Transcriptional Expression of Acidic-stress Responsive Genes in *Mycobacterium tuberculosis*

การแสดงออกของยีนตอบสนองสภาวะความเป็นกรดในเชื้อไมโคแบคทีเรียมทูเบอร์คูโลซิส

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ABSTRACT

Mycobacterium tuberculosis (Mtb) is currently major problem in public health worldwide. It is thought to infect one third of the world's population. Most of them are asymptomatic and latent infection. In order to obtain the information about the mechanism of the bacteria used for survival and adaptation during latent infection, gene expression during the growth of the bacteria in various stress conditions that mimic the growth in macrophage was studied. Acidic stress conditions of pH 4.5 and 5.0were usedin this study. RT-PCR and Real-time PCR analysis showed the expression of genes including*icl*1, *esp*D, *esp*C and*esp*A was significantly up-regulated in acidic stress condition. This result demonstrated that these genes likely to be involved in adaptation and survival in the stress condition. Study of adaptive mechanism response to stress condition could lead to discovery of new drug-target or strategy to prevent and control tuberculosis.

บทคัดย่อ

*Mycobacterium tuberculosis*ในปัจจุบันยังเป็นปัญหาที่สำคัญด้านสาธารณสุขทั่วโลกและมีประชากรโลกจำนวนถึง1 ใน 3 มีการติดเชื้อนี้โดยไม่แสดงอาการและเป็นการติดเชื้อแฝง เพื่อให้เข้าใจกลไกการดำรงชีพและปรับตัวในระหว่างการเจริญของเชื้อในระยะการติดเชื้อแฝงจึงมีการศึกษาการแสดงออกของยีนต่างๆ ในสภาวะกดดันที่คล้ายการเจริญเติบโตของเชื้อในเซลล์มาโครฟาจ ในการศึกษานี้ได้เลือกสภาวะความเป็นกรดที่ pH 4.5 และ 5.0เพื่อศึกษาการแสดงออกของยีนต่างๆ จากการศึกษาโดยวิธี RT-PCR และ Real-time PCR พบว่า การแสดงออกของยีน *icl*1 *esp*D*esp*C และ *esp*A มีการแสดงออกมากกว่าสภาวะปกติอย่างมีนัยสำคัญ ผลการทดลองนี้แสดงให้เห็นว่ากลุ่มยีนเหล่านี้น่าจะเกี่ยวข้องกับการปรับตัวและการดำรงชีวิตในสภาวะกดดัน และการศึกษากลไกการตอบสนองต่อสภาวะกดดันของเชื้อวัณโรคสามารถนำไปสู่การค้นพบเป้าหมายของยาใหม่ หรือกลยุทธการควบคุมป้องกันวัณโรค

Key Words: Acidic, *Mycobacterium tuberculosis*
คำสำคัญ: ความเป็นกรด เชื้อวัณโรค

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Introduction

One third of the world population is carrying TB infection(Deb et al., 2009). The first step in human infection with *Mycobacterium tuberculosis* (Mtb) is phagocytosis of the bacteria by macrophages in the lung (Fisher et al., 2002).Actually, phagosomal acidification is an essential for the induction of virulence factors in some pathogens and favors the survival of certain.The phagosomal acid is a major bactericidal effector mechanism of macrophages (Omar H Vandal et al., 2009).Response of acid has been studied in obligate or facultative intracellular bacterial pathogens likeMtb, resist and persist in the moderately acid environment of phagosome or phagolysosome(O. H. Vandal et al., 2009). The range of pH from 6.2 to 4.5, depend on the activation state of the macrophage infected withMtb(MacMicking et al., 2003; Schaible et al., 1998; Via et al., 1998). And the level of acidity surrounding mycobacteria in macrophage the pH containing phagosomes was in the range of 4.7 to 5.5 (Sprick, 1956).When lysosome fuse withMtb containing phagosomes in immunologically activated macrophages, the phagolysosomal pH falls to 4.5 to 5.0 (MacMicking et al., 2003; Schaible et al., 1998; Sibley et al., 1987; Via et al., 1998). When the phagosomes contain bacteria start to acidify rapidly after phagocytosis,however, the bacilli are ableto survive inside and active inhibition of phagosomal acidification. Acidification itself may be a signal used by mycobacteria to induce the expression of genes. High-throughput screening methods were used to study the gene expression ofMtb to acidify *in vitro* and *in vivo*, several genes were reported such as *icl* (isocitratelyase) (Deb et al., 2009; Fisher et al., 2002). Microarray data of Mtb gene expression under low-pH response included*esp*D(ESX-1 secretion-associated protein EspD), *esp*C (ESX-1 secretion-associated protein EspC) and *esp*A (ESX-1 secretion-associated protein EspA) (Deb et al., 2009).

Objective

The aim of this study was to select and evaluate transcriptional markers of Mtb by RT-PCR and Real-time PCR in acidic stress condition.

Methodology

Bacterial strain, Media and Growth conditions

All experiments were conducted with*Mycobacterium tuberculosis* H37Rv from the frozen stock at -70 °C and subculture once in Middlebrook7H9 (M7H9) medium (Sigma, India) before inoculation to an experimental culture (Wayne et al., 1996).The bacteriafrom frozen stock was inoculated into 20 ml of fresh M7H9 media supplemented with 2 ml of glycerol or 0.5 g of tween 80 and contained BBL™Middlebrook OADC Enrichment (BD, US) and incubated at 37 °C for 7 days, with shaking once a day. Once more the culture was then incubated into 20 ml of the M7H9 media and incubated at 37 °C for 7 days with shaking once a day. The number of bacilli present in the culture was estimated by using a plate count technique and ready to be usedinthe experiments.

The experiments were done by transferring completed stock cultures with active growing bacilli into the M7H9 media supplementedas described above and adjusted to acidic pH of both 5.0 and 4.5(Omar H Vandal et al., 2009) and incubated at 37 °C with shaking once a day.One of five tubes of experiments was then randomly selected for assessment of cell viability at 1 and 3 weeks by using dropplate technique, whereas the remaining tubes were used

forRNA extraction. The experiments were conducted in duplicate.

RNA extraction

Bacterial pellets were recovered at different time points from 20 ml samples of the culture medium by centrifugation at 4750 *g* for 20 min (4 tubes: mix together). RNA was then isolated from the cell pellets by using silica beads, Trizol RNA isolation buffer (Invitrogen, USA) (1 ml) and cell pellet were added to the matrix prior to disruptcell wall by vigorously shaking in vortex mixer about 1 min. The lysate was mixed with 200 µl chloroform, shaken vigorously for 10 s and kept at room temperature for 2 min. It was then centrifuged at 12000 *g* for 30 min at 4 °C, to separate the cell debris from the supernatant. The upper aqueous phase, which contained the total RNA, was carefully transferred into a micro-centrifuge tube. Subsequently, 500 µl of 2-propanol was added to the aqueous phase, and the mixture was incubated at room temperature for 10 min. The RNA was then pelleted by centrifugation at 12500 *g* for 30 min at 4 °C. The supernatant was discarded and the RNA pellet was washed once with 75% (v/v) ethanol. The ethanol was removed from the pellet after centrifugation at 7500 *g* for 5 min. The pellet was then air-dried about 20minand re-dissolved in 50 µlRNase-free water (0.25% DEPC) and the RNA concentration was determined spectrophotometrically at 260 nm (Haile et al., 2002).

Reverse transcriptase

The RNA was treated with Deoxyribonuclease I (Dnase I), amplification grade (Invitrogen, USA) prior to cDNA synthesis. One microlitre of 10×DNase I reaction buffer and 1 µl DNase I (1 U) were added to 1 µg total RNA and then incubated at room temperature (37 °C) for 45 min. The reaction was inactivated by the addition of 1 µl of 25 mM EDTA and heating for 10 min at 65 °C. The RNA sample was ready to use in reverse transcription, prior to amplification.

The cDNA was then synthesized from 0.5 µg of DNase I-treated total RNA in a total reaction volume of 20 µl, using SuperScript III Reverse Transcriptase (Invitrogen, USA). The reaction was performed by addition the following components to a nuclease-free microcentrifuge tube: 1 µl of 250 ng random primers, 500 ng total RNA, 1 µl 10 mMdNTP Mix (10 mM each of dATP, dCTP, dTTP and dGTP at neutral pH). Sterile deionized water to 14 µl. Heat mixture to 65 °C for 5 min and incubate on ice for at least 1 minute and then add 4 µl 5X First-Stand Buffer, 1 µl 0.1 M DTT, 1 µl of SuperScript III RT mix by pipetting gently up and down incubate at 25 °C for 5 min, 50 °C for 60 min and inactivate the reaction by heating at 70 °C for 15 min. The cDNAwas ready to be used as a template for amplification in PCR or not stored at -20 °C until used for PCR (Haile et al., 2002).

Primers for cDNA synthesis and RT-PCR

A pair of primers for each gene was designed based on the sequence obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and ordered from Ward Medic. The sequences for the primers used in this study are listed in Table 1. The 16S rRNAforward and reverse primer sequences were5'-TTG ACG GTA GGT GGA GAA GAA GC-3' and 5'-CCT TTG AGT TTT AGC CTT GCG G-3', respectively as described previously (Haile et al., 2002).

Real-time PCR

The real-time PCR mixture contained 10 µl of SsoFast™EvaGreen® Supermix (Bio-Rad Laboratory, Inc., USA), 2 µlof 0.2µMforward and

Table 1 Primer pairs used for cDNA synthesis and RT-PCR

Pair no.	Gene	Direction/position of primer	Sequence
1	16S rRNA	Forward, 469-491	5'-TTG ACG GTA GGT GGA GAA GAA GC-3'
		Reverse, 909-888	5'-CCT TTG AGT TTT AGC CTT GCG G-3'
2	*icl*1	Forward, 861-880	5'-TCG CCG ACT TGA TCT GGA TG-3'
		Reverse, 1035-1016	5'-AGC TCC TTC TGG AAC TTG GC-3'
3	*esp*D	Forward, 222-241	5'-TTT ACC GTG ACG AAT CCC CC-3'
		Reverse, 357-338	5'-CGG CAA TCA CCA GGA TCT CA-3'
4	*esp*C	Forward, 36-55	5'-GAA AAC TTG ACC GTC CAG CC-3'
		Reverse, 155-136	5'-GAT CGC CAC AGA TTC GCC TA-3'
5	*esp*A	Forward, 502-521	5'-TCA ACG CGA CTC AAC TCC TC-3'
		Reverse, 627-608	5'-TGA ACT CCC ACA CTT CTC CG-3'

reverse primers, 1µl of 40 ngcDNA and nuclease-free water added to a final volume of 20 µl.The real-timePCR was performed on the LightCycler Real-timesystem, version 2.0 (Roche,Germany). The PCR program setting included 1 cycle of pre-incubation at 94 °C for 5 min;45 cycles ofamplification, consisting of a 94°C denaturation for 30 sec, a 55°C annealing for 30 sec, and a 72°C extension for 30 sec. The data analysis was performed by using LightCycler® 480 Software.

Results

Bacterial growth in acidic culture medium

The number of proliferating bacilli based on colony forming unit counting were determined. The mycobacterial culture from week 0 to week 3, shaken once a day, indicated that the bacilli had entered into a non-replicating (NRP) stageat week 3.The cultures incubated for 1 and 3 weeks were chosen to investigate the expression of genes including *icl*1, *esp*D,*esp*C and*esp*A in active-replication and non-replication of Mtb by using RT-PCR assay.

Transcriptional expression of acidic-stress response genes

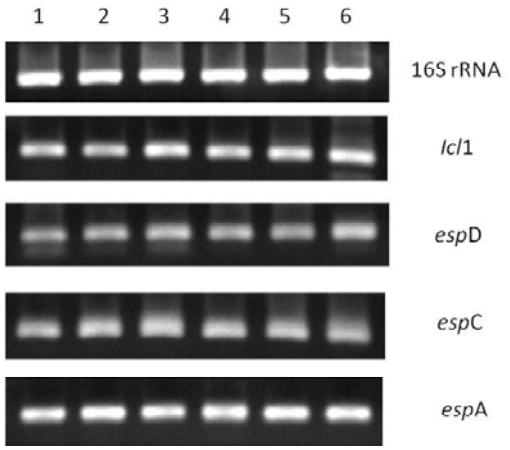

Figure 1 Gel electrophoresis profile of RT-PCRproducts using specific primer of 16S rRNA, icl1, espD, espC and espA. Lane 1: Control (normal medium) for 1 week, Lane 2: Acidic pH 4.5 for 1 week, Lane 3: Acidic pH 5.0 for 1week, Lane 4: Control (normal growth) for 3 weeks, Lane 5: Acidic pH 4.5 for 3 weeks, Lane 6: Acidic pH 5 for 3 weeks

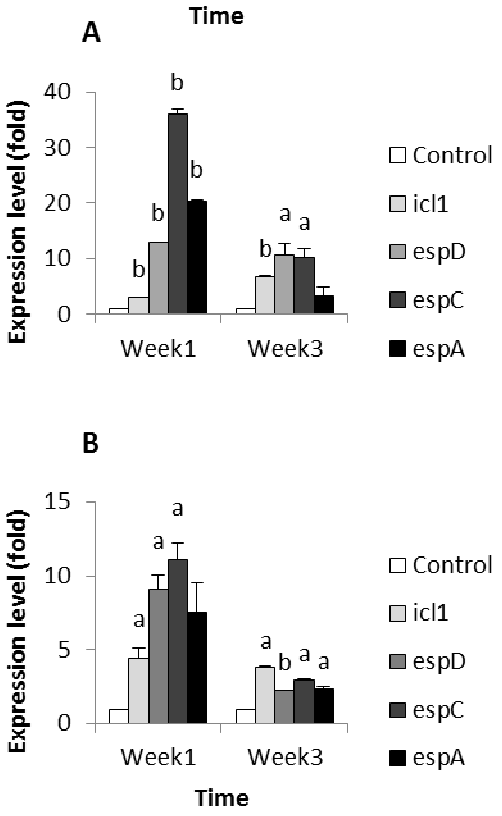

Figure 2 Gene expression level in acidic pH 4.5 (A) and pH 5.9 (B). Quantitative real-time RT-PCR was used to measure transcriptional levels of selected genes potentially involved in acidify in Mtb H37Rv. The expression level was determined in cultured of acidic pH 4.5 and pH 5.0, for 1 and 3 weeks, compared to the expression in normal medium (control). The data presented in a mean of expression fold changed which normalized by the expression level of 16S rRNA. a: p-value < 0.05; b: p-value <0.001 (T-test).

Optimization of protocol including primer design, RNA extraction, cDNA synthesis and PCR were successful as shown in the PCR products Fig. 1.

Relative transcriptional level of acidic-stress responsive genes was determined by quantitative Real-time PCR. The bacteria were cultured in acidic pH 4.5 and pH 5.0 compared to the culture in normal growth condition and 16S rRNA gene was used as the internal control (Fig. 2). All selected genes show significant up-regulated in acidic growth condition especially in week 1 showed up-regulation higher than the week 3 except *icl*1. The *esp*C gene was found highest up-regulation in both pH of week 1. Whereas, the lowest up-regulation among the selected genes were *icl*1 in week 1 and *esp*A in week 3.

Discussion and Conclusions

The expression level in acidic-stress condition of selectedMtb genes were significant up-regulated. This result confirmed the previous study using microarray screening method (Deb et al., 2009). In both pH, the expression levels of all genes in week 3 were also up-regulated but lower than those in week 1 except *icl*1. According to the plate count result, the bacteria were in NRP stage in week 3, this change in growth stage might cause the lower level of gene expression. Even almost of genes were down-regulated in week 3 but the icl1 was up-regulated in pH 4.5 and a bit down-regulated in pH 5. Previous study reported that this gene facilitated persistence of Mtb in mice. Disruption of the gene attenuated bacterial persistence and virulence in immune-competent mice (McKinney et al., 2000)

In normal growth condition, the selected genes were expressed in a certain level. This indicated that these genes have basal expression level, and they are induced in this such as stress condition for survival or adaptation.This group of genes showed higher up-regulation in pH 4.5 compared to those of pH 5. This result indicated the essential of these genes in adaptation during growth in the acidic condition.

These genes could be further studied in another stress condition for being markers of multiple-stress condition or latent stage infection. Moreover, even the functions of these genes can be predicted, molecular mechanism of these genes involved in adaptation during growth in stress condition are needed for further understanding.The latent stage associated genes can be used for the development of new TB treatment and vaccine targets.

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