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I would be very appreciated if the knowledge from this research is helpful or useful to any other researchers through their works.

Chayada Sitthidet Tharinjaroen

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LIST OF ABBREVIATIONS

Abbreviations		Term
ATCC	=	American Type Culture Collection
bp	=	Base pair
℃	=	Degree Celsius
CFU	=	Colony forming units
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
DTT	=	Dithiothreitol
IVT	=	*In vitro* transcription reaction
LB	=	Luria-Bertani
LPS	=	Lipopolysaccharide
M	=	Molarity
min	=	Minute (s)
ml	=	millilitre
mM	=	millimolar
ng	=	nanogram
nm	=	nanometer
nM	=	nanomolar
nt	=	Nucleotide
OD	=	Optical density
PBS	=	Phosphate-buffered saline
PCR	=	Polymerase chain reaction
rpm	=	Revolutions per minute
RT	=	Reverse transcription
sec	=	Second
TSA	=	Trypticase soy agar
Tris-HCl	=	Tris Hydrochloric acid

LIST OF ABBREVIATIONS (CONT.)

Abbreviations		Term
U	=	Unit
µg	=	microgram
µM	=	micromolar
µl	=	microliter
xg	=	gravity

CHAPTER I
INTRODUCTION

Mycobacterium genus is composing of *Mycobacterium tuberculosis* complex (MTBC), *Mycobacterium leprae* and Nontuberculous mycobacteria (NTM). MTBC and NTM can cause the pulmonary disease especially MTBC which is a causative agent of Tuberculosis. NTM also play a role in pulmonary tuberculosis in HIV/AIDS patient in which also show high incidence rate of infection in many countries including Thailand. Nowadays, It was reported that *Mycobacterium avium* complex (MAC) is the most common pathogen infected within HIV/AIDS patient especially *Mycobacterium avium*. MAC is including of *M. avium* and *Mycobacterium intracellulare.* Like others NTM*, M. avium* is distributed around the world as a saprophyte in environmental. Ingestion water, biofilms or Inhalation of contaminated soil was found to be a cause of infection. After *M. avium* get into the host cell, usually in enterocyte, it can live and multiple within the macrophage. In addition, it also inhibit the macrophage maturation by prevent the acidified within the macrophage environment and block the phagosome-lysosome fusion (1). The feature of infected patient is quite varies ranging from asymptomatic, lymphadenitis (in children), expectorated sputum, hemoptysis, bronchiectasis even cause mortality by disseminated infection can be found. Fever, fatigue, weight loss and loss of appetite are common unclassified features which also found in other infections. Treatment of MAC infection depends on the symptom features, typically, macrolide, rifampicin and ethambutol will be used (2). Symptom observations, radiographic of lung and culture isolation are required for identification of *M. avium*. Even though culture is a standard method for identification, but owing to the widespread of *M. avium* in environmental, the same two isolates from three sputums or by one bronchoalveolar lavage are required for confirm an infection. Furthermore, MAC is also classified as a slow grower mycobacterium which takes at least 6-8 weeks for colony formation. Biochemical testing of MAC is still controversy and time consuming. These entire make the identification is more complex, take time and sometimes incorrect identifying emerging the molecular method for overcome these problems. Data from genomic analysis can identify the difference in some genes among MTBC and even in NTM species such as insertion sequence (IS). Polymerase Chain Reaction (PCR) to amplified IS901 (3), 900 and 1245 (4) following by the Restriction Fragment Length Polymorphism (RFLP) can identified *M. avium*. Later, DNA probe assay was developed, the principle base on the

complementary of *16S rRNA* oligonucleotides specific to each mycobacterium species (5). DNA probe assay are included Genprobe (San Diego, Calif., USA), Inno LiPA (Innogenetics, Ghent, Belgium), GenoType Mycobacterium assay (Hain, Lifescience, Germany), all can identify Mycobacterium but with different number of species also base on different gene. Although, these kits will given a quick and correct identification but the expense cost is also high for affordably. Interestingly, in 2010 Tortoli and colleague (6) showed the misidentification of these kits due to the cross reactivity of probe between the mycobacterium species. Sequencing analysis is used to overwhelming the closely identical of mycobacterium genes. Nowadays, high-pressure liquid chromatography, real time PCR, real time PCR with high resolution melting (HRM) are reported as a tool for identify mycobacterium in species level. However, these techniques required the specialized instruments, skill for interpretation for accurate diagnostic.

Due to the limitations of the existing techniques for *M. avium* diagnosis, a simple, fast, and relatively inexpensive test of *M. avium* based on the ligands specifically recognizing the bacteria would be of great use in diagnosis. One class of such ligands is aptamers, which are conformational oligonucleotides capable of binding to the targets with high affinity and specificity. They are generated using the Systematic Evolution of Ligands by EXponential enrichment (SELEX) techniques, which were discovered since 1990 by Tuerk and Ellington (7, 8). Since then, aptamers have been widely used in diagnostics, therapeutics, and research because of their advantages over antibodies. In this study, we propose to develop the nuclease-resistant RNA aptamers against the whole cells of *M. avium* by constructing our own RNA libraries following by aptamer selection using SELEX process. After the candidate aptamers are identified, they will be studied for their sequences and predicted secondary structures. They will also be evaluated for the affinity and specificity to *M. avium*. The derived aptamers can be applied for using as a diagnostic tool for identify MAC infected patient with the rapid, cheap, accurate and reproducible with confidential identification. Therefore, the treatment of infected patient can be performed in time and curable. In addition, this can be impacted on the public health by controlling or monitoring the epidemiology of *M. avium* infection.

CHAPTER II
LITERATURE REVIEWS

Genus Mycobacterium recently, composing of more than 60 species and more than 100 subspecies (1) which can classified into 3 groups. First is *Mycobacterium tuberculosis* complex (MTBC) in which a causative agent of Tuberculosis in both human and animal. MTBC member are *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium bovis* bacillus Calmette-Guérin (BCG)-vaccine strain, *Mycobacterium africanum, Mycobacterium canetti, Mycobacterium microti*, *Mycobacterium pinnipedii* and *Mycobacterium caprae. M. tuberculosis* is the most common found in TB patient. Unique characteristic of bacterial in this group is the high lipid content-mycolic acid cell wall. This makes them show gram variable with Gram's stain but show the red positive cell when using acid fast staining. They are slow grower bacterium with the 18-20 hours (hr) doubling time. The infection occur by inhalation of a small droplet contains pathogenic bacilli. They are epidemic around the world especially in South Africa and South Asian pacific. One-in third of the world population is infected by this pathogen. But not all are infected; only 10% will develop the disease. Most cases are shown as latent infection with the granuloma formation in the infected lung. The disease can be shown as neither pulmonary nor extra-pulmonary appearance. Generally, TB patient will take the medicine in the first line drugs regimen:-Rifampicin, Isoniazid, Pyrazinamide, Ethambutol and Streptomycin for at least 6 months. The second is *Mycobacterium leprae,* agent causes leprosy. It is intracellular, non cultivable bacterium and also has a slow growth rate comparable to *M. tuberculosis*. Like *M. tuberculosis, M. leprae* is acid fast positive bacilli.

The last group is a majority of Mycobacterium genus, Non tuberculous Mycobacterium (NTM). They usually found in environmental such as soil and water and can be isolated worldwide. The member composing of more than 120 species (9), the most commonly found is *Mycobacterium avium* complex (MAC). Even though, NTM is shown as saprophyte in environment, some species can be pathogenic in both human and animal such as *M. avium, M. intracellulare, M. kansasii, M. paratuberculosis, M. fortuitum, M. marinum, M. ulcerans, M. smagmatis* and etc. (10). NTM is an acid fast positive bacillus and grow slower compare to other bacteria. They are distributed around the world, there were reported in many countries such as United State of America, India, China, Hong Kong including Thailand. The high

incidence rate of NTM infection was reported in Ghana with 100-150 per 100,000 cases (11). Interestingly, NTM infection is shown to be rising in USA during 1997-2007 from 20 to 47 /100,000 cases (12). In 2010, Winthrop and colleague (13) has shown the interesting data that NTM disease, the most common MAC infection, was occurred mostly in woman (59%) with median age 66. Moreover, women seem to have more 40% risk of infection than men and also most of the infected people (90%) are white, Asians/Pacific racial. In accordance to USA, other countries in Asia such as Japan, Singapore, India also Thailand have been reported that mostly NTM infection (68%) come from MAC (14). In Thailand besides MAC, *M. kansasii* were found to be the second order of NTM infection (15).

Due to the highest incidence rate of infection is MAC among NTM species, MAC will be focusing in this study. MAC is now has many members-*M. avium, M. intracellulare, M. chimaera, M. colombisense, M. vulneris, M. marseillense, M. bouchedurhonense and M. timonense* (16, 17). For *M. avium,* there are 4 subspecies which are *M. avium avium* (MAA), *M. avium hominissuis* (MAH), *M. avium paratuberculosis* (MAP) and *M. avium silvaticum* (MAS) (18, 19). Each subspecies has their own characters in which MAA and MAS is a causative agent of tuberculosis (TB)-like disease in birds (3, 20). MAP is plays a role in the Johne's disease, causing enteritis and diarrhea in the cattle (21), and also Crohne's disease in human (22). Among four subspecies of *M. avium,* MAH has impact on causing human lung infection especially in immunocompomised people such as elderly, children, untreated AID patients and the people with abnormal T cell immunity (23, 24). MAC is generally found in the air and also in water sources such as showerhead, bathtub and nozzles (25).

Pathogenesis of MAC occurred after its ability to colonize or attach the host cell (both epithelial and macrophage cells (26)); using many receptors for instance complement receptors (27), mannose receptors and fibronectin (28, 29). After penetrates, in case of macrophage, MAC can multiply, persist and prevent acidified condition within the cell. Finally, macrophage maturation following by prevent phagosome lysosome fusion is inhibited (30). After engulfment MAC, macrophage starts to secrete numerous cytokines. Key cytokines to control MAC infection are Tumor necrosis factor (TNF)-**α**, Interferon (IFN)-**γ**, Interleukin (IL)-12 (31, 32). Furthermore, Toll-like receptor (TLR) 2 (33), TLR-9 (34) and Myeloid differentiation primary response gene (MyD) 88 (33) was reported as a role in clearance of the infection. It can be stated that, the immune response which play as role in elicit MAC infection is depend on many cell types especially CD4+ T lymphocytes (T helper (Th)-1) and natural killer (NK) cells (31, 32, 35). The outcome symptom in MAC infected patient is varies and depends on a

single host gene *Nramp1* (or *Slc11a1*) which function as sequestering iron and other divalent cations from endosomal system (see review by (36)). Clinical feature of infected patient can be pulmonary and extra-pulmonary ranging from asymptomatic, chronic cough with the expectoration sputum, cylindrical bronchiectasis, distribution of small nodules in tree-in-bud pattern, Lymphadenitis to the disseminated infection (9, 37). Treatment of MAC infection depends on the symptom features and quite similar regimen to Multi Drug Resistant Tuberculosis (MDR-TB) treatment. Typically, mainly macrolide (clarithromycin or azithromycin) combined with rifampicin and/or ethambutol will be used for at least 1 year (2). Single use of macrolide is unacceptable because of the report about the macrolide resistance stain has emerging. Severely side effect such as gastrointestinal suffering, hearing loss and hepatitis can be found as long term used antibiotic (38).

Owing to the widespread of MAC in environment and can present as contaminant organism so MAC infection diagnose is complex. Symptom observations, radiography of lung and culture isolation are required for identification of MAC infection. Start from sample collection which is, usually three morning sputum, induced sputum or bronchoalveolar lavage (BAL). Acid fast staining is applied for early detection but not specific to any *Mycobacterium* species. Cultivation and biochemical testing are considered to be a standard method. Solid (Lowenstein-Jensen (LJ), Ogawa, Middlebrook 7H10/11) or liquid (Mycobacterial Growth indicator Tube; MGIT) media are widely used. Culture condition of MAC is differing from *M. tuberculosis* as optimal growth is when incubated at 30 $^{\circ}$C (38-40). Due to MAC has a slow growth rate, the colony formation can be observed for at least 6-8 weeks after inoculation which is time-consuming. American Thoracic Society (ATS) and the Infectious Disease Society of America (IDSA) had developed the guideline for NTM diagnosis. Radiographic of lung feature combined with the same two isolates from three sputum or by one bronchoalveolar lavage are required for confirm an infection (38). Even though culture is a standard method for identification, but it takes time and sometimes incorrect identifying, emerging the molecular tool for overcome these problems. Nowadays, genomic data are available. Thus, genomic analysis can identify the difference in some genes among MTBC and even in NTM species such as insertion sequence (IS), 16S-23S Internal transcribe spacer (ITS), *hsp65*, *rpoB* and 16S rRNA gene (41-43). Conventional PCR to amplify IS900 (44) and IS1245 (45) can be used to identify MAP and MAA, respectively. In addition, multiplex PCR targeting IS 901 has ability to specify MAA infection (46, 47). RFLP typing using IS1245 and IS 900 were also reported for identified MAC and MAP, respectively (48, 49). Developing real time detection by real time PCR was

shown by targeting IS 900 and F 57 (50) which can identify *M. avium* into subspecies level. Detection of NTM infection included MAC by DNA probe assay was reported. The principle of DNA probe base on the complementary of oligonucleotides to each specific gene of *mycobacterium* species (5). DNA probe assay are firstly Genprobe (San Diego, Calif., USA) targeting *16S rRNA* gene, probe were added after break cell with sonicator and detect the binding complex with a luminometer. It can detect only for one *Mycobacterium* species. Secondly, Inno LiPA (Innogenetics, Ghent, Belgium) based on different in the 16S-23S rRNA spacer region. It begins with amplification target gene by conventional PCR (with biotinylated primer) follow by hybridization with probe. This takes around 5 hr. for the total process. Inno LiPA can identify *Mycobacterium* genus and also discriminate for total 16 mycobacterial species (51). The last is GenoType Mycobacterium assay (Hain, Lifescience, Germany), multiplex PCR and then probe hybridization were performed. It has three generation of kit base on 3 different genes detection which is *gyrB* gene for MTBC infection (52), 23S rDNA (53) for detection of *Mycobacterium* spp. and NTM species. Although, DNA probe assay will give a quick and correct identification but the expense cost is also high for affordably. Interestingly, in 2010 Tortoli and colleague (6) showed the misidentification error of these technique due to the cross reactivity of probe between the *mycobacterium* species. Sequencing analysis is used to overwhelming the closely identical of mycobacterium genes. Nowadays, high-pressure liquid chromatography (HPLC) was used for mycolic acid (cell wall component) analysis to identify Mycobacterium into species level (54). Moreover, real time PCR with high resolution melting (HRM) are reported as a tool for identify mycobacterium in species level. In 2012, Kim and colleague (Patent number WO 2011149280 A3) has developed real time PCR which can differentiate *Mycobacterium* into 23 species. However, these techniques required the specialized instruments, skill for interpretation for accurate diagnostic and costly.

Due to several drawbacks of the existing diagnostic tests for *M. avium*, a simple, fast, and relatively inexpensive test of *M. avium* based on the ligands specifically recognizing the bacteria would be of great use in diagnosis. One class of such ligands is aptamers, which are conformational oligonucleotides capable of binding to the targets with high affinity and specificity. The word ‘aptamer’ is come from Latin word “aptus” means fitting and Greek word “meros” means the particle. It was first reported in 1990 by the group of Ellington in which develops unmodified RNA aptamer parallel with the group of Tuerk who developed RNA aptamer against bacteriophage T4 DNA polymerase (7, 8). The aptamer binds to its target specifically base on its complexity in the structure, stacking of aromatic ring, van der Waals

or electrostatic forces, hydrogen bonding or combination of these effected (55). In general, aptamer can be DNA, RNA, peptide, protein or modified molecule against various types of target including extracellular protein, intracellular protein, enzyme, inorganic compound, amino acid, carbohydrate, antibiotic or even the complex structure molecule such as spore or viral particle (56). Aptamer is varies in its size ranging from 20-80 bases which is bigger than peptide but smaller than antibodies fragment (svFv, Fab; (57)). This make an aptamer can access into the target epitope in which the bulky size as antibodies cannot be done. Aptamer also shows the comparable affinity incomparable to monoclonal antibodies and is in vitro chemically modified oligonucleotide which produces easier than monoclonal antibody and no need animal model.

Typically, aptamer will be identified from the varieties of oligonucleotide pool (library) through the SELEX process. Mostly, library is DNA or RNA consisting of known sequence at 5' and 3' region for 18-20 nucleotides long. In the central region is a 20-80 randomize oligonucleotides sequence, this make a library has minimal diversity for 10^{14} – 10^{15} different unique oligonucleotides which consider to be appropriated for SELEX step. The range of randomized sequence is varies and not specific. It will be designed based on the researcher, short sequence is better in case of management, cost effective and lesser post modification optimization but less complexity compare to the longer one. Even though, the longer randomized oligonucloetides has more complexity, sometime it has to be modified after aptamer were discovered to find the active part of the stand.

Basically, SELEX process (as shown in figure 1) starting from production of randomizes oligonucleotide library pool with 10^{15} different sequences. In case of DNA aptamer selection, the DNA library will be directly mixed with target or change to be single strand DNA (ssDNA) before used. In contrast to, selection of RNA aptamer, the DNA library will be amplified by the special sense primer with inserted T7 promoter and also antisense primer to get double strand DNA (dsDNA). T7 promoter will drive the *in vitro* transcription of dsDNA to be RNA via T7 polymerase enzyme before go to further steps. In the next round of SELEX, the selected RNA aptamer will be amplified by Reverse Transcriptase PCR (RT PCR) with the same primers as mention above in which the product turns to be the RNA pool for the next RNA SELEX round. Next step, target molecule will be mixed with the library pool to let it bound to target via structural specifically with high affinity. Unbound oligonucleotides will be partitioned from target bound oligonucleotides which is report to be a critical step of succession in the SELEX process (56). Elution of bound oligonucleotides will be performed later and also amplified

with the primer compatible to the flanking known sequence generated the new library pool for the next SELEX round. SELEX can be positive SELEX using target molecule for binding to the library pool. In some case, counter SELEX was performed using closely relative molecule to the target of interest to eliminate the non-specific binding of the pool (58). The SELEX process will be repeated for basically 6-20 rounds until it showed no increasing of binding affinity any further. In the last round of SELEX, each individual oligonucleotides will be determined by cloning into the vector plasmid and analysed by sequencing.

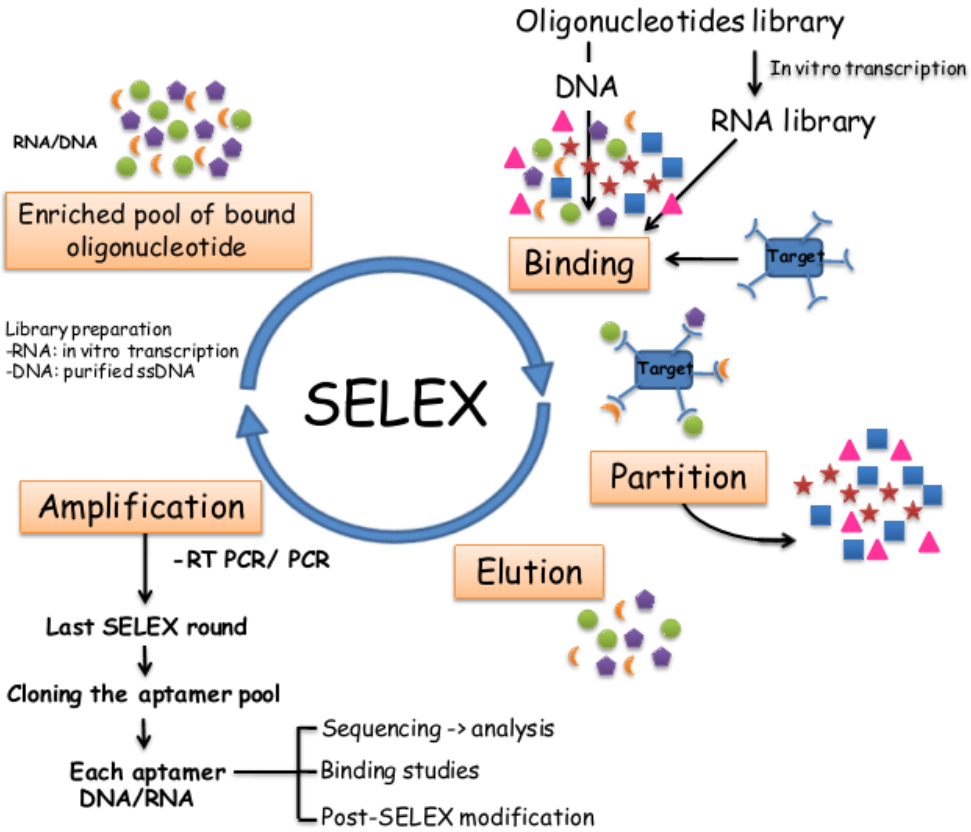

Figure 2.1 General *in vitro* selection target specific aptamer via SELEX process. Basically, SELEX starting from production of randomizes oligonucleotide library pool. In case of DNA aptamer selection, the DNA library will be directly mixed with target or change to be single strand DNA (ssDNA) before used. In contrast to, selection of RNA aptamer, the DNA library will be in vitro transcribed to be RNA via T7 promoter. Next step, target molecule will be mixed with the library pool to let it bound to target via structural specifically with high affinity. Unbound oligonucleotides will be partitioned from target bound oligonucleotides. Elution of bound oligonucleotides will be performed later and also amplified to generate the new enriched bound oligonucleotide library pool for the next SELEX round. SELEX can be positive SELEX using target molecule for binding to the library pool. The SELEX process will be repeated for basically 6-20 rounds until it showed no increasing of binding affinity. In the last round of SELEX, each individual oligonucleotides will be determined by cloning into the vector plasmid and analyzed by sequencing. The binding studies to identify the best fit aptamer are done and sometime also include the post SELEX modification step.

Due to aptamer is a conformational specific binding to the target, the relevant structures of the aptamer had to be studied using the programme on internet such as *mfold* (56). This programme predict the conformation of selected oligonucleotides based on the energy minimizing to propose the structure which part should be stem, loop, bulges or pseudoknot formation (59). Subsequently, the best fit aptamer to the target will be identified by determine the affinity constant (dissociation constant; K_D) in which represent a half occupied between protein and ligand. The more tightly binding is showed as a small K_D value usually nanomolar (ηM) to picomolar (ρM) will be accepted. Also others information are required for the aptamer and target interaction such as footprinting (60, 61), three-dimension conformational structure, studies-magnetic resonance (NMR) spectroscopy, X-ray structure analysis and crystallization (62-64).

Because the aptamers based on natural nucleotides, particularly, RNA aptamers, can be unstable and rapidly degraded when applied in vivo, several measures are used to enhance their stability. One of them is substituting the 2'-OH group of ribose with 2'-F or 2'-NH_2 group to make the RNA aptamers resistant to nuclease degradation (65, 66). In addition, to enhance the discrimination power of the closely related molecule, Locked nucleic acids (LNA) aptamer is used to hybridizing with DNA or RNA. Due to the increasing of helical thermostability in its character, make it resistant to nuclease degradation. Also, modification by truncating the strand of aptamer to narrow down the binding region in aptamer stand will be performed. Sequence truncated of the best fit aptamer is risky. Sometime the best fit aptamer loss its ability and affinity to bind specifically to the target but sometime such truncated aptamer rising the ability for binding to the target (67, 68).

Compared with antibodies, aptamers offer several advantages. Aptamers can be developed against many targets- protein, non-protein etc. even though a complex target structure-bacteria cell. It show high affinity and specifically binding to its target sometimes comparable to monoclonal antibody but it is chemically synthesized *in vitro* without any animal usage. It is reproducibility and also low immunogenicity and exhibit the long shelf life. Due to the smaller size of aptamer, it can be applied for tag with any antibiotic or siRNA, directly bound to target for intracellular detection or inhibition. In the other hand, aptamer has no standardized SELEX protocol and cannot be produced against every target molecule. It is quite unstable in the biological fluid. The successful for production is depend on many factors such as the size, complexity of library pool, quantity of the target and optimal condition in SELEX process.

The report about the production of aptamer against *Mycobacterium* species has been published. In 2007, Chen and colleague (69) had developed the aptamer (NK2) against the whole cell *M. tuberculosis* and used as a new therapeutic tool. They found that the infected cell with *M. tuberculosis* H37Rv in presence of aptamer NK2, cytokine production (IFN-γ) via CD4+T cell was improved when compared to the condition which absence the aptamer. Also with Bannantine and his group has developed the monoclonal antibodies and aptamer against MAP (70). He has produced the three aptamer against the *MAP0105c* gene which known as a unique gene found only in MAP. Although, all three aptamers showed the high affinity binding to the protein at N-terminal half, but they seem to bind to the most of *mycobacterium* strains used in this study. Later on, ssDNA aptamer against MPT64 protein of *M. tuberculosis* was reported to be a new detection tool. Aptamer against MPT64 showed the significance different for detection in group A (non-TB *Mycobacterium*, *M. bovis* BCG) and group B (*M. tuberculosis* and *M. bovis*) with 86.3% sensitivity and 88.5% specificity (71). Recently, aptamer against the specific proteins only found in *M. tuberculosis*, the 6–kDa early secreted antigen target (ESAT)-6 and the 10–kDa culture filtrate protein (CFP)-10, has been constructed and designated as CSIR 2.11. This aptamer was used to evaluate as diagnostic agent with the direct sputum. The sensitivity and specificity showed for 100% and 68.75%, respectively (72) using Youden's index. Besides the role in diagnostic tool, aptamer also showed the role in the study if pathogenesis of tuberculosis. Aptamer produce against whole cell *M. tuberculosis* can inhibit the invasion of bacterial into macrophage cell (73). Moreover, it can stimulate the secretion of cytokines, IFN-γ, IL-15, IL-17, from infected macrophage.

CHAPTER III

OBJECTIVES

3.1 Primary objective - To develop nuclease-resistant RNA aptamers against *M. avium.*

3.2 Secondary objective - To evaluate the diagnostic roles of the aptamers for *M. avium*.

CHAPTER IV
MATERIALS AND METHODS

4.1 Bacterial culture and preparation

Bacterial used in this study including *M. avium* ATCC 25291, *Mycobacterium* spp. (as shown in table 4.1). *Mycobacterium* spp. were cultured on ogawa medium and incubated in 37°C for 28 days. Before used, the confirmation test by colony morphology, biochemical test or molecular analysis were performed. The bacterium was harvested from ogawa slant and suspended into 4 ml 1X steriled PBS. Glass bead were added to the suspension and vortex for 60 sec and stranded for 15 min. Three milliliters were spun down to collected the pellet at 12,000 rpm, 1 min at RT. Then, the pellet was washed twice with 1 ml 1X steriled PBS. For using live *M. avium*, after washed, the pellet was resuspended with 1 ml 1X steriled PBS. For paraformaldehyde pretreated *M. avium,* after washed, the pellet was resuspended with 4% freshly prepared paraformaldehyde for 45 min at RT. Then, thrice washing with 1 ml 1X steriled PBS were performed and suspended with 1 ml 1X steriled PBS.

Table 4.1 List of bacterial strains used in this study

Bacterial strains	Clinical strains identified as
M. avium ATCC 25291	*M. avium* 212 (1)
M. intracellularae	*M. avium* (10)
M. fortuitum	*M. avium* I (8)
M. tuberculosis H37Ra	*M. avium* II (3)
M. chelonae	Unclassified MAC (16),
M. bovis BCG	Unclassified MAC/*M. intracellularae* (2)
M. smegmatis	*M. intracellularae* (2)
M. kansasii	*M. simiae* (1)
E. coli	*M. abcessus* (3)
Staphylococcus aureus	*M. gordonae* (2)

*in parentheses is a number of strains

4.2 Preparation of random nuclease resistant RNA library

4.2.1 Generating dsDNA library

The library template was synthesized from IDT (Integrated DNA Technologies, Inc., Ward Medic, Thailand) the single stranded deoxyoligonucleotides containing 40 nt randomized central sequence flanked by defined primer-binding sites as described in table 4.2. The ssDNA was converted into dsDNA using Polymerase Chain Reaction (PCR) in PCR buffer (10 mM Tris pH 8.3 and 50 mM KCl), 50 mM $MgCl_2$, 2 mM each dNTP, 100 µM each of T7 + 5′ primer and 3′ primer as well as 2.5 U of *Taq* polymerase (Ward Medic, Thailand). The PCR reaction was set 7 cycles: 94°C 30 sec, 60°C 2 min, 72°C 2 min. The PCR product was precipitated by ethanol precipitation and purified by QIAquick PCR purification kit (Qiagen®) as mentioned in manufacturer's instruction.

Table 4.2 Primers used in this study

T7 + 5′primer	5'-AGT AAT ACG ACT CAC TAT A**GG GAG TCG ACC GAC CAG AA**-3' (38 nt) The bold sequence is transcribed into RNA (19 nt; 74).
3′ primer	5'-ATG AGT CTA GAT GTA GAC GCA CAT A-3' (25 nt; 74)
ssDNA library	5'-AGT AAT ACG ACT CAC TAT AGG GAG TCG ACC GAC CAG AA (N40) TAT GTG CGT CTA CAT CTA GAC TCA T-3' (103 nt)
16SC	5′-TCGAAGGTGGGATCGGC-3′ (17 nt; 75)
23SG	5′-GCGCCCTTAGACACTTAC-3′ (18 nt; 75)

4.2.2 Generating nuclease resistant RNA library

The resulted dsDNA library, which contains a T7 RNA polymerase promoter at the 5′end of the sequence, was then transformed into nuclease resistant RNA library by introducing nuclease resistant nucleotides (2′-F-dCTP and 2′-F-dUTP). The *in vitro* transcription reaction was prepared by using 100 mM ATP and GTP, 50 mM 2′-F-CTP and 2′-F-UTP, 50 mM $MgCl_2$, 5x RNA transcription buffer (40 mM Tris-HCl pH 7.5, 25 mM NaCl and 1 mM spermidine), 100 mM DTT and 250 U of T7 R&D polymerase (Epicenter). The reaction was carried out 6 h at 37 °C. The remaining DNA was treated with 2 U of DNase I and further incubated 15 min. The resulting RNA was purified with QIAquick Nucleotide Removal kit (Qiagen®) and eluted

with EB buffer (10 mM Tris pH 8). The 2′-F RNA library was quantified by BioTek™ Eon™ Microplate Spectrophotometers and checked integrity by 3% agarose gel electrophoresis with ethidium bromide staining.

4.3 SELEX using Centrifugal Filter Units (Merck Millipore, Germany)

4.3.1 Coupling *M. avium* ATCC 25291 to Centrifugal Filter Units

The live *M. avium* ATCC 25291 were harvested using the cell suspension as described in 3.1 to reach the desired concentration for binding to the column. Initially, the 150 µl of the cell suspension was spun down. The pellet was washed with the binding buffer (0.5 M HEPES pH 7.4, 1 M NaCl, 0.1 M $MgCl_2$) and suspended with 100 µl of binding buffer and then, added into Centrifugal Filter Units.

4.3.2 Binding step

In this step, nuclease resistant RNA library was incubated with *M. avium* cell in Centrifugal Filter Units. Before binding, RNA library was denatured at 65°C for 1 min and allowed to cool over 5 min and added 10 µl of 10x binding buffer (0.5 M HEPES pH 7.4, 1 M NaCl, 0.1 M $MgCl_2$) and 1 µl of 10 mg/ml of Baker's yeast tRNA to make 1x reaction. The equilibrated RNA was incubated with *M. avium* cell on rotator at as described in the selection strategy (Table 4.3).

4.3.3 Washing step

After the binding step, the Centrifugal Filter Units was spun 12,000 rpm, 1 min at RT to remove the unbound RNA. The unbound RNA was washed with 1x binding buffer, for the specified volume, number of wash and period of time as described in the selection strategy (Table 4.3) on a rotator. For last wash, use pipette to completely draw all of binding buffer out from the tube of a spin column.

4.3.4 Elution step

After the last washed, 100 µl of elution buffer (8 M urea, 5 mM EDTA pH 8.0) was added and incubated at RT for 15 min on a rotator. At the end of elution, the Centrifugal Filter Units the eluate was transferred to a new 1.5-ml microcentrifuge tube and precipitated by ethanol precipitation step by adding 5 µl of 5 M NaCl (final ~ 0.25 M), 2 µl of 20 mg/ml glycogen (final ~ 400 µg/ml) and mixed well. Then, 250 µl of ice-cold 100% ethanol were addes and spun down in a refrigerated centrifuge at 12,500 xg, 30 min at 4°C. The supernatant was discarded and the pellet was washed with 500 µl of ice-cold 70% ethanol and dried for 10 -15 min.

Table 4.3 SELEX protocol for nuclease-resistant RNA aptamers specific to live *M. avium* ATCC 25291

Round	Input RNA		cell suspension (µl)	Binding volume (µl)	Washing	Note
	pmol	µg				
1	50	1.43	300	100	100 µl X 5 times, 1 min each	
2	25	0.715	300	100	100 µl X 5 times, 2 min each	
3	25	0.715	300	100	100 µl X 5 times, 4 min each	
4	25	0.715	150	100	100 µl X 5 times, 4 min each	
5	25	0.715	150	100	100 µl X 5 times, 4 min each	
6	25	0.715	150	100	100 µl X 5 times, 6 min each	
7	12.5	0.375	150	100	100 µl X 5 times, 6 min each	Counter SELEX
8	12.5	0.375	100	100	100 µl X 5 times, 6 min each	
9	12.5	0.375	100	100	100 µl X 5 times, 6 min each	
10	12.5	0.375	100	100	100 µl X 5 times, 6 min each	
11	12.5	0.375	100	100	100 µl X 5 times, 8 min each	
12	6.25	0.178	100	100	100 µl X 5 times, 8 min each	

4.3.5 Reverse transcription (RT) step

The RNA pellet was dissolved with 90 µl of water containing 3´primer (300 µM) and incubate at 65°C for 1 min and cooled on ice for 2–3 min for the purpose of primer annealing. The reverse transcription reaction was proceeded by adding 5x 1st strand buffer (0.04 M Tris-HCl pH 7.5, 10 mM NaCl and 2 mM spermidine), 10 mM dNTP, 0.1 M DTT and 0.3 µl (60 U) of Superscript™ III and incubated at 50°C for 45 min using thermal cycler (A200 Gradient Thermal Cycler, LongGene Scientific Instruments Co. Ltd., Zhejiang, China).

4.3.6 PCR reaction

After generating cDNA via reverse transcription reaction, PCR amplification was directed by using thermal cycler. The PCR reaction mix was prepared with 10x PCR (20 mM Tris pH 8.4 and 50 mM KCl), 50 mM $MgCl_2$, 100 µM T7 + 5´primer and 5 U of *Taq*; total volume 200 µl. Ethidium bromide was used as a reporter dye as it is inexpensive. The amplification was set for initially 13-14 cycles with 94°C 30 sec for denaturation, 60°C 30 sec for annealing, 72°C 2 min for elongation. The resulted PCR product was checked by 3% agarose gel electrophoresis. The resulted PCR product (100 µl) were taken to be purified with PCR QIAquick purification kit (Qiagen®) and eluted with 30 µl of EB buffer (10 mM Tris pH 8.5). The eluate (25 µl) was used for *in vitro* transcription reaction (IVT).

4.3.7 *In vitro* transcription reaction (IVT)

The IVT reaction (50 µl) was set with 5x RNA transcription buffer (0.04 M Tris-HCl pH 7.5, 10 mM NaCl and 2 mM spermidine), 100 mM ATP and GTP, 50 mM 2´F-CTP and 2'F-UTP, 50 mM $MgCl_2$, 25 µl of Qiaquick purified PCR reaction, 100 mM DTT and 62.5 U of T7 R&D polymerase. The reaction was incubated at 37°C for 3 hour. The resulted RNA was further treated with 2 U of DNase I (Promega®) and 3 µl of RQ1 buffer at 37°C for 15 min to remove extra DNA. The product was purified with Qiagen nucleotide removal kit (Qiagen®) and eluted with 50 µl of EB buffer (10 mM Tris pH 8.0). The concentration of RNA was checked with UV nanophotometer at 260 nm and also determined the ratio of 260/280 and analyzed by 3% agarose gel electrophoresis for the purpose of integrity.

The new derived RNA library was repeat incubated with target cells (*M. avium* ATCC 25291) for next selection to generate the RNA pool that selectively enriched to *M. avium* ATCC 25291 cells.

4.4 Binding assays of RNA aptamers

4.4.1 Determination of relative affinity of the aptamers to *M. avium*

Because whole bacterial cells of *M. avium* were used instead of specific targets with defined molecular weights, therefore, the dissociation constants of the aptamers could not be determined. The relative affinity was evaluated instead based on the binding assay. The assay was performed in a similar fashion to the selection process. Briefly, the target cells and the tested aptamers were incubated in column. The unbound RNAs were removed by spun down and the cells were further washed to remove any remaining non-specific RNAs. The bound aptamers were eluted. The amount of the input and eluted RNAs were determined using Real time PCR (Light Cycler 96, Roche).

Briefly, in reverse transcription reaction, the library and the interested input RNA aptamer were prepared the input samples by taken 1 µl of each input and mixed with 99 µl of urea elution buffer. Then, take 1 µl of each sample (i.e. library input sample, library eluate, interested input RNA aptamer, interested eluate RNA aptamer) and mixed with 17 µl of 3' primer/water elution mix. Next, all samples were incubated at 65°C for 1 min and cool it down on a cold metal box for 2-3 min. Master mixed of RT was prepared using RT mix and Superscript^{TM} III before dispensed 6.8 µl to each assay tube including negative control tube (NTC). Subsequently, all samples were incubated at 50°C for 45 min. In real time PCR reaction, all samples were performed in total 10 µl with triplicate reaction using 5 µl of 2x SensiFAST SYBR No-ROX kit (Lab leader), 0.05 µl of 5' primer (100 µM) and 3' primer (100 µM), 0.5 µl of cDNA from RT reaction and 4.4 µl of Water. Afterward, dispensed 32 µl of real time master mix to each tube and 1.7 µl of the corresponding cDNA from RT reaction were added. Then, transfer 10 µl of each reaction sample into the corresponding triplicate real-time PCR tube. For NTC, take 9.5 µl and 0.5 µl of RT-NTC. The amplification profile was set as 95°C for 10 min follows by 94°C 30 sec, 60°C 30 sec, 72°C 30 sec for 35 cycles. The fluorescence signal was read at 60°C and also set the dissociation curve 65°C to 95°C. The binding percentage is calculated as $2^{-(Ct\ eluate\ -\ Ct\ input)}$. The aptamers with stronger affinity show higher binding percentages.

4.4.2 Determination of the specificity of the aptamers

The binding percentages of the aptamers to the specific target *M. avium* ATCC 25291 and the non-specific targets (i.e., closely-related *Mycobacterium* spp.) are evaluated as above. Moreover, 47 NTM isolated from human samples were also used to evaluate the diagnostic role of candidate aptamer.

4.5 Aptamer characterization

4.5.1 Cloning

3´A-overhangs on the PCR product was incorporated into the linearized vector (pJET2.1), which allows the PCR inserts to ligate efficiently with the plasmid vector. The PCR products of the 6^{th} and 12^{th} selection round of live *M. avium* cell were ligated into pJET1.2 blunt cloning vector (Thermo scientific) with T4 DNA ligase in the ligation buffer and keep at 25°C for 30 min. Each 5 µl of ligation reaction is transformed into 100 µl of *E. coli* cells competent cell (TOP10F) via heat shock at 42°C, 90 s and immediately returned back onto ice for 2 min. Then, SOC medium was added to the final volume 1 ml and incubated on shaker at 37°C for 1 h. After that, the transformation reaction was separated into 100 µl each and spun down for 30 sec. The supernatant was removed to left around 100 µl of total volume. Then, the pellet was gently dissolved and spread onto the LB-Ampicillin agar plates.

After overnight incubation at 37°C, colonies were appeared on the plates. Each colony was picked and restreaked on LB-Ampicillin plate to obtain single colony. Restreaked plates were put in incubator at 37°C overnight again. Overnight cultures were grown from single colonies. Then, each colony was picked up and inoculated in LB broth with ampicillin. The solution was incubated on shaker at 37°C overnight. The bacterial cultures were transferred into 1.5 ml microfuge tube and centrifuged at 12,000 rpm for 1 min. The supernatant was discarded and plasmid DNA was extracted from pellet and purified by Nucleospin® Plasmid Extraction Kit (Macherey-nagel GmbH & Co. KG). The resulted purified plasmids were eluted with elution buffer 50 µl. Small scale PCR (10x PCR buffer, 50 mM $MgCl_2$, 2 mM dNTP, 100 µM T7 + 5´ primer, 100 µM 3´primer and 5 U of Taq polymerase) was performed to check for insert DNA sequence. PCR products were checked by 3% agarose gel with ethidium bromide staining.

4.5.2 Sequencing

The 20 plasmids with inserts of the selected clones were sent to IDT for sequencing. Each individual aptamer was developed from plasmid clones by PCR amplification with the T7 + 5´ and 3´ N40 primers, followed by *in vitro* transcription with T7 R&D polymerase.

4.5.3 Secondary structure prediction

Based on consensus sequences, the aptamers were grouped and analyzed secondary structure by mfold web server.

CHAPTER IV
RESULTS

5.1 *Mycobacterium* species were cultured and identified.

M. avium ATCC 25291, *M. tuberculosis* were cultured on ogawa medium and incubated in 37°C for 28 days. Before used, the confirmation tests were performed using PCR-RFLP. The DNA were extracted from the culture by boiling and subjected to be the template in PCR reaction using primer specific to all *Mycobacterium* species (16SC-23SG primer). Then, the PCR product (380 bp; Figure 5.1) was cut with restriction enzyme (*Hae*III). The members of MTBC were digested into 200, 150 and 25 bp. For the *M. avium*, PCR products were digested into 155, 115, 65 and 40 bp (as shown in Figure 5.1 and 5.2). Moreover, the same cultures were also tested with mtp64 strip (SD BIOLINE TB Ag MPT64 Rapid, Abbott) which is a specific antigen against *M. tuberculosis*. As expected, *M. tuberculosis* culture showed positive band and *M. avium* culture showed none of positive band in the test line (Figure 5.3). These testing can be confirmed the species of *Mycobacterium* used (*M. tuberculosis* H37Ra and *M. avium*) in this study.

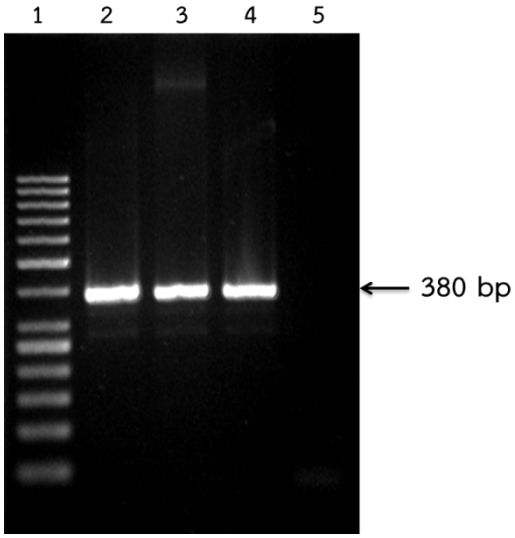

Figure 5.1 PCR products using 16SC-23SG primers (380 bp) before cutting with restriction enzyme (*Hae*III). Lane 1: 100 bp DNA ladder, Lane 2-4: PCR products using *M. avium*, *M. tuberculosis* and *M. bovis* as template, respectively. Lane 5: Negative control (NC).

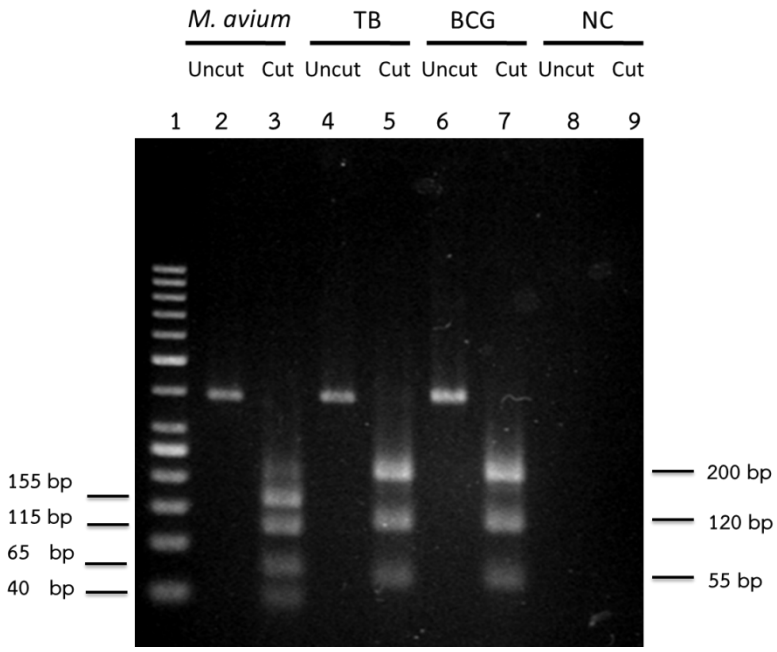

Figure 5.2 Restriction of PCR products with *Hae*III enzyme. Lane 1: 100 bp DNA ladder, Lane 2-3: Uncut and cut of *M. avium* PCR products, respectively. Lane 4-5: Uncut and cut of *M. tuberculosis* PCR products, respectively. Lane 6-7: Uncut and cut of *M. bovis* PCR products, respectively. Lane 8-9: Uncut and cut of PCR product from negative control.

Figure 5.3 MPT64 strip test. The result of MPT64 strips test when using culture supernatant from *M. tuberculosis* (upper panel) and *M. avium* (lower panel). C; control line, T; test line.

5.2 Generating nuclease-resistant RNA library

The single stranded nuclease-resistant (2′-F) RNA library template was synthesized from single stranded deoxyoligonucleotides (ssDNA) containing randomized 40 nt central sequence flanked by constant primer binding regions (T7 + 5′ primer and 3′ primer). Double stranded DNA (dsDNA) (103 nt) was generated by polymerase chain reaction (PCR) with 7 cycles of amplification. The PCR product was purified with QIAquick® PCR purification kit and eluted with EB buffer (10 mM Tris pH 8). The integrity of dsDNA was checked with 3% agarose gel electrophoresis with ethidium bromide staining and quantified by spectrophotometer (Figure 4.4). The result of purified dsDNA was 102.58 ng/µl.

The dsDNA was converted to single stranded RNA (ssRNA) (84 nt) by T7 R&D polymerase. To avoid ribonuclease digestion, modified nucleotides such as 2′-fluoro-deoxy-CTP and 2′-fluoro-deoxy UTP (2′-F-CTP and 2′-F-UTP) were introduced into *in vitro* transcription reaction. The resulted ssRNA was treated with DNase I to remove the leftover dsDNA templates. The resulted single stranded 2′-F RNA library was purified by QIAquick® nucleotide removal kit and eluted with 50 µl of EB buffer (10 mM Tris pH 8). The amount of RNA was measured with spectrophotometer and the integrity of RNA was examined on 3% agarose gel electrophoresis as well. The amount of ssRNA library was 200 ng/µl.

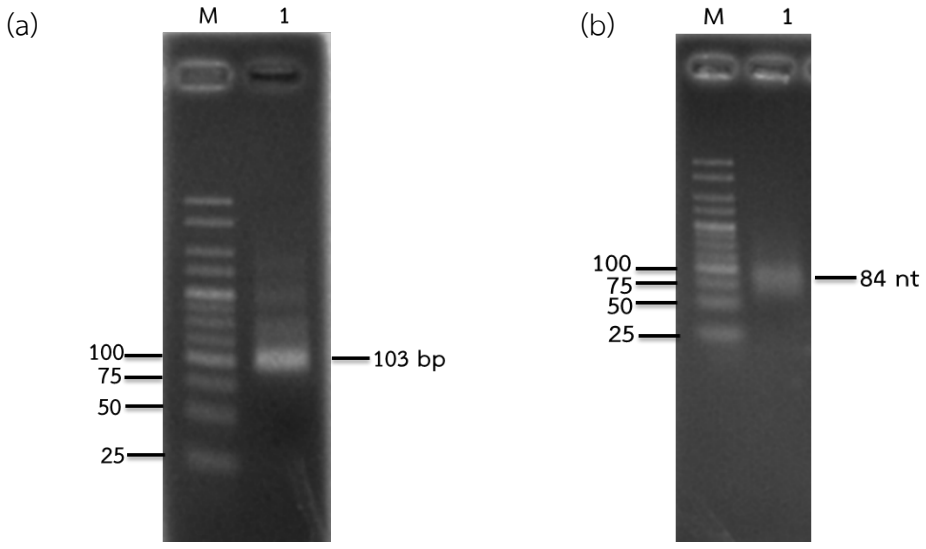

Figure 5.4 Purification of PCR products (a) and RNA (b) using QIAquick® purification kit and nucleotide removal kit of library. (a) Lane M: Low molecular weight 25-bp DNA ladder, Lane 1: purified PCR products of library. (b) Lane M: Low molecular weight 25-bp DNA ladder, Lane 1: purified RNA of library.

5.3 Nuclease resistant RNA aptamer against *M. avium* cell were produced.

5.3.1 Nuclease resistant RNA aptamer against live *M. avium* cell were produced.

Live *M. avium* cell were used as a target to produced specific RNA aptamer. The 28 days culture of *M. avium* was prepared as described in materials and methods. Then, incubated with the library aptamers in the different SELEX process in which the selection strategy was described in table 4.3. The selection processes were continuously done in total 6 rounds before applied the counter SELEX in round 7. In summary total 12 round of SELEX were done.

At first, total 8 round of SELEX were performed. The RNA aptamer from R6, R7 and R8 were selected for determination of binding affinity. Briefly, the target cells (live *M. avium* cell) and the tested aptamers (R6/R7/R8 RNA aptamer) were incubated in column. The unbound RNAs were removed by spun down and the cells were further washed to remove any remaining non-specific RNAs. The bound aptamers were eluted. The amount of the input and eluted RNAs were determined using Real time PCR (Light Cycler 96, Roche). In real time PCR reaction, all samples were performed in total 10 µl with triplicate reaction. The fluorescence signal was read at 60°C and also set the dissociation curve 65°C to 95°C. The binding percentage was calculated as $2^{-(Ct\ eluate\ -\ Ct\ input)}$. The results were clearly shown that the selected R6 RNA demonstrated the highest binding affinity against live *M. avium* cells by Real time PCR assay (Table 5.1).

Table 5.1 Binding affinity comparison of R6, R7 and R8 aptamer against live *M. avium* cell using Real time PCR

Sample Name	%binding		% Average	Ratio Binding
	1	2		
Eluted RNA library R6	3.10	3.19	3.145	
Eluted RNA R6	119.2	108.93	114.065	36.28
Eluted RNA library R7	3.10	3.10	3.10	
Eluted RNA R7	56.5	61.7	59.1	19.06
Eluted RNA library R8	2.14	-	2.14	
Eluted RNA R8	21.48	-	21.48	10.02

Furthermore, different cell types of closely related *Mycobacterium* spp. were used to further evaluate specificity of candidate aptamer; R6 RNA). The statistical analysis was done to compare the binding affinity of R6 RNA aptamer against three species of *Mycobacterium* using Mann-Whitney U test. The R6 RNA aptamer exhibited significant strong binding with *M. avium* ATCC 25291 (ratio; 77) and also slightly bound to *M. avium* 212 clinical isolate (ratio; 33) when compared to RNA library. Low binding activity was also observed when tested R6 RNA aptamer against *M. tuberculosis* H37Ra (ratio; 7.2) as summarized in table 5.2 and figure 5.5.

Table 5.2 Binding specificity of R6 aptamer against other live *Mycobacterium* spp. using Real time PCR

Sample Name	%binding		% Average	Ratio Binding
	1	2		
Eluted RNA library av. ATCC*	1.53	0.84	1.185	
Eluted RNA R6 av. ATCC*	51.27	131.9	91.585	77.29
Eluted RNA library av. 212**	0.32	0.25	0.285	
Eluted RNA R6 av. 212**	4.95	14.29	9.62	33.75
Eluted RNA library H37Ra***	1.58	0.08	0.83	
Eluted RNA R6 H37Ra***	1.9	10.06	5.98	7.2

Note: *av. ATCC: *M. avium* ATCC25291; **av. 212: *M. avium* strain 212; ***H37Ra: *M. tuberculosis* H37Ra

A)

Descriptive Statistics
Split By: Group, Target

	Mean	Std. Dev.	Std. Error	Count	Minimum	Maximum	Median
% Binding, Total	13.3	32.1	7.6	18	0.1	131.9	1.4
% Binding, Lib, 212	0.4	0.2	0.1	3	0.3	0.6	0.3
% Binding, Lib, ATCC	0.9	0.6	0.4	3	0.3	1.5	0.8
% Binding, Lib, Ra	0.7	0.8	0.5	3	0.1	1.6	0.3
% Binding, R6, 212	6.9	6.7	3.9	3	1.4	14.3	4.9
% Binding, R6, ATCC	67.5	58.0	33.5	3	19.3	131.9	51.3
% Binding, R6, Ra	3.7	4.6	2.6	3	0.3	8.9	1.9

B)

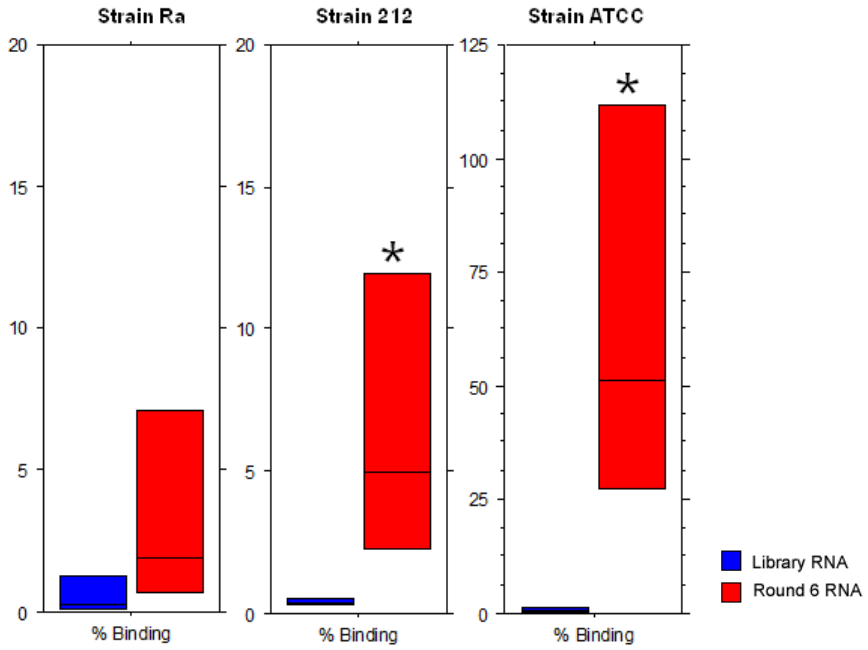

Figure 5.5 Statistical analysis of R6RNA binding affinity against three species of *Mycobacterium* using Mann-Whitney U test. To determine the specificity of candidate aptamer, R6 RNA were tested with *M. avium* ATCC25291 (strain ATCC); *M. avium* strain 212 (strain 212) and *M. tuberculosis* H37Ra (strain Ra). A) Descriptive statistic and B) Box plot graph. Asterisk mean statistical significant at p-value < 0.05. Lib; Library, R6; R6 RNA.

5.3.2 Cloning and sequencing of R6-RNA aptamer

After confirming, PCR product of potential aptamer was ligated into pJET1.2 vector and transformed into TOP10F competent cell via heat shock mechanism. The plasmids DNA from selected clones were extracted and amplified using T7-5' N40 and 3' N40 primer to further confirm the clones harboring R6-insert aptamer. Sequence analysis of 20 clones indicated the variation in the random region of aptamer which were classified into 4 groups according to their consensus sequence. As shown in figure 5.6, R6 aptamer-group 1 was composed of shared sequences containing the sequence 5' GATCTACAC 3' whereas in group 2 which were clone 24 and 25 showed the identical sequence. Group 3 and 4 were shared the homology sequence in various position throughout the random region. Moreover, the remaining clones were categorized as unclassified group (Figure 5.6).

Figure 5.6 Classification of R6 RNA aptamer sequences. The shared sequence motif was highlighted.

5.3.3 Secondary structure prediction of R6-RNA aptamer

The secondary structures of the aptamers were analyzed using a secondary-structure prediction algorithm (mfold web server). The predicted structure in the same group was quite different and indicated the complex structure elements of stem-loop. Taking group 1 of R6 aptamer as an example (Figure 5.7-5.8), all four aptamers (MA03, MA11, MA15, and MA20) shared the GAUCUACAC consensus sequence. MA03 and MA11 seem to originate from different origins but evolved to obtain the shared sequence and similar predicted secondary structures.

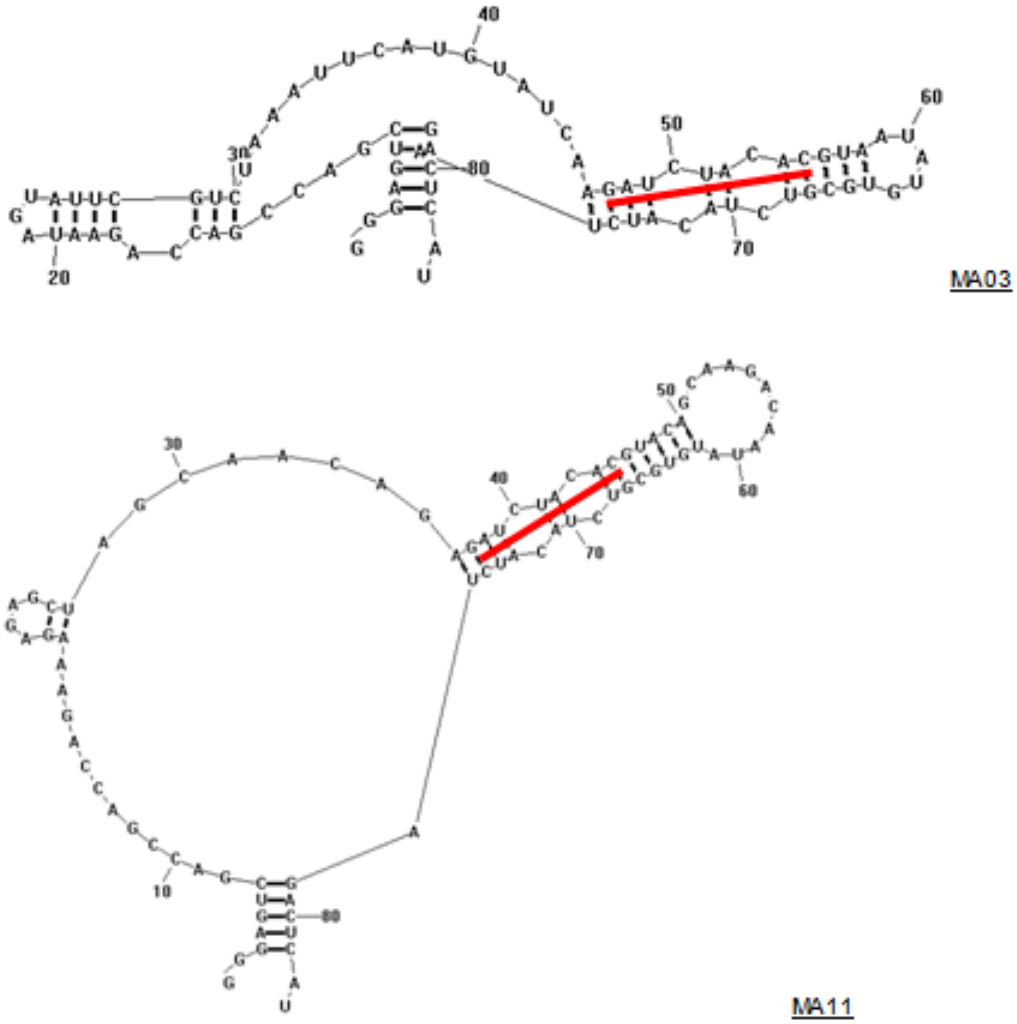

Figure 5.7 The predicted secondary structure of MA03, MA11 monoclonal aptamer

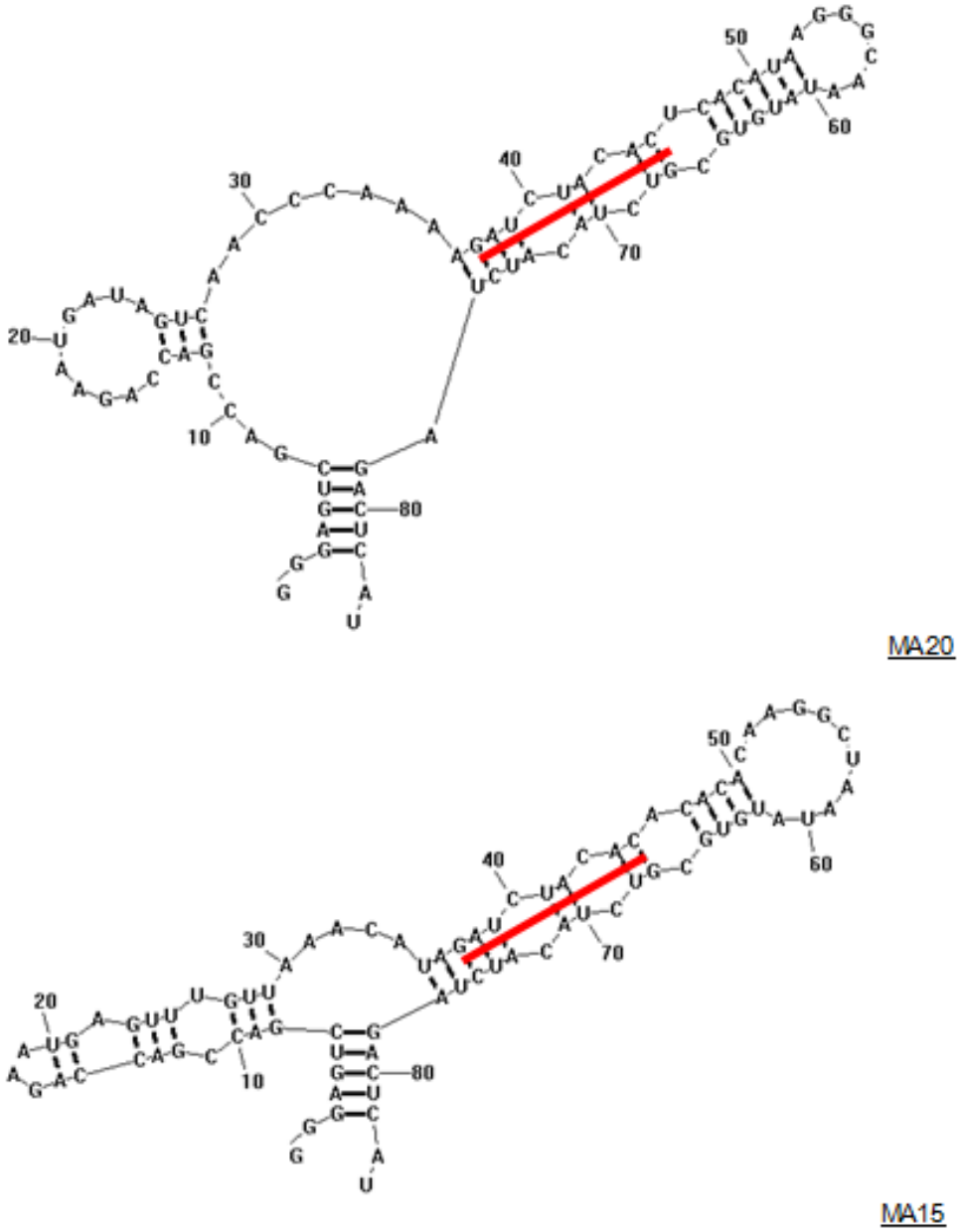

Figure 5.8 The predicted secondary structure of MA20, MA15 monoclonal aptamer

5.3.4 Binding analysis of each monoclone of R6-RNA aptamer with *Mycobacterium* spp.

To determination of relative affinity of each monoclone RNA aptamers from R6 to the target cells (live *M. avium* ATCC 25291, *M. tuberculosis* H37Ra, *M. fortuitum*, *M. intracellulare*), monoclone RNA aptamer from R6 were incubated with target cell in column. The unbound RNAs were removed by spun down and performed according to previously described.

The results were showed in the table 5.3-5.7 from total 19 clones, only 7 clones showed the significant %binding against *M. avium* ATCC25291 compare to the library including, MA01, MA02, MA03, MA05, MA07, MA19, and MA23 using Mann-Whitney U test. Then, these 7 clones were tested the specificity against other species of *Mycobacterium* (*M. tuberculosis* H37Ra, *M. fortuitum*, *M. intracellulare*). We found that all monoclone RNA aptamers can bind to the tested *Mycobacterium* spp. except MA02 which cannot bind to *M. intracellulare* compared to their libraries control. From the experiment, it can be suggested that these monoclone RNA aptamer might bind to the share epitope among *Mycobacterium* genus.

Table 5.3 Summarize of the binding specificity result of each monoclone RNA aptamer from R6 against four *Mycobacterium* spp. using Real time PCR

Monoclone	*Mycobactetium spp.*			
	M. avium ATCC	*M. tuberculosis* H37Ra	*M. intracellulare*	*M. fortuitum*
MA01	√	√	√	√
MA02	√	√	X	√
MA03	√	√	√	√
MA05	√	√	√	√
MA07	√	√	√	√
MA08	X			
MA11	X			
MA12	X			
MA15	X			
MA16	X			
MA17	X			
MA18	X			
MA19	√	√	√	√
MA20	X			
MA21	X			

Monoclone	*Mycobacterium* spp.			
	M. avium ATCC	*M. tuberculosis* H37Ra	*M. intracellulare*	*M. fortuitum*
MA22	X			
MA23	√	√	√	√
MA24	X			
MA25	X			

***X = Not significant (p –value ≥ 0.05)**

Table 5.4 Binding affinity of each monoclone RNA aptamer from R6 against *M. avium* ATCC 25291 using Real time PCR

Monoclone			%binding when tested with *M. avium* ATCC 25291								
		1	2	3	4	5	6	7	8	9	10
Group 1	MA 11	0.64	0.92	2.15	0.92	14.12					
	MA 15	1.93	3.97								
	MA 20	3.06	1.46								
	MA 23	104.45	201.78								
	MA 03	24.03	89.47	88.24	50.68	520.35	321.8	264.42	408.26	117.78	46.96
	MA 08	8.8	6.42								
	MA 21	8.75	2.83								
Group 2	MA 24	1.08	3.53	17.07	0.58						
	MA 25	4.3	6.76								
Group 3	MA 07	449.86	78.25	526.39	103.25	739.29					
	MA 17	1.69	4.58								
Group 4	MA 01	501.46	92.41								
	MA 19	19.84	56.1	78.79	0.46	402.64					
Unclassified	MA 02	88.03	54.69								
	MA 16	6.56	2.27								
	MA 05	165.42	119.7								
	MA 22	6.74	7.83								
	MA 18	1.82	2.63								
	MA 12	0.75	0.56								
Negative	L1	2.27	3.17								
library		6.96	27.16	6.36	3.06	8.88	11.11	8.79	14.06	2.37	3.51
		18.72	0.64	2.93	6.3						

Table 5.5 Binding affinity of each monoclone RNA aptamer from R6 against *M. tuberculosis* H37Ra using Real time PCR

Monoclone		%binding when tested with *M. tuberculosis* H37Ra						
		1	2	3	4	5	6	7
Group 1	MA 23	80.63	94.14	152.22				
	MA 03	51.51	425.59	378.24	49.98			
Group 3	MA 07	55.59	279.49	574.70				
Group 4	MA 01	250.15	148.06	572.05				
	MA 19	32.22	168.51	255.41				
Unclassified	MA 02	39.03	7.93	155.42				
	MA 05	68.75	181.44	260.17				
library		0.05	2.30	5.64	1.40	1.58	4.64	1.28

Table 5.6 Binding affinity of each monoclone RNA aptamer from R6 against *M. intracellulare* using Real time PCR

Monoclone		%binding when tested with *M. intracellulare*					
		1	2	3	4	5	6
Group 1	MA 23	20.25	17.47	7.96			
	MA 03	74.72	17.23	22.37			
Group 3	MA 07	23.43	39.03	23.10			
Group 4	MA 01	29.24	31.63	18.55			
	MA 19	7.45	5.80	12.13			
Unclassified	MA 02	5.25	4.27	0.90			
	MA 05	23.43	13.18	8.77			
library		3.01	0.72	1.00	0.79	1.07	0.26

Table 5.7 Binding affinity of each monoclone RNA aptamer from R6 against *M. fortuitum* using Real time PCR

Monoclone		%binding when tested with *M. fortuitum*						
		1	2	3	4	5	6	7
Group 1	MA 23	156.14	173.24	20.25	64.59			
	MA 03	45.36	119.98	39.12				
Group 3	MA 07	152.92	6.59	106.64				
Group 4	MA 01	61.25	366.25	26.11	103.97			
	MA 19	144.34	25.11	28.97				
Unclassified	MA 02	19.81	352.14	24.53	25.75			
	MA 05	5.86	18.51	17.47	283.39			
library		5.98	3.88	6.13	1.90	5.97	1.86	5.43

5.4 Counter SELEX of R6 aptamer using *M. intracellulare, M. tuberculosis H37Ra* and *M. fortuitum*

Owing to the aptamer from R6 showed non-specific binding to *M. intracellulare, M. tuberculosis* H37Ra and *M. fortuitum.* Thus, to improve the specificity of R6 aptamer, the counter SELEX using non target cells (*M. intracellulare, M. tuberculosis* H37Ra and *M. fortuitum*) were performed. The SELEX were also processed until R12 which shown the highest %binding of aptamer against *M. avium* equal to 4.75 (24.75 fold compared to library; shown in table 5.8).

Table 5.8 Ratio of binding affinity of R6-R12 aptamer compare to library against live *M. avium* cell using Real time PCR

Round	Ratio binding (compare to library)
R6	36.28
R7	0.24
R8	3.72
R9	3.18
R10	ND
R11	3.83
R12	24.75

After that, R12-RNA aptamer pool were cloned into pJET1.2 and 16 clones were sequenced as describe previously. The result show in the figure 5.9. After alignment, the R12-RNA aptamer pool were classified into 3 groups. Group 1 is clone 15, 21 and 26 (shown in red box). Group 2 is clone 2, 3 and 8 (shown in green and blue boxes). The rest of RNA aptamer clones were classified in group 3. From sequence analysis, these 3 groups of R12-RNA aptamer showed the similarity in certain motif (highlight in black). Interestingly, this motif showed similarity to group 1 of R6-RNA aptamer implied that R12-RNA aptamer might evolved from group 1 of R6-RNA.

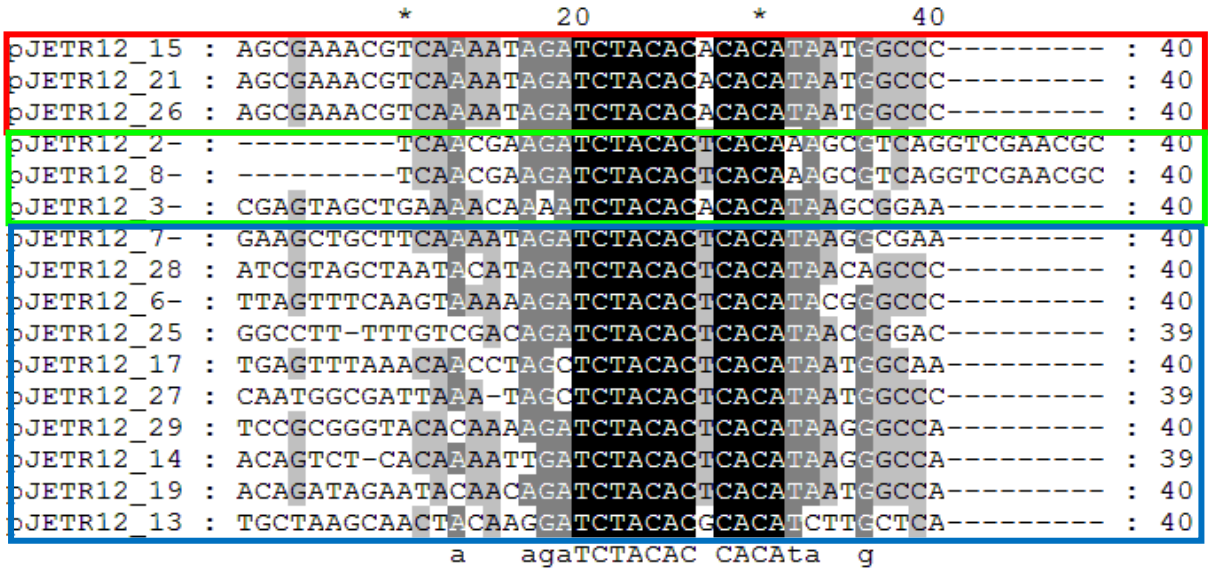

Figure 5.9 Classification of R12-RNA aptamer sequences. The shared sequence motif was highlighted.

5.4.4 Secondary structure prediction

The secondary structures of the representative aptamers from each group (MA12-8, MA12-21 and MA12-29) were analyzed using a secondary-structure prediction algorithm (mfold web server). The predicted structure of three clones were quite similar comprising of the main stem with three loops. The small different were found in the sequence and the size of the loop. The green shade area showed the randomized region of the aptamers (Figure 5.10).

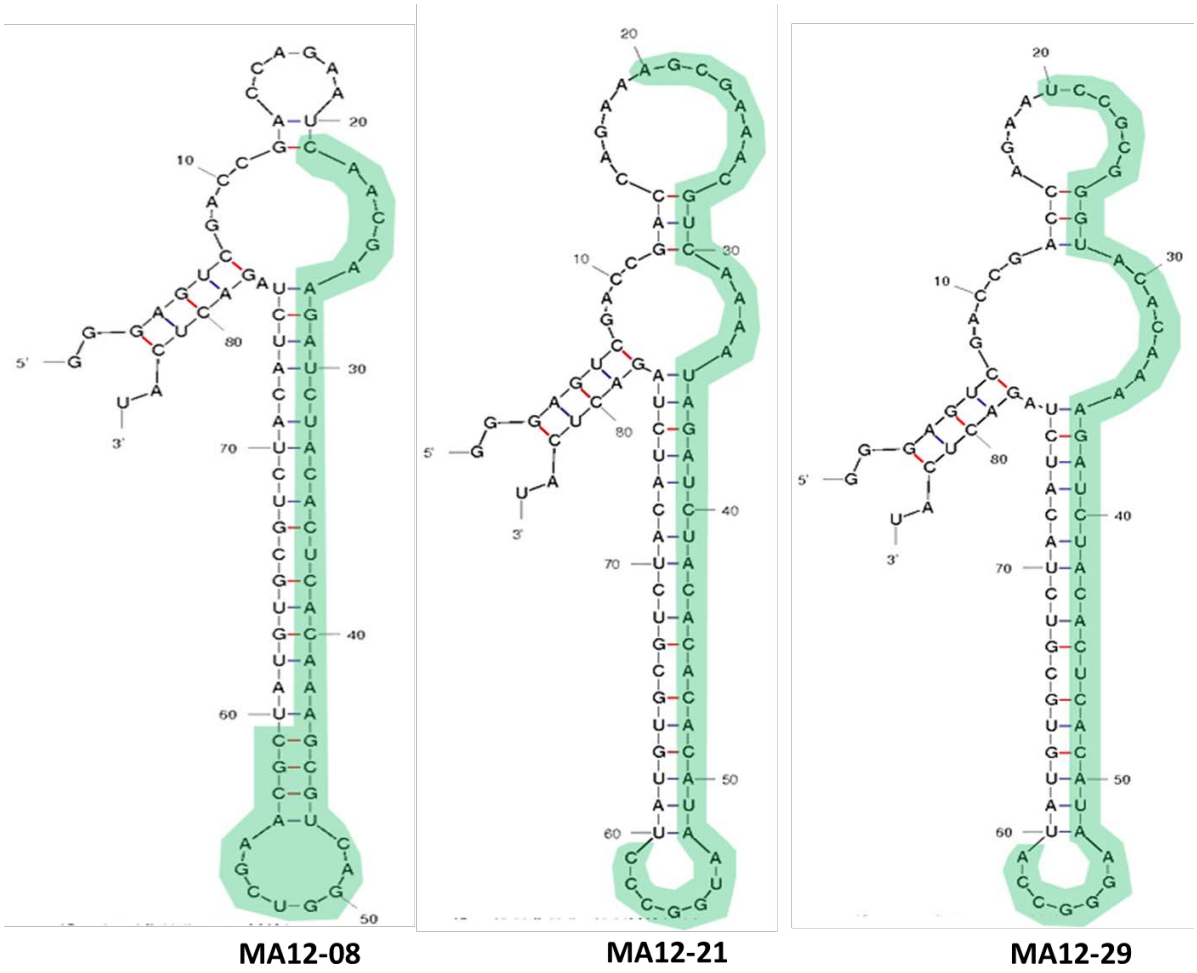

Figure 5.10 The predicted secondary structure of MA12-08, MA12-21 and MA12-29 monoclonal aptamers. The shaded areas indicate the randomized regions of the aptamers.

5.4.1 Binding analysis of representative monoclone from each group of R12-RNA aptamer with *Mycobacterium* spp.

To determination of relative affinity of each monoclone RNA aptamers from R12 to the various cells (live *M. avium* ATCC 25291, *M. tuberculosis* H37Ra, and *M. avium* 212), the representative monoclone from each group of RNA aptamer from R12 (MA12-08, MA12-21 and MA12-29) were incubated with target cells in column and performed as previously described. The results were showed in the table 5.9-5.12 that MA12-29 is the best candidate monoclone owing to it showed the significant specific bound to *M. avium* ATCC 25291 and *M. avium* 212 clinical strain but not bound to *M. tuberculosis* H37Ra.

Thus, MA12-29 were selected for further study to determine its specificity using various bacterial genus and species including *M. fortuitum, M. intracellulare, M. chelonae, M. bovis* BCG*, M. smegmatis, M. kansasii, E. coli,* and *S. aureus.* After statistical analysis using Mann Whitney U test, MA12-29 could not bind to none of these bacterial genus and species ($p<0.05$; Table 5.13). Moreover, MA12-29 was also evaluated the diagnostic role using 47 NTM human isolates. The result showed that MA12-29 could bind to 1 of *M. avium*, 3 of *M. avium* I, and 3 of unclassified MAC ($p < 0.05$; Table 5.14).

Table 5.9 Binding affinity of representative monoclone of RNA aptamer from R12 against *M. avium* ATCC25291 using Real time PCR

Monoclone		%binding when tested with *M. avium* ATCC25291							
		1	2	3	4	5	6	7	8
Group 1	MA12-08	12.64	11.52	43.92	15.71				
Group 2	MA 12-21					29.58	60.97	45.99	61.39
Group 3	MA12-29	15.45	85.23	54.44	61.39				
L1		0.07	34.62	27.28	13.83	0	3.51	0.88	60.62

Table 5.10 Binding affinity of representative monoclone of RNA aptamer from R12 against *M. avium* 212 using Real time PCR

Monoclone		%binding when tested with *M. avium* 212							
		1	2	3	4	5	6	7	8
Group 1	MA12-08	10.7	4.83	68.75	39.95				
Group 2	MA 12-21					25.81	60.83	23.97	12.04
Group 3	MA12-29	29.79	31.41	62.54	87.43				
L1		0.06	25.4	11.11	5.79	0.01	8.82	1.17	61.18

Table 5.11 Binding affinity of representative monoclone of RNA aptamer from R12 against *M. tuberculosis* H37Ra using Real time PCR

Monoclone		%binding when tested with *M. tuberculosis* H37Ra						
		1	2	3	4	5	6	7
Group 1	MA12-08	14.89	18.85	116.16	47.07			
Group 2	MA 12-21					63.26	74.03	108.89
Group 3	MA12-29		35.18	3.17	142.68			
L1		0.45	36.59	68.59	20.53	6.26	28.64	9.25

Table 5.12 Summarize of the binding specificity result of each monoclone RNA aptamer from R6 against three *Mycobacterium* spp. using Real time PCR

Monoclone	MA(12)08	MA(12)21	MA(12)29
***M. avium* ATCC 25291**	X*	√ (p=0.020)	√ (p=0.03)
***M. tuberculosis* H37Ra**	X*	√ (p=0.024)	X*
***M. avium* 212**	X*	X (p=0.076)	√ (p=0.011)

***X = Not significant (p –value ≥ 0.05)**

Table 5.13 Summarize of the binding specificity result of MA12-29 RNA aptamer against *Mycobacterium* spp. and other bacterial genus and species using Real time PCR. Statistical analysis based on Mann Whitney U test, $p < 0.05$

Bacterial strains/Monoclone	MA(12)29
M. fortuitum	X*
M. intracellulare	X*
M. chelonae	X*
M. bovis BCG	X*
M. smegmatis	X*
M. kansasii	X*
E. coli	X*
S. aureus	X*

***X = Not significant (p –value ≥ 0.05)**

Table 5.14 Summarize of the binding specificity result of MA12-29 RNA aptamer against NTM clinical isolates using Real time PCR. Statistical analysis based on Mann Whitney U test, $p < 0.05$

Clinical strains identified as	No. of isolates	No. of isolates with Significant binding
M. avium 212	1	-
M. avium	10	1
M. avium I	8	3
M. avium II	3	-
Unclassified MAC	16	3
Unclassified MAC/*M. intracellularae*	2	-
M. intracellularae	2	-
M. simiae	1	-
M. abcessus	3	-
M. gordonae	2	-

CHAPTER VI
DISCUSSIONS

Aptamer is an oligonucleotide ligand that conformational specifically bind to their target cell. The production of aptamer is generating through SELEX process. Firstly, the RNA library were created from N40 random DNA library render the diversity around 10^{14}-10^{15} aptamer sequences. The RNA aptamer is small size, low toxicity, not act as immunogen and also tolerance to pH/temperatures. Moreover, to enhance the stability the 2'-Floro, which substituting the 2'-OH group of ribose with 2′-fluoro-pyrimidine RNA to make the RNA aptamers resistant to nuclease degradation, (65, 66) were used in this study. Nowadays, the aptamer is an interested candidate biomarker for detect several pathogen in several platform. The conventional production of aptamer mostly using the specific protein-SELEX. Conversely, the cell SELEX has an advantages in many aspects. The cell SELEX platform provides the ligand interact directly to the native conformational epitope with high specificity and affinity on the surface of target cell even though the specific epitope are unidentified.

In this study, the nuclease resistant RNA aptamer specific to *M. avium* based on whole cell SELEX were first successfully selected. Starting from the generation of 2'F-RNA aptamer library with 10^{14}-10^{15} diverse molecules and then, incubated with the target cells (the standard strain *M. avium* ATCC 25291) through the SELEX process. Firstly, the aptamer from the 6^{th} round showed the highest binding affinity and were classified into 5 groups according to the nucleotide sequences. Interestingly, each aptamer showed the different behaves even it was classified in the same group due to each aptamer demonstrated the different in their specificity and also the %binding against *Mycobacterium* spp. This data implied the diversity of the aptamer sequence and structure although its shares some similar sequence. Albeit, R6-RNA aptamers can bind to the target cells, nevertheless, they also elucidated the cross interact with other *Mycobacterium* spp. after specificity testing. In this step, the aptamer-binding epitope could be the common epitope found in the *Mycobacterium* cell wall. Take into account that, *Mycobacterium* spp. is a group of lipid-mycolic acid enriched bacterial cell wall. One of the cell wall layer composing of arabinogalactan-peptidoglycan-mycolic acid core which can be found in all mycobacteria (76). From the non-specific binding R6 RNA aptamer leads to the application of these

clones in the AFB staining, in order to concentrate or enrich the *Mycobacterium* in the sample especially in scanty sputum or even in the diluted sample such as bronchial wash from elephant.

Thus, the counter SELEX were applied in R7 and went through R12. After cloning and sequencing, the three groups of the sequence are classified. The candidate aptamer, MA12-29, demonstrated the highest specificity toward the target cells but not bound to other tested *Mycobacterium* spp., *E. coli,* and *S. aureus*. This data suggesting that MA12-29 bind to the specific epitope and might be a polysaccharide molecule or lipid on the cell surface owing to lacking of binding affinity when using heat-killed *M. avium* cell as a target cell (data not shown). Interestingly, in *M. avium* subsp. *avium* (MAA) cell wall also containing the arabinogalactan-peptidoglycan-mycolic acid core surrounded by a serovar-specific glycopeptidolipids (ssGPL) found only in *M. avium* complex (76). The ssGPL consist of core nonspecific GPL (nsGPL) common found in many environmental mycobacteria, with the modification of serovar-specific oligosaccharide side chains (76). Thus from our data, it could be imply that MA12-29 might bind to the ssGPL found on the *M. avium* ATCC 25291 cell wall.

The predicted secondary structure show the loop and stem structure. Interestingly, all group of aptamer sequences elicited the same loop structure suggesting the binding domain to the target cell. From sequence analysis, R12-RNA aptamers seem likely to be evolved from group 1 R6-RNA aptamers because of the share sequences “GATCTACAC” were conserved in R12-RNA aptamers. The R12-RNA aptamers were classified into 3 groups. The representative clone from each group were selected for specificity testing. The results demonstrated that, MA12-29 showed the high specificity against *M. avium* ATCC 25291. Basically, *M. avium* can be classified into 4 subspecies which are MAA, *M. avium hominissuis* (MAH), *M. avium paratuberculosis* (MAP) and *M. avium silvaticum* (MAS) (18, 19). The MAA and MAS are the causative agent of tuberculosis (TB)-like disease in birds (3, 20). MAP is plays a role in the Johne’s disease, causing enteritis and diarrhea in the cattle (21), and also Crohne’s disease in human (22). Among four subspecies of *M. avium,* MAH has impact on causing human lung infection especially in immunocompomised people such as elderly, children, untreated AID patients and the people with abnormal T cell immunity (23, 24). In this study, derived *M. avium* was classified into *M. avium*, *M. avium* I, *M. avium* II and MAC based on the restriction pattern of their genomic DNA (75). Even though, MA12-29 were

not bound to all clinical isolated *M. avium* but it still showed the high specificity owing to the binding with 1 of *M. avium*, 3 of *M. avium* I, and 3 of unclassified MAC.

Most of aptamer reports against *Mycobacterium* spp. are specifically to *M. tuberculosis* protein such as CFP10, ESAT-6, MTP64 or whole cell *M. tuberculosis* (69). However, please be aware that NTM is also cause TB-like disease especially in immunocompromised host and require totally different drug regimen. Previous report has developed the monoclonal antibodies and aptamer against MAP (70). Three aptamers against the *MAP0105c* gene which known as a unique gene found only in MAP were produced. Although, all three aptamers showed the high affinity binding to the protein at N-terminal half, but they seem to bind to the most of *mycobacterium* strains used in the study. Even though the binding epitope on the *M. avium* surface was not identified, but it would be worth to evaluate in further experiment. In addition, it also worth to minimized the derived aptamer to identify the potential binding site of aptamer. Notice that, the produced RNA aptamer: MA12-29 is stable, easy to produce and could be applied and further developed for diagnose *M. avium* infection to reach the achievement of the efficient treatment and controlling.

CHAPTER VII
CONCLUSION

The nuclease RNA aptamer against live whole cell *M. avium* ATCC 25291 (MA12-29) were produced and evaluated for its specificity using the closely related *Mycobacterium* strains and the 47 clinical isolated NTM. The MA12-29 is the candidate aptamer with statistically specific to M. avium ATCC 25291 ($p<0.05$). Moreover, MA12-29 demonstrated the ability to bind to 5 clinical isolated identified as *M. avium* and MAC with the statistically significant ($p < 0.05$) implied that MA12-29 bind to the share epitope on the surface among this group and the standard strain. Even though, MA12-29 cannot bind to all clinical isolated *M. avium* but it also show the high specificity due to it's not bind to other strains except *M. avium* group.

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BIOGRAPHY

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EDUCATIONS

Chiang Mai University, 2001-2004:	Bachelor of Science (Medical Technology) GPA 3.72 with First Class Honors
Mahidol University, 2005-2010 :	Faculty of Medicine Siriraj Hospital Doctor of Philosophy (Immunology) GPA 3.94 Royal Golden Jubilee (RGJ)-Ph.D. program, Thailand Research Fund (TRF), 2006-2009

AWARDS

1. **The 2nd prize of poster and oral presentation** in topic of "Variation of *Burkholderia pseudomallei bimA* from Clinical samples and environmental isolates". The 120th Siriraj Medical conference, March 17-21, 2008, at Faculty of Medicine Siriraj Hospital, Bangkok, Thailand.
2. **The Student Travel Bursary** and have an Oral presentation in the topic of 'Actin-based motility of *Burkholderia thailandensis* requires a central acidic domain of BimA that recruits and activates the cellular Arp2/3 complex'. The VIth Melioidosis Congress 2010, November 30-December 3, 2010, Townsville, Australia.
3. **The Distinguished Thesis Award 2011 in Doctoral level of Biological Science from Mahidol university**
4. **Awards for Graduates with Distinctions, Round 12 Academic Year 2010**
5. **The Thesis Award 2012 in Biological Science from office of National Research Council of Thailand (NRCT)**

PUBLICATIONS

1. **Tharinjaroen CS**. Tuberculosis diagnosis: From knowledge to innovation in public health. Journal of Associated Medical Sciences. 2017;50(1):1-21.
2. Intorasoot S, **Tharinjaroen CS**, Phunpae P, Butr-Indr B, Anukool U, Intachai K, et al. Novel potential diagnostic test for Mycobacterium tuberculosis complex using combined immunomagnetic separation (IMS) and PCR-CTPP. Journal of applied microbiology. 2016;121:528-38.
3. **Tharinjaroen CS**, Intorasoot S, Anukool U, Phunpae P, Butr-Indr B, Orrapin S, et al. Novel targeting of the lepB gene using PCR with confronting two-pair primers for simultaneous detection of Mycobacterium tuberculosis complex and Mycobacterium bovis. Journal of medical microbiology. 2016;65(1):36-43.
4. Roobsoong W, **Tharinjaroen CS**, Rachaphaew N, Chobson P, Schofield L, Cui L, et al. Improvement of culture conditions for long-term in vitro culture of *Plasmodium vivax*. Malar J 2015; 14: 297.
5. **Sitthidet C**, Korbsrisate S, Layton AN, Field TR, Stevens MP, Stevens JM. Identification of motifs of *Burkholderia pseudomallei BimA* required for intracellular motility, actin binding, and actin polymerization. J Bacteriol 2011; 193: 1901-10.
6. **Sitthidet C**, Stevens JM, Field TR, Layton AN, Korbsrisate S, Stevens MP. Actin-based motility of *Burkholderia thailandensis* requires a central acidic domain of *BimA* that recruits and activates the cellular Arp2/3 complex. J Bacteriol 2010; 192: 5249-52.
7. **Sitthidet C**, Stevens JM, Chantratita N, Currie BJ, Peacock SJ, Korbsrisate S, et al. Prevalence and sequence diversity of a factor required for actin-based motility in natural populations of *Burkholderia* species. J Clin Microbiol 2008; 46: 2418-22.

RECIEVED FUNDING

- **Finished projects**

- 2012-2013 Grant for the new researcher from Faculty of Associated Medical Science, Chiang Mai University "Optimization of Confronting PCR for *Mycobacterium tuberculosis* detection"
- 2013-2014 Grant for the new researcher from Chiang Mai University "Developing and Evaluation of Confronting PCR for *Mycobacterium tuberculosis* detection in TB patient"

- 2012-2014 Grant from National Science and Technology Development Agency (NSTDA) (co-investigator) “Novel highly sensitive tuberculosis diagnostic kit using immunomagnetic separation combined two-pair confronting PCR assay”
- 2014-2018 Thailand Research Fund Grant for the New Researcher “Production of nuclease-resistant RNA aptamers against *Mycobacterium avium”*

- **Ongoing project**

- 2018-2019 National Research Council of Thailand Fund Grant for the New Researcher “Production of nuclease-resistant RNA aptamers against *Streptococcus suis”*

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2003 - present Assistant professor, Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University

1995-2002 Ph.D. in Biological Chemistry, University of Michigan, Ann Arbor.

1991-1995 Lecturer, Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University

1985-1991 M.D. (first-class honor), Faculty of Medicine Siriraj Hospital, Mahidol University

Research Interests : Medical biotechnology involving aptamers and RNA interference. Current research projects are as follows:

- Targeted Nanoparticles-Aptamer Conjugates for Hepatocellular Carcinoma therapy.
- Developing RNA aptamers for research and diagnostics of human diseases (e.g. dengue viral infection, hemoglobin diseases).
- Using the RNA interference technology for therapeutics. Currently, studying the suppression of collagen production in keloid fibroblasts using collagen-targeting siRNA/aptamer chimeras.

Publications:

1 Trakarnsanga K, Wilson MC, Heesom KJ, Andrienko TN, **Srisawat C**, Frayne J. Secretory factors from OP9 stromal cells delay differentiation and increase the expansion potential of adult erythroid cells in vitro. Sci Rep. 2018 Jan 31;8(1):1983. doi: 10.1038/s41598-018-20491-1.

2 Kooptiwut S, Wanchai K, Semprasert N, **Srisawat C**, Yenchitsomanus PT. Estrogen attenuates AGTR1 expression to reduce pancreatic β-cell death from high glucose. SciRep. 2017 Nov 30;7(1):16639. doi: 10.1038/s41598-017-15237-4.

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8 Sreekanth GP, Chuncharunee A, Sirimontaporn A, Panaampon J, **Srisawat C**, Morchang A, Malakar S, Thuwajit P, Kooptiwut S, Suttitheptumrong A, Songprakhon P, Noisakran S, Yenchitsomanus PT, Limjindaporn T.Role of ERK1/2 signaling in dengue virus-induced liver injury.Virus Res. 2014 Apr 1;188C:15-26. doi: 10.1016/j.virusres.2014.03.025. [Epub ahead of print]

9 Thuangtong R, Maneeprasopchoke P, **Srisawat C**, Vatanashevanopakorn C, Hana mornroongruang S, et al. In vitro Culture and Histological Characterization of Extracted Human Hair Follicles.J Clin Exp Dermatol Res 4:182. 2013 Sep 10. doi: 10.4172/2155-9554.1000182

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pedigrees of Southeast Asian G11778A Leber hereditary optic neuropathy. J Neuroophthalmol 2006;26(4):264-7.

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33 Xiao S, Day-Storms JJ, **Srisawat C**, Fierke CA, Engelke DR. Characterization of conserved sequence elements in eukaryotic RNase P RNA reveals roles in holoenzymeassembly and tRNA processing. Rna 2005;11(6):885-96.

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35 **Srisawat C**, Sanpakit K, Wisutsareevong W, Leenutaphong W, Permpikul C, Neungton N. A girl with a glowing tooth: a case of congenital erythropoietic porphyria. SirirajHosp Gaz 2004;56:26-32.

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39 **Srisawat C**, Engelke DR. Streptavidin aptamers: affinity tags for the study of RNAs and ribonucleoproteins. Rna 2001;7(4):632-41.

40 Jiang P, Atkinson MR, **Srisawat C**, Sun Q, Ninfa AJ. Functional dissection of the dimerization and enzymatic activities of Escherichia coli nitrogen regulator II and their regulation by the PII protein. Biochemistry 2000;39(44):13433-49.

41 Wasant P, Liammongkolkul S, **Srisawat C**. Neonatal screening for congenital hypothyroidism and phenylketonuria at Siriraj Hospital, Mahidol University, Bangkok, Thailand--a pilot study. Southeast Asian J Trop Med Public Health 1999;30 Suppl 2:33-7.

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44 Neungton N, Chearskul S, Neungton S, **Srisawat C**, Angpitakpan D, Sothanayongkul R, et al. Evaluation of new ovarian cancer markers, STN, CA 546 and CA 72-4 in Thai patients. Siriraj Hosp Gaz 1993;45:307-14.

Abstracts/presentations

1 Chotipanang K, Junnu S, Phuangphan P, Densupsoontorn N, **Srisawat C**. A microassay method for erythrocyte transketolase activity.การประชุมวิชาการโภชนาการแห่งชาติ ครั้งที่ 10 The 10th Thailand Congress of Nutrition (10th TCN 2016) 18 - 20 ตุลาคม 2559 ณ ศูนย์นิทรรศการและการประชุมไบเทค บางนา กรุงเทพฯ

2 Veerataveeporn N, Srisomsap C,Chokchaichamnankit, **Srisawat C**, Svasti J, Kalpravidh RW. Proteomic profile of coagulation proteins in β-thalassemia/Hb E patients treated with curcumioids antoxdant cocktail. The 26th Annual Meeting of The Thai Society for Biotechnology and International Conference, Mea Fah Luang University Chiang Rai , Thailand 26-29 November 2014

3 Thazin K, **Srisawat C**. GENERATION OF NUCLEASE-RESISTANT RNA APTAMERS AGAINST HEMOGLOBIN E. International Conference on Structural and Functional Genomics (ICSAFG) School of Chemical and Biotechnology, SASTRA University, Thanjavur, Tamil Nadu, India 6th & 7th January 2013

4 Siriboonpipattana S, **Srisawat C**. Human Serum Albumin Aptamers: A Potential Albumin Removal Tool for Plasma Proteome Analysis. International Conference on Structural and Functional Genomics (ICSAFG) School of Chemical and Biotechnology, SASTRA University, Thanjavur, Tamil Nadu, India 6th & 7th January 2013

5 Moongkarndi P, **Srisawat C**, Saetun P, Jantaravinid J, Peerapittayamonkol C, Soi-ampornkul R, Junnu S, Sinchaikul S, Chen ST, Charoensilp P, Thongboonkerd V, NeungtonN. Protective effect of mangosteen extract against beta-amyloid-induced cytotoxicity, oxidative stress and altered proteome in SK-N-SH cells. มหกรรมสมุนไพรแห่งชาติครั้งที่ 8, 31 สิงหาคม – 4 กันยายน 2554, อิมแพค เมืองทองธานี นนทบุรี.

6 Moongkarndi P, **Srisawat C**, Saetun P, Jantaravinid J, Peerapittayamonkol C, Soi-ampornkul R, et al. Protective effect of mangosteen extract against beta-amyloid-induced cytotoxicity, oxidative stress and altered proteome in SK-N-SH cells. Joint Conference in Medical Sciences 2011: Chula-Rama-Siriraj (JCMS2011), June 15-17, 2011 at IMPACT Exhibition and Convention Center, Thailand: 18.

7 Morchang A, Yasamut U, Netsawang J, Noisakran S, Wongwiwat W, Songprakhon P, **Srisawat C**, et al. Cell death gene expression profile: Role of RIPK2 in dengue virus-

mediated apoptosis. Joint Conference in Medical Sciences 2011: Chula-Rama-Siriraj (JCMS2011), June 15-17, 2011 at IMPACT Exhibition and Convention Center, Thailand: 9.

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9 Kirawanich P, Pausawasdi N, **Srisawat C**, Wilairat D, Neatpisarnvanit C. Numerical schemes of nanosecond pulsed electric field systems for cell apoptosis applications. ECTI-CON 2010 - The 2010 ECTI International Conference on Electrical Engineering/Electronics, Computer, Telecommunications and Information Technology , art. no. 5491543, pp. 6-10

10 Nakponetong K, **Srisawat C**, Benjasupattananun P, Suratannon C, Wachairutmanggur L , Boonchoo S, Pankaew D, Laochareonkait A, Pacharn P, Jirapongsananuruk O, Visitsun thorn N, Vichyanond P. Pharmacokinetics of epinephrine absorption via intranasal administration: a preliminary report. AAAI meeting 2010. 24th American academy of allergy, asthma, and immunology (AAAAI) annual meeting. February 26-March 2, 2010 at New orleans, LA, USA.

11 Summathed T, Manaso J, Sirivattanauksorn V, **Srisawat C**. Identification of the RNA motif capable of interacting with nickel. งานประชุมวิชาการประจำปี 2553 “โรงเรียนแพทย์ศิริราชเพื่อสังคม 120 ปีแห่งความก้าวหน้า” ระหว่างวันที่ 16 – 20 สิงหาคม 2553 ณ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

12 Panya A, **Srisawat C**, Sawasdee N, Yenchitsomanus PT, Peerapittayamongkol C. Human zinc finger and homeodomain 2 (ZHX2) and g-globin expression in erythroleukemiccell line. The 1st CMU Graduate Research Conference, November 27-28, 2009 at Chiang Mai University, Chiang Mai, Thailand.

13 Tit-oon P, Panyawirunroj T, Chuangsuwanich A, Limwongse C, **Srisawat C**. Inhibition of collagen production in keloid fibroblasts using RNA interference. The 12th National Graduate Research Conference, February 12-13, 2009 at Khon Kaen University, Khon Kaen, Thailand.

14 Neungton N, Moongkarndi P, **Srisawat C**, Jantaravinid J, Peerapittayamongkol C, Soiampornkul R, et al. Protective effect of mangosteen extract against beta-amyloid-induced apoptosis in SK-N-SH cells. Alzheimer's&dementia J 2009;5:413-4.

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19 Jantaravinid J, **Srisawat C**, Moongkarndi P, Neungton N. Protective effect of mangosteen extract against oxidative stress induced by beta-amyloid in SK-N-SH cells. Proceedings of the 12th National Graduate Research Conference by Dean of Graduate Schools of Public University (DGPU) and Khon Kaen University, February 12-13, 2009 at Faculty of Engineering, Khon Kaen University, Khon Kaen, Thailand.

20 Jantaravinid J, **Srisawat C**, Moongkarndi P, Neungton N. Protective effect of mangosteen extract against oxidative stress induced by beta-amyloid in SK-N-SH cells. Joint Conference in Medical Sciences. 2009:118.

21 Tirawanchai N, Chanarat N, Somkasetrin A, Chanchairujira T, **Srisawat C**, Peerapittaya mongkol C, et al. Study on the association of C677T and A1298C polymorphisms of the gene encoding methylenetetrahydrofolate reductase (MTHFR) enzyme and plasma homocysteine level in That hemodialysis patients. 33rd FEBS Congree & 11th IUBMB Conference, June 28-July 3, 2008 at Athens, Greece.