ORIGINAL ARTICLE

OPTIMIZATION OF RNA EXTRACTION IN *MYCOBACTERIUM TUBERCULOSIS* FOR STUDYING INTRACELLULAR GENE EXPRESSION.

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Abstract

Mycobacterium tuberculosis **is the leading cause of death due to infectious disease after Human immunodeficiency virus. There has been an upsurge in the incidence of tuberculosis since 1980s. In order to reverse this trend, there is need to understand the biology of the organism. This can be brought about by studying gene expression at transcriptional level. The success of this hinges on RNA of good quality. In this paper, five methods (hot phenol, sonication with guanidinium thiocyanate (GTC) solution, beadbeating method with Trizol, FastPrep machine with Divolab as detergent and GTC solution, and FastPrep machine with Trizol) of extracting RNA from bacteria were compared to find which of the method would be suitable for mycobacteria. The study found that physical method of lysing bacteria was necessary for extraction of RNA from mycobacteria. FastPrep machine gave the highest yield and also provided the speed necessary for optimum RNA extraction. FastPrep and Trizol as reagent for extraction of RNA was applied to macrophage infected with** *M. tuberculosis* **(H37Rv) after removing the macrophage RNA. We were able to demonstrate the expression of** *dnaK* **gene in both intracellular and broth grown bacilli. The expression of** *dnaK* **gene was found to be downregulated in macrophage compared to broth**.

Introduction

Mycobacterium tuberculosis is ranked among the leading cause of mortality and morbidity due to infectious disease. In 2006, 9.2 million people was estimated to be new cases of tuberculosis (TB) worldwide – an increase of 0.6% from previous year with 1.7 million people died from the disease [1]. Even despite the availability of anti-tuberculosis drugs, this bacterium still continue to claim more lives than any other infectious agents put together with the exception of Human Immunodeficiency virus (HIV). The resurgence in the incidence of tuberculosis since 1980s is partly due to HIV infection and the emergence of multi-drug resistance *M. tuberculosis* complex. To make matter worse, there is no effective vaccine that can curb the menace. The present Bacille Calmette Guerin (BCG) vaccine has been found to have variable vaccine efficacy [2]. For example, the BCG vaccine efficacy has been found to be 0% in India and close to 90% in United Kingdom[2-4]. This variability in vaccine efficacy has been attributed to environmental mycobacteria.

Some countries are not predisposed to the use of the vaccine for some reasons like the inability to monitor the immune status of the individuals exposed to mycobacteria.

In view of these, there is need for more anti-tuberculosis drug and new effective vaccine. But in order to do this, there is need for novel way of identifying drug target(s) and vaccine candidate. Identifying possible drug targets and vaccine candidates have been made easy with completion of *M. tuberculosis*'s genome [5] and other species of mycobacteria [6, 7]. The genetic system for studying basic biology of mycobacteria is not well developed and where it is developed, it is not easy to apply. This has been the cog in the wheel of progress. Studying gene expression of mycobacteria could provide an alternative in the sense that there is no need for well developed genetic system. Gene expression can be studied either at transcriptional level or translational level. RNA is a basic ingredient for studying gene expression at transcriptional level. RNA extraction with good quality and yields is the cornerstone of transcriptional study. There are many RNA extraction methods available but majority of them are not applicable to mycobacteria because of the inherent toughness of mycobacteria to lysis solutions that are used for lysing cells. Some of the methods do not take into consideration of the short half life of bacterial RNA. For example, the half life

of *Escherichia coli*'s mRNA has been found to be less than 1 minute. Mangan et al. (1997) developed RNA extraction for *M. tuberculosis* using a special detergent and guanidium thiocyanate [8], which might not be applicable to some of the transcription-based studies like microarray. The aim of this study was therefore to optimise RNA extraction methods for studying gene expression in *M. tuberculosis* that can be found applicable for functional genomics like microarray and individual gene expression studies.

Materials and Methods

Maintenance and culture of mycobacteria

Mycobacterial species used in this study were *M. bovis* BCG (Statens Seruminstitut vaccine strain ST1077, obtained from Evans Medical Ltd., Leatherhead, UK) and *M. tuberculosis* H37Rv (NCTC 7416/ATCC 9360) obtained from the National Culture Type Collection, Colindale, UK. Mycobacteria were grown to mid log or late log phase in Middlebrook 7H9 broth (Difco Laboratories Ltd., West Molesey, UK) supplemented with 10% albumin-dextrose-catalase enrichment (ADC; Difco) and 0.02% Tween 80 (Sigma, Poole, UK) at 37°C (in the presence of 5% CO_2 for *M. tuberculosis)* before harvesting at 0.5-1.0 OD_{600}. *M. tuberculosis* H37Rv cultures were also grown for 3 weeks at 37°C on thick 7H10 agar plates supplemented with 10% oleic acid-

albumin-dextrose-catalase enrichment (OADC; Difco) and 0.2% glycerol and then stored at 4°C. For long term storage, *M. bovis* BCG broth cultures were stored at -80°C. All work involving *M. tuberculosis* was performed in a Class I biohazard safety cabinet under Category 3 containment conditions.

Hot phenol method

Several RNA extraction methods were used to determine the optimal method for extracting RNA from mycobacteria. All equipment and solutions used in these procedures were treated with 0.1% diethyl pyrocarbonate DEPC (Sigma) as described before [9].

The hot phenol method of RNA extraction was based on the method of Von Gabain *et al.* [10]. Five millilitres of *M. bovis* BCG bacilli representing 1×10^8 colony forming units (cfu) per ml were pelleted by centrifugation at 5,000 ×g for 1 min and the supernatant was discarded immediately. The cell pellet was resuspended in 400 µl RNA lysis solution (0.15 M sucrose, 10 mM sodium acetate [pH 5.2], 1% (w/v) SDS) and quickly transferred to an Oakridge tube containing 4 ml of hot (65°C) phenol. The bacterial suspension was incubated for 10 min at 65°C with inversion of the tube twice during the incubation period. The sample was aliquoted into 1.5 ml Eppendorf tubes and centrifuged at 10,000×g for 5 min at 4°C. The supernatants were transferred to fresh

tubes containing 500 µl of phenol. The tubes were inverted several times and incubated at 65°C and centrifuged as before. The supernatant was transferred to a fresh tube containing 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1), vortexed briefly and centrifuged. The resultant supernatant was transferred to fresh tubes containing 500 µl chloroform: isoamyl alcohol (24:1), vortexed briefly and centrifuged at 10,000×g for 5 min. The supernatants were pooled and 100 µl of 3 M sodium acetate (pH 5.2; final concentration 0.3 M) was added prior to RNA precipitation. The RNA was precipitated with 2.0-2.5 volumes of absolute ethanol and placed at −20°C for at least 30 min before centrifugation at 10,000×g for 10 min at 4°C. The ethanol was discarded and the RNA pellet was suspended in 75% ethanol, centrifuged briefly, and discarded. The RNA was air dried for 15 min before being dissolved in 10 µl of DEPC treated water.

Sonication method

This method was based on the methods of Patel *et al.* [11] and Chomcynski and Sacchi [12]. One and half millilitres of *M. bovis* BCG broth culture representing 5×10^8 cfu/ml were pelleted by centrifugation at 10,000 ×g for 30 s. The bacterial pellet was immediately resuspended in 1 ml of 4 M guanidinium thiocyanate (GTC) lysis solution (containing 0.5% sodium N-

laurylsarcosine, 25 mM sodium citrate [pH 7], and 0.1 M 2-mercaptoethanol) by vigorous vortexing followed by immediate sonication using a sonicator (Heat Systems Ultrasonics) with an ultra-thin probe (4mm diameter) for 45 s with the control set at position 2. Following sonication, each tube was immediately subjected to continuous vortex mixing for 10-15 min. The lysate was left at room temperature for 3-4 hr with intermittent vortex mixing every 30 min for 3 min. Separation of RNA after bacterial lysis was performed essentially as described before [12]. The lysate was split between two 1.5 ml Eppendorf tubes and 50 µl of 2 M sodium acetate (pH 4), 0.5 ml of water saturated phenol (Invitrogen, UK) and 0.1 ml of chloroform-isoamyl alcohol (49:1) were added to the homogenate with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min. Samples were centrifuged at 10,000 ×g for 20 min at 4°C. After centrifugation, RNA present in the aqueous phase (DNA and proteins are present in the interphase and phenol phase) was transferred to a fresh tube and mixed with 0.5 ml of isopropanol to precipitate the RNA. The RNA was pelleted by centrifugation at 10,000 ×g for 20 min and dissolved in 150 µl of GTC solution and precipitated with 1 volume of isopropanol at –20°C for 1 hr. After centrifugation in a

microcentrifuge for 10 min at 4°C, the RNA pellet was resuspended in 75% ethanol, sedimented, air-dried, and dissolved in 20 µl DEPC treated water at 65°C for 10 min.

Beadbeating method

The sonication method was unsuitable for RNA extraction from *M. tuberculosis* for safety reasons due to the generation of aerosols. The use of a beadbeater circumvented this problem, as it is a closed system and could be placed in a Class I safety cabinet during its operation. One millilitre of *M. bovis* BCG culture was centrifuged at 12,000 ×g for 1 min. The cell pellet was resuspended in 1 ml of Trizol™ (Life Technologies). The suspension was transferred to a 2 ml skirted tube containing approximately 0.5 ml of 0.1 mm-size zirconia/silica (Biospec, Luton, UK) to aid the disruption of the mycobacterial cells. This suspension was beadbeater at 5,000 r.p.m for 40 s. Immediately the tube was placed on ice for 5 min before adding 0.25 ml of chloroform and mixed by vortexing for several seconds. The lysate was allowed to stand for 2 min before centrifugation at 12,000 ×g for 15 min. The upper aqueous phase was transferred to a fresh 1.5 ml Eppendorf tube and the RNA was precipitated with 0.5 ml of isopropanol. The solution was left at room temperature for 10 min before centrifuging at 12,000 ×g for 10 min. The supernatant was discarded and the RNA pellet was resuspended in 75% ethanol and centrifuged for 5 min at 8,000 ×g. The

supernatant was removed and the RNA pellet was air dried before dissolving in 20 μl of DEPC treated water.

FastPrep™ method

The FastPrep™ machine FP120 (BIO 101 Savant, obtained from Anachem Ltd., Luton, UK) was used as a physical means of disrupting the mycobacterial cells. The advantage of this machine over the bead beater is that multiple samples can be processed at any one time. RNA was extracted using a modification of Cheung *et al.'s* method [13]. *M. tuberculosis* H37Rv bacilli in mid-log phase were centrifuged at 10,000× g for 1 min and the pellets were resuspended in Trizol™ reagent. The cell suspensions were transferred to a 2 ml microcentrifuge tube to which 0.5 ml of zirconia/silica beads (0.1 mm-size) had previously been added. The tubes were shaken in a FastPrep™ machine at 6000 rpm for 20 s. Two hundred microlitres of chloroform were added to the lysate, shaken for 15 s and left at room temperature for 3 min. The mixture was then centrifuged at 12,000× g for 15 min at 4°C. The RNA in the aqueous phase (top layer) was precipitated with 0.5 ml of isopropanol and pelleted (12,000× g for 10 min). The pellet was washed with 75% ethanol, air dried for 10 min and resuspended in 10 μl of DEPC treated water.

FastPrep™/Divolab™ method

This method was developed by Mangan *et al*. [8] and it also makes use of the FastPrep™ machine but a different detergent (Divolab™) is used. This method enabled efficient lysis of the mycobacteria and recovery of RNA from the lysate resulting in a higher yield of RNA and lower DNA contamination compared to the previous methods. A culture of *M. tuberculosis* bacilli was harvested by centrifugation at 12,000 ×g for 20 s in a microcentrifuge, washed in 0.5% Tween 80 solution, repelleted and resuspended in 200 μl DEPC treated water. To a FastRNA.Kit-Blue tube [a 2 ml skirted screw-capped microcentrifuge tube with 'O' ring seal, containing acid washed 0.1 mm silica/ceramic beads] (BIO 101, obtained from Anachem Ltd., Luton, UK) were added: 500 μl detergent solution (9.6 ml Divolab™ No.1 [Diversey Hygiene, Northampton, UK], 24 ml 500 mM sodium acetate pH 4.0, 66.4 ml DEPC treated water), 500 μl acid phenol (water saturated phenol equilibrated with 50 mM sodium acetate pH 4.0), 100 μl chloroform/isoamyl alcohol (24:1) and 200 μl resuspended bacterial pellet. The tube was immediately processed in the FastPrep™ machine for 45 s at a 6.5 speed setting and left on ice for 10 min. The cell debris was removed by centrifugation at full speed in a microcentrifuge for 10 min. The aqueous phase was removed and the RNA precipitated by the addition of 500 μl

solution (0.3 ml 3 M sodium acetate pH 4.0, 49.7 ml isopropanol) at -80°C for 30 min. The sample was pelleted by centrifugation for 15 min, air dried and the RNA was dissolved in 20 μl of DEPC treated water.

DNase I treatment and testing for DNA contamination

All RNA samples were DNase I treated to destroy any contaminating DNA by incubating the RNA samples in the presence of DNase I (Pharmacia Biotech, St Albans, UK), 50 μg/ml bovine serum albumin (BSA), 60mM Tris-HCl [pH 7.5], and 10 mM $MgCl_2$ at 37°C for 30 min. The samples were transferred to a fresh Eppendorf tubes and the DNase I was inactivated by incubating the samples at 65°C for 15 min. All RNA samples were then stored at -80°C. To confirm that there was no DNA contamination, a PCR (40 μl reaction volume) was performed (see section 2.5) with the RNA samples using primers $P_{71KDTB1}$ (5′-ATTGTGCACGTCACCGCCAA-3′) and $P_{71KDTB2}$ (5′-ACCGCGGCATCAACCTTGTT-3′) to amplify a 275 bp fragment of the *M. tuberculosis dnaK* as previously described [11]. PCR was conducted for 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 2 min) and extension (72°C, 3 min) followed by 1 prolonged cycle of extension (72°C, 7 min) using a Hybaid thermal cycler. The PCR products were fractionated through a 1% agarose gel.

Determination of the yield and integrity of RNA

RNA was quantified spectrophotometrically as previously described [14]. Five microlitres of RNA was diluted 1:200 in 1 ml of distilled water. The OD was read at 260 nm and 280 nm with water as the reference. The concentration of RNA was calculated based on an OD_{260} reading of 1.0 corresponds to approximately 40 μg /ml. Thus, the concentration of RNA per ml = OD_{260} reading × dilution factor × 40 μg/ml. The purity of the RNA was determined by the OD_{260}/OD_{280} ratio where a ratio of 2.0 indicates pure RNA. The integrity of the RNA was determined by agarose gel electrophoresis against a RNA marker (BDH) comprising the eukaryotic 28S, 18S, and prokaryotic 23S, 16S, and 5S rRNA species.

RNA/DNA samples were fractionated through 1-2% agarose gels. The samples were loaded onto agarose gel with the aid of 10% of 10 × loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 5% sucrose, and 50% glycerol) with appropriate DNA/RNA size markers. Electrophoresis was performed with 0.5 × TBE buffer typically for 1 hour at 75 v in a horizontal gel electrophoresis apparatus (Invitrogen, UK) before visualisation/photography of the RNA/DNA samples with UV transillumination at 302 nm. Where appropriate, formaldehyde agarose gel

electrophoresis was performed to analyse some of the RNA samples.

Macrophage infection conditions and preparation of RNA

J774.2 macrophages infected overnight with 10:1 non-opsonised *M. tuberculosis* bacilli were used (typically, 5 x 75cm^2 tissue culture flasks). A control comprised *M. tuberculosis* bacilli growing in Middlebrook 7H9/ADC/Tween 80 broth. RNA was prepared from macrophages infected with *M. tuberculosis* using a method based on a differential lysis method kindly provided by Prof. P. D. Butcher (St. George's Hospital Medical School, London). The culture medium was discarded and the infected macrophage monolayer was resuspended in 25 ml guanidine thiocyanate solution per flask to lyse the macrophages. In order to reduce the viscosity of the solution, a long thin nosed plastic Pasteur pipette was used to squirt the lysate solution in and out with force to shear the macrophage nucleic acids. The lysates were transferred to 30 ml sterile universal tubes and centrifuged at 2,500×g for 20 min to concentrate the intracellular bacilli. The pellets of intracellular bacilli were combined using 1 ml of wash solution (1 ml 0.5 % Tween 80) and transferred to a 1.5 ml Eppendorf tube and centrifuged in a microcentrifuge at 12,000×g for 30 s. The supernatant (wash solution) was saved and placed in a fresh Eppendorf tube and stored at -80°C. The pellet of bacteria was resuspended in 200 μl of sterile DEPC

treated water. The RNA was extracted from the harvested intracellular bacilli and the extracellular bacilli control using Trizol (Invitrogen, UK) following manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription PCR was used to determine the presence of RNA transcripts in samples of interest. Total RNA (typically 250 ng) was reverse transcribed in a 20 μl reaction volume containing 0.5 mM each of dATP, dGTP, dCTP and dTTP with 1 μM of gene specific downstream primer and 100 U Superscript™ RNase H⁻ reverse transcriptase (Invitrogen, UK) in a reaction buffer of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl$_2$ and 10 mM dithiothreitol following the manufacturer's instructions. Briefly, the RNA sample, and the primer were incubated together for 15 min at 70°C and chilled on ice before adding the dNTPs (total concentration in the reaction was 0.5 mM), reaction buffer. This was incubated at 42°C for 2 min before the addition of the reverse transcriptase and cDNA synthesis was then performed at 42°C for 50 min. The reaction was stopped by incubating the samples at 70°C for 15 min. One tenth of the cDNA was then used in the PCR. The cDNA was added to the PCR reagents containing 1 μM of each gene specific primer (upstream

and downstream primers). PCR was performed as before.

Results

Several RNA extraction methods (a 'standard' method and some specifically designed to extract RNA from mycobacteria) were evaluated to determine which gave the best yield of RNA from mycobacteria. The main objective was to obtain a high yield, non-degraded RNA that would provide good transcripts for cDNA synthesis for RT-PCR analyses. The fact that prokaryotic mRNA has a very short half life of 1-3 minutes [15] was an important factor in terms of harvesting the bacilli as rapidly as possible to stabilise the mRNA and prevent as much as possible changes in gene expression. The quality of the RNA obtained using the different extraction methods are shown in Figures 1a-d and the yields are summarised in Table 1. Spectrophotometric analysis to determine purity and yield was used but where this was not possible (where small volumes were obtained, for example), agarose gel electrophoresis of the sample along side a known amount of RNA was used to determine the concentration of RNA. Difficulty in assessing the RNA yield by spectrophotometry was particularly problematic when using FastPrep™/Divolab™ method of extraction. Unreliable readings were generated and this may have been due to the detergent or the lysis buffer used to lyse the bacilli.

Table 1. Differences in the yields of RNA using different RNA extraction methods

Method	RNA yield (µg)	Number of mycobacteria used cfu/ml
Hot phenol	Nil	5×10^8
Sonication	0.50 -1.00	5×10^8
Beadbeater	0.75 - 1.50	5×10^8
Fastprep□□/Trizol□□	1.75 - 2.00	5×10^8
Fastprep□□ /Divolab□□	4.50 - 8.00	4×10^8

The RNA yields obtained with the various methods ranged from 1 µg per 5×10^8 cfu/ml of mycobacteria using the sonication method to approximately 6 µg using the FastPrep™/Divolab™ method (see Table 1). No RNA was detected using the standard method of extracting RNA from *E. coli* (the hot phenol method) reflecting the differences in the nature of the cell wall components of mycobacteria. It was clear that for efficient extraction, rapid lysis of the mycobacteria and inactivation of the

ribonucleases was essential and this could only be achieved by using the mechanical means of disrupting the bacilli in a solution containing ribonuclease inhibitors such as phenol or guanidium thiocyanate. No RNA was obtained from the methods that did not use a mechanical means of lysing the bacteria. The mark of a successful RNA extraction method is that the 16S rRNA and 23S rRNA species should be visible following formaldehyde or standard agarose gel electrophoresis. Even the RNA extraction method that produced the lowest yield of RNA (see Table 1) revealed the 16S rRNA and 23S rRNA as determined by formaldehyde gel electrophoresis (see Figure 1a). None of the methods used gave RNA free of indigenous DNA contamination (as determined by PCR of the RNA for the constitutively expressed *dnaK* gene), but contaminating DNA could be readily removed with DNase I. This step was very important in order to rule out false positive results that might be generated in the subsequent gene expression studies. To determine the integrity of the RNA extracted, RT-PCR was performed for *dnaK* and its expression was demonstrated in all samples from all the RNA extraction methods (Figure 2). RT-PCR was not performed with RNA obtained using methods that failed to reveal the presence of 16S rRNA and 23S rRNA bands.

In order to determine the main factor that influenced the efficient recovery of RNA from mycobacteria, a comparative assessment was carried out on all the RNA extraction methods using Trizol™ as the sole reagent to lyse *M. tuberculosis* and the samples were subjected to the different mechanical means of disrupting the cells (i.e. sonication, beadbeater and the Fastprep™ machine). RNA was then extracted using the procedure outlined in the beadbeating method. The FastPrep™ machine gave the highest recovery of RNA from mycobacteria while sonication method gave the least amount of RNA (Figure 3) and this efficiency could be increased further with the use of the DivolabM detergent as described in Mangan *et al.'s* method [8]. Furthermore with this method, the RT-PCR for *dnaK* showed there were more transcripts from the same volume of RNA input used in the cDNA synthesis and subsequent PCR step (Figure 3). The disadvantage of this method was the fact that only a few of the samples could be used to generate cDNA because the reverse transcriptase appeared to be inhibited by the Divolab™ detergent (data not shown). Any effort to remove the detergent resulted in the loss of RNA, which suggests that a chemical complex formation between the RNA and the detergent. Furthermore, spectrophotometric analysis to determine the RNA concentration was not possible because of this inherent problem. However, the FastPrep™/Divolab™ method was

chosen over the other methods because of the safety aspect of the operation, the higher recovery of RNA, and the consistency of the method in obtaining RNA from mycobacteria. The FastPrep™/Trizol could be used instead of FastPrep™/Divolab™ since this method did not give much inhibition of the reverse transcription process. Based on this fact, the FastPrep™/Trizol was used in extracting RNA from infected macrophages. The result (Figure 4) showed that the presence of 16S and 23S was visible on ethidium bromide stained agarose gel from RNA extracted from intracellular bacilli, but not as visible as broth grown bacilli. To ascertain the validity of using RNA from infected macrophages, competitive RT-PCR was performed to compare the expression of *dnaK* gene during intracellular infection of macrophages with broth grown *M. tuberculosis* at transcriptional level, which had previously been shown to be upregulated during intracellular infection at the protein (translational) level (Lee and Horwitz, 1995) was studied. The *dnaK* expression was investigated by RT-PCR to confirm that this gene was expressed intracellularly prior to proceeding with a competitive RT-PCR. The result showed that there was expression of *dnaK* gene by the intracellular and extracellular broth-grown

bacilli (Figure 5a). Further experiment was carried out to compare the expression of *dnaK* after equalising the RNA by competitive PCR for 16S gene, results showed that there was no much difference in the level of *dnaK* expression between the intracellular and broth-grown bacilli (Figure 5b) as the native *dnaK* amplicon shared equal intensity with the second dilution of the competitive *dnaK* amplicon in both the broth-grown and intracellular bacilli, respectively. .Quantitatively, the level of expression was found to be 1.6×10^{-4} pg in broth grown bacilli while that of intracellular macrophage was slightly less than 1.6×10^{-4} pg but more than 7.8×10^{-5} pg. In fact, if anything the gene appeared to be downregulated in intracellular bacilli after the overnight (15 hr) infection period compared to broth grown bacilli.

Discussion

RNA extraction is the cornerstone of RNA based gene expression analysis. Working with RNA can be very problematic because of the ubiquitous nature of the ribonucleases that destroy the RNA. Over the past decades this major problem has been solved with the introduction of ribonuclease inactivating agents such as DEPC, RNasin, vanadyl-ribonucleoside complexes, guanidium hydrochloride, and guanidium thiocyanate [9].
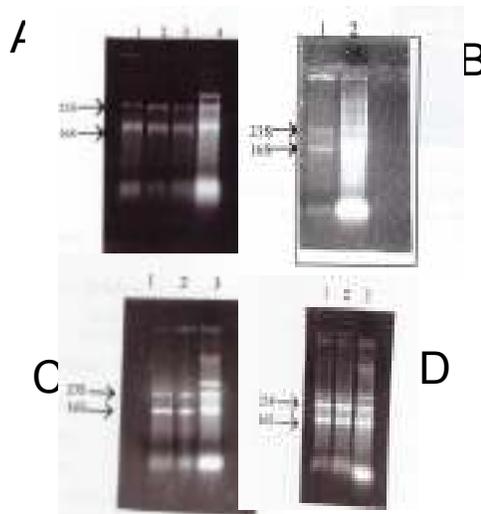
Figure 1a. RNA extracted using sonication method. Formaldehyde agarose gel electrophoresis of RNA. Lanes 1-3: RNA extracted from 9.6×10^9 *M. bovis* BCG cfu/ml (~ 2 µg RNA); lane 4: RNA size marker (5 µg).

Figure 1b. RNA extracted by the beadbeater/Trizol™ method. Formaldehyde agarose gel electrophoresis of *M. bovis* BCG RNA. Lane 1: RNA extracted from 1.0×10^8 BCG cfu/ml (~0.7 µg); lane 2: RNA size marker (2 µg).

Figure 1c. RNA extracted by the FastPrep™/Trizol™ method. Electrophoretic analysis of RNA extracted by the FastPrep™/Trizol™ method. Lanes 1 and 2: RNA extracted from 1.2×10^{10} *M. bovis* BCG cfu/ml (~1.5 µg); lane 3: RNA size marker (2 µg).

Figure 1d. RNA extracted by the FastPrep™/Divolab™ method. Electrophoretic analysis of RNA extracted by FastPrep™/Divolab™ method. Lanes 1 and 2: RNA extracted from 1.6×10^8 *M. bovis* BCG cfu/ml (~3 µg); lane 3: RNA size marker (2 µg).
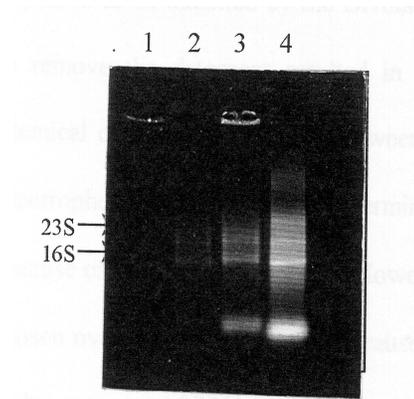

Figure 2. RNA extracted from *M. bovis* BCG using different physical methods of cell disruption. Electrophoretic analysis of RNA extracted from 1.5×10^8 cfu/ml of *M. bovis* BCG showing the yields of RNA obtained using the sonication method (lane 1: traces), beadbeating (lane 2: 0.5 µg) and FastPrep™ method (lane 3: 1.2 µg) Lane 4 : RNA size marker (2 µg).
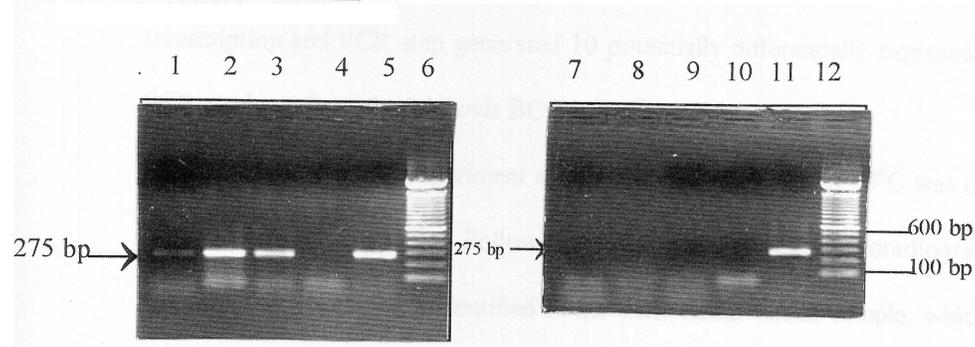

Figure 3. RT-PCR for demonstration of *dnaK* expression using RNA extracted by the different physical methods of cell disruption. Electrophoretic analysis of the RT-PCR products generated with RNA prepared from the sonication, FastPrep™, and beadbeating methods, respectively. Lanes 7, 8, and 9: DNA contamination controls for the sonication, FastPrep™, and beadbeating methods, respectively. Lanes 4 and 10: negative PCR controls; lanes 5 and 11: positive controls; and lanes 6 and 12: 100 bp DNA ladders. The arrows indicate *dnaK* 275 bp product.
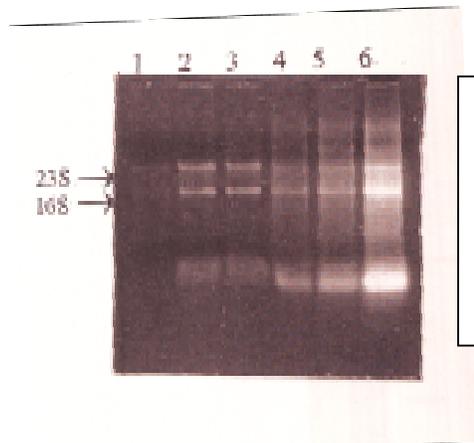
Figure 4. Typical yield of RNA from intracellular versus extracellular broth-grown M. tuberculosis (H37Rv). Agarose gel electrophoresis of RNA extracted from intracellular and extracellular broth grown *M. tuberculosis* by the FastPrep™/Trizol™ method. Lane 1: RNA from intracellular bacilli; lanes 2 and 3: RNA from broth grown bacilli; lanes 4-6: RNA size markers (1 µg, 2 µg, and 5 µg, respectively).
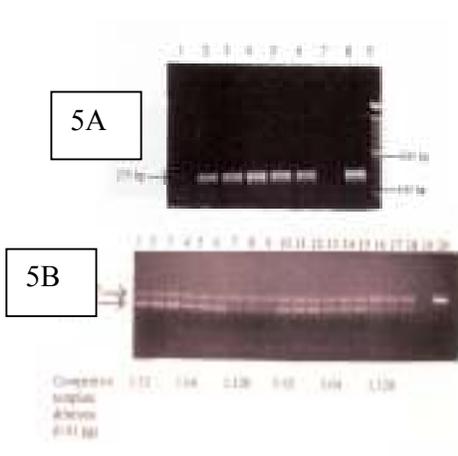

Figure 5a. Expression of *M. tuberculosis dnaK* gene during intracellular infection of macrophages. Electrophoretic analysis of RT-PCR products for *dnaK*. Lanes 1, 2, and 3: PCR products from ½, ¼, and ⅛ dilutions of the cDNA from extracellular broth grown bacilli, respectively. Lane 7: negative PCR control; lane 8: positive PCR control and lane 9: 100 bp DNA ladder. The arrow indicates the 275 bp *dnaK* product.
Figure 5b. Quantification of *dnaK* gene expression in intracellular and broth grown *M. tuberculosis* by competitive RT-PCR. Electrophoretic analysis of competitive RT-PCR products for *dnaK* gene. Various amounts of *dnaK* competitive DNA competed against a fixed concentration of native *dnaK* cDNA. Lanes 1-9 intracellular bacilli (in triplicate dilutions of the competitive template); lane 10-18: extracellular broth-grown bacilli (in triplicate dilutions of the competitive template); lanes 19 and 20: PCR negative and positive controls, respectively. The 275 and 195 bp products are the native and competitive amplicons, respectively.

Bacterial mRNA has a very short half life of approximately 1-3 min [15] which means speed is essential for the successful recovery of intact mRNA and the protection of RNA from ribonuclease (RNAse) is very important. Gene expression is accompanied by the transcription of a segment of DNA into mRNA, which later undergoes translation to protein. The way bacteria respond to their new environment is always accompanied by the expression of a new set of genes [16]. Therefore, the ability to isolate the necessary mRNA representing the genes expressed is very important.

A good RNA extraction method should be able to extract mRNA representing the genes being expressed at a particular point in time. However, not all the extraction methods can achieve this because of differences between microorganisms. The time taken to lyse mycobacteria by standard enzymatic methods (such as the use of lysozyme and detergent) will take longer than spontaneous lysis afforded by any of the machines used in this study. For example using lysozyme to lyse *E. coli*

with the hope of recovering RNA and the time involved will result in changes in gene expression and longer exposure times to RNAses and thus will not reflect true gene expression at a given time point. It is therefore important to use a method, which could lyse the mycobacteria rapidly. The first method used in this study was the hot phenol method devised for organisms such as *E. coli* and is based on the use of detergent to lyse the organism and phenol to protect the RNA from degradation. However, the fact that the RNA obtained was degraded and of a poor yield indicated that this method was inappropriate for extracting RNA from mycobacteria. The main reason for this probably lies in the differences in the mycobacterial cell wall architecture, which make mycobacterial cells more resistant to passive lysis. RNA extracted using mechanical means of lysing the bacilli was far more successful. The sonication method [11] gave good yields but failed to address the safety aspect of sonicating Category 3 pathogens and this was problematic when it comes to using *M. tuberculosis.* Moreover, the technique was time consuming. This problem was overcome by the use of the beadbeater (which also gave a better yield than the sonicator) as its compact size means it can be operated within the biohazard safety cabinet. The major drawback is that only one sample can be processed

at a time. The introduction of the Fastprep™ reciprocal shaker several years ago by Savant Instruments offered a means to process many samples at a time. However, the use of this equipment still requires the use of an appropriate lysis solution to facilitate the maximum recovery of RNA from mycobacteria. The use of the lysis solution developed by Mangan *et al.*, [8] which comprised the detergent Divolab™ was found to improve the RNA yield considerably. The major disadvantage of this method was that spectrophotometric estimation of RNA concentration was not possible, which meant that other methods of estimating the RNA yield had to be used. The *M. tuberculosis dnaK* gene encoding the HSP71 protein was chosen to evaluate the use of RNA obtained by FastPrep/Trizol method for studying gene expression. The fact that there was no increase in the level of *dnaK* expression suggests that *dnaK* is not upregulated after 15 hr of infection. This is contrary to a report where it was found to be one of the most abundantly expressed genes during intracellular infection of macrophages [17]. The discrepancy between these results probably reflects the fact that these authors studied the differential expression of *dnaK* at the translational level whereas this study determined its expression at the transcriptional level. This suggests that the control of

expression of *dnaK* is most likely to be regulated at the translational level. The HSP71 protein is a stress protein and has shown to be upregulated in response to increase in temperature *in vitro* [11]. If *dnaK* was upregulated during intracellular infection it would be expected to have a specific function in relation to the intracellular environment of macrophages. It has been shown for a wide variety of bacterial pathogens that virulence and other factors, which play a role in host-parasite interactions, are co-ordinately regulated with heat shock proteins. These stress proteins are expressed as overlapping subsets of proteins in response to environmental stimuli likely to be encountered within the infected host [16, 18].

The improvements in extracting RNA from *M. tuberculosis* made throughout the study made it possible to achieve the objective of studying the gene expression of *M. tuberculosis* during intracellular infection of macrophages as demonstrate by the ability to determine the expression level of *dnaK* gene. With this RNA extraction method (FastPrep/Trizol), it is now possible to study whole genome expression by microarrays or individual gene expression, which could provide more information in annotating some of the genes of unknown function in completed genome sequence of *M. tuberculosis* [5] and other species from the same genus. Recent study from the authors' laboratory has shown it is possible to use the RNA extracted with FastPrep/Trizol to study individual gene expression [19].

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