Mr. DHAKSHINAMOORTHY SARAVANAKUMAR was born on 25th May, 1971 at Sattur, a town near Madurai, Tamilnadu, India. He obtained his B.Sc degree in Zoology from A.J college, Madurai Kamaraj University, Madurai and M.Sc in Zoology from Madras Christian College, University of Madras, Chennai. He joined Centre for Biotechnology, Anna University, Chennai with a UGC research scholarship in 1993. He has worked on the various molecular aspects of mycobacteriology for his doctoral thesis. He has one paper to his credit and has communicated two more manuscripts. He is interested in taking up a career in molecular mycobacteriology.
Corrections / Clarifications for the comments raised by the Indian Examiner Dr. S. Vijaya, Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore.

1. In Fig. 3.12a, the legend mentions 17 lanes while the photograph of the gel shows only 16. In Fig. 3.21a, lane 6 is not described in the legend.

   In Fig. 12a, there is only 16 lanes and the lane 17 mentioned in the legend is removed. In Fig. 3.21a, the description for lane 6 is incorporated in the legend.

2. All photographs are of extremely small size. The quality of the data would be enhanced if large prints had been used. In several instances, such as Fig. 3.12 and 3.17a and b could be combined to permit the reader to get the benefit of the data more effectively.

   The suggestion of the examiner regarding the size of the photograph has been noted and will be taken into account for the future cases. As suggested by the examiner Fig. 3.12 a and b has been combined and put in the same page vide page no.87. But the Figures 3.17a and b could not be combined together as both figure could not be accommodated in one page.

3. The southern hybridization results shown in Fig. 3.4b is obviously nonspecific hybridization of the probe and does not represent a specific signal. Compare this to results shown in Fig. 3.7b where the hybridization is specific.

   The southern hybridization results shown in Fig. 3.4b is little smeary as the concentration of the DNA loaded in the gel is higher. Whereas in Fig. 3.7b it is seen as sharp band as the concentration of the DNA is low. This information is incorporated in Page No.71.

4. The major drawback of this thesis is the section dealing with development of a probe for detecting M. avium in clinical samples. The details given on page no.87 clearly reveal the poor quality of results...
obtained by other investigators using probes derived from the pMav22 sequences. Therefore it is not clear why a different region of the same stretch was attempted for a probe. Using the primers to amplify this region, it is claimed on page 95 that sequence variation within this amplified region was detected by PCR-SSCP. However, Fig.3.16a and b clearly show no shift in mobility as claimed and the minor lane to lane variations seen in these gels are clearly due to distortions in the gel, an often encountered phenomenon. The candidate has gone on to clone the PCR products and sequence 3 clones obtained from 3 isolates. The comparison of these sequences is shown in Appendix 1. The candidate has claimed that the alignment of the sequences proves that the 3 sequences differ from one another. However, even a brief scrutiny of the sequence data reveals that the quality of the sequence is so poor and is full of Ns that it is meaningless to attempt to utilise this for any worthwhile comparisons.

In order to deal with point No.4 two options are possible. One is to remove the PCR-SSCP data and redo the sequencing of the clones to obtain 100% reliable sequence data and repeat the comparison. Alternatively the candidate may delete the PCR-SSCP related figures and also the sequence alignment shown in Appendix 1 and the related section in the text. This piece of work would merit a Ph.D. degree even without this data.

As suggested by the examiner the sequence alignment shown in Appendix 1 and the related text has been removed. The PCR-SSCP related figures has been retained as it reveals some sequence variation among the different serotypes.

5. Overall, there are several errors in spelling that need to be attended to. I have pencilled a few of them.

All the spelling mistakes pointed out by the examiner has been corrected.
Corrections / Clarifications for the comments raised by the Foreign Examiner Dr. Arvind M. Dhople, Research Professor and Director, Infectious Diseases Laboratory, Florida Institute of Technology, USA.

1. The candidate should have given reasons/justifications for developing a new protocol for PCR based diagnosis of mycobacterial infection instead of adopting one of the existing protocols. Once established, the advantages of this new protocol over currently available ones should have been discussed.

The new protocol developed in this study is for processing blood samples for PCR analysis. The justification for developing the new protocol is given in page 58. The new protocol is simple straightforward, involves a visible indicator, eliminates the need for extraction of DNA using organic solvents and use of expensive enzymes to lyse bacteria. This is described in page 60. The new protocol also addresses the concern regarding safety and cost factor, which is mentioned in page 60 & 61.

2. It is a well-established fact that DNA can be detected from dead (non-viable) organisms by existing macro as well as micro methods such as PCR amplification. Thus, how one can use PCR method to monitor treatment, where bacteria become non-viable after effective treatment but still retain their DNA? Its value is only in routine diagnostic use i.e., differentiating M. tuberculosis form MOTT, and that too if appropriate internal controls are used and have included mechanisms for preventing carryover of PCR product. In the current investigations mention of both is missing.

It is clearly mentioned in page 83 that PCR could amplify DNA from dead bacilli. That is the reason why in the present study I have used this semiquantitative PCR along with two other methods i.e., sputum smear and colony counting which will pick up only viable organisms. Thus, I have evaluated the usefulness of semiquantitative PCR in accessing treatment response in tuberculosis patients in additional to the routine evaluation by
sputum smear and colony counting. This has been discussed in page 83 and 85. I have also justified this study with proper references (Kennedy et al., 1994; Palenque et al., 1995—these two clinical reports suggests PCR as a promising method for monitoring treatment response in TB patients) in page 85.

The concern regarding the carryover contamination was addressed in page 71 where we have mentioned how our simple protocol could prevent the carryover contamination problem.

3. What is the role of these new protocols and probes that the candidate claims to have developed in actual application in the fields of tuberculosis, leprosy and opportunistic mycobacterial infections in AIDS? Are these compatible for routinely being used in clinical laboratory? This problem has not yet been solved in developed countries, including United States. How it can be applicable to the needs in India?

The present investigation only suggests that PCR based diagnosis sounds promising for the early diagnosis of mycobacterial infection in the clinical specimens (sputum and blood) collected from tuberculosis and HIV infected patients. However, I have clearly mentioned in page 79 that this technology requires further refinements and reduction in the costs before it could be adopted routinely by the clinical laboratories in India. Since the problem of mycobacterial infections is of high importance to us we have to become self sufficient in developing new methods for diagnosis rather than depending on commercial probes.

4. What is the specificity of special probe developed for *Mycobacterium avium* and its efficiency compared to the existing ones? The biggest problem we have in the field of mycobacterial infections is occurrence of large percentage of false positive results. Has the candidate looked into this problem? In order to determine accurately the incidence of false positive results, one needs to have fairly large number of experimental subjects, which is not the case in present undertakings.
This, then brings the following set of questions. Nowhere in the thesis there is any mention of statistical analysis, which is absolute in any study of this nature. Why statistical analysis was not performed? No mention of Power Analysis to determine minimum number of experimental subjects needed to derive an intelligent conclusion. So, on what basis patients were selected? Was it a double blind study? Where the samples coded? What was the volume of sputum specimen from each subject and how sputa were transported from clinical settings to the laboratory?

The specificity of the probe developed for *M. avium* was demonstrated against the various standard strains in pages 87 and 88 (Figures 3.12a., 3.12b., 3.13) and was also discussed in the text (page 86 & 89). The sensitivity of the probe is less than 10 bacilli which is comparable with the other available probes. This information is incorporated in page 89.

Yes, the problem of false positiveness was taken into consideration and was widely discussed in page 70. I agree that the experimental sample number was less in the present investigation which were not statistically significant. It was for this reason why I couldn’t perform any statistical analysis for my data.

I have not performed “Power Analysis” to determine minimum number of experimental subjects needed to derive an intelligent conclusion. The number of cases (patients) selected for the examination in the present study were not based on any defined protocol. I had some limitations in getting access to the HIV patients samples and as well as samples for the follow-up study in the case of tuberculosis. However, I made every attempt to collect and examine the maximum number of available samples. It was not a double blind study and the samples were not coded. The volume of the sputum sample from each subject is 2 ml and the sputa was transported from the clinical setting to the laboratory in a sterile, well sealed container on the same day for the processing and analysis. This information is incorporated in page 37.
5. PCR-SSCP and PCR-RFLP analyses were carried out only on clinical isolates of *Mycobacterium avium*, and not from clinical specimens why?

The purpose of using PCR-SSCP and PCR-RFLP in the present investigation was to look at the sequence polymorphism exhibited by the different serovars of *M. avium*. Hence, the well characterized clinical isolates of *M. avium* (serovars 4, 8 and 9) which were received from the Imperial College of Medicine, London, UK (details of the serovars were mentioned in page 35 and 36) were analyzed by PCR-SSCP and PCR-RFLP. The PCR-SSCP and PCR-RFLP were not performed for the four clinical specimens because these were not serotyped and characterized.

6. From HIV-Positive subjects, only blood specimens were collected and not sputum specimens-why? These days pulmonary MAC infection has become very serious problem and we isolate MAC from sputum specimens more frequently than from blood specimens.

In the present investigation, most of the HIV-positive subjects suspected of having tuberculosis on the basis of clinical symptoms like infiltration and positivity by mantoux test did not expectorate sputum. Moreover, there were also report (Schluger et al., 1994) which suggest that mycobacteria could be detected from the blood samples of pulmonary tuberculosis patients (Page-29). Hence, it was decided to collect blood samples from these subjects. Eventhough, the incidence of pulmonary MAC infection is a serious problem in the United States, in India the incidence of MAC infection is very much lower than the *M. tuberculosis* infection (Our study & the study by TRC, Madras).

7. Intensity of signals were read using densitometer, and then bacterial loads were calculated. How can the candidate determine whether these bacteria were live or dead or both? Microscopic counting or coulter current counting should have been done to determine total counts and plate counts (CFUs) for viable counts, and then correlate the densitometric results.
Yes, of course microscopic counting as well as plate counts (CFU’s) for viable colony counts were done and the results were clearly summarized in page 84. The densitometric results were correlated only with the microscopic count and the plate count data. A detailed discussion was also given in pages 83 through 85.

8. In Page 29 ‘Blood Samples are easily obtainable from patients and detection of M. tuberculosis is a definite indication of active infection’ does the candidate mean that blood specimens are more reliable than sputum specimens for the detection of active infection in tuberculosis? In that case, why only sputum specimens were collected for follow-up studies (page 43)?

No, the statement doesn’t imply that blood samples are more reliable than sputum samples. This statement was actually made with reference to HIV infected subjects where disseminated mycobacterial infection (mycobacterium escaping to the blood stream from the lungs) is common in an advanced immunocompromised stage. The reference of Schluger et al., 1994 was also quoted in support of the statement in page 29.

The patients selected for the follow-up study were positive only for tuberculosis and not for HIV. Moreover all the chosen patients were clinically proven pulmonary tuberculosis cases and were expectorating sputum. Hence, the sputum sample was collected to perform microscopic examination, culturing and PCR for the follow-up study.

9. Throughout the thesis mention has been made that the results are from semiquantitative analyses, why no quantitative analyses by all three methods? One point that is not discussed, but is equally important, is the transcription-mediated amplification assay (TMA) where amplification step is isothermal (does not require thermal cycling)?

I have used the term “semiquantitative” only for the PCR analysis and not for the microscopic count and plate count. The microscopic count and the plate count are quantitative analysis which were performed by the method of
Vestal (1981) (page 43). The quantitation was not possible in PCR as there was no internal standard. Basically, the semiquantitative densitometric PCR data was correlated with the quantitative microscopy and colony count data (pages 84-85).

Yes, the rRNA based isothermal transcription-mediated amplification (TMA) assay originally developed by Gen Probe, San Diego, USA was discussed in the literature review in page 32 (2nd paragraph) with appropriate references (Abe et al., 1993; Pfyffer et al., 1994 and Bodmer et al., 1994).

10. Finally, it is with little haste that candidate has made claims of developing protocols and probes for monitoring patients (tuberculosis as well as leprosy) under chemotherapy on the basis of limited documentation. The semi-quantitative PCR test is adequate for diagnosis purposes only.

The follow-up study for monitoring treatment response was done and reported in the thesis only for tuberculosis patients and not for leprosy patients (page 80 through 85). And even in tuberculosis patients the semiquantitative PCR was done along with two other conventional methods i.e., sputum smear and colony counting and the results of all three methods have been summarized in Table 3.5 (page 84). This was also discussed in the text (page 83 and 85). I have also justified this study with appropriate references (Kennedy et al., 1994; Palenque et al., 1995- these two clinical reports suggests PCR as a promising method for monitoring treatment response in TB patients as compared to sputum smear and colony count) in page 85.

The candidate thanks the examiners for their valuable suggestions and comments.