CHAPTER VI

DISCUSSION

Since the mid-1980s, mycobacterial infections have become increasingly widespread for a number of biological and social reasons, in particular, the human immunodeficiency virus epidemic. Together with the increasing incidence of tuberculosis, the incidences of *Mycobacterium avium* diseases and other non-tuberculous mycobacterial infections have also increased [98, 104, 111]. Rapid discrimination between *M. tuberculosis* and *M. avium* is of primary important for the initiation of a correct chemotherapeutic regimen, because the two infections require different type of therapy and management [98, 111, 112]. Identification of mycobacteria to the species level by conventional biochemical tests has been fraught with a long turnaround time, loading the significant delays in diagnosis [11].

In this study, multiplex PCR and reverse hybridization was developed as assay for the rapid detection and identification of mycobacteria in clinical samples. Multiplex PCR protocol was modified from that dscribed by Wilton and Cousins [37] and Kulski et. al. [38] with modification by changing primers for amplification of *Mycobacterium* genus. Primers AFB-F and AFB-R [108] amplified the product of 640-665 bp in *Mycobacterium* genus instead of the product of 1,030 bp amplified by primers MYCGEN-F and MYCGEN-R as described by Kulski et al. [38]. These primers span region A of 16S rRNA gene that is specific at the species level. The forward primer is well conserved among a wide range of bacteria but the reverse primer (AFB-R) has limited or no homology to eukaryotic or viral sequences and most non-AFB bacteria. It matched only with several gram-positive rods that are related phylogenetically to AFB, such as *Corynebacterium, Propionibacterium, Tsukamurella, Nocardia* and *Actinomyces*, some of which are partially acid-fast and occasionally have to be differentiated from AFB [108]. It is easy to differentiate PCR products of *Mycobacterium* genus (640-665 bp), *M. avium* (173 bp), *M. intracellulare* (490 bp), and *M. tuberculosis* complex (372 bp) in this protocol. In addition, in our experience, this protocol gave better sensitivity of detection than the one described by Kulski et al. [38]

The multiplex PCR employed in this study was a rapid and inexpensive technique for identification of *M. tuberculosis* complex, *M. avium*, and *M. intracellulare* which are most commonly isolated pathogen in clinical laboratories. This protocol can be applied successfully in conjunction with hemoculture system to rapidly identify *M. avium* and *M. tuberculosis* complex directly from the culture broth in a way similar to DNA probes (AccuProbe) (Table 14). The application of multiplex PCR with AFB-positive specimen is also very successful (Table 15). In addition, this technique was easily applied to clinical isolates using simple DNA extraction method such as alkaline wash and heat lysis method.

The amplified product of *Mycobacterium* genus obtained from multiplex PCR can be analyzed by sequencing but it is not a technique that is suitable for a routine laboratory. For this reason, a reverse hybridization assay was chosen because this method is simpler than sequencing. PCR product can be hybridized with different probes simultaneously and the result obtained is easily interpreted. In this study, oligonucleotide probes specific for clinical relevant species were employed. The validity of reverse dot blot hybridization was confirmed by comparison of the results with those obtained by the usual identification methods, such as biochemical methods and AccuProbe. The comparison showed perfect agreement among the different techniques and confirmed the validity of reverse dot blot hybridization.

The benefits of the reverse dot blot hybridization method are also remarkable when the method is compared with other molecular biology-based techniques. AccuProbe directed against 16S rRNA have contributed to simplification of the procedure and shortening of the time necessary for the identification of slowly growing mycobacteria such as *M. avium, M. intracellulare, M. gordonae*, and *M. tuberculosis*. However, this method is unable to identify other clinically relevant species, such as, *M. fortuitum, M. flavescens, M. chelonae*, and *M. abscessus,* species among the opportunistic mycobacteria that were isolated in Thailand. INNO-LiPA Mycobacteria, is based on the reverse hybridization principle. The main advantage of the kit is that a range of several species can be identified by a simple PCR assay [12, 36]. However, the high cost of these assays prohibited large-scale usage in most clinical laboratories and available only in Europe at present.

The turnaround time of the multiplex PCR method was 6 hr theoretically (extraction and reaction set up, 80 min; amplification, 4 hr by conventional PCR machines; and detection, 40 min); except biopsy samples, whose the turnaround time was 24-48 hr because of the longer period of the DNA extraction.The turnaround time of the reverse dot blot hybridization was 10 hr theoretically (extraction and reaction set up; 80 min; amplification, 4 hr by conventional PCR machines; reverse dot blot analysis, 4 hr; and detection signal, 40 min). In addition, the turnaround time of the sequencing method was 12 hr to 2 weeks depending on the facilities and sequencing service provider.

The cost of the multiplex PCR and reverse dot blot hybridization were approximately 200 baht and 450 baht, respectively, including QIAamp®DNA Mini kit, reagents, but not including development cost and validation of this study. In addition, the cost of sequencing were approximately 500-1,200 baht depending on sample loads, facilities and sequencing service provider.