. Three *Penicillium* species isolates from 3 patients .Three isolates of *Actinomycetes represented* by 3 patients (Table 2)

Table -2: Number of mycotic isolates and their percentage throughout the study.

Agents of mycotic infection	No. of isolates	%
Aspergillus fumigatus	5	16.7
A. flavus	4	13.3
A. niger	4	13.3
A. terreus	3	10
Actinomycetes group	3	10
Candida albicans	4	13.3
Cryptococcus neoformans	2	6.7
Penicillium spp	3	10
Rhizopus oryzae	2	6.7
Total No. of cases	30	100 %

Sensitivity
Direct microscopy = 25/30×100% = 83.3%
Culture = 25/25 ×100% = 100%
Specificity
Direct microscopy =20/20×100% = 100%
Culture = 20/25 ×100% = 80%
Evaluating the Results of the AFB stain, L-J media And PCR Tests

Table -3: Sensitivity & specificity of direct microscopy test and sputum cultureTest of fungi.

Test		Direct microscopy		Total
		Yes	No	
Culture	Yes	(a) = 25 True positive	(b) = 5 False positive	30
	No	(c) = 0 False negative	(d) 20 True negative	20
Total		25	25	50

Validity of direct microscopy test and culturing test in Diagnosis of PMI.Two methods were used for detecting the etiological agents of PMI in sputum, direct microscopy method and culturing method. Table 6 explains sensitivity and specificity of these methods. We found that culturing method was sensitive to detecting the agents of PMI in sputum. It shows sensitive by 100%, while direct method shows 83 %(Table 3) .

Three (11) tection , table (11) . (ThreeTTTTTmethods were used for the detection of the etiological agent of pulmonary tuberculosis in the sputum, direct microscopy, culture of sputum and PCR detection. The study regard's the culture of sputum as the gold standard test to other methods.

AFB stain results versus L-J media results

From 35 positive patients by L-J media, 18 patients were positive by AFB stain , so, the sensitivity, specificity, PPV, and NPV of AFB stain were (51.4 %, 86.6%, 90%, and 43.3%), respectively (Table 33).

Table -4: Validity of AFB stain for diagnosis of TB was confirmed with results of L-J media.

Test		L-J media results		Total
		Positive	Negative	
AFB results	Positive	18 True positive	2 False positive	20
	Negative	17 False negative	13 True negative	30
Total		35	15	50

AF stain Sensitivity = 18/35 x100 = 51.4% **AF stain Specificity = 13/15 x 100 = 86.6%**
Accuracy rate = (18+13) /50 x 100 = 62 % **PPV = 18/20 x 100 = 90 %**
NPV = 13/30 x 100 = 43.3 %

PCR results versus L-J media results

From 35 positive patients confirmed by L-J media , 33 patients were positive by PCR, so the sensitivity, specificity, PPV, and NPV of PCR test were (94.2 %, 100%, 100%, and 88.2%), respectively (Table 5).

Table -5:Validity of PCR test for the diagnosis of TB was confirmed with results of L-J media.

Test		L-J media results		Total
		Positive	Negative	
PCR results	Positive	33 True positive	0 False positive	33
	Negative	2 False negative	15 True negative	17
Total		35	15	50

PCR
Sensitivity = 33/35 x100 = 94.2%
Specificity = 15/15 x 100 = 100 %
Accuracy rate = (33+15) /50 x 100 = 96 %
PPV = 33/33 x 100 = 100 %
NPV = 15/17 x 100 = 88.2 %

Culture
Sensitivity =100 %
Specificity= 88.2 %
Accuracy rate =96 %
PPV = 94.2%
NPV =100 %

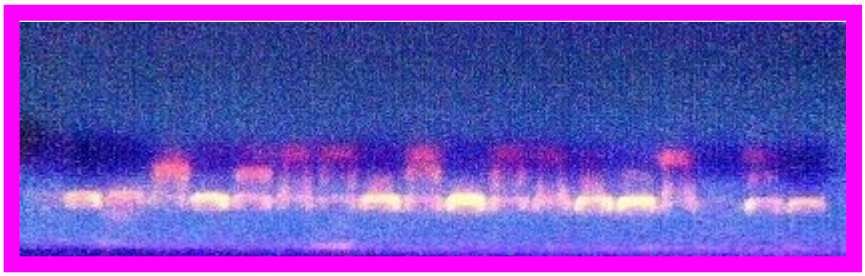

Figure: Polymerase chain reaction (PCR) amplification of a 163 bp fragment of IS6110. amplification samples was directly loaded in a 2 % agarose gel containing 0.5mg/ml ethidium bromide in electrophoresis, analysis of PCR-amplified DNA products. Lane 1: Negative control , lane 2: MTB positive control, lane 3 ,5,9,11,14,15,18, and 19 positive specimens, lanes 4,6,7,8,10,12,13,16, and 17 negative specimens.

Conclusions

The AFB stain was easy but not sufficient to diagnosis the pulmonary TB alone, whereas the use of LÖwenstein-Jensen medium sensitive enough for diagnosis of pulmonary TB, but need along time to get the results. The polymerase chain reaction (PCR) test gave a high sensitivity and specificity in comparison with other done test, with its advantages of greater speed and effectiveness than conventional detection methods. It was successfully to identify the *M. tuberculosis*, particularly when the staining for acid- fast bacilli is negative and there was a lack of growth on culture or when fresh material has not been collected for culture.

Discussion

With the similarity of clinical symptoms and roentgenographic features of pulmonary TB , PMI ,and other respiratory diseases, so the diagnosis of these infections are pose difficult and lead to misdiagnosed and complication of unwarranted chemotherapy (12).

In the present study, we found that the pulmonary infections in Al-Diwanyia and Al-Najaf provinces are distributed in to 3 categories: Tuberculosis, mixed infection (TB + Mycosis) and mycotic infection. The total number and percentages of infections were varied according to causative agent (Table 1). When comparison these results with other studies in this field as (13,14) in Al-Diwanyia province in respect with causative agents were approximately similar and they found that percent of TB (46.9%)

for (13) and (17%) for (14) and percent of mixed PMI with TB (47.14%) and (24.1%) respectively.

This may explained that the patients may had PMI but negative AFB stain and had transferred from +ve to –ve results in the period preceded the time of the study mostly under the effect of anti- TB drugs, but remain suffering from symptoms of the PMI. However, this is coordinated with reports that TB patients generally have defective defense mechanism that encourage the invasion of respiratory system by other microorganism (15).) Due to the immune response against TB which plays a fundamental role in the outcome of *M. tuberculosis* infection and the risk of developing the disease increases considerably when TB infection co-exists with an alteration in the immune system (16).

When the immune system is weak as a result to infection with some types of diseases such as pulmonary tuberculosis this is considered as encourage factor to infection with opportunistic fungi or prolonged treatment with corticosteroids (17 ,8).

Thus, the results showed that the dominance of *Aspergillus* spp was the first cause of mycotic infection with TB. Most of mycotic infections were secondary to tuberculosis.

(8,18) found that Aspergillosis was always secondary to tuberculosis. (19,8) stated that *A. fumigatus* is the most common cause of PMI, *A. flavus* is the second and *A. niger* is the third etiologigal agent of PMI. (20) isolated *A. niger* as the second cause of PMI in Babylon province. However, these results came in less percentages of occurrence than in the current study, most probably because of the different bases of patient selection for the study,i.e., the current study is not a survey study for all the attendants of the TB and chest diseases center, in fact, the selected patients were suspected to have TB.

Other fungal agents isolated in this study included Candida *albicans* , *Penicillium* spp, *Actinomycetes* group, *Cryptococcus neoformans* and *Rhizopus oryzae* (Table 2).

(21) isolated *C. albicans* from patients with respiratory diseases especially infected patients with pulmonary tuberculosis.

All of these genera isolated from patients with pulmonary tuberculosis as a secondary infections. This lead to the suggestion that tuberculosis and antibacterial treatment enhanced fungal infections. (22) reported that the use of antibacterial drugs in patients with destructive forms of pulmonary tuberculosis promotes the growth and reproduction of fungal flora in the lung tissue.

In the present study, sputum has been selected as clinical sample and four methods have been used for the detection of the etiological agents of pulmonary infections. These methods are direct microscopic examination by using 10% KOH and other stains, and culturing of the sample with tow types of media. (Table 3) explain the sensitivity and specificity of each method. Culture method was highly sensitive for the detection of fungal agents than direct examination, it was found that sensitivity was 100% and specificity was 80%, while the sensitivity for direct examination method was 83.3% and specificity was 100%. Low value of sensitivity means decreasing in false negative results (11). So it concluded that culturing method is highly accurate and convenient method for the detection of fungal agents in the clinical sample.

The results revealed that the sensitivity, specificity, PPV, NPV and accuracy rate of ZN was found to be (51.4%, 86.6%, 90%, 43.3%, 62%) respectively (Table 4), these were similar of (23, 24) However the specificity of smear examination methods should be interpreted with caution because it does not allow differentiation of *M. tuberculosis* from mycobacteria other than tubercle bacilli (MOTT) (25).

In the present study, the sensitivity , specificity, PPV, NPV and accuracy rate of the LJ culture media were found to be (100 %, 88.2 %, 94.2%, 100%, 96%), respectively (Table 5) , which were similar to (95%, 84%, 90%, 96%, 92%), respectively found by (26) Comparing to the culture results, the false positive results obtained by ZN stain were 2 cases 4% (Table 4) .These results suggest that occasionally, a sputum specimen or a smear may contain particles that are acid fast: these particles may some time resemble tubercle bacilli, *i.e.* MOTT or the precipitate of staining, which hampers reading. Because of false positive results patient have to suffer from unnecessary therapy or prolonged hospital stay and further delays in the correct diagnosis and proper treatment of other diseases, or this 2 cases were not able to be isolated on LJ medium, this may be due to the presence of non viable bacilli in sputum specimen received, while Acid fast smear examination does not discriminate between viable and non viable bacilli, also tubercle bacilli and other mycobacteria (25).

This study is in accordance to the result obtained by (27) who reported that 2-3 % of AFB specimens could not be confirmed by growth on LJ medium. In this study, false positive rate was found similar to (26). But in other studies claimed that higher false positive rate might occur in ZN staining technique (27, 28). Therefore, it is a

good laboratory practice to confirm any smear-positive or doubtful result in newly diagnosed patients. Since, false positive noted with the staining techniques was lower; a positive smear could be reliable as a good diagnostic indicator with these staining techniques.

False negative results in the staining methods were commonly due to deficiencies in the preparation of the smear such as too little materials spread on the slide or too thin / thick smears. So a negative smear should be interpreted with caution because it does not rule out the active tuberculosis. In the present study, higher percentages of patient were found to miss diagnosis when tested with sputum smear microscopy because of low sensitivity. These data supports that culture is one of the definitive diagnosis of tuberculosis that depends on the isolation and identification of *M. tuberculosis*.

Today, attention has turned to nucleic acid technology: the PCR and related techniques are rapid, specific and sensitive. However these methods require more sophisticated laboratory methods and are very usefully if being used for the routine diagnosis of TB. Specificity, sensitivity and speed of PCR test in diagnosis of TB shown in this study should encourage the use of this method in routine diagnosis of TB. We compared the performance of various tests in different clinical samples for diagnosis of TB. PCR showed the high sensitivity after LJ media as compared to other tests and was supported by other studies (29. 30).

The results revealed that the sensitivity, specificity, PPV, NPV and accuracy rate of PCR were found to be (94.2%, 100 %, 100 %, 88.2 %, 96 %) respectively (Table 5).

References

1. Plorde, J.J.(2004) .Mycobacteria , *In*: Sherris Medical Microbiology 4th ed.(Ryan ,K.J. & RAY , C.G., McGraw–Hill medical publishing division , New York, USA. Pp : 439 – 454 .

2. Beneke, E.S. & Rogers, A.L.(1996). Medical Mycology and Clinical Mycoses.Belmont, California. Star publishing Co., USA; Pp.149-204.

3. Singh,N.(2001).Trends in the epidemiology of opportunistic fungal infections predisposing factors and the impact of antimicrobial use practice of infect.Dis.,33:1692-6.

4. Haber , J. ; Kdeskova , E.; Straub, J.; Palecek,A.; Masora, I. and Klener,P.(1994). Diagnosis of systemic mycotic infection . Unitr. Lek., 40: 65-68 .

5. Angeby, KA; Hoffner, SE. and Diwan, VK.(2004). Should the' bleach microscopy method' be recommended for improved case detection of tuberculosis. Literature review and key person analysis. Int. J. Tuberc. Lung Dis., 8: 806–815.

6. World Health Organization.(2006). Anti-tuberculosis drug resistance in the world. Report No.3. Prevalence and trends. WHO/HTM/ TB / 2004.343,Switzerland. Geneva,

7. Eisenach, KD .; Sifford, MD.; Cave, MD.; Bates, JH. and Crawford, JT.(1991).Detection of *Mycobacterium tuberculosis* in sputum samples using a polymerase chain reaction . Am. Rev. Respir. Dis., 144: 1160.

8. Ellis, D. H.(1994).Clinical Mycology .The Humman's Opportunistic Mycoses. Gillingham Printers Ltd., Australia . Pp. 166.

9. Kubica, G.P.; Dye, E.; Cohn, M.L.; and Middlebrook, G.(1993).Sputum diagnosis and decontamination with N-acetyl-L-cysteine- sodium hydroxide for culture of mycobacteria . Amer.Rev. Resp. Dis., 87: 775-779.

10. Valls, J.S.; Nacente, R.B. and Coll, M.S.(1999).Hand book of microbiology culture media. 5th edition.

11. Niazi, A.D.(2000).Statistical Analysis in Medical Research. Republic of Iraq. AL-Nehrien University.P.148.

12. Randhawa, HS. and Khan, ZU.(1987).Acute pulmonary mycoses in India:Current status & laboratory diagnostic aspects. Indian J. Tuberc., 34: 3-11.

13. Al-Amry, O.J. (2005). Classification & Epidemiological study or the pulmonary mycotic infection in AL- Qadisya province. Ph.D. thesis. College of Education – Al-Qadisya University. P. 132.

14. Abood, S. W.(2005) .Diagnostic & immunologic study of pulmonaryAspergillosis using Elisa technique against some locally prepared antigens. M.Sc. thesis .College of Medicine –Al-Kufa University. P. 95.

15. Solvo eva,T .;Karaer, Z.; Ignat eva, SM. and Mizonov, V.(1991).

Diagnosiso mycotic infections in patients with tuberculosis of the respiratory organs. Probl. Tuberc., 7: 37-40.

16. Schlesinger, L.S.; Bellinger-Kawahara, C.G.; Payne, N.R.; and Horwitz,M.A.(1993). Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. J. Immunol., 144: 2771-80.

17. McGinnis, MR. (1980). Laboratory Hand Book of Medical Mycology. New york, London .USA.Kauffman, CA.(1996).Quandary about treatment of Aspergillomas persists.Lancet, 18. 347: 1640.
19. Emmons, C.; Binford, C.; Utz, J. and Kwon- Chung, J. (1977). Medical Mycology.3rd ed. Lea & Febiger Philadephia, USA.
20. Jaffer, W.(1998). Pulmonary mycotic infection in Babylone province. Ph.D.thesis, College of Science, Babylon University.
21. Al-Rubiaa, A.M.(2001).Study of fungi that isolated from respiratory tract of patients attends TB center & chest diseases in Basrah. M.Sc. thesis. College of Science, University of Basrah.
22. Solvo eva,T .;Karaer, Z.; Ignat eva, SM. and Mizonov, V.(1991).
Diagnosiso mycotic infections in patients with tuberculosis of the respiratory organs. Probl. Tuberc., 7: 37-40.
23. Bhat,K.G. & Bhat, G. (2000). Methods and Devices: Simple cold staining method for acid-fast bacilli. *Tropical Doctor* ., 30: 224.
24. Kochhar, A. (2002). Evaluation of a two step AFB cold staining method and simplified concentration technique for diagnosis of pulmonary tuberculosis. J.Commun. Dis., 34: 276.
25. Gebre, N.; Karlsson, U.; Jonsson, G.; Macaden, R.; Wolde, A.; Assfefa, A. and Miorner, H.(1997). Improved microscopical diagnosis of pulmonary tuberculosis in developing countries. Trans .R.Soc. Trop. Med. Hyg. , 91:420-1.
26. Somoskovi, A.; Hotaling, JE.; Fitzgerald, M.;Donnel, D.;Parsons, LM. And Salfinger, M. (2001). Lessons From a Proficiency Testing Event for Acid- Fast Microscopy. Chest ., 120: 250-7.
27. Jain, A.; Bhargava, A. and Agrawal, SK.(2002). A Comparative study of two commonly used staining techniques for Acid Fast Bacilli in Clinical Specimens.In.t J. Tub. , 49: 161-2.
28. Ulukanligil, M.; Aslan, G. and Sami, T.(2000).A Comparative Study on the different staining methods and number of specimens for the detection of acid fast bacilli. Mem. Inst. Oswaldo. Cruz., 95: 855-8.
29. Tiwari, V.; Jain, A. and Verma, RK.(2003). Application of enzyme amplified mycobacterial DNA detection in the diagnosis of pulmonary and extrapulmonary tuberculosis. Indian J. Med. Res., 118 : 224-8.
30. Dwivedi ,A.; Sarin, BC.; Mittar, D. and Sehajpal, PK(2003). Optimization of 38kDa based PCR assay for detection of *Mycobacterium tuberculosis* from clinical samples. Indian J. Tuberc. , 50 : 209-17.