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Full Length Research Paper

Comparison between the conventional and modern techniques used for identification of *Mycobacterium tuberculosis* complex

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Polymerase chain reaction (PCR) are rapid and simple means for the differentiation of members of *Mycobacterium tuberculosis* complex, especially *Mycobacterium bovis* and *M. tuberculosis*, where it is important to distinguish between zoonotic sources (cattle and unpasteurized dairy products) and human sources of tubercle disease. This study is aimed to evaluate the recent technique such as PCR and (BACTEC MGIT 960 ™ system) for diagnosis of *M. tuberculosis* complex among cattle in Egypt. 1180 cattle were examined during the period of 2008 to 2010 by single intradermal tuberculin test. 29 animals (2.46%) were positive reactors, the results of isolation and identification using conventional culture method Lowenstein-Jensen medium were 22 mycobacterial isolates (75.9%), 20 (68.97%) *M. bovis* and 2 (6.9%) unidentified slow grower}. The recovery rate of BACTEC MGIT 960™ system was 82.8%, while in case of Lowenstein-Jensen medium was 75.9%. The mean time for detection of *Mycobacterium* was 17.8 ± 0.9 days and 46.5 ± 0.4 days for BACTEC MGIT 960™ system and Lowenstein-Jensen medium, respectively. While the contamination rate with BACTEC MGIT 960™ system was 6.9 and 10.3% in Lowenstein-Jensen medium. PCR technique in the present study could differentiates between *M. bovis* and *M. tuberculosis* within few h rather than the long period required for the biochemical identification tests. Therefore, the use of PCR in the diagnosis of *Mycobacterium* in clinical samples is as rapid, more reliable, sensitive and specific techniques and can be used for large scale screening of *Mycobacterium* in areas where the disease is still a public health hazard as in Egypt.

Key words: Bovine tuberculosis, tuberculin test, Lowenstein-Jensen medium, BACTEC system, PCR.

INTRODUCTION

Mycobacterium bovis infects humans, causing zoonotic disease through ingestion, inhalation and less recently, by contact with mucous membranes and broken skin (Dela and Domencch, 2006) and to the epidemiological knowledge that *M. bovis* infection in cattle is on the increase in some countries including the United Kingdom (Aagaard et al., 2003). Bovine tuberculosis has been on the increase in developed countries (Grange, 2001), and with increasing incidence of tuberculosis globally, rapid and reliable diagnostic assay is required not only for detection but also for identification of the pathogenic *Mycobacteria* in clinical samples. This is essential for the promotion of diagnosis, treatment, and control of tuberculosis (Prabhakar et al., 2004).

Bovine tuberculosis is a chronic granulomatous disease mainly affecting lymph node and lung tissues of cattle. It is caused by *M. bovis*, member of *M. tuberculosis* complex (MTC) group of bacteria. MTC includes a variety

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of closely related *Mycobacteria*, namely *M. tuberculosis*, *M. bovis*, *M. canetti*, *M. africanum* and *M. microti*. At the genome level, *M. bovis* shared 99.95% identity with *M. tuberculosis* (Taylor et al., 2007).

The identification of closely related members of the MTC has remained a challenging task in diagnostic laboratories (Niemann et al., 2000). Currently, differentiation of *M. bovis* from *M. tuberculosis* is based on conventional culture and biochemical tests. In addition to being tedious and slow, current methods, such as biochemical typing, are not 100% reliable due to the advent of intermediate strains, such as niacin and TCH variant *M. tuberculosis* (Verma et al., 1987; Grange et al., 1996). Partly due to close genetic relatedness and variable biochemical patterns, definitive detection of *M. bovis* and *M. tuberculosis* up to species level, is time consuming and difficult. Methods, such as polymerase chain reaction (PCR), could be the best alternative strategy to meet this purpose (Bakshi et al., 2005). The success of PCR depends on the availability of DNA, free of contaminants that impair the amplification process (Boom et al., 1999).

The use of new techniques for the isolation and identification of *Mycobacteria* has substantially reduced the time required by the clinical laboratory to report the results, with the advantage of improved sensitivity. This achievement is mainly due to the introduction and routine use of non-radiometric techniques as BACTEC MGIT 960 **TM** system, which is fully automatic non- radiometric system based on the early detection of positive cultures in broth media (Abe et al., 1999).

Rodriguez et al. (2009) evaluated the performance of an automated BACTEC MGIT 960**TM** system, a non-radioactive, non-invasive liquid culture system for cultivation of *M. tuberculosis* complex and compared it with the conventional (L.J.) medium. They standardized p-nitro benzoic acid (PNBA) assay on BACTEC MGIT 960 **TM** system for identification of *M. tuberculosis* complex and evaluated the usefulness by comparing the results with molecular assay and sequencing. They concluded that the BACTEC MGIT 960 **TM** system with PNBA for identification of *M. tuberculosis* complex is a rapid and useful method in laboratories processing a large number of specimens.

The objective of this study is to compare the conventional culture method (L.J.) and BACTEC MGIT 960 **TM** system and polymerase chain reaction.

MATERIALS AND METHODS

Animals

Field animals

A total of 1180 cows were examined in three different governorates (El-Sharkia, El-Gharbia and El – Monefeia) by the single intradermal (SID) cervical tuberculin skin test. The positive reactor animals (29 cows) were slaughtered and examined for post mortem findings.

Samples

Tissue samples

The internal organs (livers, spleens, lungs) and lymph nodes showing tuberculous like lesions were subjected to bacteriological examination for isolation and identification of acid fast bacilli. Samples were stored in an ice box and sent as quickly as possible to the laboratory.

Processing of samples for isolation of acid fast bacilli (Marks, 1972)

Organs, lymph nodes and or tissues showing gross lesions were processed for isolation of the organism. The specimens were cut into small pieces, grinded with fine sterile sand, mixed with 2 ml sterile distilled water, grinded in the mortar. Two ml of 4% H_2SO_4 were added to the sample then incubated for ½ h at 37°C. The mixture was diluted with 16 ml sterile distilled water and centrifuged at 3000 rpm/20 min. The obtained sediment was resuspended in 0.5 ml sterile distilled water and inoculated onto Lowenstein-Jensen slants and incubated at 37°C in inclined position for overnight then vertically for at least 6 to 8 weeks with weekly examination starting from three days post inoculation.

Isolation of acid fast bacilli

Conventional culture method [Lowenstein-Jensen medium (L.J.)] (Marks, 1972)

After processing of samples, the obtained sediment was resuspended in 0.5 ml sterile distilled water and inoculated onto Lowenstein-Jensen slants and incubated at 37°C in inclined position for overnight then vertically for at least 6 to 8 weeks with weekly examination starting from three days post inoculation.

BACTEC MGIT 960 system^{TM} **(Hines et al., 2006)**

Only 15 ml of BACTEC MGIT growth supplement were reconstituted a lyophilized vial of BBL MGIT PANTA antibiotic mixture. MGIT tubes were labeled with the specimen number and only 0.8 ml of the growth supplement / MGIT PANTA mixture were added to the MGIT 960**TM** liquid culture tubes to reduce the growth of contaminant bacteria and supplement the growth of bacteria. After then, 0.5 ml of processed specimens was added to MGIT 960^{TM} liquid culture tubes. All MGIT 960 tubes were incubated at 37°C into BACTEC MGIT 960^{TM} instrument for 6 weeks. The instrument was automatically monitoring the fluorescence which was emitted within each tube every h.

Interpretation

MGIT 960 culture tubes were interpreted as positive if the instrument signaled positive within 6 weeks and smear was made to detect acid fast bacilli by the Ziehl – Neelsens method.

MGIT 960 culture tubes were interpreted as negative if the instrument did not signal positive by the end of sixth week of incubation.

Polymerase chain reaction

Extraction of DNA

The standard and bacteriollogically positive strains were grown in

Table 1. Results of the recovery rates of *Mycobacteria* from slaughtered tuberculin positive cows by BACTEC MGIT 960 ™ system and conventional culture method.

Type of lesions (No.)	BACTEC MGIT 960 system		Conventional culture system (L.J.)	
	N	*%	N	%*
1. Visible lesions (22)	19	86.4	18	81.8
A. Localized:				
- Respiratory (pulmonary L.N + lung tissue) (9)	8	36.4	8	36.4
- Digestive (liver + mesenteric L.N) (4)	3	13.6	3	13.6
- Mixed (liver + lung tissue + L.N) (6)	5	22.7	4	18.2
B. Generalized (3)	3	13.6	3	13.6
2. Non visible lesions (7)	5	71.4	4	57.1
Total (29)	24	82.8	22	75.9

*%calculated according to the type of lesions.

10 ml tryptic soya broth (TSB) at 37°C for 24 h. The overnight cultures were centrifuged at 3000 RPM for 5 min and the supernatant were decanted carefully. The bacterial pellets were washed three times with phosphate buffer saline pH 7.2 and resuspended in 400 µl tris-EDTA buffer (pH 8.0) and heated in water bath at 100°C for 20 min. There were left to cool at room temperature and centrifuged at 14,000 rpm for 10 min. An aliquot of 5 µl of the supernatant was used as template DNA in the PCR.

Primers used for differentiation of M. bovis and M. tuberculosis

Two sets of primers pairs were used, the first one was (TB1F: 5´- GAC ATG TAC GAG AGA CGG CAT GAG -3´ and TB1R: 5´- AAT CCA ACA CGC AGC AAC CAG-3´) which could amplify 700 base pair fragments of RD4 specific for *M. tuberculosis*. The second one was (TB2F: 5´- TCT TGC GGC CCA ATG AAT -3 and TB2R: 5´- GGT GTG ATT TGG TGA GAC GAT -3´) which could amplify 800 base pair fragments of RD8 specific for *M. bovis*.

DNA amplification

PCR amplifications were performed in a final volume of 50 µl in micro-amplification tubes (PCR tubes). The reaction mixtures consisted of 5 µl of the DNA template, 5 µl 10 × PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl2, 50 mM KCl, 20 mM (NH4) 2SO4), 1 µl dNTPs (40 µM), 1 µl of 1U Ampli Taq DNA polymerase, and 1 µl of 25 pmol from the forward and reverse primers of both primer pairs and the volume of the reaction mixture was completed to 50 µl using DDW. The thermal cycler was adjusted as follows: Initial denaturation at 94°C for 5 min., followed by 35 cycles of (denaturation at 94°C for 1 min., annealing at 62°C for 1 min. and extension at 72°C for 1 min.). Final extension was carried out at 72°C for 10 min and the PCR products were stored in the thermal cycler at 4°C until they were collected.

Agarose gel electrophoresis

The PCR products were tested for positive amplification by the agarose gel electrophoresis previously reported by Sambrook et al. (1989) using Sui molecular weight markers.

RESULTS

Results of the isolation of *Mycobacteria* from tuberculin positive cows by BACTEC MGIT 960 ™ system and the conventional culture method

The results in Table 1 and Figure 1 showed that out of 29 processed tissue samples from tuberculin positive cows, 24 (82.8%) were recovered by BACTEC MGIT 960 ™ system and 22 (75.9 %) by conventional culture method (L.J.).

It is clear that, 19 (86.4%) mycobacterial isolates were recovered from 22 cows showing visible lesions, while 5 (71.4%) of *Mycobacteria* were isolated from 7 cows without any visible lesions (NVL) by using BACTEC MGIT 960 ™ system. However, when conventional culture method was used, 18 (81.8%) were isolated from 22 cows showing visible lesions and 4 (57.1%) *Mycobacteria* were isolated from 7 cows without any visible lesions.

The mean time for detection of mycobacteria using the BACTEC MGIT 960 ™ system and the conventional culture method (L.J.)

Table 2 shows the mean time for detection of mycobacterial isolates using BACTEC MGIT 960 ™ system and the conventional culture method.

For the BACTEC MGIT 960 ™ system, the recovery of the organism required only 17.8 days (S.E. 0.9), while the conventional culture method required 46.5 days (S.E. 0.4).

The contamination rate of the BACTEC MGIT 960 ™ system and conventional culture method

The contamination rate of each method was displayed in

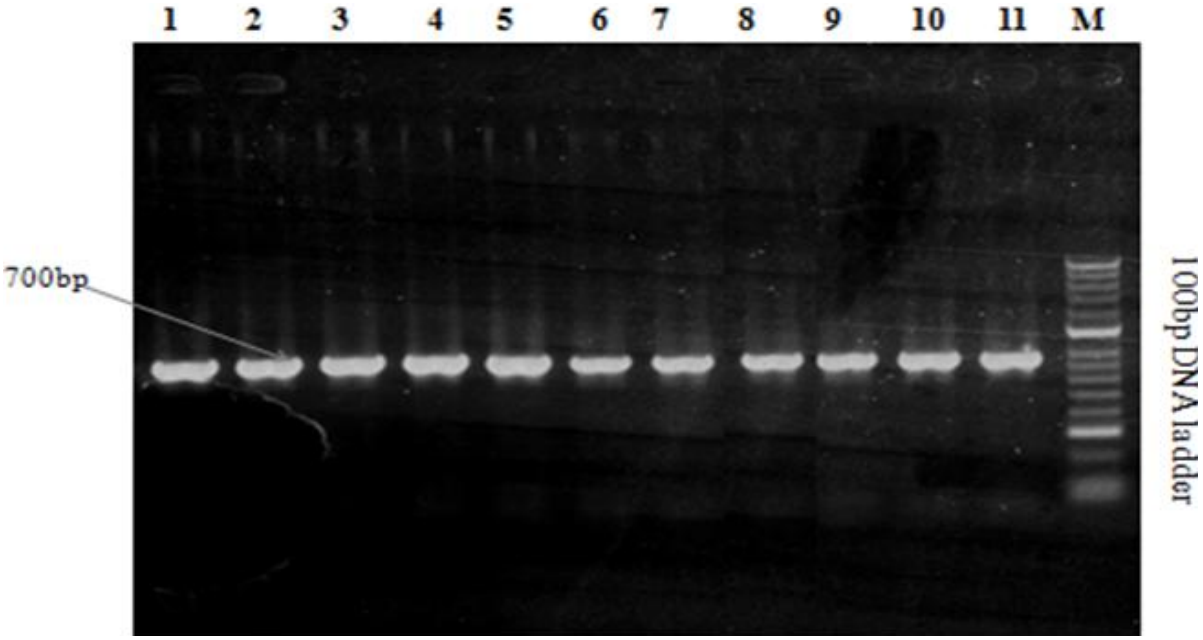

Figure 1. Agarose gel electrophoresis showing amplification of 700 bp fragments of RD4 specific for *M. tubdrculosis*.

Table 2. Comparison between the mean time for detection of *Mycobacteria* using the BACTEC MGIT 960 ™ system and conventional culture method.

Culture system	Days to detection
BACTEC MGIT 960 ™	17.8 ± 0.9
Conventional culture method (L.J.)	46.5 ± 0.4

± Standard of Error (S. E.).

Table 3. Comparison between the contamination rates of the BACTEC MGIT 960 ™ and conventional culture method.

Media system	Number of contamination / total number of samples	Percentage of contamination (%)
BACTEC MGIT 960 ™	2/29	6.9
Conventional culture method (L.J.)	3/29	10.3

Table 3. The contamination rate of the MGIT 960 ™ system was 6.9%, while the contamination rate of the conventional culture method was 10.3%.

Results of Polymerase chain Reaction

Results of amplification of RD4 region conserved in M. tuberculosis using TB1F and TB1R primer pair

PCR assay was conducted to recognize *M. tuberculosis* depending on the amplification of RD4 region conserved in *M. tuberculosis* using TB1Fand TB2R primer pair. The PCR could detect all the *M. tuberculosis* strains previously identified by bacteriological examination, and amplification of 700bp fragments specific for RD4 region were observed with *M. tuberculosis* isolates as shown in Figure 1.

Results of amplification of RD8 region conserved in M. bovis using TB2F and TB2R primer pair

The PCR assay was carried out to amplify the RD8 region specific for *M. bovis* using TB2F and TB2R primers. Amplification of 800 bp fragments specific for RD8 region were observed with all strains of *M. bovis* as shown in Figure 2.

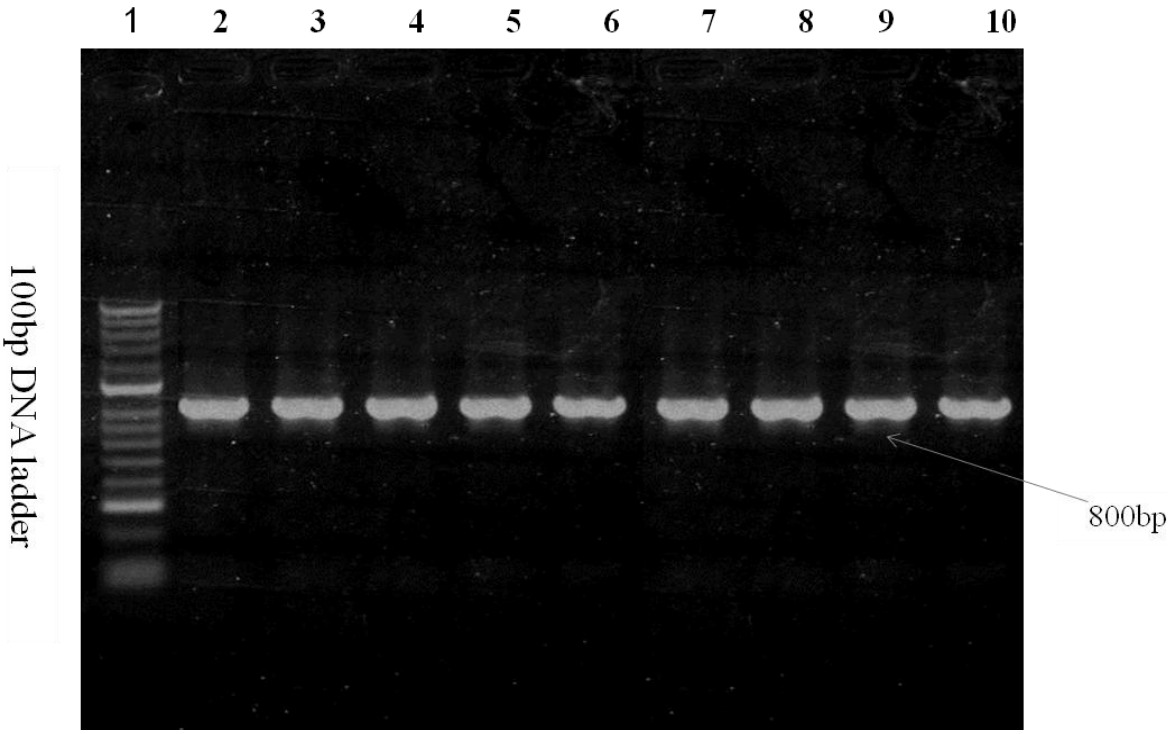

Figure 2. Agarose gel electrophoresis showing amplification of 800 bp fragments of RD8 specific for *M. bovis*.

DISCUSSION

The diagnosis of bovine tuberculosis in live animals mainly depends on clinical manifestations of the disease, skin testing, staining with Ziehl-Neelsen (Z.N.) stain and more recently by molecular methods. Subsequent identification of the pathogen has been made by culturing and biochemical tests (Mishra et al*.,* 2005). The Z.N stain is a very rapid method, but lacks specificity and cannot be used to distinguish the various members of the *Mycobacteriaceae* family, while culturing usually requires 4 to 8 weeks to obtain good growth (Vitale et al., 1998). Although tuberculin skin testing has been a hallmark of bovine tuberculosis eradication campaigns and has been effective in reducing the prevalence of bovine tuberculosis in most developed countries, problems do exist with such tests as it lacks sufficient sensitivity and specificity (Palmer et al., 2006).

The BACTEC MGIT 960™ system is fully automated, high capacity, non-invasive instruments, which requires neither needles nor other sharp instruments (Rishi et al., 2007). The present study was carried out to compare one such automated system (BACTEC MGIT 960™ system) with conventional culture method L.J. for the isolation of *Mycobacteria*. It is clear from Table 1 that out of the 29 processed samples from tuberculin positive cows, 24 (82.8%) had positive culture for *Mycobacteria* by BACTEC MGIT 960™ system and 22 (75.9%) had positive culture for *Mycobacteria* by the conventional culture method (L.J.).

The results of this study demonstrated that the number of *Mycobacteria* recovered in BACTEC MGIT 960™ system was greater than those recovered using conventional culture method (L.J.). The obtained results are in agreement with those reported by Hanna et al. (1999) which showed that the BACTEC MGIT 960™ system had a recovery rate that is greater than that in BACTEC 460 TB ™ system and in solid media. Tortoli et al. (1999) showed that the overall rates of recovery obtained with the BACTEC MGIT 960™ system and BACTEC 460 TB ™ systems were clearly higher than those achieved with solid media. Moreover, Dongsi and Dunnc (2002) found that the BACTEC MGIT 960™ system consistently provided better recovery of all *Mycobacterium* species from a variety of clinical specimens than the traditional L.J. slants did. In addition, Hines et al. (2006) showed that the BACTEC MGIT 960™ system had a higher recovery rate of *M. bovis* (122/129) than the solid media system (96/129).

There was a low positivity rate shown by conventional culture method (L.J.) in these studies. It could be because of the fact that sample slants were grossly contaminated and considered negative, whereas in BACTEC MGIT 960™ system, since the smear were made from all instrument positive MGIT 960 tubes, it was found that there were samples which had both contamination, as well as mycobacterial growth in them. Such tubes were considered positive by BACTEC MGIT

960™ system (Rishi et al., 2007).

Beside higher isolation rate, even the time to detect *Mycobacteria* was shorter on the BACTEC MGIT 960™ (17.8 ± 0.9) days than the conventional culture method (46.5 ± 0.4) days as shown in Table 2. The present study's results are in agreement with those recorded by Tortoli et al. (1999) which showed that the mean detection time was significantly shorter for methods that made use of a liquid medium than those that made use of the L.J. medium. Moreover, Hines et al. (2006) recorded that the BACTEC MGIT 960™ had a significantly lower mean time to detection (15.8 ± 0.8) days than BACTEC 460 TB (28.2 ± 1.0) days and solid media (43.4 ± 1.0) days and finally Rishi et al. (2007) found that the time to detect *Mycobacteria* was shorter on the BACTEC MGIT 960™ than on the L.J. medium, average being 9.66 days with BACTEC MGIT 960™ and 28.81 days with L.J. medium.

For the time consuming point of view, PCR technique in the present study differentiates between *M. bovis* and *M. tuberculosis* within two days rather than the long period required for the biochemical identification tests. These results are in agreement with those reported by David et al. (1989) who assessed that PCR has distinct advantage over the traditional culture techniques. Moreover, Liebana et al. (1995) reported that the PCR technique is much faster than culture and reduce the time for diagnosis from several weeks to two days. So the PCR can be used for large scale screening of TB in areas where the disease is still a public health hazard as in Egypt, test and slaughtered police to eradicate bovine tuberculosis, rigidity of meat inspection, efficient pasteurization or boiling of milk and public health eradication.

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REFERENCES

Aagaard G, Govaerts M, Limei MO, Andersen P, Pollock JM (2003): Genomic approach to identification of M. bovis diagnostic antigens in cattle. J. Clin. Microbiol., 41 : 3719 – 3728.

Abe CK, Hirano K, Wada M (1999). Comparison of the newly developed MB redox system with *Mycobacterium* Growth Indicator Tube (MGIT) and 2% Ogawa egg media for recovery of mycobacteria in clinical specimens. Kekkaku (Japanese). 74: 707 – 713.

David HL , Freebault VL, Thorel MF (1989). Methods of laboratoire pour mycobacteriologie clinique, Commission des laboratories de Reference et d' Experties de l' institute Pasteur. Paris. pp. 156-166.

Dela R, Domencch R (2006). Human *M. bovis* infection in the United

Kingdom: Incidence, Risk, control measures and review of the zoonotic aspects of bovine tuberculosis. Tuberculosis, 68: 77 -109.

Dongsi L, Dunnc M (2002). Comparizon of the automated *Mycobacteria* Growth Indicator Tube system (BACTEC MGIT 960) with Lowenstein Jensen medium for recovery of *mycobacteria* from clinical specimens. Microbiol. Infect. Dis., 118: 542 – 545.

Grange JM (2001). *M. bovis* infection in human beings.Tuberculosis 81: 71-78.

Hanna BA, Ebrahim A, Elliott B, Morgan MA, Novak SM, Gerdes SR, Aclo M, Dunbar DF, Holmes TM, Rexer CH, Savtny C, Vannier AM (1999). Multicenter evaluation of the BACTEC 960 system for recovery of *Mycobacteria*. J. Clin. Microbiol., 37: 748- 752.

Hines N, Payeur JB, Hoffman LJ (2006). Comparison of the recovery of *Mycobacterium bovis* isolates using the BACTEC 460 TB system and Middle brook 7H10 and 7H11 solid media. J. Vet. Diagn. Invest., 18: 243 – 250.

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

Marks J (1972). Ending the routine guinea pig test. Tuberculosis 53: 31-34.

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: 5670–5678.

Palmer MV, Waters WR, Thacker TC, Greenwald R, Esfandiari J, Lyashchenko KP (2006). Effects of different tuberculin skin-testing regimens on gamma interferon and antibody responses in cattle experimentally infected with *Mycobacterium bovis*. Clin. Vaccine Immunol., 13: 387– 394.

Prabhakar A, Mishra A, Singhal VM, Katoch SS, Thakral JS, Tyagi, Prasad NM (2004). Use of the hup B gene encoding a histone –like protein of *M. tuberculosis* as a target for detection and differentiation of *M. tuberculosis* and M. bovis. J. Clin. Microbiol., 42: 2724-2732.

Rodriguez C, Shenai S, Sadami M, Sukhadia N, Jani M, Jbani K, Sodha A, Mchtn A (2009). Evaluation of the BACTEC MGIT 960 TB system for recovery and identification of *Mycoabcterium tuberculosis* complex in a high through put tertiary care centre. Indian J. Med. Microbiol., 27: 217-221.

Rishi S, Sinha P, Pal N (2007). A comparative study for the detection of *Mycobacteria* by BACTEC MGIT 960, Lowenstein Jensen media and direct AFB smear examination. Indian J. Med. Microbiol., 25: 383 - 386.

Sambrook J, Fritscgh EF, Meniates (1989). Molecular Colning: A laboratory manual. Vol.1. Cold Spring Harbor Laboratory press, New York. pp. 114-120.

Taylor MG, Danny RW, Si Palmer KJ, Glyn H (2007): Rapid detection of *Mycobacterium bovis* DNA in cattle lymph nodes with visible lesions using PCR. BMC Vet. Res., 3: 12.

Tortoli E, Cichero P, Piersimoni C, Simonett MT, Gesu G, Nista D (1999). Use of BACTEC MGIT 960 for recovery of *Mycobacteria* from clinical specimens: Multicenter study. J. Clin. Microbiol., 37: 3578 – 3582.

Vitale F, Giuseppina C, Letizia M, Stefano R, Gesualdo V, Santo C (1998). Detection of *Mycobacterium tuberculosis* Complex in Cattle by PCR Using Milk, Lymph Node Aspirates, and Nasal Swabs. J. Clin. Microbiol., 36: 1050–1055.