STUDIES ON LABORATORY DIAGNOSIS OF MYCOBACTERIA

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Nasser Fathy Hassan Ahmed

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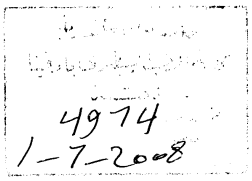

" Studies on Laboratory Diagnosis of Mycobacteria"

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List of Abbreviations

AFB	:	Acid Fast Bacilli
AIDS	:	Acquired Immunodifficiency Syndrome
BCG	:	Bacillus Calmette and Guerin
CMI	:	Cell mediated immune response
DOT	:	Directly Observed Therapy
DPI	:	Days Post Infection
DTH	:	Delayed Type Hypersensitivity
DNA	:	Deoxyribonucleic acid
ELISA	:	Enzyme Linked Immunosorbent Assay
ESAT-6	:	Early Secretory Antigenic Target-6
FAO	:	Food and Agricultural Organization
GGT	:	Gluteraldehyde Gelification Test
GOVS	:	General Organization for Veterinary Services
HIV	:	Human Immunodeficiency Virus
ID	:	Intradermal
ICT	:	Immunochromatographic
IFT	:	Indirect Fluorescent Antibody Test
IgG	:	Immunoglobulin-G
INOS	:	Inducible nitric oxide synthase
LAM	:	Lipoarabinomannan
LJ	:	Lowenstein Jensen
LN	:	Lymph Node
M	:	Mycobacteria
M.bovis	:	Mycobacterium bovis
MDR	:	Multi Drug Resistance
MIC	:	Minimal Inhibitory Concentration
MOTT	:	Mycobacteria other than tuberculosis
M .Tuberculosis	:	Mycobacterium tuberculosis
MPB70	:	Mycobacterial Protein of M. Bovis 70
MW	:	Molecular weight
N	:	Normality
OD	:	Optical Density
PAGE	:	Polyacrylamide gel electrophoresis
PA-HRP	:	Protein A Horse Radish Peroxidase
PBS	:	Phosphate buffer saline
PCR	:	Polymerase Chain Reaction
PH	:	Hydrogen Ion Concentration
PHA	:	Phytohaemagglutinin
PM	:	Post Mortem

PPD	:	Purified Protein Derivatives
RNI	:	Reactive nitrogen intermediates
SA	:	Sonicated antigen
SDS	:	Sodium dodecyl sulphate
SICT	:	Single Intradermal Cervical Tuberculin Test
TB	:	Tuberculosis
TBS	:	Tri –base sodium chloride solution
TCH	:	Thiophene 2-Carboxylic Acid Hydrazide
TU	:	Tuberculin Unit
USA	:	United States of America
WHO	:	World Health Organization
ZN	:	Ziehel Neelsen

INTRODUCTION

1-INTRODUCTION

Today, tuberculosis has become the most important communicable disease in the world, with over 8 million cases of pulmonary tuberculosis occurring each year, 95 % of which are in developing countries.

WHO estimated that there were 8.8 million new cases of TB in 2003, of which 3.9 million were smear positive and 674000 were infected with human immunodeficiency virus (HIV). An estimated 1.7 million people died from TB in 2003.

Bovine tuberculosis remains an important disease in many countries of the world causing significant economic losses and proving difficult to control .The causative agent, *M. bovis* is also responsible for tuberculosis in other animals and its transmition to humans constitutes a public health problem (**Hardie and Waston, 1992**)

The ability of the respectively pathogenic species of *M.species* to infest naturally one or more heterologus hosts makes tuberculosis in any species of animals a potential threat to others, including human being.

The fact that organism of cattle tuberculosis (*M. bovis*) is capable of infesting a diversity of species such as swine, cats, dogs, canneries, parrots and human being emphasizes the necessity of a comprehensive plan if tuberculosis is to be successfully controlled or eradicated.
Like wise the human type (*M. tuberculosis*) is capable of introducing tuberculosis in cattle, swine, dogs and parrots.

These facts make it evident that to eliminate tuberculosis in one species and ignore the disease in others is not likely to solve the larger problems, but a comprehensive plan is necessary if tuberculosis is to be successfully controlled or eradicated.
For this reason, rapid diagnosis & identification of mycobacterial isolates, along with rapid and simple susceptibility testing of all isolates of mycobacteria, has become critical for the prevention of the spread of organisms

Diagnosis of the mycobacterial infection is based on the traditional method with ZN stain ,but this method does not allow identification at the species level and is low of sensitivity ,where it requires a relatively large number of bacteria (more than 10,000/ml) to be present in the sample(**Eisenach et al., 1991**)

Culturing of organisms has a specificity that approach 100 % and permits susceptibility testing of the isolates ,but the main disadvantage that growth of the organism may take 6-8 weeks ,culture techniques also require viable organisms ,and this can be a problem when tissues are inadequately handled (**Nolte and Metchock, 1995**)

Tests based on the polymerase Chain Reaction (PCR) have shown more promising for mycobacterial detection in clinical samples (**Pao et al., 1990**). PCR has been evaluated for the detection of tubercle bacilli from a range of specimens and seems to have sensitivity equal or greater than that of the culture method (**Brisson-Noel et al., 1989, Del**

Portillo 1991, Pierre et al., 1993). Also **Zvizdic et al (1999)** stated that modern tests such as PCR shortened diagnostic duration from several weeks to one or two days.

The aim of this work : to compare between the different methods either conventional or recent for diagnosis of Tuberculosis in animals and human being.

So, to achieve this aim the following points are done:-

1-Collection of samples (Tissue, Sputum, and Blood) from suspected diseased cases

2- Direct staining with Acid Fast stain (Z.N)

3-Isolation & Identification of the causative microorganism

4- Immuno chromatographic test on the collected sera

5-Using the PCR (polymerase chain reaction) for diagnosis

2-Review of Literature

2.1- Historical

Koch (1882) announced the discovery of the organism causing tuberculosis , a certain reaction was obtained in Guinea pigs suffering form tuberculosis when they were injected with dead organisms of the disease. This reaction, which became known as Koch's phenomenon, was not obtained when healthy Guinea pigs were inoculated with dead organisms of tuberculosis. He concluded that the reaction was setup by some substance common to both dead and live organisms of tuberculosis which was later named as tuberculosis.

Rabinowitsch (1897) injected market butter intraperitoneally into guinea pigs, then isolated an acid fast organism and called it "butter A

Moeller (1898) isolated two acid-fast microorganisms; the first was the "timothy grass bacillus" or grass bacillus No.1 as it was isolated from timothy bag and the second microorganism was the " mist bacillus" since it was isolated from animal excreta. In 1899, he had isolated a third acid-fast microorganism from plant dust and called it "grass bacillus No. 2"

Thomson (1932) used the term saprophytic for all mycobacterium other than three types of tubercle bacilli *(M. tuberculosis, M. bovis* and *M. avium intracellular).*

Julin (1960) criticized the name atypical mycobacteria and preferred the name anonymous mycobacteria.

Paull (1973) used the term opportunistic mycobacteria in his environmental study on mycobacteria.

Dekantor (1994) reported to WHO that tuberculosis due to *M.bovis* has increased among the rural slaughter house workers from 1.6 % in 1982 to 6.2 % in 1988

EL-Sabban et al., (1992) found that the proportion of human disease due to *M.bovis* ,vareied from 0.4 % to 6% in different hospitals in Egypt. He added that out of 50 patients with pulmonary tuberculosis, 47 cases (94 %) were human type and 3 cases (6%) were bovine type at ABBASSIA Chest Hospital

Samiha (1995) declared that in many advanced countries tuberculosis remains an important cause of death than all communicable diseases.

Dolin (1999) pulmonary proved that tuberculosis is one of the most important health problems in the world, with an estimated 8 million new cases of tuberculosis and 1.9 million deaths having occurred in 1997.

Bering (2001) assessed that tuberculosis is the leading infectious cause of death.

Khasnobis et al., (2002) assessed that every minute, some where in the world 4 people die from tuberculosis.

2.2- Diagnosis of tuberculosis :

Diagnosis of tuberculosis based on measurement of either cell mediated immune response (CMI) like tuberculin test, lymphocyte blast genesis assay (LBA) and gamma in interferon assay or humeral immune response like enzyme linked immunosorbent assay (ELISA) and immuno chromatographic using different highly purified antigens or antigen detection (Z.N stain , Isolation , identification , and PCR)

2.2.1. Direct Detection of TB by using Z.N stain:

David (1970) Recorded that there must be 5.000 to 10.000 bacilli per milliliter of sputum to permit 50 % chance to detect even one or two bacilli in the entire smear , where as culture techniques have been estimated to detect 10 to 100 viable mycobacteria per milliliter of sample.

Rickman and Moyer (1980) Stated that microscopy is the first step in identification.

Nolte and Metchok (1995) Recorded that mycobacteria are usually identified by microscopic examination , culture charcter ,and for differentiation purpose, biochemical profile . They added that a wide variety of acid fast staining procedure is available. The most classic is carbol fuchsin Zeil-Neelsen stain, AFB appear as pink , red, thiny ,slightly curved and short or long rods.

Farnia et al., (2001) improved the results of direct smear microscopy, by using the mucus-digesting quality of chitin in tuberculosis (TB) laboratories. For this purpose, a total of 430 sputum specimens were processed by the *N*-acetyl-L-cysteine concentration, sodium hypochlorite (NaOCl) liquefaction, chitin sedimentation, and direct microscopy methods. Then, the smear sensitivity for acid-fast bacillus detection by chitin-treated sputum was compared with the sensitivity of smears prepared by other methods. The results showed that the chitin solution took less time to completely homogenize the mucoid sputum than did the *N*-acetyl-L-cysteine and NaOCl methods. The *N*-acetyl-L-cysteine concentration method demonstrated sensitivity and specificity levels of 83 and 97%, respectively. In comparison, the sensitivity of chitin sedimentation was 80%, with a specificity of 96.7%. The NaOCl liquefaction method showed a sensitivity of 78%, with a specificity of 96%. Finally, the sensitivity of direct microscopy was lower than those of the other tested methods and was only 46%, with a specificity of 90%. The chitin and NaOCl liquefaction methods are both easy to perform, and they do not require additional equipment (centrifuges).

Van Deun et al., (2005) Made comparative between performance of variations of carbolfuchin staining of sputum smears for AFB under field conditions and they found that Kinyoun cold staining sensitivity was unsatisfactory in field clinics. The sensitivity of the WHO/IUATLD recommended 0.3 fuchsin for 5 min was not significantly different from the original 1 % ZN for 5 min , but 1% for 15 min hot staining might be superior.

Kiraz et al.,(2006) determined the performance of the FASTPlaqueTB test, based on bacteriophage amplification technology, by comparison with the BACTEC 460 TB

culture system, the Löwenstein-Jensen (LJ) medium culture method and Ziehl-Neelsen (ZN) staining. Of 400 sputum specimens studied in , 19 were excluded due to contaminant growth. The FASTPlaqueTB test was performed according to the manufacturer's instructions. They found that: Only 42 of the 381 specimens examined were positive on at least one test: 30 were positive with ZN staining, 34 with LJ medium, 36 with the FASTPlaqueTB test and 39 with BACTEC 460 TB. The combination of BACTEC 460 TB and LJ medium culture was considered the gold standard. The sensitivity and specificity were 70.7% and 99.7% for ZN staining, 87.8% and 100% for the FASTPlaqueTB test, 82.9% and 100% for LJ, and 95.1% and 100% for BACTEC 460 TB.

Kathuria , et al., (2006) Reported that on 30 cases of cutaneous tuberculosis and along with the routine stains, ZN and periodic acid Schiffs staining was carried out in all cases. On cytology, out of 9 cases of lupus vulgaris, 89% showed cohesive epithelioid cell granulomas with or without chronic inflammatory infiltrate; however, acid fast bacilli (AFB) could be demonstrated only in 22.2% on cytology while none on histopathology. Of 19 cases diagnosed as Scrofuloderma, 79% showed caseation necrosis with or without granulomas, 10.5% revealed granulomas with acute inflammatory infiltrates. AFB was demonstrated in 78.9% cases on cytology when compared with 15.8% on histopathology

Renata et al., (2007) evaluated the impact of respiratory specimen dilution on MTD test accuracy in a public health laboratory. The difference in MTD test sensitivity between the dilution and conventional methods was 15.9% (P = 0.001) for smear microscopy-positive specimens and –3.6% (P = 0.38) for smear microscopy-negative specimens. (Specimen dilution has been proposed as a strategy to minimize amplified *M. tuberculosis* direct (MTD) test inhibition)

Makunde et al., (2007). Studied candidates included individual patients presenting with cough <3 weeks (Group I) and > or =3 weeks (Group II). Sensitivity and specificity of the bleach method was calculated and compared at 100% using the ZN staining technique as the standard. A total of 171 patients (94 males, 77 females) with mean age 34.9 years (SD +/- 12.9) were recruited. Fifty-eight patients had coughed for <3 weeks while 113 had coughed for 23 weeks. Smear-positive TB in Group I was 13.8% (95% CI = 5-23) while in Group II was 25.7% (95% CI = 21-29). Using the bleach method, the prevalence of smear-positive TB in Group II was 28.3% (CI 95% = 20-36). This was an increase in smear-positivity rate of 15.6% as compared to the ZN technique. These results suggest that the use of bleach technique "on the spot" improve the sensitivity of tuberculosis diagnosis among patients with a history of coughing of over three weeks. However, further studies in different settings are recommended to validate the technique.

Fabre , et al., (2007). evaluated the Patho-TB kit (Anda Biologicals, France) as an alternative for the fastidious search for acid-fast bacilli by the Ziehl-Neelsen method. Three hundred and ten samples from 189 patients were collected between July 2005 and March 2006, these were divide between 301 pulmonary and 9 extrapulmonary samples. The Patho-TB tests consists of a filtration step on a cassette followed by an immuno-chromatographic revelation. Samples were decontaminated by the Kubica method; after neutralization, an aliquot of the centrifuged pellet was separated for evaluation of the Patho-TB test. The rest was used for direct microscopic examination and cultures on solid and liquid medium. Positive results with auramine were always confirmed by the ZN staining. Analysis of the results per sample gave the follows results: 91.1% sensitivity and

85.5% specificity compared to 91.8% and 100% respectively or microscopy. Sensitivity of the Patho-TB test rose to 93.7% when only the MTB complex was considered. Per patient, the Patho-TB was found to be 96.4% sensitive and 86% specific. By comparison the sensitivity of microscopy was 94.5% and its specificity 100%. Positive and negative values were respectively 90.6% and 94.4% for the Patho-TB while they were 100% and 92.9% for microscopy. It is concluded that the Patho-TB test gives good performances; it is easy to use and very easy to determine the results.

Wright , et al., (2008) Reported that, on 200 children, and 25 (12.5%) aspirates were inadequate. Cultures were positive in 79/175 (45%); *M. tuberculosis* was identified in 61 and *M. bovis* BCG in 18 cases. Using culture as the gold standard, the concordance of the different techniques was as follows: cytomorphology 70%, ZN staining 73%, and autofluorescence 68%. Using an alternative gold standard (culture positive and/or suggestive cytomorphology plus positive autofluorescence or ZN smear), the "true" diagnostic performance of the various techniques was as follows: cytomorphology-sensitivity 78%, specificity 91%, positive predictive value (PPV) 93%, ZN staining - sensitivity 62%%, specificity 97%, PPV 97%; autofluorescence-sensitivity 67%, specificity 97%, PPV 97%; and culture-sensitivity 75%, specificity 100%, and PPV 100%. FNAB was shown to provide a rapid and definitive diagnosis in the majority of cases of suspected tuberculous lymphadenitis in children, based on cytomorphology and identification of the organism.

Varello , et al., (2008) Reporeted that , histologic methods were compared with mycobacterial culture as reference test on suspected lymph node samples from 173 cattle reacting positive in antemortem tests. Histopathology demonstrated high sensitivity (93.4%) and specificity (92.3%), while ZN sensitivity and specificity were respectively 33.9% and 100%. There was good agreement between histopathology and bacterial culture, suggesting that histopathologic examination is a reliable tool for rapid diagnosis in countries where active tuberculosis eradication programs allow the prompt identification and elimination of reactor cattle. Histopathology permits identification of typical mycobacterial lesions and its differentiation from other causes

2.2.2. Isolation and identification of Acid Fast Bacilli (AFB) :

Castro and Nemoto (1972) examined 252 mesentric and mediastinal lymph nodes of apparently healthy cattle for mycobacteria and isolated 19 strains (7.5%). Typing of which revealed group II (2 strains), group III (14 strains), and group IV (3 strains). They concluded that these species are not so much of pathogenic important as cause of non – specific reaction to the intra-dermal tuberculin test.

Kamel (1972) isolated 248 mycobacterial strains from human cases with pulmonary TB lesions, of these strains he found 16 (6.4%) *M. bovis* and 227 (91.5%) human type of mycobacteria.

Osman (1974) in Egypt, examined 225 lymph nodes show tubercluous – like lesions collected, from buffaloes and cows. They concluded that the incidence of acid-fast bacilli in cows and buffaloes was 34.22% and 27% respectively. The most frequent atypical mycobacteria isolated from cattle *M. Smegmatis* (28.56%) *M. fortuinm*(14.28).

Pruchard, et al (1975) ., recorded that tuberculous lesions were representing 75 (27%) of 281 slaughter cattle, lesions were most common in retropharyngeal lymph nodes, lungs and bronchial and mediastinal lymph nodes. Of 21 (28%) isolates of mycobacteria obtained, 20(26.7%) were *M. bovis* and one isolate was atypical mycobacterium (1.3%). There was no simple relationship between the result of the tuberculin test and postmortem finding.

Claxton et al.,(1979) recorded that out of 642 lesions considered to be tuberculous 62% yielded *M.bovis* and 4.51% other mycobacteria (OM).
M. bovis and (OM) were recovered also from 0.6% and 3.65% respectively of 165 cattle which gave tuberculin reaction but without visible lesions at slaughter.

Elsabban (1980) in Egypt bacteriologically examined random samples of tuberculous and congested lymph nodes and isolated *M. bovis* from (71%) and 2.37% respectively.

Sami (1980) stated that in Assiut governorate 63% of patient attending TB dispensaries come from rural areas. He added that in cases of pulmonary TB of human being, the bovine bacillus was isolated from 5.4% of Cairo dispensaries lived in the abattoir areas.

WHO (1982) Mentioned that for preliminary identification of tubercle bacilli ,the following characteristic must be taken in consideration : tubercle bacilli do not grow in primary culture in less than 3-4 weeks to give vesible growth . the colonies are buff colored and rough, having appearance of cauliflower and they do not emulsify in the saline used for making smears as it gives a granular suspention and it added that there is no single test that will differentiate *M.tuberculosis* from other mycobacteria.
The following tests when used in combination with the characteristics described before will enable the identification of more than 95 % of *M.tuberculosis* ,Niacin and nitrate reduction positive but catalase negative and grow on L.J medium containing THC but no growth on L.J medium containing P-nitobenzoic acid.

Gallo et al., (1983) rerecorded that out of 1287 positive reactors 117 (9.1%) had tuberculous lesions and mycobacteria were recovered from 374 (29.1%). Typing revealed 71 (5.5%) *M.bovis* (69 yielded from reactors with T.B. lesions), other mycobacteria (OM) were recovered from 373 (23.6%) reactors (all yielded from NVL reactors).

Krishnaswami and Mani (1983) examined 60 positive reactors slaughtered in India and isolated acid fast bacilli from 16 positive reactors.
Two of these were identified as *M . tuberculosis*. The source of infection was attributed to animal attendant suffering from pulmonary tuberculosis.

Small and Thomson (1986) studied the efficiency of bovine PPD tuberculin in the single caudle fold test, among 76 feral buffalo calves. 15gave a positive skin reaction and 61 gave a negative reaction , *M. bovis* was isolated from all animals with tuberculous lesions .

El-Sabban et al., (1992) reported that about 86 mycobacterial cultures were isolated from the cerebrospinal fluid of patients suspected to be infected with tuberculous meningitis one was *M. bovis* (1.2%).

Neill et al., (1994) isolated *M.bovis* from respiratory secretion and lymph nodes of 15 skin test – negative cattle on extensive postmortem examination , typical tuberculous lesions were found in 7 of them ; concluded that cattle with early tuberculosis infection not being detected by traditional tuberculin testing.

Dekantor and Rittaco (1994) recorded that between 1982-1984, a nation wide bacteriological survey was performed in Argentina where sputum species were examined from 7700 patients with pulmonary tuberculosis. *M. bovis* was identified in species from only 49 (0.6%) of these patients. In Santa Fe province, during 1984-1989 *M. bovis* was responsible for 2.4% to 6.2% of human cases and 64% of patients were slaughter louse or rural workers.

Baron et al., (1995) Reported that colonies of human tubercle bacilli generally appear on egg media after 2-3 weeks at 37 C .growth first appears as small,dry friable colonies that are rough with characteristic buff color. After several weeks, these increases in size (5 to 8 mm), typical colonies have flat irregular margin and a cauliflower center , while *M.bovis* requires longer incubation periods 4-8 weeks , and appear as tinny, translucent,smooth , pyramidal colonies. They added that the first test must always be performed on colonies growing on mycobacterial media is acid fast stain to confirm that the colonies are indeed mycobacteria.

Nolte and Metchok (1995) Recorded that mycobacteria are usually identified by microscopic examination ,culture charcter ,and for differentiation purpose, biochemical profile . they added that a wide variety of acid fast staining procedure is available. The most classic is carbol fuchsin Zeil-Neelsen stain ,AFB appear as pink ,red thiny slightly curved and short or long rods.

Zvizdic, et al., (1999) Stated that the diagnosis of tuberculosis depends on many factors but essentially on the detection of mycobacteria, their identification to the species level, and their susceptibility to major anti tuberculous drugs. The classic tests (direcrt examination by Z.N ,conventional culture and antibiotic susceptibility tests) are combined with modern tests such as PCR . They added that these modern tests shortened diagnostic duration from several weeks to one or two days. The sensitivity of PCR test was 97% for Z.N positive , 68% for Z.N negative samples while specifity was 99% either for Z.N positive or negative samples.

Chien , et al., (2000) recorded that a total of 124 mycobacterial isolates (114 Mycobacterium tuberculosis and 10 non-tuberculous mycobacteria) were detected. The recovery rates were 94% (117/124) with BACTEC MGIT 960 and 75.8% (94/124) with LJ. The rates of contamination for each of the systems were 5.5% with BACTEC MGIT 960 and 4.1% with LJ. The TTDs for mycobacteria were 10.7 days with BACTEC MGIT 960 and 30.6 days with LJ. Excluding the non-tuberculous mycobacteria, the TTDs for M. tuberculosis were 11.1 days with BACTEC MGIT 960 and 30.7 days with LJ.

Piersimoni , et al., (2001)studied the recovery rate of acid-fast bacilli (AFB) and the mean time to their detection from clinical specimens was determined by using the MB/BacT system. Data were compared to those assessed by the radiometric BACTEC 460 system (B460) and by culture on Löwenstein-Jensen (L-J) solid medium. A total of 2,859 respiratory and extra pulmonary specimens were processed by the N-acetyl-L-cysteine (NALC)-NaOH method using two different concentrations of sodium hydroxide; 1.5% was adopted in study design A (1,766 specimens), and 1.0% was used in study design B (1,093 specimens). The contamination rates for MB/BacT were 4.6% (study design A) and 7.1% (study design B). One hundred seventy-nine mycobacterial isolates were detected by study design A, with 148 Mycobacterium tuberculosis complex (MTB) isolates and 31 non tuberculous mycobacteria (NTM) isolates. Overall recovery rates were 78.8% for MB/BacT ($P = 0.0049$), 64.2% for L-J ($P < 0.0001$), and 87.1% for B460, whereas they were 84.5, 70.9, and 91.2%, respectively, for MTB alone. A total of 125 mycobacteria were detected by study design B, with 46 MTB and 79 NTM. Overall recovery rates by the individual systems were 57.6% ($P = 0.0002$), 56.8% ($P = 0.0001$), and 80% for MB/BacT, L-J, and B460, respectively, whereas the rates were 91.3, 78.3, and 97.8% for MTB alone. By study design A, the mean times to detection of smear-positive MTB, smear-negative MTB, and NTM were 11.5, 19.9, and 19.6 days, respectively, with the MB/BacT; 8.3, 16.8, and 16.6 days, respectively, with the B460; and 20.6, 32.1, and 27.8 days, respectively, with L-J medium. By study design B, the mean times were 15.1, 26.7, and 26 days with the MB/BacT; 11.7, 21.3, and 24.8 days with the B460; and 20.4, 28.7; and 28.4 days with L-J medium

Kristian Ängeby et al., (2001) evaluated a new nitrate reductase assay (NRA) that depends on the ability of *M. tuberculosis* to reduce nitrate to nitrite. The reduction can be detected using specific reagents, which produce a color change. they tested a panel of 57 *M. tuberculosis* strains with various resistance patterns. The bacteria were inoculated on Löwenstein-Jensen medium, either without drugs or with rifampin, isoniazid, streptomycin, or ethambutol and with potassium nitrate (KNO_3) incorporated. After incubation for 7, 10, or 14 days, the reagents were added and nitrate reduction, indicating growth, could be detected by a color change. Sensitivities to and specificities for drugs as determined by the NRA method compared to those determined by the BACTEC 460 method were 100 and 100% for rifampin, 97 and 96% for isoniazid, 95 and 83% for streptomycin, and 75 and 98% for ethambutol, respectively. The results were in the majority of the cases available in 7 days. The evaluated method is rapid and inexpensive and could correctly identify most resistant and sensitive *M. tuberculosis* strains. It has the potential to become an interesting alternative to existing methods, such as the proportion and BACTEC methods, particularly in resource-poor settings.

Mirovic , Lepsanovic .(2002) Reported that two hundred and fifty-one (10.8%) mycobacterial isolates [190 Mycobacterium tuberculosis complex (MTBC) and 61 non-tuberculous mycobacteria (NTM)] were detected. Of these 251 isolates, 234 (93.2%; 181 MTBC and 53 NTM) were detected in MB/BacT and 169 (67.3%; 154 MTBC and 15 NTM) on LJ. The mean (median) times to detection of MTBC by MB/BacT and LJ were 13.8 (13) and 22.1 (20) days, respectively, while overall contamination rates were 7.7% and 8.1%, respectively.

Van Deun et al., (2005) Made comparative between performance of variations of carbolfuchin staining of sputum smears for AFB under field conditions and they found that Kinyoun cold staining sensitivity was unsatisfactory in field clinics. The sensitivity of the WHO/IUATLD recommended 0.3 fuchsin for 5 min was not significantly different from the original 1 % ZN for 5 min , but 1% for 15 min hot staining might be superior.

Venkataswamy, et al., (2007), Reported that among the total 2325 CSF specimens processed by both methods, *M. tuberculosis* was isolated from 256 specimens. The isolation rates were 93% and 39% for the BACTEC system and LJ medium respectively. Both the media supported growth in 32% of the culture-positive specimens. BACTEC system alone yielded growth in 61% and LJ alone in 7%, of the culture-positive specimens. Among 205 isolates tested for drug susceptibility 81% were sensitive to all the drugs tested and 19% were resistant.

Falconi et al., (2008) evaluated the recovery rate and mean time for detection (TTD) of mycobacteria between 2 culture media: the VersaTREK system and the Löwenstein-Jensen medium (LJ). Clinical specimens were processed using the standard N-acetyl-L-cysteine (NALC)-NaOH method, and then inoculated onto VersaTREK system and LJ slants. Of 1510 specimens cultured, a total of 200 mycobacterial isolates (159 Mycobacterium tuberculosis and 41 no M. tuberculosis mycobacteria) were detected. The recovery rates were 84.8% (168/198) for the VersaTREK system and 89.4% (168/188) for LJ ($p=0.2$); while the contamination rates were 4.2% for the VersaSATREK system and 7.4% for LJ ($p<0.001$). The TTDs for mycobacteria spp. were 18.2 (+/-11.4) d for the VersaTREK system and 27.9 (+/-10.9) d for LJ ($p<0.001$). The TTDs for M. tuberculosis were 19.8 (+/-11.2) d for the VersaTREK system and 27.3 (+/-10.2) d for LJ ($p<0.001$). The difference in TTD between smear-positive and smear-negative specimens for Mycobacterium spp. was 15.9 (+/-10.0) vs 23.0 (+/-12.5) d, and for M. tuberculosis 16.7 (+/-9.5) vs 28.4 (+/-11.1) d for the VersaTREK system. The VersaTREK system significantly reduces the TTDs of mycobacteria detection, which is clinically relevant.

2.2.3.Diagnosis of TB by immunochromatographic serological test

Del Prete, et al., (1998) evaluated MycoDot, a new commercially available serological test, for the detection of immunoglobulin G antibodies to lipoarabinomannan (LAM), a glycolipid common to mycobacteria. Serum samples from 102 non-human immunodeficiency virus (HIV)-infected patients with no previous history of tuberculosis and with suspected active pulmonary (66) and/or extra-pulmonary (36) tuberculosis were investigated; 50 HIV-negative healthy subjects, sputum culture-negative, tuberculin skin test negative and with no history of tuberculosis, were used as controls. RESULTS: In 28 patients with microbiologically ascertained tuberculosis 25/28 serum samples were positive, whereas the test was negative in two patients with renal tuberculosis and in one with pulmonary tuberculosis.

Pottumorthy, et al., (2000) evaluated simultaneously seven serological tests, two immunochromatographic tests, ICT Tuberculosis and rapid test TB, and five enzyme-linked immunosorbent assays, TB IgA EIA, Pathozyme-TB complex, Pathozyme-myco IgG, Pathozyme-myco IgA, and IgM, with 298 serum samples from three groups of individuals: 44 patients with active tuberculosis, 204 controls who had undergone the Mantoux test (89 Mantoux test-positive and 115 Mantoux test-negative controls), and 50

anonymous controls. The sensitivities of the tests with sera from patients with active tuberculosis were poor to modest, ranging from 16 to 57%. All the tests performed equally with sera from subgroups of those with active tuberculosis, those with pulmonary (33 patients) versus extrapulmonary (11 patients) disease, and those who were smear positive (24 patients) versus smear negative (12 patients) ($P > 0.05$). The specificities of the tests ranged from 80 to 97% with sera from the Mantoux test controls and 62 to 100% with sera from the anonymous controls. The TB IgA EIA had the highest sensitivity (57%) with sera from patients with active tuberculosis, with a high specificity of 93% with sera from the Mantoux test controls, but a very poor specificity of 62% with sera from the anonymous controls. Overall, ICT Tuberculosis followed by P-MYCO IgG had the best performance characteristics, with sensitivities of 41 and 55%, respectively, with sera from patients with active tuberculosis and specificities of 96 and 89%, respectively, with sera from the Mantoux test controls and 88 and 90%, respectively, with sera from the anonymous controls. By combining all the test results, a maximum sensitivity of 84% was obtained, with reciprocal drops in specificities to 55 and 42% for the Mantoux test controls and anonymous controls, respectively. The best combination was that of ICT Tuberculosis and P-MYCO IgG, with a sensitivity of 66% and a specificity of 86% for the Mantoux test controls and a sensitivity and specificity of 78% for the anonymous controls. While a negative result by any one of these tests would be useful in helping to exclude disease in a population with a low prevalence of tuberculosis, a positive result may aid in clinical decision making when applied to symptomatic patients being evaluated for active tuberculosis.

Steingart, et al., (2002) studied the usefulness of commercial serological antibody based tests in the diagnosis of extrapulmonary tuberculosis In a comprehensive search, 21 studies that reported data on sensitivity and specificity for extrapulmonary tuberculosis were identified. These studies evaluated seven different commercial tests, with Anda-TB IgG accounting for 48% of the studies. The results showed that (1) all commercial tests provided highly variable estimates of sensitivity (range 0.00-1.00) and specificity (range 0.59-1.00) for all extrapulmonary sites combined; (2) the Anda-TB IgG kit showed highly variable sensitivity (range 0.26-1.00) and specificity (range 0.59-1.00) for all extrapulmonary sites combined; (3) for all tests combined, sensitivity estimates for both lymph node tuberculosis (range 0.23-1.00) and pleural tuberculosis (range 0.26-0.59) were poor and inconsistent; and (4) there were no data to determine the accuracy of the tests in children or in patients with HIV infection, the two groups for which the test would be most useful. At present, commercial antibody detection tests for extra pulmonary tuberculosis have no role in clinical care or case detection.

Reddy, et al., (2002). used the 38, 63, 64, 14, 59-kDa antigens of *M. tuberculosis* to develop a rapid immunochromatographic test kit. This study evaluates the diagnostic potential of the rapid test kit using TB positive and TB negative serum samples from various hospitals in India. The samples were obtained from patients infected with or exposed to bacteria and viral pathogens. The results demonstrated that the combination of antigens improved the diagnostic specificity and sensitivity. The specificity of the test was 99.42% with sensitivity of 98.52% ($n = 241$). In case of multiple infections, the specificity was 93.15% with a low sensitivity of 73.52% $n = 141$). The test kit may offer an improved alternative to purified protein derivative (PPD). This rapid TB test kit may be a useful tool for first-line testing of suspected cases, epidemiological studies and in designing a quality health system to reduce health hazards in resource-poor countries.

Bartoloni, et al., (2003) determined specificity, sensitivity and predictive values of a rapid immunochromatographic assay (ICT tuberculosis) for the diagnosis of tuberculosis (TB) in an Italian clinical setting, and to identify tentative new guidance for the interpretation of test results. The ICT tuberculosis test is an immunochromatographic test based on the detection of IgG antibodies directed against five highly purified antigens secreted by *M. tuberculosis* during active growth. Sera from 60 patients with active pulmonary (48 sputum smear-positive and six sputum smear-negative cases) and extra pulmonary (six cases) TB were obtained. Personal, anamnestic and clinical data were investigated and recorded for each patient. The control groups comprised 156 subjects: 40 healthy individuals, half of them *M. bovis* BCG-vaccinated, and 116 patients with mycobacterial diseases other than TB (five cases), with non mycobacterial lung diseases (30 cases), with non mycobacterial non lung diseases (30 cases), with non mycobacterial diseases and rheumatoid factors positivity (30 cases), and with asymptomatic HIV infection (21 cases). For 21 individuals the test was simultaneously performed with both serum and whole blood sample. The overall sensitivity and specificity were 56.7% and 90.4%, respectively. The sensitivity for pulmonary TB patients was 61.1% (66.7% for smear- positive and 16.7% for smear-negative cases) and 16.7% for extrapulmonary TB patients. The difference between ICT results in pulmonary TB patients and control subjects was statistically significant ($P < 0.0001$). The analysis of the positive ICT tests revealed that samples with strong color intensity (>/=++) and specific antibodies bound to antigens immobilized on line D were significantly more frequent in TB patients than in controls ($P = 0.001$ and $P= 0.027$, respectively). ICT test results with the presence of at least three visible lines were more often observed in the TB patients than in controls, although not reaching statistical significance ($P = 0.052$). No difference was observed between the results of the ICT test performed both on serum and whole blood sample. The ICT tuberculosis test was confirmed to be rapid and easy to perform without requiring special equipment, both on serum and whole blood sample.

HyeCheong koo, et al., (2005) used a synthetic peptide derived from early secretory antigenic target 6 (ESAT6-p) and a recombinant major secreted immunogenic protein (rMPB70) of *M. bovis* in an enzyme-linked immunosorbent assay (EIA), an immunochromatographic assay (ICGA), and a latex bead agglutination assay (LBAA). Sera from noninfected, *M. bovis*-infected, or *M. avium* subsp. *paratuberculosis*-infected (by natural and experimental routes) animals were evaluated. Receiver operating characteristic analysis comparing optical density values from the EIA with results of bacterial culture or skin test, the reference test, established suitable cutoff values for assessing sensitivity and specificity. The EIA and LBAA, respectively, had sensitivities of 98.6 and 94.8%, specificities of 98.5 and 92.6%, and kappa values of 0.97 and 0.88 with ESAT6-p. The EIA, ICGA, and LBAA, respectively, had sensitivities of 96.8, 83.0, and 86.7%, specificities of 90.1, 99.4, and 97.8%, and kappa values of 0.87, 0.85, and 0.83 with rMPB70. Examination of serial samples of sera collected from experimentally *M. bovis*-infected cattle and deer revealed that ESAT6-p-specific responses developed early after infection whereas responses to rMPB70 developed later in the course of disease. The advantage of the LBAA and ICGA as initial tests for multiple species is a rapid reaction obtained in 2 to 3 h by LBAA or 20 min by ICGA without species-specific secondary antibodies under field conditions, thus allowing immediate segregation of suspect animals for further testing before culling.

2.2.4. Diagnosis of TB by using PCR :

Dominiaue, et al., (1990) isolated an insertion sequence-like element, 1S6110 from *M. tuberculosis* cosmid library as a repetitive sequence. This insertion sequence was found to be specific to mycobacteria belonging to the *M. tuberculosis* complex. For detection and identification of *M. tuberculosis* complex in uncultured specimens, oligonucleotids derived from the 1S6110 sequence were used as primers and probes in PCR studies. The results obtained were consistent with results of classical identificatoin procedure, bacteriological data and clinical criteria.

Pao, et al., (1990) used the PCR assay to identify mycobacterial DNA sequences in uncultured clinical specimens. Results from analysis of cultured bacteria and clinical specimens showed that PCR was sensitive and specific both in detecting mycobacteria and in differentiating *M. tuberculosis* and BCG from other species of It is concluded that the PCR method with the primers reported here may become a useful tool in the early and rapid detection of mycobacterial infections in uncultured clinical specimens.

Shankar, et al., (1990) carried out a PCR for detection of A/f tuberculosis complex using two oligonucleotide primers choused from gene sequence coding for the (MPB64). They evaluated the specificity of the assay with a battery of DNA templates from eukaryotic and prokaryotic sourcees. Amplification was seen only with *M. tuberculosis* complex and not with any of the other DNA templates tested. This provides an advantage in that diagnosis can be achieved by agarose gel electrophoresis without the need for confirmation by hybridization, Of 10 PCR positive specimens 8 were also positive by smear and culture, the other 2 were negative by smear and culture.

Sjobring, et al., (1990) developed a PCR for the specific detection of mycobacteria belonging to the Al tuberculosis complex by using a single primer pair derived from the nucleotide sequence of protein antigen b of *M. tuberculosis*, they achieved specific amplification of a 419 - bas- pair DNA fragment in *M. tuberculosis* and *M. bovis*. The PCR assay could detect 419-base-pair sequence in samples containing few mycobateria. They suggested that this technique could be applied to clinical specimens for early and specific diagnosis of tuberculosis.

Brisson-Noel, et al., (1991) carried out a comparison between the PCR and the standard microbiology method used for diagnosis of tuberculosis. Specimens were tested for the presence of *M. tuberculosis* complex and atypical mycobateria in two assays one based on amplification of the 65KDa gene and the other on the 1S6110 insertion sequence. For the 489 samples that did not contain inhibitors of the amplification reaction PCR findings correlated well with bacteriologial and/or clinical data in 476 (97.4%). Six PCR results turned out to be false negatives, 3 to be false positives and 4 to be mis-identification of strains.

Manjunath, et al., (1991) developed a PCR for the specific detection of All. tuberculosis complex. Primers were designed to amplify a 240 bas pair region in the MPB64 protein coding gene (nts-460-700).
From among 15 different DNA templates tested (including 10 species of mycobactria) PCR amplified the DNA from *M. tuberculosis* complex only, demonstrating its exquisite

specificity, sensitivity studies using serial ten-fold dilutions of M tuberculosis bacilli determined the limit of detectability to be 10 organisms. They recorded that none of the specimens in the control gorup (26 known non-tuberculous specimens) were positive by PCR but out of 117 specimens (received at the tuberculosis diagnostic service), 19 were culture positive for (mycobacteria and identified as M. tuberculosis. All of them were PCR positive.

Kolk, et al., (1992) developed a PCR for direct detection of *M. tuberculosis* complex in clinical samples. They recorded that PCR was positive for all 32 culture-positive and Ziehl-Neelsen staining (ZN) positive samples, 10 of 12 culture-positive and ZN-negative smaples, and 4 culture-negative and ZN-positive, samples. PCR detected *M. tuberculosis* complex bacteria in 35 of 178 culture and ZN-negative samples.

Barry, et al., (1993) used the PCR and DNA probe for detection of *M. bovis* in bovine blood. They concluded that the DNA oligonucleotide probe from the spacer region between the 16s and 23s ribosomal RNA genes which is specific to the *M. tuberculosis* complex combined with a PCR assay showed that blood samples from non- infected cattle gave negative results and that the assay detected *M. bovis* cells at a level of 100 microorganisms per milliliter. The results also suggested that a DNA probe assay is feasible for the direct detection of *M. bovis* DNA, from both lysed and viable organisms, in the blood of infected cattle.

Shawar, et al., (1993) compared between the PCR and the microbiological methods used for diagnosis of tuberculosis. A 317-bP segment within the M tuberculosis-specific insertion sequence 1S6110 was amplified. The detection limit of the PCR assay for cultured mycobacteria was 50 cells per reaction by ethidium bromide-stained agarose gel electrophoresis and 5 cells 'per reaction by hybridization with an oligonucleotide probe conjugated with either diagoxigenin or alkaline phosphatase (AP).
Compared with culture, PCR showed sensitivities and specificities of 55 and 98%, respectively, for agarose gel electrophoresis and 74 and 95% ,respectively, for the AP procedure. Despite this slow sensitivity, a rapid positive PCR results was accurate and clinically useful.

Stuart, et al., (1993) simplified the PCR by developing two methods of sample preparation and by using a colorimetric method for product detection. This method was as sensitive and specific as agarose gel electrophoresis for detection of PCR product. They recorded that the PCR of samples prepared by the chaotrope-silica method had a sensitivity of 75% and a specificity of 100% whereas PCR of samples prepared by the chloroform method had a sensitivity of 92% and specificity of 100% when compared with the sensitivities and specificities of the combined classical microbiological methods for the diagnosis of tuberculosis. It is concluded that the PCR was at least sensitive as microscopy but had a greater specifity because samples with atypical mycobacteria were not detected by PCR

Bal, et al., (1994) used a modified culture medium and the polymerase chain reaction for detection o f *M. bovis* in 27 samples from lesions in 13 dairy cattle slaughtered because of positive tuberculin tests, 19 (70%) were positive on both microscopie examination of concentrated smears and culture on BM medium and another 6 on culture only. The PCR using.Is 1081 primers showed 26 of the samples were positive for

M.bovis. From one PCR- negative but culture- positive sample *M. non chromogenicum* was isolated

Noordhoek, et al., (1994) mentioned that several studies were done in clinical laboratories and such results illustrate that PCR is a good test for detecting *M. tuberculosis* complex (DNA) even when small numbers of bacteria are present in the clinical specimens.

Frothingham, et al., (1994) in a study carried out on the *M. tuberculosis* complex includes the four species *M.tuberculosis, M.bovis, M. africanum* and *M.microti,* by PCR, sequenced 13 *M. tuberberculosis* complex strains in the 16S to 23S rDNA internai transcribed spacer (1TS). The ITS has a high rate of nucleotide substitution and ITS was amplified by using 30 cycles of PCR. Previous reports found three nucleotide substitutions in the ITS between two *M. tuberculosis* complex strains. In contrast, the authors found the same ITS sequence in all 13 *M. tuberculosis* complex strains (includine all four species and *M. bovis* BCG). This finding confirms the conservation of 16S rDNA sequence and the high DNA-relatedness found in previous studies. By the usual criteria, the four species of the *M. tuberculosis* complex would be considered a single species. In a phylogenetic analysis based on the ITS sequence, the four species of the *M. tuberculosis* complex were distinct from non tuberculous mycobacteria. The ITS contains at least seven potential sites for oligonucleotide probes with specificity for the *M.tuberculosis* complex.

Cajal , et al., (1994) Reported that ,The DNA amplification method tested is a useful technique for the detection of *M. bovis* and other pathogenic *M. tuberculosis* complex microorganisms directly from tissue samples. By this PCR method, 71.4% of the samples from animals that were confirmed to be infected with *M. bovis* by culture were found to be infected by PCR within 2 days. The technique has a distinct time advantage over the traditional methods of identification, because by this technique specimens could be screened in 1 to2 days. In samples which were positive by PCR, these results were obtained on the second day, which is up to 8 to 20 weeks before confirmation could be obtained by traditional methods.

Liebana, et al., (1995) developed. a simple, rapid method for the extraction of DNA from bovine tissue samples and used in a PCR designed for the diagnosis of tuberculosis. Tissues from 81 cattle from tuberculosis-infected herds (group 1) and 19 cattle from tuberculosis-free herds (group 2) were tested in this PCR, and the results were compared with those of conventional culture. The PCR assay detected 71.4% of the culture- positive animals from group 1. Tissue from all animals in group 2 were negative in the PCR assay and by culture. The described method could be used as a rapid screening technique which would be complementary to culture of tissue specimens for the routine diagnosis of bovine tuberculosis. The PCR technique is much faster than culture and reduces the time for diagnosis from several months to 2 days. It also provides for the detection of *M. bovis* when rapidly growing Mycobacterium spp. are present in the sample and may be able to detect the presence of *M. bovis* in samples even when organisms have become nonviable.

Kulski, et al., (1995) carried out using a multiplex PCR to detect and identify members of the genus Mycobacterium, *M. avium, M. intracellulare, and M. tuberculosis.* Three different methods of extracting mycobacterial DNA from blood culture fluid were

compared for use with the multiplex PCR. Mycobacterial cells were pelleted from a small aliquot of blood culture fluid by centrifugation, and the DNA was extracted from cells by heat lysis or a sodium iodide-isopropanol or a phenol-chloroform method. DNAs of different sizes were amplified from a region of the MPB70 gene of *M. tuberculosis* (372 bp) and from a region of the 16S rRNA gene of members of the genus Mycobacterium (1,030 bp), *M. intracellulare* (850 bp), or *M. avium* (180 bp) as a multiplex PCR in a single tube. The amplified DNA products were detected by agarose gel electrophoresis and ethidium bromide staining in all 41 (100%) positive cultures after sodium iodide-isopropanol extraction, in 18 (44%) after heat lysis, and in 5 (12%) after phenol-chloroform extraction. Of the 41 positive cultures, 38 were identified as *M. avium* and 2 were identified as *M. intracellulare* by both routine methods and multiplex PCR. The remaining mycobacterium was identified as *M. intracellulare* by routine methods and as *M. avium* by the multiplex PCR. Another six blood cultures that were negative for the presence of acid-fast bacilli after Ziehl-Neelson staining were also negative by PCR.

Szewzyk, et al., (1995) reported that forty- nine isolates of *M. bovis* from humans and animals in Sweden were analyzed by restriction fragment length polymorphism (RFLP) patterns probed by the insertion element IS6110. Most isolates had patterns indicating the presence of only one or two genomic copies of the IS 6110 insertion element. This simple type of pattern was found in all human isolates. In contrast, isolates from *M.bovis* infections in five herds of farmed deer in Sweden showed a specific RFLP pattern with seven bands, indicating seven copies of the IS6110 sequence. Continued RFLP studies of the new Swedish *M. bovis* isolates can reveal possible transmission of this deer strain to other animals or humans.

Schirm, et al., (1995) reported that the conventional tests for detection of *M. tuberculosis* complex, culture and microscopy, are undoubtedly much cheaper than the PCR system or related systems. However, none of these conventional methods combine a reasonable sensitivity with a reasonable test time, as the PCR systems do since the PCR system is rapid, very user-friendly and the sensitivity problem might make PCR system a real gain for *M. tuberculosis* diagnostics.

Rodriguez, et al., (1995) used the PCR for species- specific Identification *of M.bovis.* They reported that the detection limit of the PCR is 10 fg of purified *M. bovis* DNA, corresponding to about 2 bacilli. The assay was also useful for identifying the bacilli directly from uncultured biological samples including milk.

Wards, et al., (1995) developed a PCR to detect *M.bovis* in tissues.They could detect as few as 10 organisms from pure culture and between 200-500 organisms from tissues spiked with cultured organisms. The method was tested on 110 selected tissues recovered from post mortem examination of a variety of animals (cattle, deer, pigs, ferrets, possums and sheep). Fifty-three of 58 samples diagnosed as *M. Bovis* culture positive including all samples containing microscopically visible acid- fast bacilli, were positive on duplicate testing by PCR. Five of 52 culture negative samples were also positive by PCR including 3 which contained large numbers of acid-fast organisms. Ten of the culture negative samples came from animals in a herd known to be free of bovine tuberculosis and all these were negative by PCR.

Bascunana and Belak (1996) developed a novel assay based on nested polymerase chain reaction (PCR) and restriction enzyme analysis of PCR products for the rapid detection and idntification of *M. bovis* and *M. avium-M. intracelluar* species in formalin-fixed, paraffin-embedde tissue (PET) specimens. They concluded that the nested PCR assay was very sensitive and could detect 5 to 10 fg of extracted mycobacterial DNA. It is suggested that PCR combined with an internal control of amplification and restriction enzyme analysis of the amplicons provides a rapid, sensitive and reliable method for routine diagnostic laboratories to detect and identify *M. bovis* and *M. avium-M.intracellular* in (PET) specimens.

Neimark, et al., (1996) developed a rapid PCR assay that types strains of *M. tuberculosis* by generating distinct DNA fingerprints directly from primary cultures. This assay allows strain identification analogous to that achieved by the standard restriction fragment length polymorphism method, and fingerprints are obtained in less than 8 h. This assay does not require subculturing, DNA purification, restriction digestion, Southern blotting, or nucleic acid hybridization. Rapid and precise identification of *M. tuberculosis* strains permits immediate molecular epidemiologic studies. The assay can be converted to a computer- automated system by employing fluorescently labeled PCR primers and the Perkin-Elmer DNA sequencer so that unknown-specimen fingerprints are identified by computer comparison to a database of *M. tuberculosis* strain fingerprints.

Noordhoek, et al., (1996) .reported that , reliable detection of *M. tuberculosis* in clinical samples by nucleic acid amplification techniques is possible, but many laboratories do not use adequate quality controls. This study underlines the need for good laboratory practice and reference reagents to monitor the performance of the whole assay, including pretreatment of clinical samples

Richter, et al., (1996) Reporeted that in 191 Tanzanian patients admitted to hospital with suspected extrapulmonary tuberculosis (TB), TB was diagnosed in 158 patients; the remaining 33 patients had neither microbiological nor clinical evidence of TB. *M. tuberculosis* was detected in the blood of 25 patients, in 92% by a polymerase chain reaction (PCR) technique and in 52% by culture of Buffy coat cells. The presence of mycobacterial DNA or *M. tuberculosis* bacteria in peripheral blood (positive culture) was significantly associated with HIV infection; it was detected in 22 (21.4%) of 103 HIV-seropositive patients compared to only 3 (3.5%) of 55 HIV-seronegative patients ($p < 0.009$). In two-thirds of the patients with mycobacteraemia, TB can be detected by simple smears from other organ sites. In patients with suspected extra pulmonary tuberculosis in whom smears from the infected site are negative or not available, PCR on blood will confirm the diagnosis within 24 hours in one third of the cases.

Foigueira, et al., (1996) used method based on DNA amplification and hybridization for the rapid detection of *M. tuberculosis* in blood samples from 38 hospitalized patients (15 human immunodeficiency virus [HIV] positive and 23 HIV negative) in whom localized or disseminated forms of tuberculosis were suspected. In 32 of these patients, the diagnosis of tuberculosis was eventually confirmed by conventional bacteriological or histological procedures. M. tuberculosis DNA was detected with the PCR technique in the peripheral blood mononuclear cells from 9 of 11 (82%) HIV-infected patients and in 7 of 21 (33%) HIV- negative patients ($P < 0.01$), while M. tuberculosis blood cultures were positive in 1 of 8 (12.5%) and 1 of 18 (5.5%) patients, respectively.

PCR was positive in all cases with disseminated disease in both HIV- negative and HIV-positive patients and also in the HIV-positive patients with extra pulmonary tuberculosis. Seven samples from patients with documented illness other than tuberculosis and 12 specimens from healthy volunteers, including seven volunteers with a recent positive purified protein derivative test, were used as controls and had a negative PCR. These results suggest that detection of *M. tuberculosis* DNA in peripheral blood mononuclear cells may be a useful tool for rapid diagnosis of disseminated and extra pulmonary forms of TB especially in an HIV-positive population.

Talbot, et al., (1997) mentioned that the attenuated bacillus Calmette-Guerin (BCG) vaccine strain is derived from a virulent strain of *M. bovis.* BCG is difficult to differentiate from other strains of *M. bovis* and other members of the *M. tuberculosis* complex by conventional methods. Recently, a genomic region designated RDl was fund to be present in all virulent *M. bovis* and *M. tuberculosis* strains tested but deleted from all BCG strains tested. With this information, a multiplex PCR method was developed to detect the RDl deletion. A large collection of BCG and other *M. tuberculosis* complex strains from diverse host and geographic origins was tested. RDl was deleted in 23 of 23 BCG strains. RDl was present in 129 of 129 other *M. tuberculosis* complex strains. This multiplex PCR method can be used as a tool for the rapid and specific identification of BCG.

Miller, et al., (1997) used the PCR for detection of *M. bovis* in formalin-fixed, paraffin-embedded tissues of cattle and elk. The primers used for PCR amplified a 123-bp fragment of IS 6110, an insertion sequence that is specific for organisms in the *M. tuberculosis* complex (M *tuberculosis, M. bovis, M.microti* and *M. africamim).* The PCR test detected this sequence in tissues from 92 of 99 tuberculosis cases in non decalcified tissues from cattle and 3 of 4 elk. In 80 tissues, the positive results were obtained using material prepared by immersion of paraffin sections in water containing a detergent, followed by alternating boil/freeze cycles. The remaining positive results were obtained with DNA isolated from the crude tissue extracts by proteinase digestion and phenol/chloroform purification. Accuracy of the IS6110 PCR test was demonstrated by negative test results on 31 tissues that had either non-mycobacterial granulomas organulomatous lesions by other mycobacteria. It is concluded that a PCR test can usually provide a rapid diagnosis of tuberculosis when applied to paraffin sections that have characteristic lesions and acid-fast organisms.

Scorpio, et al., (1997) developed a rapid PCR- single- strand conformation polymorphism (SSCP) assay to differentiate *M. bovis* from *M tuberculosis* strains, based on the detection of a single characteristic point mutation in the pyrazinamidase (PZase) gene of *M. bovis.* Eighty seven of 89 *M. bovis* strains could be distinguished from *M. tuberculosis* strains. It is suggested that the development of a rapid PCR- SSCP test for distinguishing *M. bovis* from *M. tuberculosis* will be useful for monitoring the spread of bovine tuberculosis to humans in areas where bovine TB is endemic and for directing the treatment of human TB eaused by M. *bovis.*

Cousins, et al., (1998) reported that DNA fingerprinting tehniques were used to type 273 isolates *of M. bovis* from Australia, Canada, the Irish Republic and Iran. The results of restriction fragment length polymorphism (RFLP) analysis with DNA probes from IS 6110, the direct repeat (DR), and the polymorphic GC- rich sequence (PGRS)

were compared with those of a new PCR- based method called spacer oligonucleotide typing (Spoligotyping) developed for the rapid typing of *M. tuberculosis.* It is concluded that spoligotyping may have advantages for the rapid typing of *M. bovis.* but it needs to be made more sensitive.

Zanini ,et al., (1998) developed a simple method for DNA extraction of *M. bovis* from milk and detection of the bacterium by PCR. Digestion of milk sample vvith proteinase K at 56 °C and phenol extraction, followed by ethanol precipitation and PCR. The amplification pattern obtained was analyzed with primers BW8-BW9 which amplified a 248-bp insertion sequence (IS 1081) in strains of *M.bovis.* By using the BW8- BW9 primers, a minimum of 103 c.f.u. could be detected on silver- stained PAGE gels. The procedure was validated by PCR analysis of milk in tuberculin positive animals. It is suggested that this method can be used for routine diagnosis of *M. bovis* in milk samples.

Rodriguez, et al., (1999) carried out a PCR for amplification of a 500- base-pair fragment from cultured isolates of *M. bovis.* All the isolates (121 isolates obtained from cattle in different regions and four additional isolates belonged to the *M. tuberculosis* complex) tested were PCR- positive, rendering the expected 500- bp and giving a correlation of 100% with previous microbiological characterization. It is concluded that this assay may be useful for diagnosing and identifying *M. bovis* in cattle.

Moussa (2000) camparing the results of PCR and that of ELISA of 45 slaughtered cattle, the PCR could detect all the animals which yielded *M. bovis* on bacteriological examination but the ELISA could detect 22 animals only in a sensitivity of 100% and 88% respectively which indicate the higher sensitivity of PCR in comparison with ELISA, also all the 14 animals with positive ELISA titer and negative by PCR revealed no *M. bovis* on bacteriological examination, which indicate the lower specificity of ELISA in comparison with the PCR technique. It is concluded that the PCR could differentiated the different mycobacterium isolates in a very short time. The nested PCR is a reliable, fast, high sensitive and highly specifie test for identification of mycobacterium isolates and for direct detection of *M. bovis* in tissue in two days.

Rossi, et al., (2000) developed an easy and rapid detection assay for the diagnosis of mycobacterial diseases. This is a PCR-hybridization assay based on selective amplification of a 16S rRNA gene sequence using pan-*Mycobacterium* primers followed by hybridization of the amplification products to biotinylated *M. tuberculosis* and *M. avium*-specific probes. A total of 55 mycobacterial isolates were tested. For all isolates, results concordant with those of conventional identification methods were obtained. Moreover, a developed method for extraction of DNA from Ziehl-Neelsen-positive smears which allows the recovery of intact target DNA in PCR-hybridization assay. the method was able to confirm all culture results for 59 Ziehl-Neelsen-positive smears from clinical specimens (35 sputum, 11 lymph node biopsy, 6 stool, 4 pus, 2 urine, and 1 pericardial fluid specimens). These data suggest that, the PCR-hybridization assay, which is simple to perform and less expensive than commercial probe methods, may be suitable for the identification of *M. tuberculosis* and *M. avium.* It could become a valuable alternative approach for the diagnosis of mycobacterial infections when applied directly to DNA extracted from Ziehl-Neelsen-positive smears as well.

Nashwa (2000) recorded the detection of *M. bovis* in milk samples from positive tuberculin cows and human sputum of clinically suspected pulmonary tuberculosis by using polymerase chain reaction assay (PCR).

Taylor, et al., (2001) compared conventional PCR with Light Cycler PCR,where Nucleic acid sequence capture extraction was coupled with LightCycler PCR amplification and product detection using real-time fluorescence for rapid, definitive detection of *M. bovis* in lymph node specimens from 38 cattle with bovine tuberculosis lesions. PCR amplification of sequence-captured DNA using both a conventional heating block thermocycler and a LightCycler thermocycler was compared with culture and histopathological analyses. Conventional PCR enabled detection of 26 of 28 culture-positive specimens (93%) in approximately 9 h, and the LightCycler PCR detected 20 of 28 culture-positive specimens (71%) in only 30 min. Specific confirmation of *M. tuberculosis* complex DNA was achieved by LightCycler PCR amplification using Syb Green 1 and an M. tuberculosis complex-specific Cy5-labeled fluorescence resonance energy transfer probe. The system described here enabled rapid and specific laboratory confirmation of *M. tuberculosis*, and this is the first report of the detection of *M. bovis* in tissues using LightCycler PCR. The fluorescence technology used in the study has potential to allow development of a high-throughput molecular diagnostic test for bovine tuberculosis.

Said, et al., (2001) evaluated 25 *M. tuberculosis* isolates from patients at a major Egyptian reference hospital in Assiut, Egypt, who had been treated for at least 1 year for tuberculosis. Typing patterns (IS*6110*) were diverse, and multidrug resistance was found among 11 (44%) of the isolates. Mutations associated with antimicrobial drug resistance were found in rpoB, katG, rpsL, and embB in the resistant isolates

Urvashi, et al., (2001) Reported a study examines the diagnostic utility of the polymerase chain reaction (PCR) in samples of bone marrow aspirate in 85 patients presenting with diverse clinical symptoms. Using primers specific for *M. tuberculosis*, tubercular etiology was detected in 33% of patients clinically suspected of tuberculosis while culture on Lowenstein-Jensen medium grew *M. tuberculosis* in only one patient (2.5%). None of these patients had been diagnosed by microscopy. Clinical improvement with ATT was observed in 85% of the patients with positive PCR. PCR demonstrated much higher sensitivity and specificity, thereby facilitating early therapeutic decisions for suspected extra pulmonary tuberculosis.

Garcia, et al., (2002) developed a 5'-exonuclease fluorogenic PCR assay in a single-tube balanced heminested format that simultaneously detects *M. tuberculosis* complex (MTC) and members of the *Mycobacterium* genus (MYC) using the 16S ribosomal DNA target directly on clinical samples. One hundred twenty-seven clinical samples (65 smear negative and 62 smear positive) with a positive culture result from 127 patients were tested, including 40 negative control specimens. The finding of both a positive MTC and probe value and a positive MYC probe value confirmed the presence of MTC or mycobacteria with a 100% positive predictive value. However, a negative value for MTC or MYC did not discount the presence of mycobacteria in the specimen. Interestingly, the addition of the MYC probe allowed the diagnosis of an additional 7% of patients with tuberculosis and rapid screening of non tuberculous mycobacteria (NTM). Thus, over 75% of the patients were diagnosed with mycobacterial disease by PCR. The

sensitivity was much higher on smear-positive samples (90.3%) than smear-negative samples (49.2%) and was slightly higher for MTC than NTM samples. With regard to the origin of the sample, MTC pulmonary samples gave better results than others.

Miller, et al., (2002) studied a real-time PCR assay using the LightCycler (LC) instrument for the specific identification of *M. tuberculosis complex* (MTB) was employed to detect organisms in 135 acid-fast bacillus (AFB) smear-positive respiratory specimens and in 232 BacT/ALERT MP (MP) culture bottles of respiratory specimens. The LC PCR assay was directed at the amplification of the internal transcribed spacer region of the *Mycobacterium* genome with real-time detection using fluorescence resonance energy transfer probes specific for MTB. The results from the respiratory specimens were compared to those from the Amplicor *M. tuberculosis* PCR test. Specimens from MP culture bottles were analyzed by Accuprobe and conventional identification methods. MTB was cultured from 105 (77.7%) respiratory AFB smear-positive specimens; 103 of these samples were positive by LC PCR and Amplicor PCR. Two samples negative in the LC assay contained rare numbers of organisms; both were positive in the Amplicor assay. Two separate samples negative by Amplicor PCR contained low and moderate numbers of AFB, respectively, and both of these were positive in the LC assay. There were 30 AFB smear-positive respiratory specimens that grew mycobacteria other than tuberculosis (MOTT), and all tested negative in both assays. Of the 231 MP culture bottles, 114 cultures were positive for MTB and all were positive by the LC assay. The remaining 117 culture bottles were negative in the LC assay and grew various MOTT. This real-time MTB assay is sensitive and specific; a result was available within 1 h of having a DNA sample available for testing.

Mirza, et al., (2003) compared detecting *M. tuberculosis* DNA in peripheral blood mononuclear cells (PBMC-PCR) with standard *M. tuberculosis* diagnostic techniques or a lymph node PCR (LN-PCR) for the diagnosis of tuberculosis aimed at lymphadenitis In this study carried out in Karachi, Pakistan, Thirty-seven (77%) specimens from 48 patients with clinical or diagnosis of tuberculosis lymphadenitis were positive by cytology [17/48 (35%) with no acid fast bacilli (AFB) (suggestive), and 20/48 (42%) with AFB (positive) in direct smears], 30 (63%) by PBMC-PCR, 16 (33%) by LN-PCR, and 13 (27%) by culture. All controls were negative, with the exception of one false-positive LN-PCR. These data suggest the PBMC-PCR may be helpful in the diagnosis of tuberculous lymphadenitis

Niyaz , et al., (2003) Reported a PCR test based on insertion sequence IS1081 was developed to detect *M. tuberculosis* complex organisms in the peripheral blood. The method was applied to blood samples from immunocompetent individuals with localized pulmonary tuberculosis. Seven of 16 (43.75%) blood samples were found to be positive for the circulating DNA copies of *M. tuberculosis* complex.

Elvira , et al., (2004) evaluated a novel DNA strip assay, GenoType MTBC, for differentiation of *M. tuberculosis* complex species from 77 positive liquid cultures in clinical practice. Species identification (*M. tuberculosis* [71 strains], *M.bovis* subsp. *bovis* [5 strains], and *M. africanum* subtype I [1 strain]) results were identical to conventional results. The sensitivity was slightly higher for this test than for the AccuProbe assay.

Mishra, et al., (2005) reported that the degree of concordance between the PCR-RFLP assay and the microbiological characterization was 99.0% ($P < 0.001$). A nested PCR (N-PCR) assay was developed, replacing the PCR-RFLP assay for direct detection of *M. tuberculosis* and *M. bovis* in bovine samples. The N-PCR products of *M. tuberculosis and M. bovis* corresponded to 116 and 89 bp, respectively. The detection limit of mycobacterial DNA by N-PCR was 50 fg, equivalent to five tubercle bacilli. *M. tuberculosis* and/or *M. bovis* was detected in 55.5% (105/189) of the samples by N-PCR, compared to 9.4% (18/189) by culture. The sensitivities of N-PCR and culture were 97.3 and 29.7, respectively, and their specificities were 22.2 and 77.7%, respectively. The percentages of animals or samples identified as infected with *M. tuberculosis or M. bovis* by N-PCR and culture reflected the clinical categorizations of the cattle (P of <0.05 to <0.01). Mixed infection by N-PCR was detected in 22 animals, whereas by culture mixed infection was detected in 1 animal.

Prasad, et al., (2005) designed a specific nested-PCR (N-PCR) assay, based on the hupB gene of *M. tuberculosis* (Rv2986c) and *M.bovis* (Mb3010c) as a method to differentiate these closely related species. The present paper deciphers the utility of this assay for identification of pathogenic Mycobacteria in clinical samples. Extra-pulmonary clinical samples obtained from cattle and humans were investigated. Pre-dominance of *M. tuberculosis* (15.7%) and *M. bovis* (26.8%) was seen in humans and cattle, respectively. However, more importantly, both mycobacterial pathogens (mixed infection) were identified in a number of samples. In humans 8.7% of the samples and 35.7% in cattle were classified as mixed infection. The detection of mixed infection with the mycobacterial pathogenic duo in humans and bovines denotes the prospect of potential transmission of these pathogens from humans to cattle (zoonosis) and vice versa (reverse zoonosis).

Taylor, et al., (2007) Reported that The minimum detection limits of the IS1081 method was < I genome copy and for the RD4 PCR was 5 genome copies. Both methods can be readily adapted for quantitative PCR with the use of SYBR Green intercalating dye on the RotorGene 3000 platform (Corbett Research).Initial testing of field samples of bovine lymph nodes with visible lesions (VL, n = 109) highlighted two shortfalls of the molecular approach. Firstly, comparison of IS1081 PCR with the "gold standard" of culture showed a sensitivity of approximately 70%. The sensitivity of the RD4 PCR method was 50%. Secondly, the success rate of spoligotyping applied directly to clinical material was 51% compared with cultures. A series of further experiments indicated that the discrepancy between sensitivity of detection found with purified mycobacterial DNA and direct testing of field samples was due to limited mycobacterial DNA recovery from tissue homogenates rather than PCR inhibition. The resilient mycobacterial cell wall, the presence of tissue debris and the paucibacillary nature of some cattle VL tissue may all contribute to this observation. Any of these factors may restrict application of other more discriminant typing methods. A simple means of increasing the efficiency of mycobacterial DNA recovery was assessed using a further pool of 95 cattle VL. Following modification of the extraction protocol, detection rate with the IS1081 and RD4 methods increased to 91% and 59% respectively.

Bakshi, et al., (2007) Validated a rapid and easy single tube multiplex-PCR (m-PCR) assay for the unequivocal differential detection of *M. bovis* and *M. tuberculosis*. Oligonucleotide primers were based on the uninterrupted 229-bp sequence in the *M. bovis*

genome and a unique 12.7-kb insertion sequence from the *M. tuberculosis* genome, which is responsible for species-specific genomic polymorphism between these two closely related pathogens. The m-PCR assay was optimized and validated using 22 *M. bovis* and 36 *M. tuberculosis* clinical strains isolated from diverse host species and 9 other non-tuberculous mycobacterial (NTM) strains. The designed primers invariably amplified a unique 168-bp (M. *bovis*-specific) and 337-bp (M. *tuberculosis*-specific) amplicon from *M. bovis and M. tuberculosis* strains, respectively. The accuracy of the assay, in terms of specificity, was 100%, as none of the NTM strains tested revealed any amplification product. As little as 20 pg of genomic DNA could be detected, justifying the sensitivity of the method. The m-PCR assay is an extremely useful, simple, reliable and rapid method for routine differential identification of cultures of *M. bovis* and *M. tuberculosis.* This m-PCR may be a valuable diagnostic tool in areas of endemicity, where bovine and human tuberculosis coexist, and the distinction of *M. bovis* from *M. tuberculosis* is required for monitoring the spread of *M. bovis* to humans.

David, et al., (2007) described the application of the BD ProbeTec ET direct tuberculosis system for the detection of *M. bovis* in bovine lymph node tissues. Compared to traditional culture, the overall sensitivity, specificity, and positive and negative predictive values of the BD ProbeTec were 87, 100, 100, and 87%, respectively.

3-Material & Methods

3.1. Materials:

3.1.1. Samples:

3.1.1.1. Animal samples:

a- Tissue

-The tissue samples were collected from Alexandria abattoir at Alameria City.,The mesenteric lymph nodes had tuberculous like lesions were collected (50 cases), and each sample was taken in clean plastic bag, kept in ice box and transferred for laboratory for direct Z.N staining, Isolation of Acid Fast Micro organism, and PCR

b-Blood

- About 10 ml of blood were collected aseptically in sterile tube (from each of the 50 cases), 5 ml Blood lifted 30 min at room temp for clotting then centrifuged at 3000 RPM /15 min., Serum was aspirated, labeled and kept at -20 C until use.
- Other 5 ml blood added to citrated tube (100 micron Na.citate solution + 5 ml blood) to get citrated whole blood
- Citrated blood tube was centrifuged at 3000 RPM/ 15 min , upper plasma layer discarded then the Buffy coat was aspirated , labeled and kept at -20 C until use for PCR

3.1.1.2. Human samples:

A-Sputum:

-Three morning sputum specimens were collected from 40 patients (positive X-ray TB) during 3 consecutive days from each patient in clean tightly closed plastic disposable containers. Samples were labeled, identified and then transported to laboratory as soon as possible for bacteriological examination of mycobacterial micro organisms.

b- Blood

- About 10 ml of blood were obtained aseptically in sterile tube(from each of the 40 cases), 5 ml Blood lifted 30 min at room temp for clotting centrifuged at 3000 RPM /15 min. Serum was aspirated, labeled and kept at -20c till use.
- Other 5 ml blood added to citrated tube (100 micron Na.citate solution + 5 ml blood) to get citrated whole blood
- Citrated blood tube was centrifuged at 3000 RPM/ 15 min , upper plasma layer discarded then the Buffy coat was aspirated , labeled and kept at -20 c till use

3.1.2. Materials used for preparation and isolation of AFB (Acid Fast Bacilli):

3.1.2.1. For animal tissues (Marks, 1972).

- 4% sulfuric acid (H_2So_4).
- Sterile distilled H_2O.
- Lowenstein Jensen medium.
- McCartney bottles.
- Sterile glass powder and mortar

3.1.2.2. For sputum (Petroff, 1915):

- 2% NaOH solution
- Sterile 0.85% NaCl or sterile distilled H_2O.
- Hydrochloric acid (HCl).
- Phenol red indicator.

3.1.3 Reagent used for Z.N staining :

- Strong carbolfuchsin
- Sulphoric acid 20 % as decolorizing reagent
- Methylene blue 0.3% as counter stain
- Dist.water to rinse smear

3.1.4. Media and reagents of biochemical tests:

3.1.4.1. Niacin test (George and Berlin, 1990):

- L.J. medium.
- Sterile distilled water.
- Niacin test strips (Difco).

3.1.4.2. Nitrate reduction test (Vestal, 1975):

- Sodium nitrate ($NaNo_3$) 0.085g
- Potassium dihydrogen phosphate (KH_2Po_4) 0.117g
- Disodium hydrogen phosphate ($Na_2HPo_4.12H_2O$) 0.485g
- Distilled water 100ml

- HCl (1:1) dilution
- Sulfanilamide (0.2g dissolved in 100ml H_2O)
- 0.1g N.naphthayl ethylene diamine dihydrochloride(dissolvedin100mldistilled water).

3.1.4.3. Hydrolysis of Tween 80 test (Kubica, 1973):

a. Phosphate buffer, 0.067M, pH 7 — 100ml
   61.1ml M/15 Na_2HPo_4 — 9.47 g/L
   38.9ml M/15 KH_2Po_4 — 9.09 g/L
b. Tween 80 — 0.5ml
c. Neutral red, 0.1% aquous — 2ml.

The a, b and c reagents were mixed and dispensed in about 3ml in screw capped tubes, autoclaved and stored in refrigerator in dark container to protect from spontaneous hydrolysis.

3.1.4.4. Arylsulfatase test (Kubica, 1973):

- Tripotassium phenolphthalein disulfate — 65mg
- Glycerol — 1mg
- Dubos oleic agar base — 100ml

Phenolphthalein and glycerol were added to 100ml of melted Dubos oleic agar and dispensed in 2ml amounts screw capped culture tubes, 5.3g sodium carbonate was added to 100ml H_2O.

3.1.4.5. Iron uptake test (Vestal, 1975):

Sterile Fe ammonium citrate 20% aqueous.

3.1.4.6. Catalase test (Kubica, 1973):

- Hydrogen peroxide — 30%.
- Tween 80 — 10%

Sterile at 121°C / 10 minutes and stored at 4°C and shacked before use.

Equal amounts 30% H_2O_2 and Tween 80 in amounts needed, mixed just before use.(only used freshly prepared) and any Tween peroxide mixture left was discarded.

3.1.4.7. Sensitivity to thiophene-2 carboxylic acid hydrazide (TCH) (Tsukamura, 1967):

TCH was inoculated into L.J. medium in conc. of 10ug/ml.

3.1.4.8. 5% sodium chloride tolerance test (Silcox, 1981):

- L.J. medium containing 5% NaCl.

3.1.4.9. Growth on MacConkey agar (Kubica, 1973):

- Special MacConkey agar(without crystal violet).

3.1.4.10. Urease test (Kubica, 1973):

Was prepared according to **Kubica,1973**

3.1.5. Materials used in McFarland Nephlometer standard:

According to **(Finegold and Martin, 1982)**

Turbidity standard:

- Sulfuric acid 1%.
- Barium chloride 1%.

Turbidity No. 1 ($3X10^8$) □ No. 0.5 ($1.5X10^8$).

3.1.6 Materiales used for Immunochromatography

- Disposable spicemen droppers

- Test devices: The TB Tuberculosis Rapid Test Device (Whole Blood/Serum/ Plasma) is a qualitative . solid phase two-site immunoassay for the detection of anti-TB antibodies in whole blood ,serum or plasma specimens .the membrane is pre-coated with TB recombinant antigen(specific antigen for *M.tuberculosis*) on the test line region of the Device.

-Buffer (for whole blood only)

3.1.7 Reagent and Materials used for PCR

MATERIALS:

Samples:

• Lymph Nodes (mesenteric LN).

• Blood and Buffy coat.

***Materials used for DNA extraction from tissues:**

Ez-10 spin column genomic DNA isolation kit (for animal) (Bio-Basic Inc.

Canada, Cat # BS427) the kit consists of :-

1. ACL solution for lysis of the tissues
2. PBS solution for sample dilution if necessary
3. AB solution for binding the DNA onto the membrane of the spincolumn
4. Proteinase K (10mg/ml) for digestion of the tissues
5. Washing buffer
6. Elution buffer
7. EZ-10 column
8. Nuclease free 2 ml collecting tubes.

***Materials used for DNA extraction from blood and Buffy coat:**

Biospin Blood Genomic DNA Mini-Prep Kit (BioFlux cat # BSJ040 1 00001S80) the kit consists of:

1. Proteinase K solution 10mg/ml
2. LB buffer for lysis of the cells
3. G binding buffer for binding the DNA to the spin column membrane
4. G washing buffer
5. Elution buffer
6. Spin column

Materials used for PCR:

a- BioReady Taq Pac (bioFlux cat# BSA09M2) that contains:

- Taq enzyme 5U/µL
- 10X reaction buffer with 15mM MgCl2
- 25mM MgCl2
- 6X Loading day
- DD water

b- Deoxynucleotide mix (dNTPs): (Stratagene , Cat. No. 200415).

Diluted before use from stock concentration {100mM (25mM each)} to the working stock {4mM (1mM each)} by mixing 20 ul of dNTPs mix with

480 ul distilled water and stored at -20oC till use.

c- Q-Solution 5X: (Stratagene).

d- Oligonucleotide primers:

Synsithized by BioBasic, Germany, purified by salting out and concentrated to 100 pg/ul. The primers designated based on the IS6110 gene sequence

e- Primer:

~Primer 1 (Sense):

5′- ATG TCA GGT GGT TCA TCG AG-3′

~ Primers 2 (Antisense):

5′- TGG CCG GTC GTG CGA TT-3′

Buffers and reagents used for agarose gel electrophoresis: (according to Sambrook et al., 1989)

□ **Ethedium bromide (EBr):**

It was prepared in stock solution, 10mg/ml by dissolving one tablets (10mg/tab) in 1ml-distilled water, vortex, stored in dark at +4oC. To be used at 0.5 ug/ml final concentration.

□ **50X electrophoresis buffer:**

(Tris-acetate EDTA, pH 8)

Tris-base..... 242.0 g

Glacial acetic acid..... 57.1 ml

EDTA (0.5 M pH 8)..... 100 ml

Bidist. Water to ....1000 ml

□ **1% agarose gel:**

Agarose..... ..... 1 g

1X TAE..... .....100 ml

The solution was heated but not boiled in a microwave till dissolving, left to cool to 60oC, and then 5ul of EBr solution was

added. The solution was poured in the casting tray of electrophoresis apparatus and left to solidify.

□ **DNA markers:**

1. Gene ruler 100bp DNA ladder 0.5µg/µl (Fermentas cat#SM0241).
2. *HindIII* digested λ phage DNA marker

3.2. Methods:

3.2.1. Preparation and isolation of acid fast bacilli:

3.2.1.1. From animal tissues (Marks, 1972):

The tissues had tuberculous like lesions were put in sterile morter containing sterile sand. After the fat was trimmed .the suspected material was cut into small pieces. Two ml of sterile distilled water were added to crushed material, homogenized and ground till a suspension was obtained.

Two ml of 4% H_2So_4 were diluted with 16ml sterile distilled water ,incubated at 37 C for 30 min with periodic shaking and centrifuged at 3000 rpm/20 min. the supernatant was poured off into 5% phenol solution and the obtained sediment was exposed for neutralization using phenol as colour indicator, made direct smear and also inoculated into 4 LJ slants. The inoculated LJ slants were incubated at 37°C /8 weeks and examined daily for one week then examined once weekly for 8 weeks. On the other hand, the smears were stained with Z.N stain technique and examined microscopically for acid fast microorganisms. The suspected growing colonies was picked up and the pure culture was suspected for identification , morphology ,culture character and biochemically

3.2.2.2. From sputum:

Samples processing were carried out according to (**Petroff, 1915**)

Specimens were collected from patients suspected to hade pulmonary TB subjected to the following steps:

- Direct smear was done and stained with Ziel Neelsen stain.
- Digestion-decontamination was done to every specimen as follows:
    - Equal volume of 2 % NaOH solution was added to 1ml of specimen in 50 ml screw capped test tube.
    - Test tube was shacked vigorously on vortex /1-2 min. and was left stand 15 min. for digestion at room temperature.
    - Centrifugation at 3000 rpm /15 min.
    - Supernatant was decanted and sediment was neutralized with HCl-phenol red indicator until indicator changes to persistant yellow.
    - A smear was taken from sediment and stained with Z-N method of staining.
    - Three LJ slants were inoculated each with 3 drops of neutralized sediment.
    - The inoculated LJ slants incubated at 37 C / 8 weeks and observed as in case of animal tissue and the growing suspected colonies were purified and identified

3.2.3. Identification of acid-fast bacilli:

The suspected growing colonies were picked up and subjected to purification and identification . the purified colonies were identified according to Physiochemical characteristics (**Kubica, 1973**).

a. Morphology:

Smears were made from suspected colonies, allowed to dry and heat fixed. The fixed smears were stained with Z-N method of staining and examined under oil immersion objective lens to detect the acid fast micro organisms , shape, size and arrangement.

b. Growth rate:

Isolates were inoculated onto LJ slants and incubated aerobically at 37°C for 7 days. Slants were examined daily to detect growth. Rapid growers grow within 7 days while slow growers grow thereafter.

c. Pigmentation:

Two LJ medium slants were inoculated with each isolate. One was wrapped in aluminum foil and both were incubated at 37°C. when unwrapped showed growth, the aluminum foil was removed from the other and growth colour was recorded. The growth in wrapped slant was exposed to daylight for one hour and examined on next day for any change in colour.

d. Growth at different degrees of temperatures:

LJ medium slants were inoculated with each isolate and incubated at 28°C, 37°C, 45°C and 52°C and examined after 7 days for growth.

3.2.4. Biochemical identification:

a. Niacin test (George and Berlin, 1990)

- 1.5ml distilled water were added to the culture showing good growth.
- Stabbing through the medium (with 1ml sterile pipette) containing the growth to permit niacin extraction.
- Culture tube was slanted so the surface of the medium was in horizontal position and covered with liquid, allowed to soak /20-30 min.
- Using sterile capillary pipette 0.5ml of extract was removed and transferred to a test tube.
- The uninoculated medium was used as negative control while the inoculated medium was used as positive control.
- Niacin test strip with arrow downward was dropped into each tube and stoppered immediately.
- Tubes were shaken gently and repeatedly /5-10 min.

- Colour of the extract was compared after 10-15 min against white background.
- Appearance of yellow colour in extract of tested culture and positive control was considered as positive test, while no colour indicate negative test.

b. Nitrate reduction test (Vestal, 1975):

- Several drops sterile water placed sterile screw capped test tube.
- Loopfull of actively growing isolate from the test culture was emulsified in water.
- Mixture of 2ml M/100 sodium nitrate buffer and M/45 phosphate buffer was added to the emulsified organs mixed by shaking and incubated at 37°C / 2 hours.
- Adding 1 drop of 1:1 dilution HCl to acidify the test culture.
- Two drops sulphanilamide solution followed by 2 drops of 0.1% N-naphthalyl ethylene diamine dihydrochloride solution were added to each tube respectively.
- Development of faint pink to red colour within 30-60 sec. was considered positive for the test.

c. Hydrolysis of Tween 80 test (Kubica, 1973):

Loopful of actively growing isolate of test culture was placed in the Tween 80 incubated at 35-37°C / 10 days and observed for colour change initially after 3 days and then daily. Positive test ; change of substrate colour from straw yellow to pink.

d. Arylsulfatase test (Kubica, 1973):

Each strain was inoculated into 5ml of Tween albumin broth and incubated at 37°C / 7 days. 0.3ml of that culture was inoculated into 0.001 M tripotassium phenolphthalein disulfate solution and incubated for 3 days /37°C. after 3 days, 6 drops of 2N sodium carbonate solution were added to each tube and change in colour was recorded immediately (pale pink – red: positive).

e. Iron uptake test (Vestal, 1975):

- Each LJ slant was inoculated with 1 drop of slightly turbid suspension of mycobacterial species to be tested and incubated till faint growth is visible.
- 2-3 drops ferric ammonium citrate were added to surface of the slant.
- Slants were incubated and examined daily for 21 days maximum.
- Appearance of colonies with rusty brown-pigmentation was considered positive test. No change in colour is negative test.

f. Catalase test (Kubica, 1973):

Spot test:

Two drops freshly mixed Tween-peroxide solution were added to colony of the suspected isolate on tube of culture medium and observation for 4-5 sec. for evaluation of bubbles.

Rapid bubbles : strong positive

Slow bubbles : weak positive

Lack of bubbles : negative test.

g. Semiquantitative test:

0.1ml of 7 days liquid culture of tested organism was inoculated onto surface of LJ medium tube and incubated at 37°C /2 weeks. Caps on culture tubes must be loosen to permit adequate exchange of air. One ml freshly prepared Tween-peroxide solution (1:1) (1% Tween 80 and 30% H_2O_2) was added and left upright for 5 min. height of bubbles column above surface of the culture medium was measured and recorded.

h. Test for heat stable catalase at 68 °C (pH 7/ 20 min):

Several colonies of tested organism were emulsified in 0.5ml M/15 phosphate buffer (pH 7) in small test tube. Tube was placed in water bath at 68°C /20 min., then removed and allowed to cool at room temperature. Half ml freshly prepared tween-peroxide mixture were added and observed for bubbles on the surface of the fluid. Negative test is recorded after 20 min. presence of bubbles is positive result.

i. Sensitivity to thiophene 2-carboxylic acid hydrazide (TCH) (Tsukamura, 1967):

The L- J medium containing 10mg TCH was inoculated with loopful of barely turbid broth culture of the organism to be tested and streaked to obtain isolated colonies. Then incubated in 5% Co_2 for 14-21 days then examined the growth. Good growth of the organism on the medium was positive test.

j. 5% sodium chloride tolerance test (Silcox, 1981):

LJ medium containing 5% NaCl was inoculated with 0.1ml of bacterial suspension then incubated at 30°C /4 weeks.

k. Growth on to MacConkey's agar medium (Kubica, 1973):

Loopful of 7 days mycobacterial broth culture was streaked on the surface of MacConkey agar plate to obtain isolated colonies. Culture was incubated at 35°C and examined for growth after 5-11 days.

l. Urease test:

Loopful of the tested colonies of mycobacteria was added to 0.5ml of urease broth substrate, mixed to emulsify, incubated at 35°C /3 days. Colour change from amber yellow to pink indicated positive result .

3.2.5-Methods of Immunochromatographic test:

1-Remove the test device from the sealed pouch and use it as soon as possible.

2-For Serum or Plasma specimens : transfer 3 drops of serum or plasma to the specimen well (S) of test device and then start the timer

For Whole Blood specimens : transfer 3 drops of Whole Blood to the specimen well (S) of test device , then add 1 drop of Buffer and start the timer.

3-Wait for the colored line(s) to appear , the result should be read at 10 minutes . Do not interpret the result after 30 minutes.

POSITIVE : Two distinct lines appear .One line should be in the control region (C) and another line should be in the test region (T)

NEGATIVE : One colored line appear in the control region (c)

INVALID : Control line fails to appear.

*** N.B (principle)**

The TB Tuberculosis Rapid Test Device (Whole Blood/Serum/ Plasma) is a qualitative . solid phase two-site immunoassay for the detection of anti-TB antibodies in whole blood ,serum. or plasma specimens .the membrane is pre-coated with TB recombinant antigen on the test line region of the Device. During testing the anti-TB antibodies . if present in whole blood ,serum. or plasma specimens react with the particles coated with TB recombinant antigen. The mixture migrates upward on the membrane chromatographically by capillary action to react with TB recombinant antigen on the membrane and generate a colored line. The presence of this colored line in the test region indicates a positive result. While its absence indicates a negative result. To serve as a procedural control . a colored line will always appear in the control line region indicating that proper volume of specimen has been added and membrane wicking has occurred

3.2.6-Method of PCR

DNA extraction:

DNA extraction from tissues:

The DNA was extracted using EZ-10 spin column genomic DNA isolation kit according to the manufacture instruction. Briefly, 30mg of the tissue sample was placed in 1.5 ml centrifuge tube and lysed with 300µl of ACL lysis buffer and 20µl of proteinase K (20mg/ml) at 55°C/5hours. The solution was then centrifuged at 14000rpm/5min

and the supernatant was placed in the EZ spin column. The DNA was bind to the membrane using 300µl AB solution, and centrifuged at 4000rpm/2min.washing done twice using 500µl of washing buffer followed by centrifugation at 10000rpm/2min. finally the DNA was eluted with 50µl of the elution buffer provided with the kit and the DNA was collected by centrifugation of the spin column at 14000rpm/2min after brief incubation at 50°C /5 min. the eluted DNA was visualized under UV transillumination and kept at -20°C till used *HindIII* digested λ phage DNA was used as a marker.

DNA extraction from blood and Buffy coat:

The DNA was extracted using Biospin Blood Genomic DNA Mini-Prep Kit according to the manufacture instruction. First the samples were digested with 2µl/1ml of proteinase K at 55°C/10 min to which 200µl of LB buffer was added. After digestion, 200µl of G binding buffer was added followed by 50µl absolute ethanol and the all mixture was transferred to the spin column and centrifuged at 10000rpm/2min. 200µl of G binding buffer was again added and the column was centrifuged as before. The column was then washed twice each with 600µl of washing buffer and centrifuged as before. Finally the DNA was eluted with 100µl of elution buffer by centrifugation at 14000rpm/2min and kept at -20°C till used.

Polymerase Chain Reaction:

To detect presence of the *M. complex IS6110* gene was amplified using the eluted DNA as a tamplet. A pair of primer was designed according the published sequence in the gene bank.

The PCR reaction was prepared as followed in a 0.2 ml PCR tubes:

DNA (100ng/µl) 2µl

Forward primer..... 1µl

Reverse primer……..…….. 1µl

Taq polymerase (1U/µl)…….…….. 2µl

10X reaction buffer…….. 5µl

dNTPs mix……..……..2µl

Q solution…….. 10µl

Nuclease free water ……up to ……50µl

The esat6 gene was amplified in T gradient (Biometra, Germany) using the following parameters:

Initial denature……..96°C/2min

Denaturing……95°C/45sec (39 cycle)

Annealing……60°C/30sec (39 cycle)

Extension……72°C/30sec (39 cycle)

Final extension…….……72°C/10min

The size of the amplicon was determined by electrophoresis on 1% agarose and visualized under UV transillumination Gene ruler 100bp DNA ladder was used as a marker.

Genomic DNA extraction:
A pronounced clear band were seen all processed samples that represents the genomic DNA of both the tissues and the mycobacteria (if present)

PCR:
A clear band migration at 327 bp (base pair) were seen which represents the amplified IS6110 gene.

4-RESULTS

4.1 -Results of direct staining by Z.N stain;

4.1.1- Z.N staining on Animal samples

As shown in table (1), A 50 mesenteric lymph node had tuberculous lesion were examined. Dry fixed smear were made from the sediment after treatment for isolation of acid fast micro organism and stained with Z.N technique. 19 samples were positive (Acid Fast Micro organism) and 31 were negative, with a percentage of (38 %) and (62%) respectively

4.1.2. Z.N staining on human Sputum

A 40 sputum sample were examined by Z.N staining smear technique, 22 samples were positive (Acid Fast Micro organism) and 18 were negative, with a percentage of (55 %) and (45%) respectively . as shown in table (2)

Table (1) - Z.N staining on animal tissues (Mesenteric L.n)

Number of tested samples	Positive		Negative	
	No.	%	No.	%
50	19	38	31	62

% : were calculated according to the total number of tested samples(50)

Table (2) Z.N staining on Human sputum

Number of tested samples	Positive		Negative	
	No.	%	No.	%
40	22	55	18	45

% : were calculated according to the total number of tested samples(40)

4.2- Results of Isolation

4.2.1-Results of isolation from animal tissue samples

As shown in the table (3), on culturing of the 50 samples on specific mycobacterial medium. 32 samples gave mycobacterial growth and 18 samples no growth was obtained, with a percentage of (64%) and (36%) respectively.

4.2.2-Results of isolation from sputum samples

On culturing of the 40 samples on specific mycobacterial medium. 27 samples gave mycobacterial growth and 13 samples no growth was obtained, with a percentage of (67.5%) and (32.5%) respectively. As shown in table (4)

Table (3) -results of isolation from animal tissue samples

Number of tested samples	Positive		Negative	
	No.	%	No.	%
50	32	64	18	36

% : were calculated according to the total number of tested samples(50)

Table (4) -results of isolation from human sputum samples

Number of tested samples	Positive		Negative	
	No.	%	No.	%
40	27	67.5	13	32.5

% : were calculated according to the total number of tested samples(40)

4.3- Results of identification of the recovered acid fast isolates:

4.3.1. Morphological identification

The recovered suspected colonies were examined morphologically using dry fixed stained film (ZN). All the covered isolates were AFB arranged in cord like picture *(M.bovis)*. The smears of human recovered isolates were long and curved bacilli (*M.tuberculosis*) and the numbers of bacilli were scanty.

4.3.2. b. Culture characteristics

The colonies of *M.bovis* had white, smooth with scanty growth

On the other hand the colonies of *M.tuberculosis* had yellowish, rough surface with luxuriant growth

4.3.3. Biochemical identification:

The biochemical identification of the recovered isolates (32) from bovine samples indicated that all isolates were *M.bovis*.

On the other hand the biochemical identification of the recovered isolates (27) from human samples indicated that all isolates were *M.tuberculosis*

As shown in the table (5)

Table (5) :The results of the biochemical reaction of the isolates recovered from animal & human samples

Number of Isolates	Biochemical identification								Growth on		
	Nitrate reduction	Catalase activity		Aryl sulphatase	Tween 80 hydrolysis	Urease	Niacin test	Iron uptake test	5% NaCl tolerance 28oC	MacConkey agar	TCH 10mg
		Semi quantitative	PH 7 68o C	3 days							
Bovine (32) *M.bovis*	-	< 45	-	-	-	+	-	-	-	-	-
Human (27) *M.tuberculosis*	+	< 45	-	-	-	+	+	-	-	-	+

+ : mean positive reaction

- : mean negative reaction

4.4.1- Results of using Immuno Chromatographic test on animal samples

The 50 animal serum samples were examined by immuno chromatographic test. 15 samples gave faint lines (weak positive) and 35 were negative with a percentage of (30%) and (70%) respectively. As shown in the table (6)

*The positive result appear in the photo (1)

4.4.2. Results of using Immuno chromatographic test on human samples;

The 40 human serum samples were examined by immuno chromatographic test. 10 samples gave positive and 30 were negative, with a percentage of (25%) and (75%) respectively. As shown in the table (7)

*The positive result appear in the photo (2)

* The samples gave positive result were collected from active TB patients and serum samples of these cases were collected before treatment.

Table (6) -Results of Immuno chromatographic test on animal blood samples ;

Number of collected blood samples	Positive		Negative	
	No.	%	No.	%
50	15	30	35	70

% : were calculated according to the total number of tested samples(50)

Photo 1

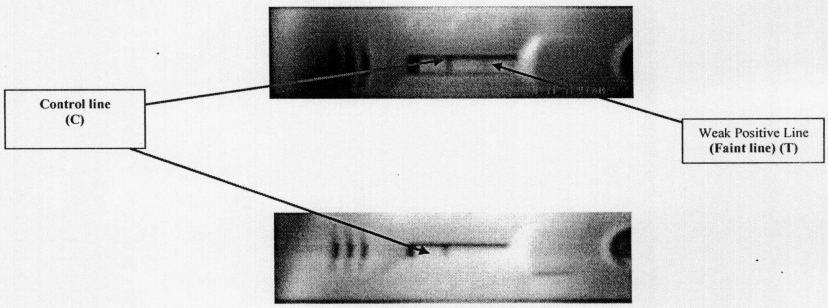
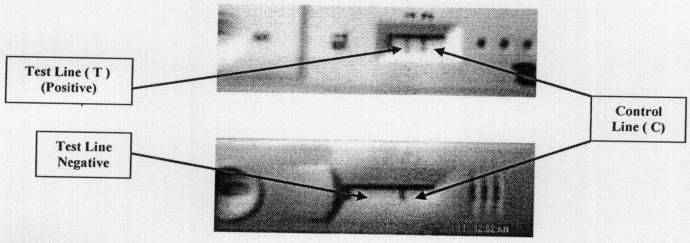


Table (7) - Results of Immuno Chromatographic test on Human samples

Number of collected blood samples	Positive		Negative	
	No.	%	No.	%
40	10	25	30	75

% : were calculated according to the total number of tested samples(40)

Photo (2)



4.5.1. Results of PCR on Buffy coat

4 samples (lanes 1, 2, 3, 4) were positive (each has band at 327 bp) From total tested 6 Buffy coat samples with an incidence of 66.6 %

On the other hand 2 samples (lanes 5 & 6) were negative had no bands with an incidence of 33.3 % .as shown in table (8)

*The result was demonstrated in photo (3)

Table (8) - results of PCR on Buffy coat

Number of tested samples	Positive (Buffy coat)		Negative (Buffy coat)	
	No.	%	No.	%
6	4	66.6	2	33.3

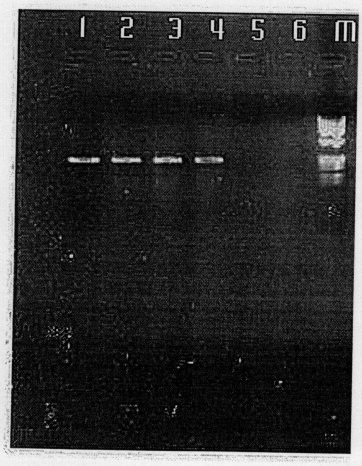
% : were calculated according to the total number of tested samples(6)

photo 3: results of PCR on Buffy coat

a- lanes 5 and 6 have no bands (Negative)

b- Lanes 1 ,2, 3 , 4 each has band at 327 bp (Positive)

c- Lane M is the standard



4.5.2. Results of PCR on animal tissues (Mesenteric L.n)

8 samples (lanes 7,8,9,10, ..) were positive (each had band at 327bp) from total tested 10 tissue samples with an incidence of 80 % .

On the other hand 2 samples (lane 11) were negative (had no band) with an incidence of 20 %. As shown in table (9) and photo (4)

Table (9): Results of PCR on animal tissues (Mesentric L.n)

Number of tested samples	Positive		Negative	
	No.	%	No.	%
10	8	80	2	20

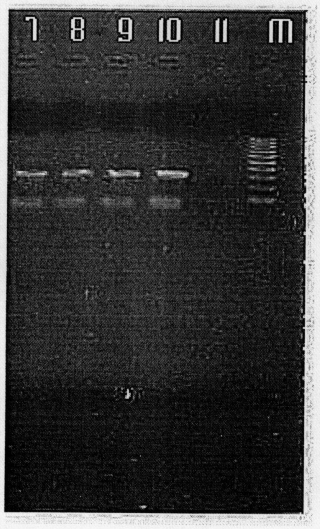
% : were calculated according to the total number of tested samples(10)

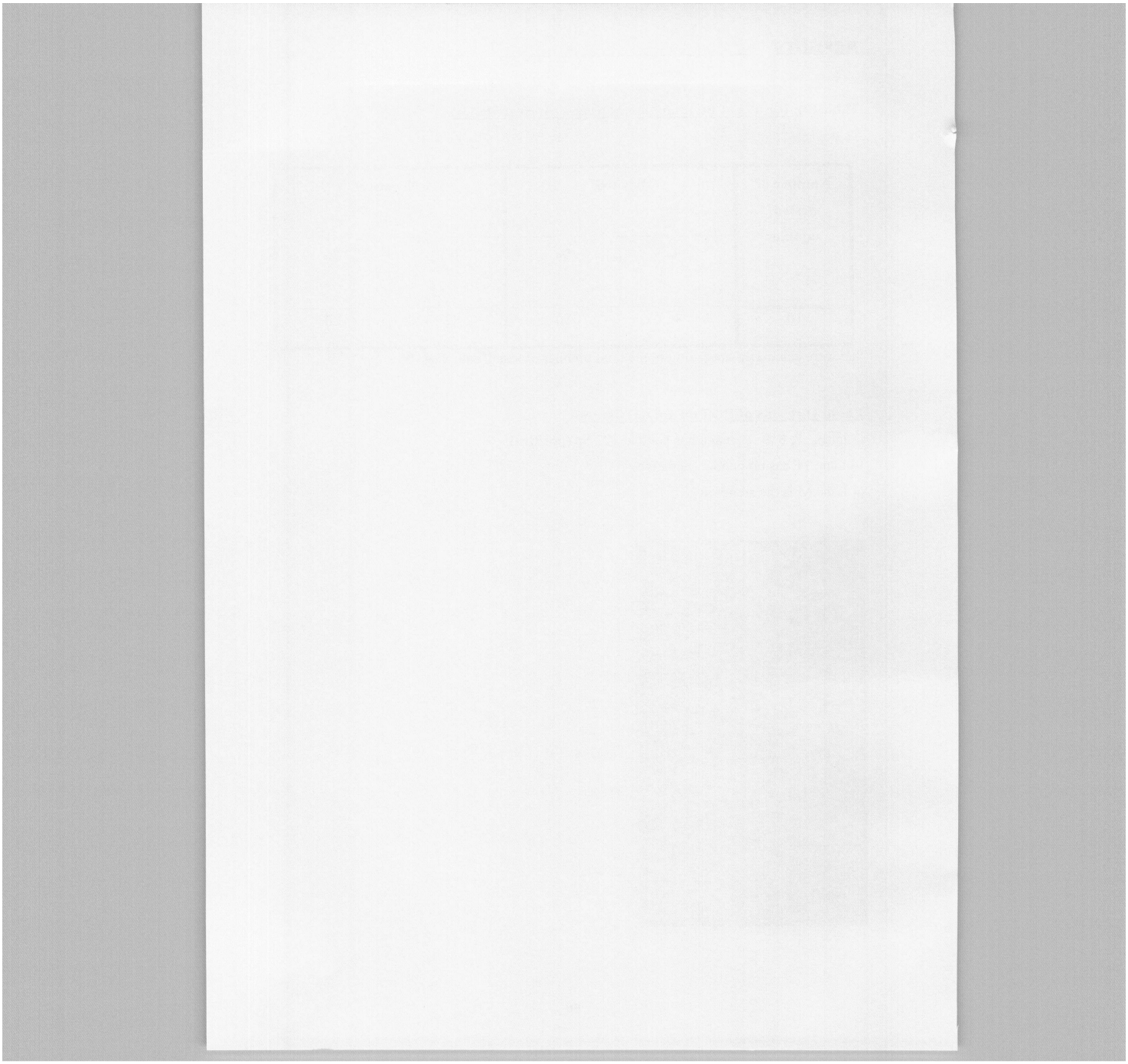
Photo 4: results of PCR on animal tissues

a- lanes 7, 8 ,9 ,10 each has band at 327 bp (positive)

b- Lane 11 has no band (negative)

c- Lane M is the standard





5. Discussion

Tuberculosis remains to be one of the most prevailing and devastating diseases of man and animals, in spite of great efforts done for control and eradication.

Confirmation of diagnosis of TB need the isolation and identification of the causative microorganism, however the culture results usually require 6-8 weeks. The presence of characteristic lesion is very beneficial **(Corner, 1994).**

The fact that organism of *M. bovis* is capable of infesting a diversity of species such as swine, cats, dogs, canneries, parrots and human being emphasizes the necessity of a comprehensive plan if tuberculosis is to be successfully controlled or eradicated. Like wise the human type (*M. tuberculosis*) is capable of introducing TB in cattle, swine, dogs and parrots.

Eradication programs of bovine TB among cattle lead to decrease incidence of disease in human (**Collins and Grange, 1983**).

Adequate sterilization or disinfection research medical devices is essential to prevent cross contamination and possibility of infection transmission (**Jette, et al., 1995**).

In table (1), Direct microscopic examination using ZN method of staining revealed 19 positive cases out of 50 examined animals tissue smears (38%) . On the other hand as shown in table (2) ZN staining revealed 22 positive cases out of 40 examined human sputum smears (55%), these results were nearly agree with **Varello, et al., (2008)** who reported that the percentage of positive smears stained with ZN method was 33.9 % ,but different from those reported by **Benjamin and Lipsky (1984)** who reported that the percentage of positive smears stained with ZN method of staining was 25%, also, **El-Hadidi, (1994)** revealed 27 positive smears out of 121 examined smears (22.3%). Our findings were also differ than those of **Yousef, (1997)** and **Sobhi, et al. (2000)** who found that using of Z.N stain gave results reach up to 86% and 78% respectively , also differ than those of **Kiraz, et al., (2006)** and **Wright, et al., (2008)** who found that using of Z.N staining gave results reach up to 70.7 and 73% respectively, and these variation may be due to difference in the number of population included in other studies that might affect the percentage of the results.

Culture procedures are generally believed to be more sensitive than the microscopic examination for the detection of mycobacteria in clinical specimens. So, its advantage is obvious in cases where specimens contained only small amounts of tubercle bacilli and for isolation of tubercle bacilli for further identification of the organisms and for the performance of drug susceptibility tests (**Ratman and March, 1986**).

Culturing of 50 animal tissues samples on Lowenstein-Jensen medium (LJ) medium gave 32 isolates in a percentage of 64% as shown in table (3) . On the other hand Culturing of 40 sputum samples on LJ medium gave 27 isolates (67.5%) as shown in table (4) . These results were higher than that of **Benjamin and Lipsky, (1984)** who reported that every 10000 suspected pulmonary tuberculosis gave 3500 positive cultures (35%) and **El-Hadidi, (1994)** also reported that out of 121 sputum samples gave 38 isolates (31.4%) but our result nearly agree with **Sobhi, et al. (2000)** who found that out of 300 sputum samples, 226 were culture positive (75.3%).also our result nearly agree with **Mirovic and Lepsanovic.,(2002) ,Falconi, (2008)** and **Wright, et al., (2008)** who found that the

positive cultures were 67.3% ,89 % and 75% respectively ,the difference in the present studies might have been resulted from the discarded contaminated cultures and patient conditions (type of infection, course of treatment.). The false negative culture equal to 32.5% (13 out of 40). **Rickman and Moyer, (1980)** owed the cause of this false negative culture may be due to administration of effective chemotherapy ,usage of overly harsh procedures in specimens decontamination, that might inhibit mycobacterial growth and allow overgrowth of the medium with non-mycobacterial organisms (contamination), defective culture media which failed to support growth of mycobacteria present in clinical specimens and any delay between specimens collection and processing that allow progressive dying of the organisms.

In table (5), the recovered (32) isolates obtained from animal tissues were identified as *M. bovis* according to the morphological characters, growth rate, growth at different temperatures and biochemical identification. On the other hand the recovered (27) isolates obtained from human sputum were identified as *M.tuberculosis*

By immunochromatographic test (New Technique) on animal serum samples (As shown in table 6) , a total number of 50 animal serum samples were tested by immuno-chromatographic test and found that 15 sample were weak positive with an incidence of 30 % , where the test was non specific for *M.Bovis* but specific mainly for *M.Tuberculosis* , but there are some antigenic relationship between different species of Mycobacteria need for specific antigen for *M.bovis* help in the validation of such new technique.

Costello, et al.,(1997) and Ritacco et al.,(1991) ,probably due to the use of complex bacterial extracts containing antigens expressed by pathogenic and environmental mycobacteria. It is possible that diagnostic assays can be improved by using antigens unique to the pathogens, such antigens should be validated in easy performed test ,that can be used in the field without any preparation or treatment for the samples ,and there is no need for any other tools or equipments such as immunochromatographic assay such test can be used and performed easly and in the same time can differentiate between the infection with the different types of mycobacteria when used with a specific antigens.

The same finding also noted by **Pottumorthy, et al.,(2000) and Bartoloni, et al.,(2003).** Also, **HyeCheong koo et al.,(2005)** used a major secreted immunogenic protein (rMPB70) of M.bovis in an immunochromatographic assay (ICGA) for testing the animals infected with the mycobacterium (by natural and experimental routes) and found that the test had sensitivity and specificity reached up to 86.7% and 97.8% respectively within 20 minutes under the field conditions ,by this way can allowing the immediate segregation of suspect animals.

Concerning the human samples (As shown in the table 7) , 10 samples were positive from total tested 40 serum samples with an incidence of 25% (Active TB form before treatment) .On the other hand, 30 samples were negative with an incidence of 75% (due to withdrawal of antibodies after beginning coarse of specific Tuberculous drug.)

Using of PCR considered as a good test for detecting of mycobacterium(DNA) even when a small numbers of bacteria are present in the clinical samples such finding recorded by **Noordhoek, et al.,(1994), Frothingham, et al.,(1994)** and **Cajal, et al.,(1994)**

As shown in table (8) , 4 samples were positive from total tested 6 Buffy coat samples with an incidence of 66.6 % (Mononuclear cells detect viable mycobacteria in peripheral blood correlated with active form), this result nearly agree with **Mirza, et al.,(2003)** Who revealed 30 positive cases PCR on peripheral blood mononuclear cells out of 48 examined samples (63%),but our result were different than **Richter, et al.,(1996) ,Urvashi ,et al.,(2001),** and **Niyaz, et al.,(2003)** who found that the positive PCR on peripheral blood were 92% ,33% and 43.75% respectively.

From the results recorded in table (9) , 8 samples were positive from total tested 10 tissue samples with an incidence of 80 % ., also **Pao, et al.,(1990)** used PCR assay in the identification of mycobacterium directly from the clinical specimens ,our results nearly coincided with those reported by **Brisson-Noel, et al.,(1991) , Cajel, et al.,(1994),**and **Miller, et al., (1997),**they detected the mycobacterium in the clinical samples in a percentage of 97.4% ,71.4% and 92.9% respectively ,also **Moussa, (2000)** noted that the using of PCR gave 100% in the detected of all infected animals but found that the ELISA test was 88% only.

Our results also agree with **David, et al., (2007)** who reported that the sensitivity of PCR on animal tissues were 87%, and **Taylor, et al .,(2007)** who recorded that the positive result of PCR on animal tissues were 91%

Recommendations :

It can be concluded that:

-Percentage of TB infection increase from year to another

-The ICT tuberculosis test was confirmed to be rapid and easy to perform without requiring special equipment, both on serum and whole blood. This rapid TB test kit may be a useful tool for first-line testing of suspected cases, epidemiological studies and in designing a quality health system to reduce health hazards in resource-poor countries.

- It is recommended that further studies on the immunological characteristic of mycobacterium antigen are needed to improve the efficiency of ICT tuberculosis test and to evaluate its specificity and sensitivity on large scale.

- The PCR technique is much faster than culture and reduces the time for diagnosis from several months to 2 days. It also provides for the detection of *M. bovis* when rapidly growing Mycobacterium spp. are present in the sample and may be able to detect the presence of *M. bovis* in samples even when organisms have become nonviable.

-Detection of Mycobacterium DNA in peripheral blood mononuclear cells (Buffy coat) may be a useful tool for rapid diagnosis of disseminated and extra pulmonary forms of TB.

6-Summary

Tuberculosis caused by *M. bovis* represent a major zoonotic and as a cause of economic losses. Egypt is one of the developing countries that suffer from chronic infectious diseases such as bovine tuberculosis.

Tuberculosis also still represents the main cause of morbidity and mortality so the goal of eradication has become more distance both in the third world and in the developing countries.

This study was carried out to compare between conventional and recent techniques for diagnosis of TB

The results showed the following:-

From 50 mesentric lymph node had tuberculous lesions (animal tissues) stained with Z.N technique . 19 samples were positive (Acid Fast Micro organism) and 31 were negative, with a percentage of (38 %) and (62%) respectively

On the other hand, 40 human sputum samples (X –Ray positive) were examined by Z.N staining smear technique . 22 samples were positive (AFB) and 18 were negative, with a percentage of (55 %) and (45%) respectively

On culturing of the 50 animal tissue samples on specific mycobacterial medium , 32 samples gave mycobacterial growth and 18 samples no growth was obtained , with a percentage of (64%) and (36%) respectively.

On the other hand culturing of the 40 human sputum samples on specific mycobacterial medium . 27 samples gave mycobacterial growth and 13 samples no growth was obtained, with a percentage of (67.5%) and (32.5%) respectively.

The biochemical identification of the recovered isolates (32) from bovine samples indicated that all isolates were *M.bovis.*

On the other hand the biochemical identification of the recovered isolates (27) from human samples indicated that all isolates were *M.tuberculosis*

The 50 animal serum samples were examined by immuno chromatographic test . 15 samples gave faint lines(weak positive) and 35 were negative with a percentage of (30%) and (70%) respectively.

On the other hand The 40 human serum samples were examined by immuno chromatographic test . 10 samples gave positive and 30 were negative , with a percentage of (25%) and (75%) respectively.

By using PCR technique on human Buffy coat, 4 samples were positive from total tested 6 Buffy coat samples with an incidence of 66.6 %

On the other hand 2 samples(lanes 5 & 6) were negative with an incidence of 33.3 % .

PCR technique on animal tissues, 8 samples were positive from total tested 10 tissue samples with an incidence of 80 % .

On the other hand 2 samples were negative with an incidence of 20 %.

7-References

Bakshi C.S., Shah D.H., Verma R, Singh R.K., Malik, M.(2007)
Rapid differentiation of *M. bovis and M. tuberculosis* based on a 12.7-kb fragment by a single tube multiplex-PCR.
Vet. Microbiol. 30;123(1-3):282.

Baron , E.J.Peterson, L.R. and finegold, S.M. (1995)
Mycobacteria in Diagnostic Microbiology.
Bailey and Scotts 9th ed. P. 590- 633 Mosby.

Bal, G.H., Hwang, H.S.and Shon, B.W. (1994)
Detection of *M. bovis* lesions in slaughtered dairy cattle using a modified culture & PCR
J.Vet. Public - Health, 18 (3): 183-190.

Barry, T., Glennon, M., Smith, T. and Gannon, F. (1993):
Detection of *M. bovis* in bovine blood by combined PCR and DNA probe methods.
Vet. Ree, 132:66-67.

Bartoloni A, Strohmeyer M, Bartalesi F, Messeri D, Tortoli E, Farese A, Leoncini F, Nutini S, Righi R, Gabbuti A, Mazzotta F, Paradisi F. (2003)
Evaluation of a rapid immunochromatographic test for the serologic diagnosis of tuberculosis in Italy.
Clin. Microbiol. Infect. ;9(7):632-9.

Bartoloni A, Strohmeyer M, Bartalesi F, Messeri D, Tortoli E, Farese A, Leoncini F, Nutini S, Righi R, Gabbuti A, Mazzotta F, Paradisi F.(1998)
Detection of anti-lipoarabinomannan antibodies for the diagnosis of active tuberculosis.
Int J Tuberc Lung Dis. **2**(2):160-3.

Bascunana, C.R. and Belak, K. (1996):
Detection and identification of mycobacteria in formaiin-fixed, paraffine-embedded tissues by nested PCR and restriction enzyme analysis.
J. Clin. Microbiol., 34 (10): 2351-2355.

Benjamin, L.A. and Lipsky, J.A. (1984):
Factors affecting the clinical value of microscopy for acid-fast bacilli.
Rev. Infect. Dis., 6: 214-216.

Benjamin, R.G., Debann, S.M., Ma, Y. and Daniel, T.M. (1984):
Evaluation of mycobacterial antigens in an enzy linked immunosorbant assay for the serodiagnosis of tuberculosis.
J. Med. Microbiol., 18: 309-318.

References

Bering, S.E. (2001):
The role of fluoroquinolones in tuberculosis today.
Drug, 61(1): 9.

Brisson-Noel, A., Giequel, B., Nassif, X. and Hance, A.J. (1989):
Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples.
Lancet., 4: 1069-1071.

Brisson-Noel, A., Aznar, C, Chureau, C, Nguyen, S., Pierre, C, Bartoli, M., Bonete, R. and Garrigue, G. (1991):
Diagnosis of tuberculosis by DNA amplificaiton in clinical practice evaluation.
Lancet, 339: 364-366.

Castro, A.F.P.,and Nemoto, H.(1972):
Occurrence of in the lymph nodes of apparently healthy slaughtered cattle in Sao Paulo,
Brazil. Revista de Microbologia 3,2,75-78

Cajal, Madrid, and Pharma Gen, 28760 Tres Cantos, Madrid, Spain(1995)
Simple and Rapid Detection of *M.tuberculosis*
Complex Organisms in Bovine Tissue Samples by PCR
j.clin.Microbiology , Jan. 1995, p. 33–36 .

Claxton,P,D; Emamens ,G.J.and Mylarea,P.J.(1979):
Laboratory diagnosis of bovine tuberculosis
Aust. Vet. J. 55,11,514-520.

Collins, C.H. and Grange, M. (1983):
The bovine tubercle bacillus, a review.
J. Apl. Bact., 55: 13-29.

Corner, L.A. (1994):
Post mortem diagnosis of *M. bovis* infection in cattle.
Vet. Microbiol., 40: 53-63.

Cousins, D.Y., Williams, S., Liebana, E., Aranaz, A., Bunschotcn, A., Embden, J. van., Ellis, T. and Van- Embden, J. (1998):
Evaluation of tour DNA typing techniques in epidemiological investigations of *M.bovis*.
J. Clin. Microbiol., 36 (1): 168-178.

Costello, E; O'Reilly, P.F. Yearslay, D.K; O'Grady, D.P; Collins, J.D; Monaghan, M.L; and Bassett, H.F (1997):
A study of enzyme-Linked immunosorbent Assay for the diagnosis of tuberculosis in cattle.
In. Vet.J.50.35-38

References

Chedore P, Jamieson FB. (1999)
Routine use of Gen-Probe MTD2 amplification test for detection of *M. tuberculosis* in clinical specimens in a large public health mycobacteriology laboratory. Diag. Microbiol. Infect Dis 35: 185-91.

Chien HP, Yu MC, Wu MH, Lin TP, Luh KT.(2000)
Comparison of the BACTEC MGIT 960 with Löwenstein-Jensen medium for recovery of mycobacteria from clinical specimens.
Int. J. Tuberc. Lung Dis. ;4(9):866-70.

David C. Bean, Andrea Hills, Terry Ryan, and John Aitken(2007)
Evaluation of the BD ProbeTec ET System for Direct Detection of *M. bovis* in Veterinary Specimens
Journal of Clinical Microbiology, p. 3434-3435, Vol. 45, No. 10

David, H.L. (1976):
Probability distribution of drug resistant mutant in unselected populations of M. tuberculosis.
Appl. Microbiol., 20: 810-814.

Dekantor, I.N. and Ritacco, V. (1994):
Bovine tuberculosis in Latin America and Caribbean; current status control and eradication programs.
Vet. Microbiol., 40(1-2): 5-14.

Del Prete R, Picca V, Mosca A, D'Alagni M, Miragliotta G. (1998)
Detection of anti-lipoarabinomannan antibodies for the diagnosis of active tuberculosis.
Int. J. Tuberc. Lung Dis. 1998 Jun;2(6):521-2.

Del Portillo, P., Murillo, L.A. and Patarroyo, M.E. (1991):
Amplification of a species- specific DNA fragment of *M. tuberculosis* and its possible use in diagnosis.
J. Clin. Microbiol.,29: 2163-2169.

Dominiaue, T., Anne, B.N., Veronione, V.L., Simon, N., Jean- Lue, G. and Brigitte, G. (1990):
Characterization of a *M. tuberculosis* insertion sequence, IS 6110 and its application in diagnosis.
J. Clin. Microbiol., 28 (12): 2668-2673.

Dolin, P. (1999):
Global burden of tuberculosis. Estimated incidence, Prevalence, and mortality by country.
J.A.M.A., 282: 677-686.

Eisenach, K.D., Siiford, M.D., Cave, M.D. and Cravvford, J.J. (1991):
Detection of *M. tuberculosis* in sputum samples using a PCR. Am. Rev. Res. Dis., 144 (5): 1160-1163.

Elvira R., Michael W., Anne-Marie F., and Sabine R.-G. (2004)

Usefulness of the GenoType MTBC Assay for Differentiating Species of the *M. tuberculosis* Complex in Cultures Obtained from Clinical Specimens J. Clin. Microbiol. ,42: 4303-4306.

El-Hadidi, G.S. (1994):
Differential identification of pathogenic mycobacteria isolated from patients attending Ismailia Chest Hospital.
M.Sc., Microbiol., Fac. of Med., Suez Canal Univ.

El-Sabban, M.S.; Lotfy, O.; Awad, W.M.; Soufi, H.S.; Mikhail, D.G.; Hammam, M.; Dimitri, R.A. and Gergis, S.M. (1992)
Bovine TB and its extent of spread as a source of infection to man and animals in A.R. of Egypt.
Proc. of the Int. Conf. on Ani. TB in Middle East, Cairo (1992),

Falconi FQ, Suárez LI, López Mde J, Sancho CG.(2008)
Comparison of the VersaTREK system and Löwenstein-Jensen medium for the recovery of mycobacteria from clinical specimens.
Scand J Infect Dis. ;40(1):49-53.

Farnia, F. Mohammadi, Z. Zarifi, D. J. Tabatabee, J. Ganavi, K. Ghazisaeedi, P. K. Farnia, M. Gheydi, M. Bahadori, M. R. Masjedi, and A. A. Velayati(2001)
Improving Sensitivity of Direct Microscopy for Detection of Acid-Fast Bacilli in Sputum: Use of Chitin in Mucus Digestion
J. Clin. Microbiol. 40: 508-511.

Fabre M, Gérôme P, Maslin J, Hervé V, Vong R, Carpentier G, Soler C.(2007)
Assessment of the Patho-TB kit for diagnosis of tuberculosis
Pathol Biol (Paris). 55(10):482-5.

Finegold, S.M. and Martin, W.J. (1982):
In Bailey and Scott's, Diagnostic Microbiology, Sixth edition.
The C.V. Mosby Company, USA.

Foigueira, I., Delgado, R. and Palenque, E. (1996):
Rapid diagnosis of *M. Tuberculosis* bacteremia by PCR.
J. Clin. Microbiol., 34(3): 512-515.

Frothingham(1995)
Differentiation of strains in *M.tuberculosis* complex by DNA sequence polymorphisms, including rapid identification of M. bovis BCG
J. Clin. Microbiol., 1995 33: 840-844

Garcia-Quintanilla, A., Gonzalez-Martin, J., Tudo, G., Espasa, M., Jimenez de Anta, M. T. (2002).
Simultaneous Identification of Mycobacterium Genus and *M. tuberculosis* Complex in Clinical Samples by 5'-Exonuclease Fluorogenic PCR.
J. Clin. Microbiol. 40: 4646-4651

References

Gallo, J.A.G.; Veitia, F.; Remon, S. and Delgado, L. (1983):
Mycobacteria isolated from cattle reacted to tuberculin tests.
Revista-Cubana de Ciencias Vet., 14(3): 173-176.

George, O. and Berlin, W. (1990):
Diagnostic Microbiology. Cited from Bailey and Scott's J.E. and Fineglod M.S., 8th edu. The C.V. Mos by company mycobacteria. Chapter 41.

Hardie, R.M. and Watson, J.M. (1992):
M. bovis in England and Wales: Past, Present and Future.
Epidemiol. Infect., 109: 23-33.

Hye Cheong Koo, Yong Ho Park, Jongsam Ahn, W. Ray Waters, Mitch V. Palmer, Mary Jo Hamilton, George Barrington, Abdelaziz A. Mosaad, Kun Taek Park, Woo Kyung Jung, In Yeong Hwang, Sang-Nae Cho, Sang Jae Shin, and William C. Davis(2005)
Use of rMPB70 Protein and ESAT-6 Peptide as Antigens for Comparison of the Enzyme-Linked Immunosorbent, Immunochromatographic, and Latex Bead Agglutination Assays for Serodiagnosis of Bovine Tuberculosis
J. Clin. Microbiol. 43: 4498-4506.

Jette, L.P.; Ringuette, L.; Ishak, M.; Miller, M. and Saint-Autoines, P. (1995):
Evaluation of three glutaraldehyde-based disinfectants used in endoscopy.
J. Hosp. Infect., 30: 295-303.

Julin, (1960):
Method for groups and typing of mycobacteria . 3 differentiation of anonymous mycobacteria in to groups, subgroups or types.
Acta, Path. Microbio . Scand., 50:195-207.

Kamel, Y.Y. (1972):
Prevalence of mycobacterium tuberculosis var bovis among human pulmonary effection in Egypt.
M.D. Vet. Thesis in Zoonosis Assiut University.

Kathuria P, Agarwal K, Koranne RV.(2006)
The role of fine-needle aspiration cytology and Ziehl Neelsen staining in the diagnosis of cutaneous tuberculosis.
J Ayub Med Coll Abbottabad. 18(2):25-8.

Khasnobis, S.; Escuyer, V.E. and Chatterjee, D. (2002):
Emerging therapeutic targets in tuberculosis: post genomic era.
Expert Opinion Ther. Targets, 6(1): 21.

Kiraz N, Et L, Akgun Y, Kasifoglu N, Kiremitci A.(2006)
Rapid detection of Mycobacterium tuberculosis from sputum specimens using the FASTPlaqueTB test.
Diagn Cytopathol. 34(12):826-9.

References

Kulski, C. Khinsoe, Pryce T., and Christiansen K. (1995)
Use of a multiplex PCR to detect and identify *M. avium and M. intracellulare* in blood culture fluids of AIDS patients
J. Clin. Microbiol. , 33: 668-674.

Koch, R. (1882):
Der aetiologie der tuberculose.
Bert. Klin. When chr., 19: 221-239.

Kubica, G.P. (1973):
Differential identification of mycobacteria VII. Key features for identification of clinically significant mycobacteria.
Am. Rev. Resp. Dis., 107: 9-21

Kolk, A.H., Schuitema, A.R., Kuijper, S., Embden, J.D. and Hartskeerl, R.A. (1992):
Detection of M. tuberculosis in clinical smaples by using PCR and non radio active detection system.
J. Clin. Microbiol., 30(10): 2567-2575.

Kristian Ängeby, Lisbeth Klintz, and Sven E. Hoffner(2001) :
Rapid and Inexpensive Drug Susceptibility Testing of Mycobacterium tuberculosis with a Nitrate Reductase Assay
J. Clin. Microbiol. 40: 553-555.

Kubica GP(1980).:
Correlation of acid-fast staining methods with culture results for mycobacteria.
Bull Int Union Tuberc 55(3-4): 117-124.

Liebana, E., Aranaz, A., Mateos, A., Vilafranca, M., Domingo, M. and Dominguez, M. (1995):
Simple and rapid detection M. tuberculosis complex organisms in bovine tissue samples by PCR.
J. Clin. Microbiol., 33 (1): 33-36.

Liebana, E., Aranaz, A., Francis, B. and Cousins, D. (1996):
Assessment of genetic markers for species identification within the *M. tuberculosis* complex.
J. Clin. Mirobiol., 34 (4): 933-938.

Manjunath, N., Shankar, P., Rajan, L., Bhargava, A., Saluja, S. and Shriniwas, R. (1991):
Rapid diagnosis of *M tuberculosis* by PCR.
Tubercle, 72 (1): 21-27.

Marks, J. (1972):
Ending the routine guinea pigs test.
Tubercle., 53: 31-34.

References

Makunde WH, Makunde RA, Kamugisha LM, Mgema SG, Liwa A. (2007)
Improved microscopy diagnosis of pulmonary tuberculosis using sodium hypochlorite concentration technique in Tanga, Tanzania.
Int J Tuberc Lung Dis. 11(8):904-8.

Miller, J., Jenny, A., Rhyan, J., Saari, D. and Suarez, D. (1997):
Detection of *M.bovis* in formalin-fixed, paraffin embedded tissues of cattle by PCR amplification.
J. Vet. Diagn. Investigation, 9 (3): 244-249.

Miller, N., Cleary, T., Kraus, G., Young, A. K., Spruill, G., Hnatyszyn, H. J. (2002).
Rapid and Specific Detection of *M. tuberculosis* from Acid-Fast Bacillus Smear-Positive Respiratory Specimens and BacT/ALERT MP Culture Bottles by Using Fluorogenic Probes and Real-Time PCR.
J. Clin. Microbiol. 40: 4143-4147

Mirza, S., Restrepo, B. I., Mccormick, J. B., Fisher-Hoch, S. P. (2003).
Diagnosis of tuberculosis lymphadenitis using PCR on peripheral blood mononuclear cells.
Am. J. Trop. Med. Hyg .,69: 461-465

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(11):5670-8.

Mirovic V, and Lepsanovic Z.(2002)
Evaluation of the MB/BacT system for recovery of mycobacteria from clinical specimens in comparison to Lowenstein-Jensen medium. ClinMicrobiolInfect.;8(11):709-14.

Moeller A. (1898):
Microorganisms , die den tuberklbacillen, venvant sind und bei.
Deut Med. Wsclir., 24:376-379. cited by Kubira et al.,(1963).
Am.Rev.Ses.do:718-720.

Moussa, I.M.I. (2000):
Detection of Mycobacterium tuberculosis bovine type among isolates or directly in affected tissues by using nested PCR. Ph.D. Thesis (Microbiology),
Fac. Vet. Med., Cairo University.

Nashwa, O'Khalifa (2000):
Study on zoonestic TB caused by polymerase chain reaction.
Vet. Med. J. Giza, Vol. 48(4): 659-666.

References

Niyaz A., Ashok K. M., Utpal M., Virender K. Batish, and Sunita Grover(2003)
PCR-Based Rapid Detection of *Mycobacterium tuberculosis* in Blood from Immunocompetent Patients with Pulmonary Tuberculosis
Am. J. Trop. Med. Hyg., 69(5), pp. 461-465

Neimark, M., Ali Baig, and Carleton S.(1996)
Direct identification and typing of Mycobacterium tuberculosis by PCR. J. Clin. Microbiol. 34: 2454-2459.

Neill, S.D.; Cassidy, J.; Hanna, J.; Mackie, D.P.; Pollock, J.M.; Clements, A.; Walton, E. and Brysson, D.G. (1994):
Detection of infection in skin test negative cattle with an assay for bovine interferon gamma.
Vet. Rec., 135(6): 134-135.

Noordhoek, G.T., Kolk, A.H.T., Byune, G., Catty, D., Dale, J.W., Fine, P.E.M., Godfrey-Fausset, P., Clio, S.N., Shinnick, T., Svenson, S.B., Wilson, S. and Van Embden, J.D.A. (1994):
Sensitivity and specificity of PCR for detection of *M. tuberculosis* complex: a blind comparison study among seven laboratories.
J. Clin. Microbiol., 32: 277-284.

Noordhoek, JD van Embden, and AH Kolk(1996)
Reliability of nucleic acid amplification for detection of *M.tuberculosis*: an international collaborative quality control study among 30 laboratories
J. Clin. Microbiol. 34: 2522-2525.

Nolte,and Metchock,B (1995).
Mycobacteria in (Manual of Micrbiology) 6th ed P 400-437
Ameican socity of Microbiology press,Washington ,D.C

Osman, K.M. (1974):
Studies on acid-fast microorganism in some demostic animals with special reference to atypical mycobacterial group.
Ph.D. Thesis, Cairo University.

Pao, C.C., Yen, T.S.B., You, J.B., Maa, J.S., Fiss, E.H. and Chan-Hsiung (1990):
Detection and identification of *M. tuberculosis* by DNA amplification.
J. Clin. Microbiol., 28(9): 1877-1880.

Paull, A. (1973):
An environmental study of the opportunistic mycobacteria.
Med.Lab .Techn,. 30:11-190.

Petroff, S.A. (1915):
A new and rapid method for isolation and cultivation of tubercle bacilli directly for the sputum and feces.
J. Exp. Med., 21: 38-42.

References

Pierre, C, Olivier, C. and Lecossier, D. (1991):
Diagnosis of primary tuberculosis in children by amplification and detection of mycobacterial DNA.
Am. Rev. Respir. Dis., 147(2): 420-424.

Piersimoni C, Scarparo C, Callegaro A, Tosi CP, Nista D, Bornigia S, Scagnelli M, Rigon A, Ruggiero G, Goglio A.(2001)
Comparison of MB/Bact alert 3D system with radiometric BACTEC system and Löwenstein-Jensen medium for recovery and identification of mycobacteria from clinical specimens: a multicenter study.
J Clin Microbiol. ;39(2):651-7.

Pottumarthy S, Wells VC, Morris AJ. (2000)
A comparison of seven tests for serological diagnosis of tuberculosis.
Department of Microbiology, Green Lane and National Women's Hospitals, Auckland, New Zealand
J. Clin .Microbiol.;38(6):2227-31

Prasad HK, Singhal A, Mishra A, Shah NP, Katoch VM, Thakral SS, Singh DV, Chumber S, Bal S, Aggarwal S, Padma MV, Kumar S, Singh MK, Acharya SK.(2005)
Bovine tuberculosis in India: potential basis for zoonosis. Tuberculosis (Edinb). ;85(5-6):421-8.

Pruchard, D.G; Francis, D. A; Gripp, R; Harding R.B. Jones E.B. mintern ,C .and Me Govern , P.T.(1975):
An abattoir survey of bovine tuberculosis in the Karamoja region of Uganda.
British Veterinary Journal 131,1,120-127.

Ratman, S. and March, S.B. (1986):
Effect of relative centrifugal force and centrifugation time on sedimentation of mycobacteria in clinical specimens.
J. Clin. Microbiol., 23: 582-585.

Rabinowitsch, L. (1897):
Zur frage das vorkommens von Tuberklbazillen in der marktbutter
Hyg. Infektiokrankh.,26:90-111.
Cited by Fray and Hagan , J . Inf. Dis. (1931) ,44:497-506.

Reddy JR, Kwang J, Lechtenberg KF, Khan NC, Prasad RB, Chengappa MM.(2002)
An immunochromatographic serological assay for the diagnosis of *M. tuberculosis*.
Comp Immunol Microbiol Infect Dis. ;25(1):21-7.

References

Renata L. Guerra, James F. Baker, Roya Alborz, Derek T. Armstrong, Julia A. Kiehlbauch, Marcus B. Conde, Susan E. Dorman, and Nancy M. Hooper(2007)
Specimen Dilution Improves Sensitivity of the Amplified *M.tuberculosis* Direct Test for Smear Microscopy-Positive RespiratorySpecimens
J. Clin. Microbiol. 46: 314-316.

Rickman, T.W. and Moyer, N.P. (1980):
Increased sensitivity of acid-fast smears.
J. Clin. Microbiol., 11: 618-620.

Ritacco, V., Lopez, B., Kantor, I.N., Barrera, L., Errico, F., Nader, A. and De-Kantor, I.N. (1991):
Receprocal cellular and humoral immune responses in bovine tuberculosis.
Res. Vet. Scien., 50 (3): 365-367.

Rodriguez, J.G., IMejia, G.A., Portillo, P., Murillo, L.A. and Delportillo, P. (1995):
Species-specific identification of *M. bovis* by PCR.
Microbiology, 141: 2131-2 138.

·ez, J.G., Fissanoti, J.C., Portillo, P., Patarroyo, M.E. and Del-Portillo, P. (1999):
Amplification of a 500-base-pair fragment from cultured isolates of *M. bovis*,
J. Clin. Microbiol., 37(7): 2330-2332.

Rossi, M. C., Gori, A., Zehender, G., Marchetti, G., Ferrario, G., De Maddalena, C., Catozzi, L., Bandera, A., Esposti, A. D., Franzetti, F. (2000).
A PCR-Colorimetric Microwell Plate Hybridization Assay for Detection of *M. tuberculosis* and *M. avium* from Culture Samples and Ziehl-Neelsen-Positive Smears.
J. Clin. Microbiol. 38: 1772-177

Sami, A. (1980):
The natural TB research project.
The Egypt. J. Chest Dis. And Tuberc., Vol. 23, No. 2, P. 3.

Said Abbadi, Heba G. Rashed, Glenn P. Morlock, Charles L. Woodley, O. El Shanawy, and Robert C. Cooksey(2001)
Characterization of IS6110 Restriction Fragment Length Polymorphism Patterns and Mechanisms of Antimicrobial Resistance for Multidrug-Resistant Isolates of *M. tuberculosis* from a Major Reference Hospital in Assiut, Egypt
J. Clin. Microbiol. 39: 2330-2334

Samiha, M.A (1998)
Seminar on tuberculosis in Egypt, past and present

References

Steingart KR , Henry M, Laal S, Hopewell PC, Ramsay A, Menzies D, Cunningham J, Weldingh K, Pai M.*(2002)*
An immunochromatographic serological assay for the diagnosis of *M. tuberculosis.*
Comp Immunol Microbiol Infect Dis. 2002 Jan;25(1):21-7.

Sambrook, J., Fritscgh, *E.F.* and Meniates, T. (1989):
Molecular cloning: a laboratory manual, 2nd. Ed. Cold Spring Harbor, 141.

Schirm, J., Ooten-Drop, A.B. and Mulder, G. (1995):
Comparison of Amplicor, In house PCR and conventional culture for detection of M. tuberculosis in clinical samples.
J. Clin. Microbiol., 33: 3221-3224.

Scorpio, A., Collins, D., Whipple, D., Cave, D., Bates, J. and Zhang,Y. (1997):
Rapid differentiation of bovine and human tubercle bacilli based on a characteristic mutation in the bovine pyrazinamidase gene.
J. Clin. Microbiol., 35(1): 106-110.

Shankar, P., Manjunath, N., Lakshmi, R., Aditi, B. and Shriniwas, P.S. (1990):
Identification of *M. tuberculosis* by PCR.
Lancet, 335 (8686): 423.

Shawar, R.M., Fouad, A.K., Nataraj, A. and Clarridge, E.J. (1993):
Detection of *M. tuberculosis* in clinical samples by two-step PCR and non isotopic hybridization methods.
J. Clin. Microbiol., 31(1): 61-65.

Silox, V.A. (1981):
Identification of clinically significant mycobacterium fortuitum complex isolates.
J. Clin. Microbiol., 14(6): 686-691.

Skuce, D Brittain, MS Hughes, and SD Neill(1996)
Differentiation of *M. bovis* isolates from animals by DNA typing
J. Clin. Microbiol. 34: 2469-2474.

Sjobring, U., Michael, M., Asebengard, A. and Hakan, M. (1990):
Polymerase chain reaction for detection of *M.tuberculosis.*
J. Clin. Microbiol., 28 (10): 2200-2204.

Small, K.J. and Thomson, D. (1986):
The efficiency of bovine PPD tuberculin in the single caudal fold test to detect tuberculosis in water buffaloes.
Buffalo Bulletin, (1986), 5: 3, 62-64.

Sobhi, F.; Latif, S.A.; Aref, S. and El-Hadidy, A. (2000):
Multi drug resistant mycobacteria among Egyptian patients and evaluation of mycobacteria growth indicator tube method in testing for antituberculous drug susceptibility.

Egyptian J. of Medical Microbiology, 9: 356-357.

Stuart, M.W., Ruth, M., Pamela, M.N., Peter, D., Godfrey, F. and Alister, V. (1993):
Progress toward a simplified PCR and its application to diagnosis of tuberculosis.
J. Clin. Microbiol., 31(4): 776-782.

Szewzyk, R., Svenson, S. Hoffner S.E, Bolske, G., Wahlstrom, H., Englund, L., Engvall, A. and Kallenius, G. (1995):
Molecular epidemiological studies of *M. bovis* infections in humans and animals in Sweden.
J. Clin. Microbiol., 33 (12): 3183-3185

Taylor GM, Worth DR, Palmer S, Jahans K, Hewinson RG.(2007)
Rapid detection of Mycobacterium bovis DNA in cattle lymph nodes with visible lesions using PCR.
BMC Vet Res. 2007 Jun 13;3:12.

Taylor, M. J., Hughes, M. S., Skuce, R. A., Neill, S. D. (2001).
Detection of *M. bovis* in Bovine Clinical Specimens Using Real-Time Fluorescence and Fluorescence Resonance Energy Transfer Probe Rapid-Cycle PCR.
J. Clin. Microbiol. 39: 1272-1278

Talbot, E.A., Williams, D.L. and Frothingham, R. (1997):
PCR identification of *M. bovis* BCG.
J. Clin. Microbiol., 35(3): 566-569.

Thomson, H,M.; (1932):
Studies on saprophytic acid fast bacteria. Amer. Rev. Tuberc., 26:162-178.

Tsukamura, M. (1967):
Identification of mycobacteria.
Tubercle, 48: 311-338.

Urvashi B. Singh*, N. Vijaya Bhanu, V. Naga Suresh, Jyoti Arora, Tanu Rana, and Pradeep Seth (2001)
Utility of PCR on diagnosis of TB from samples of bone marrow aspirate
Int J Tuberc Lung Dis. 2001 Aug;5(8):754-62.

VanDeun A, Hamid Salim,Aung KJ, Hossain MA,Chambugunj N, Hawria (2005)
Performance of variations of Carbol Fuchsin staining of sputum smear for AFB under field conditions .
Int.J.tubercle lung disease 9(10):1127-23

Venkataswamy M.M., Rafi W., Nagarathna S.,and Ravi V, (2007)
Comparative evaluation of BACTEC 460TB system and Lowenstein-Jensen medium for the isolation of M. tuberculosis from cerebrospinal fluid samples of tuberculous meningitis patients.

Indian. J. Med. Microbiol. ,25(3):236-40.

Vestal, A.L. (1975):
Procedures for the isolation and identification of mycobacteria.
DHEW. Publication No. (CDC), 75-8230.

Varello K, Pezzolato M, Mascarino D, Ingravalle F, Caramelli M, Bozzetta E.(2008)
Comparison of histologic techniques for the diagnosis of bovine tuberculosis in the framework of eradication programs
J .Vet. Diagn .Invest., 20(2):164-9.

Wright C.A., Van der Burg, M., Geiger, D., Noordzij J.G., Burgess S.M., Marais B.J.(2008)
Diagnosing mycobacterial lymphadenitis in children using fine needle aspiration biopsy: Cytomorphology, ZN staining and autofluorescence-Making more of less.
Diagn. Cytopathol. ;36(4):245-251

Wards, B.J., Collins, D.M., Lisle, G.W. and De-Lisle, G.W. (1995):
Detection of *M. bovis* in tissues by polymerase chain reaction.
Vet. Microbiol., 43 (2-3): 227-240.

WHO Technical Report on TB (1982):
Series No. 671. cited from tubercle, the international T. of TB, Vol. 63, Sept (1982), No. 2.

WHO (1998) Laboratories.
Services in tuberculosis control Part 3 : Geneva, swizerland.

Yousef, S.E.E. (1997):
Serological diagnosis of pulmonary tuberculosis.
MSc. D. "Microbiol.", Fac. of Med., Cairo Univ..

Zanini, M.S., Moreira, E.C., Lopes, M.T., Mota, P. and Salas, C.E. (1998):
Detection of *M. bovis* in milk by PCR.
J. Vet. Med. Series B., 45 (8): 473-479.

Zvizdic,S,Dizdarenic,Z and Rivanavic Z (1999)
Modern methods in diagnosis of tuberculosis PCR
Med. Arch, 53(1) :13-7.

الملخص العربى

يعتبر مرض السل من أهم الأمراض التي تنتقل من الحيوان إلى الإنسان حيث تعتبر الماشية العائل الرئيسي لهذا المرض و توجد معظم حالات الإصابة بالسل في البلاد النامية و منها جمهورية مصر العربية و انتشار مرض السل البقرى فى الإنسان يكون ملازما بصورة واضحة لانتشاره في الحيوان ٠

كذلك يعتبر التشخيص المبكر و العلاج و تعقيم الأدوات من أهم العناصر في السيطرة على هذا المرض المعدي و الخطير.

وفى هذه الدراسة:

تم عمل مقارنة لتشخيص مرض السل بين طرق التشخيص الحديثة والطرق الاعتيادية:

- عن طريق صبغة ميكروب السل(زيل نيلسون) على الغدد الليمفاوية للجاموس(عدد ٥٠ حالة بها إصابة ب الغدد الليمفاوية) كانت نسبة الحالات الايجابية ٣٨% ونسبة الحالات السلبية ٦٢% ٠ أما بالنسبة لعينات المخاط الخاصة ب الإنسان (٤٠ عينة لمرضى مصابين ب الدرن)) كانت نسبة الحالات الايجابية ٥٥% ونسبة الحالات السلبية ٤٥%.

- عند عمل المزرعة لعزل ميكروب الدرن كانت نسبة الحالات الايجابية للحيوانات ٦٤% ونسبة الحالات السلبية ٣٦% ٠ أما بالنسبة لعينات المخاط الخاصة بالإنسان كانت نسبة الحالات الايجابية٦٧،٥% ونسبة الحالات السلبية ٣٢،٥%

- عند إجراء الاختبارات الكيميائية للميكروب المعزول أسفرت النتائج أن الميكروب المعزول من الحيوانات كن ميكروب السل الحيوانى٠ وأن الميكروب المعزول من الإنسان كان ميكروب السل البشرى

- تم استعمال اختبار جديد وسريع على المصل(السيرم) للكشف عن وجود الأجسام المضادة لميكروب السل وكانت النتائج كالأتي: بالنسبة للحيوانات كانت النتيجة الايجابية ٣٠% والسلبية ٧٠% ٠ بالنسبة للإنسان كانت النتيجة الأيجابية٢٥% والسلبية ٧٥%

- تم عمل اختبار ال بى سى أر على عينات الأنسجة للحيوانات فكانت النتيجة الأيجبية ٨٠%

و تم عمل اختبار أل بى سى أر على عينات الدم للإنسان (طبقة الخلايا البيضاء) فكانت النتيجة الإيجابية ٦٦،٦%

لجنة الإشراف:

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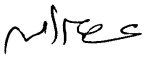

دراسات على طرق تشخيص ميكروب السل

رسالة مقدمة من

ط.ب/ ناصر فتحى حسن أحمد

بكالوريوس العلوم الطبية البيطرية

كلية الطب البيطرى جامعة الأسكندرية ١٩٩٠

للحصول على درجة

الماجيستير فى العلوم الطبية البيطرية

ميكروبيولوجيا (بكتريولوجيا)

لجنة المناقشة والحكم على الرسالة

موافقون

الأستاذ الدكتور / عبد العزيز عبد الخالق مسعد

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كلية الطب البيطرى السادات – جامعة المنوفية

الأستاذ الدكتور / حلمى أحمد السيد تركـــــى

أستاذ الميكروبيولوجيا المتفرغ – كلية الطـــب

البيطرى – جامعة الأسكندريـــــــــــة

الأستاذ الدكتور / محمد على عقيلـــــة مرعـــى

أستاذ ورئيس قسم الميكروبيولوجيـــــــــــا

كلية الطب البيطرى – جامعة الأسكندريـــــــة

(والمشرف على الرسالـــــــــــــة)

التاريخ ١٠/ ٥ /٢٠٠٨

دراسات على طرق تشخيص ميكروب السل

رسالة علمية

مقدمة إلى الدراسات العليا بكلية الطب البيطري- جامعة الإسكندرية
استيفاء للدراسات المقررة للحصول على درجة

الماجستير في العلوم الطبية البيطرية

في

الميكروبيولوجى

مقدمة من

ط ٠ب ٠ ناصر فتحى حسن أحمد

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