

**The Development of a Rapid Diagnostic System for Difficult to
Culture Human Pathogens**

By

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LIST OF TABLES

Table		Page
1.1	An average estimation of the burden of disease caused by tuberculosis in 2011 reported by WHO. This summary has been adapted from Global Tuberculosis Report 2012 (www.who.int/tb/publications/global_report/) and represents the rate per 100,000 populations ^a (Dias et al. 2012).	6
1.2	An estimation of MDR-TB cases reported in 2011 from WHO regions. This data was adapted from the Global Tuberculosis Report 2012 (www.who.int/tb/publications/global_report/). Direct measurements are still in progress therefore, the data supplied is estimation from the actual incidence (Dias et al. 2012).	9
1.3	List of selected <i>Mycobacterium</i> spp. showing variation in size and genome composition from species to species. This data was analysed and summarized from Genome Atlas Database (http://www.cbs.dtu.dk/services/GenomeAtlas).	14
1.4	Most frequent human diseases caused by common RGM group/species.	21
1.5	Commercially available NAA assays for the detection of <i>Mycobacterium</i> spp. from clinical specimens.	34
3.1	List of primers used in this study for detection of <i>M. smegmatis</i> (NCIMB8548), environmental isolates VS/02 and genomic DNA from <i>M. tuberculosis</i> (ATCC [®] 25177).	99
3.2	The purity and concentration of DNA extracted from archived slides by using the scraping techniques.	117
3.3	The DNA amplification correlates with fixation, staining and DNA extraction methods by using nested PCR.	122
3.4	Average of C _q value of samples detected using nested real-time PCR from primary amplicons of enumerated <i>M. smegmatis</i> cells catapulted from archived glass slides. The C _q values correlate with the numbers of cells successfully catapulted and primarily amplified using touchdown PCR.	146

LIST OF TABLES

Table		Page
3.5	The concentration and purity of DNA extracted using different methods and subjected to a pre-amplification step using MDA REPLI-g [®] UltraFast Mini kit.	151
3.6	Comparison of the sensitivity for detection of <i>rpoB</i> gene from <i>M. smegmatis</i> cells, isolated from archived glass slides, using nested touchdown and nested real-time PCR detection assay.	155
4.1	List of primers used in this study for detection of <i>M. smegmatis</i> (NCIMB8548) for RNA based work.	184
4.2	The concentration and purity of RNA extracted from two isolates of <i>M. smegmatis</i> and determined using the NanoDrop 2000 spectrophotometer.	194
4.3	The concentration and purity of RNA extraction using combined methods of Lysing matrix-A step, GeneJet kit, gDNA eliminator spin column (RNAeasy Plus Mini kit) and “on-column” DNase treatment from <i>M. smegmatis</i> (NCIMB 8548), fixed on glass slides and quantified using the NanoDrop 2000 spectrophotometer.	196
4.4	The C _q values of primary amplicons detected using 16S rRNA gene specific primers for nested real-time PCR. The C _q values correlate with the RNA extracted from <i>M. smegmatis</i> fixed on glass slides by different types of slide processing, isolated and primarily amplified using PCR.	201
4.5	The C _q values of the primary amplicons detected using <i>rpoB</i> gene specific primers for nested real-time PCR. The C _q values correlate with the RNA extracted from <i>M. smegmatis</i> fixed on glass slides by different type of slides processing isolated and primarily amplified using PCR.	206
4.6	The C _q values of cDNA amplification using a 16S rRNA-sequence-specific probe. The C _q values correlate with the RNA extracted from <i>M. smegmatis</i> fixed on glass slides, by different types of slide processing, isolated and primarily amplified using PCR.	211

LIST OF FIGURES

Figure		Page
1.1	The incident rate of tuberculosis, reported from 1990 to 2011, from 22 classified high burden countries (HBCs) within the WHO region (Dias et al. 2012). These figures indicate that the tuberculosis incidence has been falling since the peak recorded in mid-1990s among 22 HBCs. This was for most countries except for several regions of Africa as shown from the graph pattern.	7
1.2	An estimated number of tuberculosis and death (in millions) reported between 1990 to 2011 globally (Dias et al. 2012). The falling rates of tuberculosis cases are shown globally from mid-2000. Despite the number of tuberculosis incidence cases increasing, death rates have been reportedly declining every year.	8
1.3	Schematic diagram of <i>Mycobacterium</i> showing the main components of the outer and inner layer of cell walls. The presence of mycolic acid in the complex of covalent-linked MA-AG-PG is a unique character for identification of the genus <i>Mycobacterium</i> and plays an important role as a permeability barrier (Medjahed, Gaillard & Reyrat 2010).	13
1.4	The flow-chart shows natural cause progression of tuberculosis and general plan-of-detection in immunocompetent and immunocompromised individual following exposure to tubercle bacilli by inhalation.	17
2.1 (a - b)	The morphology of <i>M. smegmatis</i> observed on Nutrient agar plates after 48 hours incubation at 37°C. (a) The small colonies with a white to yellow colour were observed on the Nutrient agar plate; (b) Negative control of sterile Nutrient agar in which no colonies had grown.	51
2.2 (a - d)	The <i>M. smegmatis</i> observed in broth growth media. Changes of broth turbidity were compared with the non-inoculated broth media (-ve control) incubated for 24 and 48 hours at 37°C. Pellicle and sediment were observed in both broth media inoculated by colonies and culture suspension respectively after 24 hours incubation. The sedimentation was obvious in the BHI broth after 48 hours. (a) The <i>M. smegmatis</i> grown in Nutrient broth observed after 24 hours; (b) The <i>M. smegmatis</i> grown in Nutrient broth observed after 48 hours; (c) The <i>M. smegmatis</i> grown in BHI broth observed after 24 hours; (d) The <i>M. smegmatis</i> grown in BHI broth observed after 48 hours.	52

LIST OF FIGURES

Figure		Page
2.3 (a - b)	The <i>M. smegmatis</i> cells incubated for 48 hours, observed under the LCM at 63× magnification. (a) The red coloured of <i>M. smegmatis</i> cells stained by using standard Ziehl-Neelsen procedures; (b) The colourless and the very light-red coloured <i>M. smegmatis</i> cells stained by using 37°C carbol-fucshin dye solution.	55
2.4	Growth curve of <i>M. smegmatis</i> in Nutrient and BHI broth measured by optical density (OD ₆₀₀) versus incubation time (hour). The doubling time is shown by the dashed arrow for both growth media. The t ₁ was 24 hours post-inoculation and t ₂ was the end of exponential growth.	57
2.5 (a – b)	Ziehl-Neelsen stained slides of <i>M. smegmatis</i> isolating process. This uses a scraping technique and showed that the largest amount of cells were recovered from the slides observed under LCM at 63× magnification. (a) Smear before isolation; (b) Smear after isolation by using the scraping method.	59
2.6 (a – b)	LPC application of LCM performed on the un-smear PALM [®] PEN-membrane slide which ensures the efficiency of LPC or the laser-cutting (microdissection) application. (a) Prior to laser pulse being applied; (b) LPC application applied shown by black arrows indicating the laser function.	62
2.7 (a – b)	The <i>M. smegmatis</i> smear was mounted and stained by using the Ziehl-Neelsen standard method on a PALM [®] PEN-membrane slide which was observed and isolated at 63× magnification. (a) <i>M. smegmatis</i> cells before LPC; (b) <i>M. smegmatis</i> cells after LPC.	63
2.8 (a - b)	Isolation of <i>M. smegmatis</i> cells from a thick smear Ziehl-Neelsen stained slide observed and isolated at 63× magnification. (a) Thick smear Ziehl-Neelsen slides before any laser pulses have been applied; (b) The laser-cutting or microdissection application was used on the smear and showed a slightly clear zone indicated by the black arrow. The LPC application is shown by white arrow.	66

LIST OF FIGURES

Figure		Page
2.9 (a - c)	Isolation of <i>M. smegmatis</i> cells fixed and stained by Ziehl-Neelsen standard protocols. The cells were observed and isolated from the glass slides by using LPC application at 63× magnification. (a) <i>M. smegmatis</i> cells before catapulting; (b) The clear zone has shown the cells were catapulted by LPC; (c) The PCMs collected in the ddH ₂ O was re-examined at 63 × magnification with no re-staining.	67
2.10 (a - c)	<i>M. smegmatis</i> cells stained by Ziehl-Neelsen observed and isolated from glass slides using the 63× magnification of LCM. (a) Ziehl-Neelsen stained slides before catapulting; (b) The selected area was catapulted by using LPC application; (c) PCMs collected in the PTA was re-examined to ensure cells were successfully collected.	72
2.11 (a - c)	The PCMs of individual <i>M. smegmatis</i> cells observed under TEM and catapulted into 1% PTA from archived glass slides. (a) Several catapulted individual cells; (b) Several different shapes of post-catapulted cells; (c) An intact single cell successfully catapulted and isolated using LPC.	74
2.12 (a - b)	A <i>M. smegmatis</i> cell grown and isolated from the Nutrient broth (48 hours, 37°C). This was used to compare the shape and dimension with the PCMs isolated by LCM. (d) Several <i>M. smegmatis</i> cells with slight different dimensions; (e) a single cell of <i>M. smegmatis</i> .	76
2.13 (a - b)	LPC application for isolating Gram-negative bacteria <i>P. aeruginosa</i> PAO1 fixed and stained by Gram-staining on glass slides at 63× magnification. (a) <i>P. aeruginosa</i> PAO1 stained smear before catapulting; (b) The catapulting action has removed the top layer of the smear displaying different colour intensity at the catapulting zone compared to (a).	79
2.14 (a - b)	Gram-stained slides of mixed bacteria cultures (<i>P. aeruginosa</i> PAO1 and <i>M. smegmatis</i>). These showed that the LPC application is able to isolate different type of bacterial cells. (a) The mixed-cultures of bacteria before catapulting; (b) The mixed-culture of bacteria after catapulting. LPC was used to isolate cells of interest selectively.	80

LIST OF FIGURES

Figure		Page
3.1 (a - c)	The agarose gel electrophoresis (1.5%) shows the amplification of DNA extracted from <i>M. smegmatis</i> (NCIMB8548), environmental isolate (VS/02) and genomic DNA from <i>M. tuberculosis</i> (ATCC [®] 25177). (a) Primary PCR using primers BMS738aF and 1311R amplified a 599 bp product; (b) Nested PCR using primers BnMS1100bf and 1184br amplified 103 bp product; (c) Nested PCR using primer BnMS949bf and 1105br which amplified a 179 bp product. Lanes 1 and 6: 100 bp ladder, lane 2: <i>M. smegmatis</i> (NCIMB8548), lane 3: environmental isolate (VS/02), lane 4: genomic DNA <i>M. tuberculosis</i> and lane 5: negative control.	109
3.1 (d - e)	The agarose gel electrophoresis (1.5%) shows the amplification of DNA extracted from <i>M. smegmatis</i> (NCIMB8548), environmental isolate (VS/02) and genomic DNA from <i>M. tuberculosis</i> (ATCC [®] 25177). (d) Primary PCR using primers MS-rpoB-F2 and R2 which amplify a 450 bp product; (e) Nested PCR using primers MS(in-9) rpoB-F and R which amplify a 148 bp product. Lanes 1 and 6:100 bp ladder, lane 2: <i>M. smegmatis</i> (NCIMB8548), lane 3: environmental isolate (VS/02), lane 4: genomic DNA from <i>M. tuberculosis</i> (ATCC [®] 25177) and lane 5: negative control.	111
3.1 (f - g)	The agarose gel electrophoresis (1.5%) shows the amplification of DNA extracted from <i>M. smegmatis</i> (NCIMB8548), environmental isolate (VS/02) and genomic DNA from <i>M. tuberculosis</i> (ATCC [®] 25177). (f) Primary PCR using primers MycoF and R which amplify a 764 bp product; (g) Nested PCR by using primers Myco-F7-in and R7-in which amplify a 271 bp product. Lanes 1 and 6:100 bp ladder, lane 2: <i>M. smegmatis</i> (NCIMB 8548), lane 3: environmental isolate (VS/02), lane 4: genomic DNA from <i>M. tuberculosis</i> (ATCC [®] 25177) and lane 5: negative control.	112
3.2	Correlation of DNA concentration of unstained slides with extraction and fixation methods.	116
3.3	Correlation of DNA concentration with staining and extraction methods from air-dried slides.	120
3.4	Correlation of DNA concentration with staining and extraction methods from heat-fixed slides.	121

LIST OF FIGURES

Figure		Page
3.5 (a - e)	<p>Agarose gel electrophoresis showing an approximate 176 bp nested PCR product amplified with primers BnMS949bf and 1105br. DNA was extracted by using different extraction method. (a) DNA extracted from Ziehl-Neelsen (hot stain) stained slides; (b) DNA extracted from Ziehl-Neelsen (37°C stain) stained slides; (c) DNA extracted from <i>Mycobacteria</i> fluorescent stain kit stained slides; (d) DNA extracted from Gram-stained slides; (e) DNA extracted from unstained slides. The 100 bp ladder showed in lanes 1 and 12. Lanes 2 to 5 showing the air-dried samples and 8 to 11 showing the heat-fixed samples. Lanes 2 and 8: heat-shock; lanes 3 and 9: heat-shock followed by ethanol precipitation; lanes 4 and 10: QIAamp[®] DNA Micro kit; lane 5 and 11: Chelex-100; lane 6: negative control; lane 7: positive control.</p>	124
3.6 (a - b)	<p>Dual parameter dot plot (i) and histogram (ii) showing the distribution of FSC and dual-staining uptake of an exponential culture of bacteria. The red and green colour of the dot plot indicated TO (0.5 µg/ml) and PI (1.3 mg/ml) taken up by cells. The FSC determined the size of cell ranging from 0.5 to 40 µm (a) The total number of <i>M. smegmatis</i> cells - 489,420 enumerated from 15 µl culture; (b) The total number of <i>P. aeruginosa</i> PAO1 cells - 438,525 enumerated from 15 µl cultures. The histogram (ii) indicated that all cells were determined and there was no interference from background noise.</p>	127
3.7 (a - b)	<p>The observation of post-enumerated bacteria cell cultures under the fluorescence microscope, 40× magnification using blue light for excitation. Results indicate that targeted bacteria cells were stained using TO (0.5 µg/ml) and PI (1.3 mg/ml). The green and red staining represents live and dead cells respectively. (a) Population of <i>M. smegmatis</i> cells showing live and dead cells; (b) Population of <i>P. aeruginosa</i> PAO1 cells showing live and dead cells.</p>	128
3.8 (a - b)	<p>This figure shows that it is possible to enumerate pre-stained cells recovered from glass slides using FCM. The cells were isolated using LCM and were stained with TO (0.5 µg/ml) and PI (1.3 mg/ml). The incubation time was 40 minutes at room temperature to allow for the dyes absorption into the cell wall before employing the FCM. (a) A dot plot (i) analysis demonstrates the uptake of FCM staining dyes by PCMs. The histogram (ii) analysis indicates various sizes of samples for enumeration; (b) <i>P. aeruginosa</i> PAO1 catapulted cells from Gram-stained glass slides.</p>	131

LIST OF FIGURES

Figure		Page
3.9 (a – c)	Observations of post-enumerated samples under the fluorescence microscope, 40 × magnifications, using blue light for excitation. The results indicate the prolonged incubation time has increased the pre-stained PCMs cells permeability to absorb the dyes showed by arrow. (a) <i>M. smegmatis</i> ; (b) <i>P. aeruginosa</i> PAO1; (c) The background with the unclear staining indicates that, several cells could not allow the dye absorption resulting in dye diffusion.	132
3.10 (a – f)	A 10-fold serial dilution of catapulted <i>M. smegmatis</i> cells stained using TO (0.5 µg/ml) and PI (1.3 mg/ml) which are subjected to FCM for enumeration. The dot plot analysis (i) shows that the cells were enumerated according to the dilution factor. The total number of cells per µl is reduced for each counting. The dual-parameter histogram (SSC versus FSC) shows uniformity of a bell-shape peak and indicates the subjected PCMs were enumerated without background interference (a) 10 ⁻¹ (b) 10 ⁻² (c) 10 ⁻³ (d) 10 ⁻⁴ (e) 10 ⁻⁵ (f) 10 ⁻⁶ .	135
3.11 (a - e)	A set of catapulted Ziehl-Neelsen stained <i>M. smegmatis</i> cells isolated from glass slides using LCM. The pre-stained <i>M. smegmatis</i> cells were stained with TO (0.5 µg/ml) and PI (1.3 mg/ml). The incubation time was 40 minutes at room temperature to allow the dye absorption into the <i>M. smegmatis</i> cell wall before employing the FCM. The post-enumerated cells were collected and subjected to DNA extraction to ensure the amplifiable DNA was recovered.	138

LIST OF FIGURES

Figure		Page
3.12 (a – c)	<p>The absolute quantification to evaluate the LOD from assay was performed using amplicons from primary touchdown PCR. The DNA was extracted from fresh cultured <i>M. smegmatis</i> (standard samples). This external standard was performed to detect false negative result and determine primers efficiencies. (a) Nested real-time PCR amplification pattern shows the limit of the carrying capacity for detection and was progressively decreased at 10^{-4} dilution of template concentration. i: 10 ng/μl; ii: 1 ng/μl; iii: 10^{-1} ng/μl; iv: 10^{-2} ng/μl; v: 10^{-3} ng/μl; vi: 10^{-4} ng/μl; vii: non-template control (NTC); (b) SYBR Green I melting curve analysis shows that no non-specific product was amplified; (c) Post-quantification analysis shown on 1.5% agarose gel electrophoresis. A 179 bp product was amplified using nested primers (BnMS949bf and 1105br) specifically for the <i>rpoB</i> gene of <i>M. smegmatis</i>. Lanes 1 and 9: 100 bp ladder; lane 2: 10 ng/μl; lane 3: 1 ng/μl; lane 4: 10^{-1} ng/μl; lane 5: 10^{-2} ng/μl; lane 6 10^{-3} ng/μl; lane 7: 10^{-4} ng/μl; lane 8: non-template control (NTC).</p>	142
3.13 (a - c)	<p>Detection of <i>M. smegmatis</i> cells from archived glass slides using real-time LightCycler nested PCR. This produced 176 bp <i>rpoB</i> gene-specific product. (a) Nested real-time PCR detects approximately 200 enumerated of <i>M. smegmatis</i> cells catapulted from glass slides by LCM. The DNA templates were amplified by using touchdown PCR. The C_q relates to the amount of template originated from the number of catapulted cells; (i) 3182 cells (ii) 1578 cells (iii) 795 cells (iv) 397 cells (v) positive control (vi) 198 (vii) negative control (NTC); (b) A single peak of T_m Calling analysis showed that a specific product could be amplified with no inhibitory effect; (c) Agarose gel (1.5%, 4°C) showing a 176 bp nested PCR product amplified with primers BnMS949bf and 1105br. DNA sequencing of amplified materials confirmed the amplified product to be the <i>rpoB</i> gene of <i>M. smegmatis</i>. Lanes 1 and 9: 100 bp ladder, lane 2: 3,182 cells, lane 3: 1,578, lane 4: 795, lane 5: 397, lane 6: 198, lane 7: positive control, lane 8: negative control.</p>	144

LIST OF FIGURES

Figure		Page
3.14 (a - b)	A agarose gel electrophoresis (1.5%, 4°C) showing the MDA using REPLI-g [®] UltraFast Mini kit of catapulted <i>M. smegmatis</i> cells, isolated from archived glass slides using LCM. Samples were extracted using different methods to determine the most appropriate DNA extraction method for low number of catapulted cells. (a) Undiluted post-MDA reaction; (b) 1:25 dilution of post-MDA reaction. Lanes 1 and 6: 100 bp ladder; lane 2: heat-shock; lane 3: heat-shock followed by ethanol precipitation; lane 4: QIAamp [®] DNA Micro kit; lane 5: positive control; lane 6: negative control	152
3.15 (a - b)	A primary touchdown PCR of post-MDA reaction showed no visible product on agarose gel electrophoresis (1.5%, 4°C). (a) Amplification was performed using an undiluted post-MDA reaction; (b) Amplification was performed using the 1:25 dilution of post-MDA reaction. Lanes 1 and 7: 100 bp ladder; lane 2: heat-shock; lane 3: heat-shock followed by ethanol precipitation; lane 4: QIAamp [®] DNA Micro kit; lane 5: positive control; lane 6: negative control.	153
3.16 (a - b)	A nested touchdown PCR of post-MDA reaction showing non-visible or a low intensity 176 bp product on agarose gel electrophoresis (1.5%, 4°C). (a) The primary PCR was performed using an undiluted post-MDA reaction in which no amplification product was observed; (b) The primary PCR was performed using a 1:25 dilution of post-MDA reaction showing low intensity of amplification at lanes 2 and 3. Lanes 1 and 7: 100 bp ladder; lane 2: heat-shock; lane 3: heat-shock followed by ethanol precipitation; lane 4: QIAamp [®] DNA Micro kit; lane 5: positive control; lane 6: negative control.	154

LIST OF FIGURES

Figure		Page
3.17 (a - c)	<p>Nested real-time PCR using primers BnMS949bf and 1105br for the detection of 30 <i>M. smegmatis</i> cells isolated from glass slides. The post-MDA reaction was primarily amplified by touchdown PCR using an undiluted post-MDA reaction amplicons. The undiluted primary amplicon was used as a template for the nested real-time PCR detection system. The analysis was carried out based on different DNA extraction methods; i: heat-shock; ii: heat-shock followed by ethanol precipitation; iii: QIAamp[®] DNA micro kit. (a) The C_q pattern of amplification showed the detection of samples at different C_q values. A dilution (10⁻⁶) of the positive control was used to increase the efficiency of detection; (b) T_m Calling analysis showed two melting peaks generated from the melting curve after amplification. The products were then analysed using agarose gel electrophoresis; (c) Agarose gel electrophoresis (1.5%, 4°C) shows the 176 bp nested real-time product. Lanes 1 and 7: 100 bp ladder; lane 2: heat-shock; lane 3: heat-shock followed by ethanol precipitation; lane 4: QIAamp[®] DNA Micro kit; lane 5: positive control; lane 6: negative control.</p>	157
3.18 (a - c)	<p>Nested real-time PCR using primers BnMS949bf and 1105br for the detection of 30 <i>M. smegmatis</i> cells isolated from glass slides. A template from the post-MDA reaction, primarily amplified using touchdown PCR was used. The primary amplification was performed using the 1:25 dilution of post-MDA reaction and an undiluted amplicon was used for nested real-time PCR detection system. The analysis was carried out based on different DNA extraction methods; i: heat-shock; ii: heat-shock followed by ethanol precipitation; iii: QIAamp[®] DNA micro kit. (a) The C_q pattern of amplification showed the detection of samples at different C_q value. The diluted (10⁻⁶) positive control was used to increase the efficiency of detection; (b) A single peak of T_m Calling analysis indicates that the product was specifically amplified. The products were further analysed using agarose gel electrophoresis; (c) Agarose gel electrophoresis (1.5%, 4°C) shows the 176 bp nested real-time PCR amplification product. Lanes 1 and 7: 100 bp ladder; lane 2: heat-shock; lane 3: heat-shock followed by ethanol precipitation; lane 4: QIAamp[®] DNA Micro kit; lane 5: positive control; lane 6: negative control.</p>	160

LIST OF FIGURES

Figure		Page
3.19	The C_q pattern comparison analysis of nested real-time PCR post-detection by using C_q values obtained from amplification of undiluted and diluted (1:25) post-MDA reaction amplicons.	161
4.1	The agarose gel electrophoresis (1.0%, 4°C) shows the 23S and 16S rRNA extracted from total RNA of <i>M. smegmatis</i> (NCIMB8548) and environmental isolates (VS/02) cultures using three kinds of extraction method. The amplicon brightness and the smears shown on the gel are associated with the RNA extraction method used and the RNA degradation due to the presence of chromosomal DNA (ChDNA). Lane 1: 1 kb ladder, lanes 2 and 3: NCIMB8548 and VS/02 extracted using GeneJET RNA purification kit, lanes 4 and 5: NCIMB8548 and VS/02 extracted using combined methods of Lysing matrix-A, gDNA eliminator spin column (RNeasy Plus Mini kit) and GeneJET RNA purification kit, lanes 6 and 7: NCIMB8548 and VS/02 extracted using combination methods of Lysing matrix-A, gDNA eliminator spin column (RNeasy Plus Mini kit), GeneJET RNA purification kit and “on-column” DNase treatment.	193
4.2	The agarose gel electrophoresis (1.0%, 4°C) showing the 23S and 16S rRNA extracted from total RNA of <i>M. smegmatis</i> (NCIMB8548) isolated from glass slides using combined methods of Lysing matrix-A, gDNA eliminator spin column (RNeasy Plus Mini kit), GeneJET RNA purification kit and “on-column” DNase treatment. The low amplicon brightness shown on the gel could be due to the RNA degradation. Lane 1: 1 kb ladder, lane 2: Ziehl-Neelsen stained slide sample, lane 3: frozen-Ziehl-Neelsen stained slide sample, lane 4: unstained slide sample.	195

LIST OF FIGURES

Figure		Page
4.3 (a – c)	<p>Nested real-time PCR of <i>M. smegmatis</i> (NCIMB8548) cells isolated from glass slides using 16S rRNA gene-specific primers. (a) The C_q of primary amplicons observed at cycle 25; (i) Ziehl-Neelsen stained slide sample (ii) frozen-Ziehl-Neelsen stained slide sample (iii) unstained slide sample (iv) positive control (v) negative control (NTC); (b) T_m Calling analysis showing a single narrow peak of amplification product. The stable DNA amplification is shown by melting curve analysis with no by-product generated during the process; (c) Agarose gel (1.5%, 4°C) showing 166 bp nested real-time PCR amplified product. Lanes 1 and 7: 100 bp ladder, lane 2: Ziehl-Neelsen stained slide sample, lane 3: frozen-Ziehl-Neelsen stained slide samples, lane 4: unstained slide samples, lane 5: positive control, lane 6: negative control (NTC).</p>	199
4.4 (a – c)	<p>Nested real-time PCR of <i>M. smegmatis</i> (NCIMB 8548) cells isolated from glass slides using <i>rpoB</i> gene-specific primers. (a) The C_q of primary amplicons observed at cycle 30; (i) Ziehl-Neelsen stained slide sample (ii) frozen-Ziehl-Neelsen stained slide sample (iii) unstained slide samples (iv) positive control (v) negative control (NTC); (b) T_m Calling analysis showing an additional weak, broad peak with T_m of 77°C which arose from a non-specific by product from the unstained sample labelled iii (red line). (c) Agarose gel (1.5%, 4°C) showing a 176 bp nested real-time PCR product amplified. Lanes 1 and 7: 100 bp ladder, lane 2: Ziehl-Neelsen stained slide sample, lane 3: frozen-Ziehl-Neelsen stained slide sample, lane 4: unstained slide sample, lane 5: positive control, lane 6: negative control (NTC).</p>	204
4.5 (a – b)	<p>Amplification of cDNA of <i>M. smegmatis</i> (NCIMB 8548) cells isolated from glass slides using a 16S rRNA-sequence-specific probe. (a) The C_q has shown the cDNA was amplified from all samples and the first detection was visible at $C_q > 20$; (i) Ziehl-Neelsen stained slide sample (ii) frozen-Ziehl-Neelsen stained slide sample (iii) unstained slide sample (iv) positive control (v) negative control (NTC); (b) Agarose gel (1.5%) showing the amplified 67 bp nested real-time PCR product. Lanes 1 and 7: 50 bp DNA ladder, lane 2: Ziehl-Neelsen stained slide sample, lane 3: frozen-Ziehl-Neelsen stained slide sample, lane 4: unstained slide sample, lane 5: positive control, lane 6: negative control (NTC).</p>	209
5.1	<p>Flow-chart of conventional detection system for MTBC versus system model developed in this study.</p>	226

ABSTRACT

The World Health Organization (WHO) has outlined a TB-free World strategy that aims to reduce tuberculosis prevalence by 2015. That could be achieved through the development of a specific and rapid detection system. In countries with a high incidence of tuberculosis, detection is solely based on sputum smear microscopy using Ziehl-Neelsen stain and chest radiography. This is followed by cultivation, which requires up to 12 weeks for confirmation. The diagnostic test available is insensitive, laborious, lacks specificity and does not even give unequivocal proof of infection. Consequently, this study was conducted to obtain confirmatory diagnostic information from Ziehl-Neelsen stained smears on glass slides. The fast-grower, non-pathogenic *Mycobacterium smegmatis* was used as the model organism due to similarity to the pathogenic strain, *Mycobacterium tuberculosis*. Laser capture microscopy (LCM) is primarily used as a microdissection tool in studies involving tissues and membranes. This technology was able to isolate intact individual cells fixed on archived glass slides regardless of how the smears and staining have been performed, confirmed by transmission electron microscopy (TEM) observation. Typically between 100 – 1,000 catapulted cells are enumerated using flow cytometry (FCM). A series of DNA extraction techniques which are heat-shock, heat-shock followed by ethanol precipitation and QIAamp[®] DNA Micro kit were compared and optimized for the lowest number of post-catapult cells. To maintain the detection of lowest number of catapulted cells, the *rpoB*-gene specific primers were designed for amplification using nested real-time PCR. Routinely, this system was able to detect as few as 30 catapulted cells per assay. This result demonstrated that it is possible to isolate the bacteria from glass slides and subsequently perform downstream molecular applications regardless of any

inhibitory factors. In conclusion, we strongly recommend that this system may offer improved specificity and speed of tuberculosis detection with lower risk of exposure to infection through the use of stained slides.

ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
CDC	Centres for Diseases Control and Prevention
cDNA	copy DNA/complementary DNA
C _q	Quantification cycle
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside Triphosphate
FCM	Flow cytometry
G	gram(s)
HIV	Human immunodeficiency virus
L	litre(s)
LCM	Laser Capture Microscope/Laser Capture Microscopy
MDA	Multiple displacement amplification
mg	milligram(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
mRNA	messenger RNA
µg	microgram(s)
µl	microlitre(s)
µm	micrometer(s)
NAA	Nucleic Acid Amplification Assay
NCBI	National Center for Biotechnology Information
ng	nanogram(n)
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PI	Propidium Iodide
PCM	Post-catapult material(s)
PMIT	Photomultiplier tubes
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
<i>rpoB</i>	gene that encodes β -subunit of bacterial RNA polymerase
RT-PCR	Reverse Transcription PCR
ddH ₂ O	double distilled water
sp./spp.	species
<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	tris-borate-EDTA
TE	Tris-EDTA
TO	Thiazole orange
tRNA	total RNA
U	unit
WHO	World Health Organization

TABLE OF CONTENTS

ACKNOWLEDGEMENT	iii	
DECLARATION	iv	
LIST OF TABLES	v	
LIST OF FIGURES	vii	
ABSTRACT	xviii	
ABBREVIATIONS	xx	
Chapter	Page	
1	General introduction.	1
1.1	An overview of the global tuberculosis (TB) incidence.	2
1.2	Description of the genus <i>Mycobacterium</i> .	10
1.3	Epidemiology and transmission.	15
1.4	Clinical significance.	18
1.4.1	Slowly-growing mycobacteria (SGM).	18
1.4.2	Rapidly-growing mycobacteria (RGM).	19
1.5	A standard diagnosis of <i>M. tuberculosis</i> from clinical specimens.	24
1.6	A variety of modern diagnostic methods for detection and identification mycobacterial infections.	25
1.6.1	Biological and biochemical methods.	25
1.6.1.1	Microscopy techniques.	25
1.6.1.2	Culture techniques.	27
1.6.2	Immunodiagnostic test for tuberculosis.	28
1.6.3	Nucleic acid amplification (NAA) assays.	29
1.7	Aim and objectives.	37
2	The potential of laser capture microscopy as an isolation tool for isolating bacterial cell from slides.	38
2.1	Introduction.	39
2.2	Aim and objectives.	42
2.3	Materials and methods.	43
2.3.1	Bacterial strains.	43
2.3.1.1	<i>Mycobacterium smegmatis</i> (<i>M. smegmatis</i>).	43
2.3.1.2	<i>Pseudomonas aeruginosa</i> PAO1.	43
2.3.2	Smear preparation.	43
2.3.3	Acid fast staining (Ziehl-Neelsen).	44
2.3.4	Gram-staining.	44
2.3.5	Slides scraping.	45
2.3.6	Laser capture microscopy (LCM).	45
2.3.7	Transmission Electron Microscopy (TEM).	46
2.3.7.1	Sample preparation for TEM from fresh culture.	46
2.3.7.2	Sample preparation from post-catapult materials (PCMs).	47

2.3.8	Growth curve of <i>Mycobacterium smegmatis</i> .	47
2.4	Results	49
2.4.1	<i>M. smegmatis</i> : observation from the growth media.	49
2.4.2	Cell morphology of <i>M. smegmatis</i> observed under LCM.	54
2.4.3	A growth curve study of <i>M. smegmatis</i> in Nutrient and brain heart infusion (BHI) broth media.	56
2.4.4	Isolating Ziehl-Neelsen stained <i>M. smegmatis</i> from slides by using scraping method.	58
2.4.5	An optimization study for isolating bacterial cells fixed on glass slides.	60
2.4.5.1	Isolation of <i>M. smegmatis</i> cells fixed on the PALM [®] PEN-membrane slide.	60
2.4.5.2	Isolation of <i>M. smegmatis</i> cells fixed on normal glass slides.	64
2.4.6	TEM showing that intact <i>M. smegmatis</i> cells are recovered from glass slides following LCM treatment.	69
2.4.7	Optimization of the LCM for isolation of other bacteria fixed on glass slides.	77
2.5	Discussion.	81
3	The development of molecular detection system for bacterial cells isolated from glass slides by laser capture microscopy technique.	87
3.1	Introduction.	88
3.2	Aim and objectives.	93
3.3	Materials and methods.	94
3.3.1	Bacterial strains and cultivation conditions.	94
3.3.1.1	<i>Mycobacterium smegmatis</i> (<i>M. smegmatis</i>).	94
3.3.1.2	<i>Pseudomonas aeruginosa</i> PAO1.	94
3.3.1.3	Control DNA.	94
3.3.2	Stain inhibition study.	94
3.3.2.1	Slide preparation.	95
3.3.2.2	Acid fast staining (Ziehl-Neelsen)	95
3.3.2.3	Gram-stain.	95
3.3.2.4	Fluorescent stain kit for <i>Mycobacteria</i> (<i>Mycobacteria</i> fluorescent stain kit) (Sigma-Aldrich, UK).	95
3.3.2.5	Isolation of bacterial cells.	95
3.3.2.6	Molecular detection of <i>M. smegmatis</i> cells scraped from glass slides.	96

3.3.3	DNA extraction.	96
3.3.3.1	DNA extraction using FastDNA [®] Spin for soil kit.	96
3.3.3.2	Heat shock.	97
3.3.3.3	Heat shock-ethanol precipitation.	97
3.3.3.4	Chelex-100	97
3.3.3.5	QIAamp [®] DNA Micro kit (Qiagen, USA).	98
3.3.4	Multiple Displacement Amplification (MDA).	98
3.3.5	Primers.	98
3.3.6	Polymerase Chain Reaction (PCR).	102
3.3.6.1	Hot-start PCR.	102
3.3.6.2	Primary PCR.	102
3.3.6.3	Nested PCR.	103
3.3.6.4	Nested touchdown PCR.	103
3.3.6.5	Nested real-time PCR.	103
3.3.7	Pre-washing for inhibitory removal.	104
3.3.8	Agarose gel electrophoresis.	104
3.3.9	Purification of DNA fragments.	105
3.3.10	Sequencing of amplicons.	105
3.3.11	Analysis of nucleotides.	106
3.3.12	Flow cytometric quantification (FCM).	106
3.3.13	Statistical analysis.	107
3.4	Results	108
3.4.1	Primer validation.	108
3.4.2	Stain inhibition study and optimization of DNA extraction methods for <i>M. smegmatis</i> recovered from glass slides.	113
3.4.3	Optimization of FCM for quantification of cells isolated from glass slides.	125
3.4.4	Enumeration of cells isolated from archived glass slides using FCM.	129
3.4.5	The detection sensitivity of post catapulted <i>M. smegmatis</i> cells using a touchdown and real-time nested PCR method.	139
3.4.6	Determination of the minimum number of cells that could be detected by using combinations of MDA-nested PCR assay.	147
3.5	Discussion.	162
4	Investigation of the presence of RNA extracted from <i>Mycobacterium smegmatis</i> isolated from glass slides.	173
4.1	Introduction.	174
4.2	Aim and objectives.	178

4.3	Materials and methods.	179
4.3.1	Bacterial strains and cultivation conditions.	179
4.3.1.1	<i>Mycobacterium smegmatis</i> (<i>M. smegmatis</i>)	179
4.3.2	Smear preparation.	179
4.3.3	Frozen smear preparation.	179
4.3.4	Modification of acid fast staining (Ziehl-Neelsen) from standard protocols.	179
4.3.5	Isolation of <i>M. smegmatis</i> cells from slides by scraping method.	180
4.3.6	RNA extraction.	180
4.3.6.1	Cell disruption using mechanical and enzymatic procedures.	181
4.3.6.2	RNA purification.	181
4.3.7	DNase treatment.	182
4.3.7.1	On-column DNase treatment.	182
4.3.7.2	DNase treatment of eluted RNA.	182
4.3.8	Primers.	183
4.3.9	cDNA synthesis.	186
4.3.10	Primary polymerase chain reaction (primary PCR).	186
4.3.11	Nested PCR.	187
4.3.12	Real-time PCR.	187
4.3.12.1	Nested real-time PCR.	187
4.3.12.2	Real-time PCR using a pre-designed probe.	188
4.3.13	Pre-washing for inhibitory removal.	188
4.3.14	Agarose gel electrophoresis.	188
4.3.15	Confirmation of amplified DNA.	189
4.4	Results.	190
4.4.1	Optimization of total RNA extraction methods for <i>M. smegmatis</i> cells isolated from glass slides.	190
4.4.2	Optimization of RT-PCR and RT-nested real-time PCR for the detection of RNA using 16S rRNA gene specific primers.	197
4.4.3	Primary and nested real-time PCR to detect RNA Using <i>rpoB</i> gene-specific primers.	202
4.4.4	16S rRNA-sequence-specific probe binding assays for the detection of RNA extracted from <i>M. smegmatis</i> cells, isolated from glass slides.	207
4.5	Discussion	212
5	General discussion.	219
6	Future work and recommendations	227

Appendix (A - D)	230
References	250

Chapter 1

General introduction.

1.1 An overview of the global tuberculosis (TB) incidence.

Tuberculosis is a major health problem caused by *Mycobacterium tuberculosis* complex (MTBC) with many new cases reported each year which claim almost 2 million deaths (Dagneu et al. 2012, Dias et al. 2012). Many countries are affected by tuberculosis (Table 1.1). In 2011, 85% of cases were reported from Africa and Asia with the major prevalence, 59%, contributed from India and China alone. Meanwhile, a small proportion of tuberculosis incidence reported was by the Americas (3%), the European region (4.3%), Eastern Mediterranean (7.7%) and Western Pacific (21% (reported in 2010) (Dias et al. 2012, Small 2012). Tuberculosis is ranked as the eighth leading cause of death in low- and middle income countries. However, it becomes the third biggest killer after HIV/AIDS and ischemic heart disease among adult populations (age 15 to 59) (Lessells et al. 2011, Noens et al. 2011, Balakrishnan et al. 2012). *M. tuberculosis* infection is transmitted by the inhalation of infected aerosol droplets generated by people with pulmonary disease, through coughing. Transmission by other methods has not been reported.

Although tuberculosis is curable, the available treatment regimes slow the infection rate but do not eliminate this infection from the population. This is indicated when reported tuberculosis cases continued to increase every year even though the number per 100,000 population decreased by 1% (Dias et al. 2012, Fitzpatrick et al. 2012). Treatment, using first-line drugs to treat *M. tuberculosis* among susceptible patients, does have an effect within 6 months. For the non-susceptible patient, second-line drug treatments are introduced which are used to treat multidrug-resistant tuberculosis (MDR-TB) strains (Ahmad 2011). Despite the presence of second-line drugs which are able to overcome non-susceptible patients,

the high cost and side effects of their use result in cases of MDR-TB not being eradicated.

Tuberculosis cases also relate to the spread of HIV/AIDS. In the 22 high-burden countries (HBCs) classified by WHO (accounting for 80% of tuberculosis cases) showed the tuberculosis cases reported from 1990 to 2011 are mostly contributed from the HIV-positive patient (Figure 1.1) (Dias et al. 2012, Fitzpatrick et al. 2012). According to WHO, one in four deaths from tuberculosis is HIV related. The diagnoses of HIV-positive patients are more difficult to treat as opposed to non-HIV-positive patients. In 2007, almost 1.37 million new tuberculosis cases were reported among HIV infected people alone. In addition, due to the low curable rate of people living with HIV, the mortality rate increased among these cases. The highest number of HIV-related tuberculosis cases peaked in Africa during the 1980s and steadily increased to 2004 (Blanc et al. 2009). The extensive treatment and monitoring then slowed the epidemic. Although new tuberculosis cases had reportedly decreased among the WHO region, the absolute numbers of cases increased along with the growth of population.

From 2009 to 2011, the majority of tuberculosis incidence and deaths were reported from less-developed regions of world countries with the highest death rates recovered from the poorest region of Africa, India, China and Southeast Asia (approximately 20 to 40 per 100,000 population) (Small 2012, Fitzgerald, Sterling & Haas 2010). This may be due to low income countries having limited access to health-care systems, high exposure to unhealthy dwelling, poor-nutrition, HIV infection, diabetes mellitus, and unhealthy life styles like smoking and drug abuse (El Khechine et al. 2009, Balakrishnan et al. 2012). Dating from 1990, the post-communist Eastern European region and former Soviet Union countries, were also

known as high tuberculosis burden areas where reported cases increased until 1998 (approximately 20 to 40 rates per 100,000 population). There was a decline of 8.5% in mortality rate shown from 1998 to 2011 (Dias et al. 2012). This trend has been related to improvements in political and socio-economic status of former Soviet Union dependents. Globally, mortality rates of tuberculosis are successfully decreasing as reported in 2011 among 22-HBCs (Figure 1.2).

The rise of MDR-TB and extensively drug-resistant TB (XDR-TB) cases have become a major concern of the WHO. This may be due to the determination of successful current strategic planning to control the spread of disease. There were an estimated 630,000 cases of MDR-TB among the world's 12 million tuberculosis cases reported in 2011. The actual number of cases and incidence trend is currently under investigation (Dias et al. 2012). Therefore the assessment of data for each country is still not fully accessible. However, an estimated number of MDR-TB cases reported, based on region, has been provided by WHO. Data was obtained from affected countries (Table 1.2). This surveillance data is crucial due to the chronic effects of MDR-TB infection.

Existing diagnostic methods for MDR-TB are slower and take longer to perform in comparison to diagnostics for MTBC common infection. Second-line drug treatment for MDR-TB is expensive due to the long treatment regime required to ensure effectiveness and prevent recurring infections. Although the death rate recorded in the WHO region from 1990 to 2011 has decreased, the actual number of deaths from HIV-related tuberculosis cases still may be greater than recorded. This may be due to no tuberculosis diagnosis from HIV patients but in such cases death is often not recorded as deaths due to tuberculosis. Many studies indicated that tackling the prominent factors of tuberculosis incidence allows the improvement of

tuberculosis controls. This involves many different intervention strategies which are largely dependent on political power and public awareness.

Table 1.1: An average estimation of the burden of disease caused by tuberculosis in 2011 reported by WHO. This summary has been adapted from the Global Tuberculosis Report 2012 (www.who.int/tb/publications/global_report/) and represents the rate per 100,000 population^a (Dias et al. 2012).

Region	Population (thousand)	Mortality ^b	Prevalence	Incidence	HIV prevalence in incident TB cases (%)
Africa	857,382	26	293	262	41
America	943,039	2.2	35	28	17
Eastern Mediterranean	608,628	16	170	109	2.1
European	899,500	5.0	56	42	8.0
Southeast Asia	1,830,361	26	271	189	5.0
Western Pacific	1,808,797	6.9	138	92	3.1

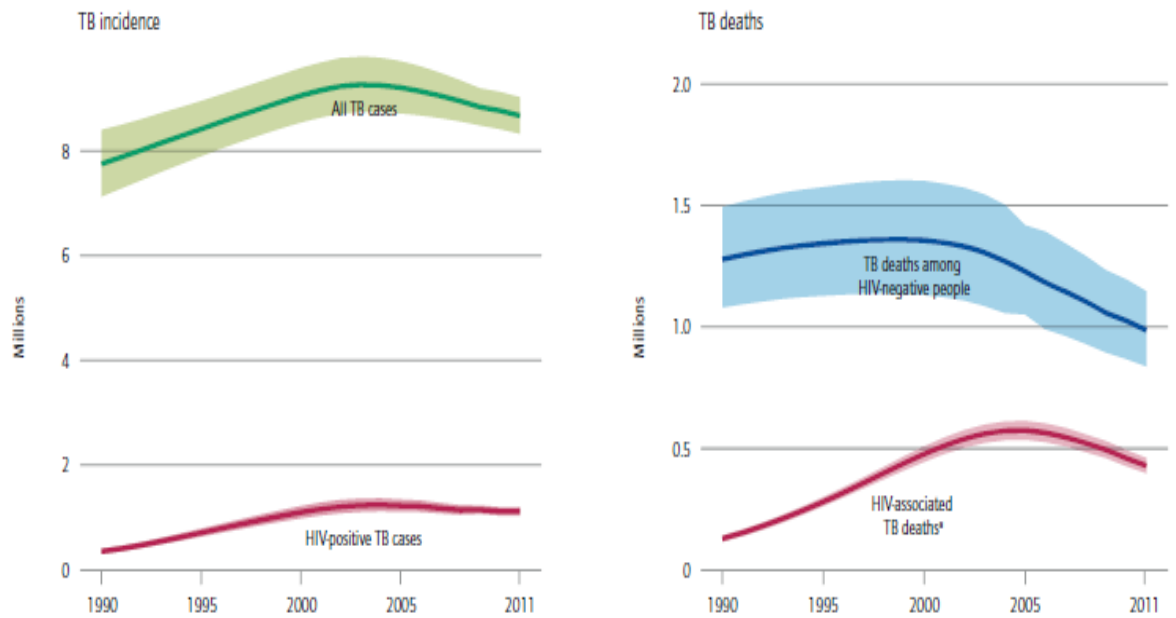
Note:

^a = The value is the point categorized as “best” estimation at lower and upper bounds of the 95% uncertainty interval.

^b = Mortality excludes deaths among HIV-positive tuberculosis cases. Deaths among HIV-positive tuberculosis cases are classified as HIV deaths according to the International Classification of Diseases (ICD-10).



Figure 1.1: The incident rate of tuberculosis, reported from 1990 to 2011, from 22 classified high burden countries (HBCs) within the WHO region (Dias et al. 2012). These figures indicate that the tuberculosis incidence has been falling since the peak recorded in the mid-1990s among 22 HBCs. This was for most countries except for several regions of Africa as shown from the graph pattern.



^a HIV-associated TB deaths are classified as HIV deaths according to ICD-10.

Figure 1.2: An estimated number of tuberculosis cases and death (in millions) reported between 1990 to 2011 globally (Dias et al. 2012). The falling rates of tuberculosis cases are shown globally from mid-2000. Despite the number of tuberculosis cases increasing, death rates have been reportedly declining every year.

Table 1.2: An estimation of MDR-TB cases reported in 2011 from WHO regions. This data was adapted from the Global Tuberculosis Report 2012 (www.who.int/tb/publications/global_report/). Direct measurements are still in progress therefore, the data supplied is an estimation of the actual incidence (Dias et al. 2012).

Region	Estimation of new TB cases with MDR-TB (%)	Confidence interval	Estimation of previous treated TB cases with MDR-TB (%)	Confidence interval
Africa	2.9	0.1-6.2	11	3.4-18
America	2.0	0.8-3.3	11	4.5-18
Eastern Mediterranean	3.4	0.1-10	30	6.9-53
European	5.1	10-20	44	40-49
Southeast Asia	2.1	1.8-2.5	16	12-19
Western Pacific	4.8	3.4-6.1	22	18-26

1.2 Description of the genus *Mycobacterium*.

Currently, there are almost 200 mycobacterial species that have been described by molecular techniques (Pfyffer, Palicova 2011). *Mycobacterium* is the only genus in the family of Mycobacteriaceae. This aerobic bacterium contains various morphological shapes based on growth condition. Some species such as *Mycobacterium smegmatis* and *M. tuberculosis* are able to grow in a limited oxygen environment. It has typical rod-shape or slightly curve with cell dimensions of 0.2 to 0.6 μm by 1 to 10 μm (Pfyffer, Palicova 2011).

This genus cell wall is unique and contains a high proportion of a complex lipid that prevents access of common aniline dyes. This complex cell wall comprises an outer and inner compartment. The outer compartment contains protein and lipid. The lipid-linked polysaccharides (outer cell wall) consist of lipoarabinomannan (LAM), lipomannan, phthiocerol dimycocerosate, trehasole and phosphatidylinositol mannoside (Draper 1998, Hett, Rubin 2008). In addition, the sulfolipid is only present in *M. tuberculosis*. The outer compartment of the cell wall is soluble and has a role for interacting with the host immune system. The insoluble inner compartment of the cell wall consists of peptidoglycan (PG), arabinogalactan (AG) and mycolic acid (MA). These are linked together by covalent bonds and contribute to the core component of mycobacteria cell wall (Cole et al. 1998, Draper 1998, Toney et al. 2010).

Mycobacteria are grouped neither as Gram-positive nor Gram-negative bacteria. They have some unique qualities that are divergent from members of the Gram-positive group, which is that the cell wall contains mycolic acids. A prototypical Gram-positive bacterial cell wall contains a thick PG layer (10 to 20

sheets) with a size range of 20 to 80 nm for each sheet. This is contrary to a Gram-negative bacterial cell wall which consists of 1 to 10 thin layers (approximately 10 nm thickness of each layer) of PG (Figure 1.3). In contrast, a *Mycobacterium* cell wall consists of between 4 to 15 nm thickness of PG associated with teichoic acids, lipoteichoic acids and MAs which does not significantly show a distinctive layer of PG (Brennan 2003, Hett, Rubin 2008).

Mycobacteria are commonly described as acid-fast, implying that after staining they resist decolourization with acidified alcohol and also with strong mineral acid. The common staining procedure used is Ziehl-Neelsen staining (Pfyffer, Palicova 2011) which makes the bacteria stain red. The property of acid-fast, due to the waxy materials in the cell walls is particularly important to recognizing the *Mycobacterium* genus and primarily determines the permeability of cell walls. It is composed of α -alkyl and long β -hydroxyl-fatty acids (60 to 90 carbons for each chain) and is linked to AG. A study by Rao et al. (2005) has shown that a component of MAs (*trans*-cyclopropanation) suppresses inflammation caused by *M. tuberculosis* infection (Rao et al. 2005, Hett, Rubin 2008).

The *Mycobacterium* genus is also grouped as high G+C content bacteria with an average of 61 to 71 mol% G+C for all member species except 57 mol% for *M. leprae*. The genome size varies from species to species ranging from 4 to 7 million base pair (bp) (Table 1.3). Ninety percent (90%) of the genome represents coding regions that potentially encode for more than 6000 proteins (Cole et al. 1998, Zakham et al. 2011). Natural division and growth of *Mycobacterium* species differs based on slowly-growing to rapidly-growing characteristics. Slowly-growing *Mycobacterium* (SGM) requires more than 7 days for visible growth to appear as opposed to rapidly-growing *Mycobacterium* (RGM) which requires less than 7 days

when grown on Löwenstein-Jensen (L~J) medium. However, the RGM may require more than a week if grown from a clinical specimen (Brown-Elliott, Wallace 2011).

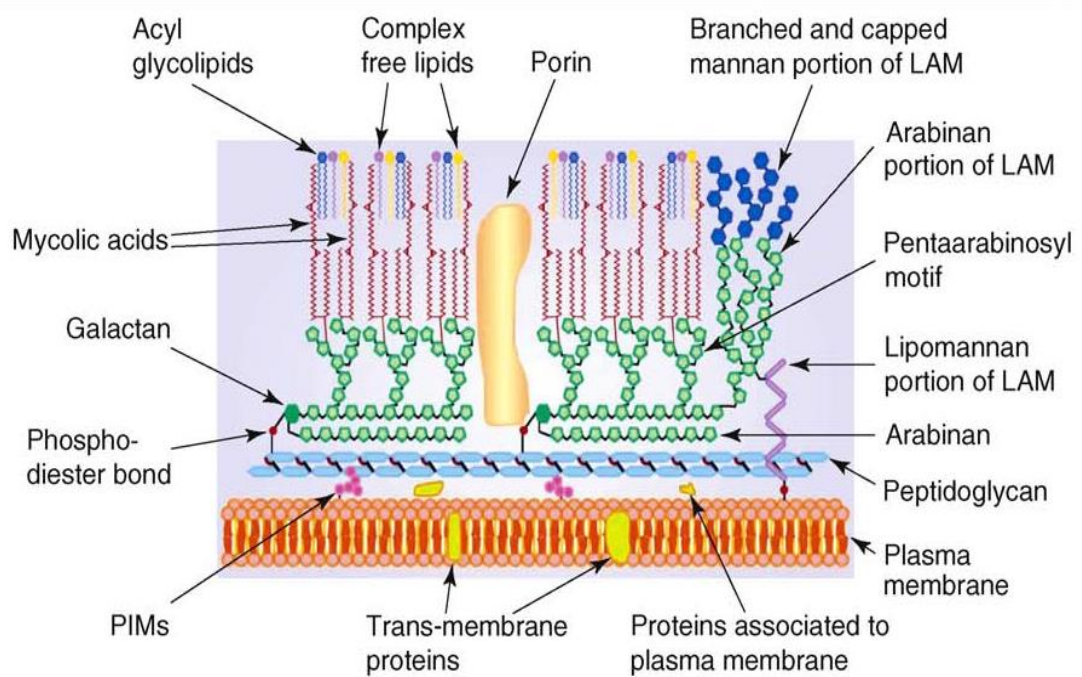


Figure 1.3: Schematic diagram of *Mycobacterium* showing the main components of the outer and inner layer of cell walls. The presence of mycolic acid in the complex of covalent-linked MA-AG-PG is a unique character for identification of the genus *Mycobacterium* and plays an important role as a permeability barrier (Medjahed, Gaillard & Reyrat 2010).

Table 1.3: List of selected *Mycobacterium* spp. showing variations in size and genome composition from species to species. This data was analysed and summarized from Genome Atlas Database (<http://www.cbs.dtu.dk/services/GenomeAtlas>).

Organism	Total size (bp)	Number of genes	Number of estimated proteins	G+C (%)	16S rRNA count	23S rRNA count	NCBI project ID
<i>M. abscessus</i>	5,090,491	4,941	1,030	64	1	1	15691
<i>M. avium</i> 104	5,475,491	5,120	1,069	69	1	1	88
<i>M. bovis</i> BCG str. Pasteur 1173P2	4,374,522	3,988	3,464	66	1	1	1173P2
<i>M. leprae</i> Br4932	3,268,071	3,720	1,202	58	1	1	31271
<i>M. marinum</i> M	6,660,144	5,452	1,222	66	1	1	16725
<i>M. smegmatis</i> str MC ² 155	6,988,209	6,716	5,614	68	2	2	92
<i>M. tuberculosis</i> H37Ra	4,419,977	4,034	1,096	66	1	1	18883
<i>M. ulcerans</i> Agy99	5,805,761	4,241	1,378	65	1	1	16230

1.3 Epidemiology and transmission.

Members of the *Mycobacterium* genus are incapable of replicating in inanimate environments. For the MTBC and *M. leprae*, replication occurs in tissues of human and warm-blooded animals. The risk of infection is dependent on the load of the bacillus that has been inhaled, levels of infectiousness, person-to-person contacts and the immune level of the potential host. This air-borne pathogen is transmitted from an active pulmonary tuberculosis patient by expectoration (cough). The droplet nuclei, approximately 1 to 5 μm in size “meander” in the air and are transmitted to the susceptible person by inhalation (Figure 1.4).

The primary route of infection is the lungs. Tuberculosis can also infect other vital organs of human body such as kidney, spine and brain. Due to the relatively small size of the droplets inhaled into the lungs, the infection penetrates the defence system of bronchi and enters the terminal alveoli. These are then engulfed by alveolar macrophage and dendritic cells. The cell-mediated immune response arrests the multiplication of *M. tuberculosis* and stops infection. An infected person with a strong immune system is able to combat the infection within 2 to 8 weeks post-infection, when the active cell-mediated immune response alleviates multiplication of *M. tuberculosis*. However, in some patients, the tubercle bacilli are latently infected with asymptomatic appearance. This occurs when the tubercle (infected macrophage or granuloma) is not completely eradicated by the immune system. Therefore, the tubercles remain in the system for a longer period of time. This type of infection is called latent tuberculosis.

The latently infected person is non-infectious however the infection can be detected by tuberculin skin test (TST), and interferon gamma (IFN- γ) release assays (IGRA). While latent tuberculosis has a low potential to cause recurrent infection

(10%), it has been reported that reactivation of latent tuberculosis (active tuberculosis) may possibly happen after years of post-infection. The risk of reactivation is greater in HIV infected patients (15%) (Ahmad 2011). Studies have shown that, the pathogen enables the intracellular replication before responses of the immune system occurs in the lymph nodes and other extra pulmonary sites. This is an extraordinary ability that *M. tuberculosis* has to allow persistence and avoid eradication by host-immune system (Ahmad 2011, Obregon-Henao et al. 2012).

Unlike *M. tuberculosis*, non-tuberculous mycobacteria (NTM) are live in moist habitats such as lakes, rivers and damp-soil. For example, *M. avium* complex (MAC), *M. genavense*, *M. kansasii*, *M. xenopi*, *M. simiae*, *M. gordonae* and some RGM have been recovered from tap water (Han, De & Jacobson 2007, Esteban et al. 2008, Stout et al. 2011). Some NTM play important roles in nosocomial disease and pseudo-outbreaks. A recent study showed that NTM may be isolated from skin, upper respiratory tract, intestinal tract and genital tract and shows no symptoms to individuals (Lim, Kim & Yang 2012). Although the presence of NTM is not significant it indicates effects of infection and due to their ubiquity nature, their clinical significance is worth noting.

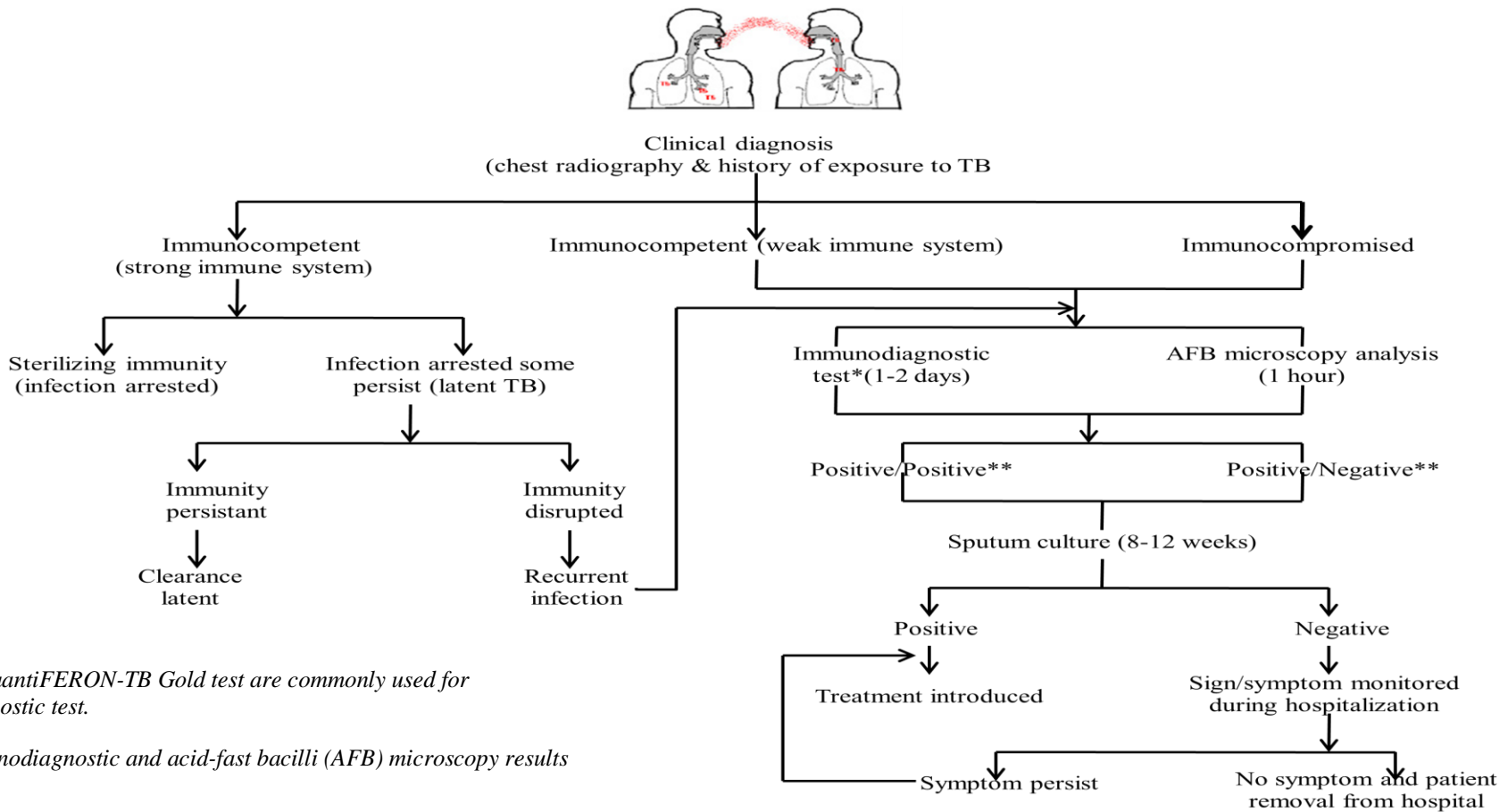


Figure 1.4: The flow-chart shows natural cause progression of tuberculosis and general plan-of-detection in immunocompetent and immunocompromised individual following exposure to tubercle bacilli by inhalation.

1.4 Clinical significance.

1.4.1 Slowly-growing mycobacteria (SGM).

The higher prevalence of MTBC is known to involve several factors such as poverty, individual movement from high-risk countries, close-contact with infected tuberculosis patients and reactivation of latent tuberculosis among HIV infected persons (Pfyffer, Palicova 2011, Knechel 2009). MTBC consists of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG (bacillus Calmette-Guérin), *M. africanum*, *M. caprae*, *M. microti*, *M. canettii* and *M. pinnipedii*. They share genetic homology with no significant variation between sequences (~0.01 to 0.03%) although they have characterized difference with phenotypes (Homolka et al. 2008). Although all members of MTBC cause infection of humans, *M. bovis*, *M. bovis* BCG, *M. microti* and *M. pinnipedii* commonly infect warm-blooded animal as their primary host. It is likely that transmission occurs from animal to human (de la Rua-Domenech 2006, Leung et al. 2008, Kiers et al. 2008). Primarily, the animal to human tuberculosis transmission cases have been found from people who have worked or resided in the particular environment (Miltgen et al. 2002, Cvetnic et al. 2007, Romero et al. 2007, Kiers et al. 2008, Schoepf et al. 2012).

In adults, tuberculosis progression is slow. Tuberculosis infection shows several significant clinical manifestations in pulmonary and extrapulmonary sites (Knechel 2009). Prolonged coughing, severe weight-loss, night sweats, low-grade fever, dyspnoea and chest pain are clinical symptoms indicated from pulmonary infections. The extrapulmonary manifestation of *M. tuberculosis* infection includes meningitis, pleuritis, pericarditis, synovitis, cervical lymphadenitis and infections of the skin, joints, bones and internal organs (Fitzgerald, Sterling & Haas 2010, Ahmad

2011). In HIV infected tuberculosis patients, both types of clinical manifestation are significant due to the rapid progression of infection.

M. africanum and *M. canetti* tuberculosis infection is mainly reported in tropical Africa (Miltgen et al. 2002, de Jong, Antonio & Gagneux 2010). It can be transmitted to western regions by migration of infected persons that used to live in Africa. Studies have shown that *M. africanum* can be subdivided into type I (*M. bovis*-like) and II (*M. tuberculosis*-like) based on chromosomal deletion distribution and geographic origin (Mostowy et al. 2004, de Jong, Antonio & Gagneux 2010). Infected patients have shown similar pulmonary clinical features regardless of type. Studies by de Jong et al. (2007) showed that a smear-positive tuberculosis patient identified various clinical and epidemiology differences between *M. africanum* type II and *M. tuberculosis*. Patients infected with this strain more likely showed major lung affects which were observed on chest X-rays regardless of a cough duration manifestation and occurred in the group of older, HIV infected and malnourished patients (de Jong et al. 2007). *M. canetti* tuberculosis infection have also been highly reported in Africa with similar clinical features of the *M. africanum* infection. Although the main reservoir of *M. canetti* is unknown, patients show *M. tuberculosis* tuberculosis pulmonary clinical features and also lymphadenitis in tuberculosis infected children. Most of the cases have been reported from the continent of Africa (Miltgen et al. 2002).

1.4.2 Rapidly-growing mycobacteria (RGM).

The RGM are grouped as NTM and are opportunistic pathogens that can grow within 7 days in specific growth media. To date, more than 130 species of RGM have been found. Among these, several species have a clinical significance

and contribute to major health care-associated pseudo-outbreaks worldwide. Although many species have been identified as RGM, studies have shown that non-pigmented RGM, *M. fortuitum*, *M. chelonae* and *M. abscessus* are pathogenic and responsible for disease in humans (Han, De & Jacobson 2007, Chan et al. 2010).

Several cases have been reported due to the presence of RGM, commonly *M. fortuitum*, *M. chelonae* and *M. abscessus* and including less common (>10 clinical isolates or cases) human pathogenic RGM species; *M. phocacium*, *M. mucogenicum* and *M. smegmatis* group (known as sensu stricto). The common RGM species/groups cause health care-associated pseudo-outbreak diseases and are described in table 1.4. A study by Gayathri et al. (2010) has shown the *in vitro* antibiotic susceptibility testing among 148 RGM isolates and has indicated that a majority of RGM were sensitive to Amikacin (98%), Gatifloxacin (91%), Moxifloxacin (87%), Ciprofloxacin (76%) and Norfloxacin (Gayathri et al. 2010). Although the majority of health care-associated pseudo-outbreak diseases are treatable using antibiotics, the emergence of multidrug-resistant *M. abscessus* strains worsen the pseudo-outbreak infection scenario due to the most-virulent and chemotherapy-resistant strains (Leao et al. 2009, Gayathri et al. 2010).

Table 1.4: Most frequent human diseases caused by common RGM group/species (continued on next page).

Organism	Infection/disease	Clinical features	Potential host
<i>M. fortuitum</i> group	Post-traumatic wound	Localized cutaneous, localized cellulitis or abscesses	Normal or healthy patient undergone surgery
	open fracture following bone and joint surgery,	Osteomyelitis, arthritis	Metal puncture wound and motor accident patient related cases
	Catheter infections	Bacteremia	Immunosuppressed patients receiving corticosteroid
	Post-keratoplasty and LASIK surgery	Keratitis	Normal or healthy patient undergone surgery
	Cosmetic/salon equipment contamination	Furunculosis	Normal or healthy patient exposed to contamination
<i>M. chelonae</i>	Post-surgical trauma	Desseminated cutaneous,	Immunosuppressed patients receiving corticosteroid
	Catheter infections	Hematogenous spread/bacteremia	Immunosuppressed patients receiving corticosteroid
	Chronic pulmonary infection	Lipoid pneumonia	Older non-smoking women on long-term corticosteroids therapy patient
	Post-keratoplasty and LASIK surgery	Keratitis	Normal or healthy patient undergone surgery

Table 1.4: Most frequent human diseases caused by common RGM group/species (continued on next page).

Organism	Infection/disease	Clinical features	Potential host
<i>M. abscessus</i>	Post-traumatic wound	Localized cutaneous	immunosuppressed patients receiving corticosteroid
	Post-surgical wound	Chronic disseminated cutaneous	Immunosuppressed patients
		Otitis	Ear tube placement surgical patients
	Pulmonary	Chronic lung infection	Cyctic fibrosis, <i>M. avium</i> complex infected patient, non-smoking older women with bronchiectasis. Chronically immunosuppressed patients receiving corticosteriod
	Catheter infections	Multiple draining cutaneous nodules at lower extremities	Immunosuppressed patients receiving corticosteroid
	Post-keratoplasty and LASIK surgery	Keratitis	Normal or healthy patient undergone surgery
<i>M. smegmatis</i> group	Post-surgical or medical procedures, face-lift plastic surgery	Cellulitis, localized abscess,	Normal or healthy patient undergone surgery
	Post-surgical open-fractures wound	Osteomyelitis	Normal or healthy patient undergone surgery

Table 1.4: Most frequent human diseases caused by common RGM group/species.

Organism	Infection/disease	Clinical features	Potential host
<i>M. mucogenicum</i>	Catheter infection	Bacteremia	Immunosuppressed patients receiving corticosteroids
<i>M. fortuitum</i> biovarint complex	Post-traumatic wound, Post-surgical open-fractures wound	Osteomyelitis	Metal puncture wound and motor accident patient related cases

LASIK= Laser-assisted in situ keratomileusis.

1.5 A standard diagnosis of *M. tuberculosis* from clinical specimens.

The most frequent type of sample in which the presence of *M. tuberculosis* is detected is respiratory expectorate or sputum. Samples are taken from potential patients after clinical manifestations are confirmed by chest X-rays, except for the HIV infected and elderly patients that do not show the typical pulmonary clinical features (Perkins, Cunningham 2007, Richter, Brown-Elliott & Wallace 2011). Conventionally, three sputum samples collected from persistent coughing patients are processed to ensure a large number of bacilli. Usually, there are a small numbers of bacilli present in body fluid samples. Therefore, the sample is suspended in sterile saline (0.85%) or bovine albumin (0.2%) and centrifuged ($\geq 3,000 \times g$, 15 minutes) prior to inoculation of the sediment.

M. tuberculosis detection from pre-processed samples is performed by acid-fast staining to identify the presence of AFB. This is followed by culturing on solid media. Due to the slow-growing character of *M. tuberculosis*, at least 4 to 8 weeks are required for visible growth on solid media. The CDC has recommended that positive results from AFB smears must be reported after 24 hours of specimen receipt (Watterson, Drobniowski 2000, Knechel 2009). The standard culture media used in many diagnostic laboratories for identification of *M. tuberculosis* isolated from sputum samples is Löwenstein-Jensen (L~J) or Kirchner solid/liquid media and Middlebrook (7H9, 7H10 and 7H11) formulation. Following confirmation of tuberculosis from standard protocols, an AFB microscopy analysis must be performed to ensure the prescribed treatment is successful. However, it is unnecessary to obtain three samples to discontinue respiratory isolation.

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