Optimization of Mycobacterial Diagnostic Systems for Using in A Microfluidic Chip

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ABSTRACT

The microfluidic technology is a novel tool for detection pathogen due to perform easily and preferable at point-of-care. Nevertheless, system in this device still involve with inter-related problems such as amount of target cell and detection system of their presence. Hence, we aimed to optimize the mycobacterial diagnostic systems that appropriate for using in microfluidic chip. *M. smegmatis* mc^2155, *Mtb* H37Ra (ATCC 25177), and drug-resistant strains were used as the target bacteria. Two types of magnetic bead techniques; namely Dynabeads and TB beads were evaluated to isolate bacteria prior detection. Mycobacterial growth detection and antimycobacterial susceptibility testing were also performed. All viability and/or death cells were determined by SYTO9/PI dyes staining and examining with inverted fluorescent microscope. Dynabeads®-conA has high capturing efficiency than TB-Beads™. The capability of Dynabeads®-conA for binding relied on buffer solution and ratio between beads and cells. For antimycobacterial susceptibility testing presented after observed for 10 days, all Mtb H37Ra cells died in rifampicin-containing media and amount rifampicin-resistant strain in rifampicin-containing media quite equally when compared with cell control. This study shows that the Dynabeads®-conA is appropriate for this detection system. Five mM Tris pH 7.0 is selected due to non-self-agglutination with divalent cations and amount of magnetic beads and cell densities affected to the binding efficacy. The ratio of MtbH37Ra concentration affected to binding efficiency that was significantly increase when used 10^7 beads/mL with 10^4 CFU/mL of *Mtb* H37Ra. Furthermore, this detection system that used dyes staining of SYTO9/PI provide the antimycobacterial susceptibility result within 10 days because of dyes staining can penetrate pass through cell wall to stain with nucleic acid. Therefore, this optimization gives essential information for further use to test with the microfluidic chip.

Keywords: Magnetic bead, Mycobacterial cell, SYTO9/PI

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Introduction

Tuberculosis (TB) is a severe airborne infectious disease caused by *Mycobacterium tuberculosis (Mtb*) which normally affects to the lungs called pulmonary tuberculosis but it can affect to the other sites of body as well (extrapulmonary TB). The current TB situation which reported by World Health Organization (WHO) in 2016, TB is leading cause of death globally especially in HIV/AIDS patients. From WHO global tuberculosis report in 2016, people worldwide approximately 10.4 million are fell with TB including people who infected with HIV about 1.2 million of all new TB cases. Moreover, there were an estimated 480,000 new cases of multidrug-resistant TB (MDR-TB) and an about 100,000 people with rifampicin-resistant TB (RR-TB). Furthermore, TB deaths were approximately 1.4 million and 0.4 million deaths who infected with HIV. Even though amount of TB deaths fell about 22% since 2000 to 2015, TB retained one of the top 10 causes of death globally (WHO, 2016).

For the effective control of TB, it composes of timely diagnosis and treatment strategy. Before TB can be treated, a diagnosis method needs to be made in time and efficient, preferable at point-of-care (POC), and performing field-friendly tools and accuracy. The POC definition is contentious, however, it implies that capability of diagnosis at the point where patient consultation and symptom occurs, especially the ability to translate the outcome into the same day treatment. The WHO and Stop TB Partnership have earmarked 2015 as a target year for developing a simple POC test for TB (Dheda *et al*., 2013). In addition, WHO has strategy for post-2015 by focusing on the intensive research and innovation development for early diagnosing TB that will decline the TB mortality rate by have vision to reach the target in 2035 (WHO, 2016). Nowadays, the method which uses for detecting TB remain inadequate to low-resource setting. The most common TB diagnostic tool is direct microscopy by sputum smear method. This process is relatively rapid, inexpensive but low sensitivity specifically is less sensitive in children and HIV co-infected patients. Another method is culture of Mtb from clinical sample which is the gold standard most sensitive but normally require 3-8 weeks (Mathur *et al*., 2008). Furthermore, the conventional diagnosis of drug-resistant TB depends on bacterial culture and drug susceptibility testing, time consuming and cumbersome method. Hence, during this time the patient may be inappropriately treated, drug-resistant strains may continue to spread and resistance may increase (WHO, 2014).

However, the rapid culture-based drug susceptibility testing (DST) are developed such as commercial liquid culture drug susceptibility testing for example Mycobacterial Growth Indicator Tube (MGIT), the most popular semi-automated culture system, it consists of Middlebrook7H9 broth mixing with growth supplement (oleic acid, albumin, dextrose, catalase or OADC) and antibiotic mixture called PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethroprim, Azlocillin) that necessary to suppress other bacterial and fungal contaminations. The MGIT tube contains an oxygen-quenched fluorochrome embedded in silicone at the bottom of the tube. During bacterial growth, free oxygen is depleted. So the fluorochrome is no longer inhibited, resulting in fluorescence within tube when visualized under UV light. Other rapid culture-based DST which are commercially such as BacT/Aleart3D, VersaTrek, Fast Plaque, TK-medium, and E-test. In addition, the other methods culture-based DST like microscopic observation drug susceptibility (MODS), thin-layer agar (TLA), colorimetric redox indicator methods; MTT,

AlamarBlue, rezasurin, and nitrate reductase assays (NRA) are the in-house methods of rapid culture-based DST (Moore *et al*., 2004; Caviedes *et al*., 2000; Moore *et al*., 2006; Robledo *et al*., 2006).

Although these methods were approved by the WHO, it cannot provide the result within same-day while patients are still at the hospital and also require infrastructure, operator training, and standardization before using. By contrast, several nucleic acid amplification test (NAAT) technologies are commercially available for the laboratory-based TB diagnosis which can rapidly detect small quantities of DNA through many different amplification methods such as the Cobas Amplicor and Light Cycler Mycobacterium Detection kits (all produced by Roche, San Deigo, CA, USA), INNO-LiPA Rif.TB line probe assays (Innogenetics, Ghent, Belgium), Genotype MTBDR*plus* (Hain Lifescience, Nehren, Germany), Capilia TB-Neo rapid detection and speciation assays (Taunus, Numazu, Japan), and Xpert MTB/RIF assay (Cepheid) (Dheda *et al*., 2013). In part of Xpert MTB/RIF test, this is a fully automated real-time polymerase chain reaction assay which simultaneously detect Mtb and rifampicin drug resistance. It performed optimally on expectorated sputum specimens, using the disposable cartridge and provides the accurate outputs in less than two hours (Boehme *et al*., 2010). Thus, the patients can be offered treatment properly on the same day. Because of the potential properties, WHO endorsed and strongly recommended to use GeneXpert assay for the initial diagnostic test in adults and children presumed to have MDR-TB or HIV-associated TB (WHO, 2014). Nonetheless, as the accuracy of many NAAT is generally similar but these tools are still need infrastructure, operator training, and expensive cost such a Xpert MTB/RIF that limited affordability in high burden settings (capital outlay of about USD 17,000 and USD 10 per cartridge) (Dheda *et al*., 2013) and lack of isoniazid-resistance readout which a lot of patients are risk and remaining unsuitable remedy. Recently, Gene Xpert Omni®, the new version diagnostic equipment has developed for point-of-care purpose. This tool will be used for Mtb detection and rifampicin-resistant testing which use instead Xpert MTB/RIF cartridges. By WHO will bring this new platform to assess in 2016. Furthermore, another innovation now emerging called Xpert Ultra®. It has two functions, one is anticipated to replace the previous tool (Xpert MTB/RIF), another is substitute the culture conventional method (WHO, 2014).

For the optimal use in many low-resource settings that do not have the means or infrastructure, it is also significant for test to be low-cost or cost-effective, easy-to-use, rapid, simple interpretation, and stable when transported together with can store under extreme condition (Yager *et al*., 2006). Consequently, microfluidic technology may now ideally appropriated in developing world (Ali *et al*., 2006) that can resolve these challenges because of fast and POC detection of pathogens (Li *et al*., 2014; Cai *et al*., 2014; Watkins *et al*., 2013). In addition, the microfluidic system is a potential tool that used widely in many application such as biomedical device (Sackmann *et al*., 2012; Gopalakrishnan *et al*., 2015), clinical analysis (Chang *et al*., 2015; Hung *et al*., 2014), and high throughput screening (Cao *et al*., 2012). The concept of microfluidic is that fluids can be preciously manipulated by using a microscale device built with technologies (Whitesides *et al*., 2006). This system allows miniaturization and integration of variety function of diagnostic tests. The devices include being simple to perform and preparation of some quantitative and qualitative output which can be measured with low-cost and omnipresent equipment such as mobile-phone, camera, or scanner (Gopinath *et al*., 2014; Preechaburana *et al*., 2014; Ephraim *et al*., 2015; Prue *et al*., 2013). In addition, the material used to make the device are inexpensive, easy to eliminate by avoiding the

hazardous contamination, and scalable manufacture. A new technology for cell-based assays called Kit-on-a-lid Assays (KOALA) which simplified complex microfluidic systems, therefore get rid of specialized equipment and technical training (Guckenberger *et al*., 2015).

For retrospective study of Chin *et al.* in 2011, they did the ELISA-like assay on the microfluidic chip used blood from a lancet puncture and detected with inexpensive photodetectors as a readout. The results were successfully diagnosed HIV in all 70 samples which obtained from hospital in Rwanda. In two recent papers in 2014, one is He *et al.* aimed to present a microbead-based microfluidic platform for bacterial detection and antibiotic susceptibility testing. The results presented this system can detect *Escherichia coli* O157 within 30 minutes at a cell density range from 10^1- 10^5 CFU/μL. Additionally, it evaluated the effect of three antibiotics within 4-8 hours. The other is study of Jing *et al.* that they fabricated the integrated microfluidic system for airborne *Mycobacterium tuberculosis* capture, enrichment, and developed the rapid bacteriological immunoassay. Thus, the detection time was declined to less than 50 minutes including enrichment process for 20 minutes and analysis of immunoreaction about 30 minutes. In addition, the study of Zelenin *et al.*in 2015 that presented a microfluidic-based for diagnosis the blood-stream infections *(*BSI*)* and performed drug susceptibility testing. The result showed that this device can accomplish complete the blood-cell lysis, whereas the bacterial cells are available for 100% that promptly recovered for downstream analysis.

Thereby, the microfluidic technology could detect the presence of pathogens rapidly and give additionally clinical information such as antibiotic susceptibility. However, this system must involve with two distinct but inter-related problems including amount of target pathogen cells and detection system of their presence. To solve these challenge problems, the first way is isolate the target cells and another way, devise a suitable platform for quantification and detection. By the isolation method that not only increase cell concentration but also discard the interfering other species as well (Sengupta *et al*., 2008).

Recently, the KOALA microfluidic platform integrates the antimicrobial susceptibility testing (AST) part that is essential step so as to determine the antibiotic sensitivity of pathogens in the clinical specimen such as blood, sputum, or urine (Chen *et al*., 2010). Thus, it will provide the drug resistant information to the physicians find the therapeutic route properly and as soon as possible together with prescribe the antimicrobials correctly (Murray *et al*., 2015). Normally, the gold standard AST of *M. tuberculosis* is still rely on the broth dilution involves co-culture on the media that time-consuming. So, the microfluidic chip is a novel approach in point-of-care device for rapid AST that will decline the multidrug-resistant situation later (Hawkey *et al*., 2008). Additionally, it will carry the advantage of potential reagent and analyte usage (Kalashnikov *et al*., 2014) and can perform AST with many kinds of drugs simultaneously. In several cases, this device indirectly measure the bacterial growth in presence of drugs, resulting in the outcome will relate with phenotype resistance (Pulido *et al*., 2013; Corona *et al*., 2013). Lately, a number of microfluidic system which performing with AST have been reported and illustrated the effective of this device for giving the data of antibiotic resistance quickly (Boedicker *et al*., 2018).

Hence, it may be possible that the microfluidic KOALA technology will developing for *M. tuberculosis* identification along with drug susceptibility testing for first-and-second-line antituberculous drugs in the future.

Because of their small size and other benefits can be incorporated into a portable device that could provide the meaningful and fulfill significant need directly towards the developing world.

Objectives of the study

1. To evaluate the capturing efficacy of mycobacterial cells using the magnetic technique which are commercially available including TB Beads™ and Dynabeads.

2. To determine viability of mycobacterial cells by SYTO9/PI staining kit and inverted fluorescence microscope both in the absence and the presence of anti-tuberculous drugs.

Methodology

Mycobacterial cell culture

M. smegmatis mc^2155, *Mycobacterium tuberculosis* H37Ra (ATCC 25177), isoniazid and rifampicin-resistant strains will be used as the target bacteria. These strains are retrieved from Drug-resistant Tuberculosis Research Laboratory, Siriraj Foundation under patronage to HRH Princess Galyanivadhana KromLoung Narathiwas Rajnakarindth (Drug-resistant Tuberculosis Research Fund), Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. The mycobacterial strains were cultured on Loewenstein-Jensen medium slant (Biomedia, Thailand) and incubated at 36±1 ^{o}C until confluent growth is obtained within 3-4 days for *M. smegmatis* and 3-4 weeks for Mtb H37Ra and drug-resistant strains respectively.

Preparation of mycobacterial cells suspension

Pipette Middlebrook7H9+tween80 about 3 drops and add into glass beads-containing tube (glass beads size 4 mm Ø for 5 beads, tube size 20x150 mm). Use inoculating loop to get *M. tuberculosis* H37Ra colonies for 2 loops and mix thoroughly by vortexing. Add 6 mL of Middlebrook7H9 + tween80 into the tube, mix by vortexing, and leave at the room temperature for 20 minutes. After that get cell suspension 5 mL into plastic container and use syringe and needle (No.26) to pull up and down cell suspension about 50 times. Then, get cell suspension from plastic container about 5 mL and filtrate pass through Millex® syringe filter units, disposable, Durapore® PVDF, pore size 5 µm to another plastic container. Transfer this cell suspension to glass tube (16x150 mm), measure McFarland, and adjust to McFarland No.2 by using distilled water. Then perform serial ten-fold dilution 10^7, 10^6, and 10^5 CFU/mL respectively.

Magnetic bead preparation

For TB-Beads™, this kit contains 2X TB-Beads Solution and Elution buffer that used to evaluate the bound mycobacteria. In addition, the kit requires wash-solution (0.04% or 10 mM sodium hydroxide; NaOH). Before using 2X TB-Beads Solution must be inverted and shake vigorously until homogeneous. After that, dilute with distilled water to make 1X solution and make serial dilution to 1/4X for further testing. The diluted TB-Beads solution is stable for one week at room temperature. In part of Dynabeads® MyOne™ Streptavidin T1 (Dynal Biotech, Norway), this beads has characteristic of hydrophobic superparamagnetic polystyrene beads with 1.05 µm in diameter. It enables effective and instant capture of any biotinylated molecules. By in this study concanavalin A (conA) (Sigma, USA) was used as a biotinylated molecules that require a transition metal such as a calcium ion (Ca^{2+}) and manganese

ion (Mn^{2+}) for binding. For conA preparation, reconstitute conA lyophilized powder in 5 mM Tris buffer pH 7.0. For preparation Dynabeads®, washed three times in PBS pH 7.4 to remove azide, then add conA into washed Dynabeads equal volume of both in PBS at room temperature using gentle rotation. Wash the conA-coated beads 4-5 times in PBS containing 0.1% bovine serum albumin (BSA). Resuspend the ConA-coated beads with Tris buffer and perform serial dilution of beads to 10^7 beads/mL and keep in 2-8 °C.

Antimycobacterial drugs preparation

Prepare mycobacterial growth indicator tube (MGIT) that contains 7 mL of Middlebrook 7H9 broth by adding 0.8 mL of OADC/PANTA mixture and aliquot 5 mL to another tube. After that add 1 drug disc of isoniazid (1 µg/disc) and rifampicin (5 µg/disc) (BBL, USA) that provide final concentration to be 0.2 and 1 µg/mL respectively. Then, incubate MGIT tube in the dark, overnight at 2-8 °C for drug dispersion thoroughly the media.

Cell viability dye staining and inverted fluorescence microscopy

The LIVE/ DEAD® *Bac*light™ Bacterial Viability Kits (L7012, Invitrogen, USA) for microscopy and quantitative assays contains the dye stains solutions in dimethylsulfoxide (DMSO) separately with 3.34 mM SYTO9 dye and propidium iodide (PI). For SYTO9, excitation/emission spectra is about 480/500 nm and 490/635 nm for PI. Combine equal volumes of SYTO9 and PI (1:1) in a microcentrifuge tube and mix thoroughly. Add 3 µL of the dye mixture for each mL of bacterial suspension in 0.85% NaCl. Mix thoroughly and incubate at room temperature in dark for 15 minutes. Get cell suspension about 50 µL then drop on the glass slide and cover with the cover slip. Inverted fluorescence microscopy (Nikon, ECLIPSE Ti) and 40X magnification objective was used to randomly observe cell viability both live and dead cell for 10 fields in oblique line of glass slide.

Efficiency of magnetic beads in capturing mycobacterial cells

For TB-Beads™, 1/4X TB-Beads Solution about 100 µL mixed with mycobacterial cell suspension with concentration ranging from 10^7 - 10^4 CFU/mL in equal volume in microcentrifuge tube. Leave tube for 2 minutes at room temperature to enable the mycobacteria to be captured to beads. Separate the target bacteria-TB-Beads™ with a magnet by remaining the magnet to be captured for 1 minute. Use pipette to transfer the supernate to another microcentrifuge tube and bring the tube to centrifuge at 12,000 rpm for 5 minutes, discard supernate, resuspend the cell pellet in 0.85% NaCl and transfer to another tube. Use the Elution buffer to elute cell from beads to get binding cell. Transfer the solution from microcentrifuge tube of step both binding and unbinding and stain with SYTO9/PI and observing under the inverted fluorescence microscope. In case of Dynabeads®-conA, beads were added with calcium ion (Ca^{2+}) and manganese ion (Mn^{2+}) and mycobacterial cell suspension with concentration ranging from 10^7 - 10^4 CFU/mL in equal volume. Incubate at room temperature for 2 hours. Separate the target bacteria- Dynabeads®-ConA with a magnet for 1 minute. Use pipette to transfer the supernate to another microcentrifuge tube and bring the tube.

Antimycobacterial susceptibility testing

Mtb H37Ra and isoniazid and rifampicin drug-resistant strains were cultured in media with OADC/PANTA and drug-containing media respectively by using cell suspension of these cells at 10^5 and 10^4 CFU/mL about 10 µL and 90 µL of media and rifampicin -containing media by performing in 96-well plate. Then,

observe for 10 days by dividing into two groups. The first group will stain with 0.3 µL of SYTO9/PI dyes mixture and the second will add lysozyme 0.1 µL for 5 minutes before staining with 0.3 µL of SYTO9/PI dyes mixture. Then take cell suspension about 50 µL drop on the glass slide and cover with the cover slip. Inverted fluorescence microscopy (Nikon, ECLIPSE Ti) and 40X magnification objective was used to randomly observe cell viability both live and dead cell for 10 fields in oblique line of glass slide (triplicate, three occasions).

Results

Efficiency of magnetic beads in capturing mycobacterial cells

For Evaluation the magnetic beads techniques, two magnetic beads; TB-Beads™ and Dynabeads®-conA which concentration ranging from 1X (~3 x 10^9 beads/mL for TB-Beads™ and ~7-10 x 10^9 beads/mL for Dynabeads®-conA) to half and quarter folds were compared for capturing *M. smegmatis* mc^2155 cells at 10^7 CFU/mL and observed under the inverted fluorescence microscopy. The results presented Dynabeads®-conA concentration showed strong signaling of cell for fluorescent detection than TB-Beads™ which presented low fluorescent signal causing hard to detect those cells. In addition, the Elution buffer of TB-Beads™ interfere dye staining process resulting in no fluorescent signal.

In part of optimization Dynabeads®-conA in capturing *Mtb* H37Ra cells, from the previous experiment, Dynabeads®-conA is a suitable isolation method for this detection system. Capturing efficacy was performed by evaluating amount of binding and unbinding cell of *Mtb* H37Ra by using *M. smegmatis* mc^2155 as a control together with determined by staining with SYTO9/PI and inverted fluorescence microscopy. The ratio of *Mtb* H37Ra concentration affected to binding efficiency of Dynabeads® - conA (Fig.3). Binding efficiency was significantly increased when optimized Dynabeads®-conA at 10^7 beads/mL with 10^4 CFU/mL of *Mtb* H37Ra cell suspension which showed greatest capturing ability and followed with cell densities at 10^5 CFU/mL. While 10^6 and 10^7 CFU/mL have binding capacity not differently. (P = 0.05, Duncana)

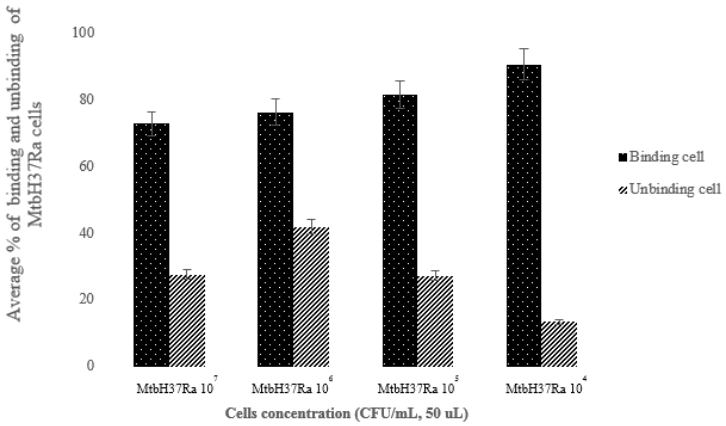

Figure 1 Efficiency of Dynabeads®-conA in capturing *Mtb* H37Ra cells when stained with SYTO9/PI and observed cell viability under the inverted fluorescence microscope. Each cell concentration was obtained about 50 µL and dropped on glass slide. Then observed for 10 fields and calculated the average percent binding and unbinding cell.

Antimycobacterial susceptibility testing

After observed for 10 days, amount of *Mtb* H37Ra live cell (as a sensitive strain) in isoniazid and rifampicin-containing media were decreased significantly when compared with cell control. By cell concentration at 10^5 CFU/mL, all cell died when exposed to drug for 10 days. A few cell were found at 10^6 CFU/mL. Meanwhile, rifampicin-resistant strain in rifampicin-containing media showed their amount equally with cell control.

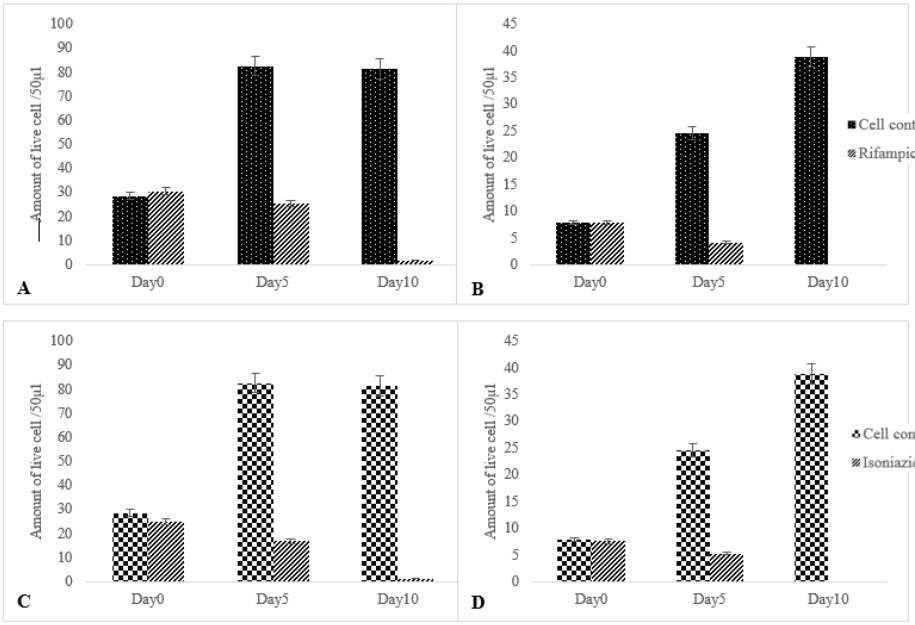

Figure 2 Antimycobacterial susceptibility testing of *Mtb* H37Ra in rifampicin-containing media compared with cell control which has only media at cell concentration A) 10^6 CFU/mL and B) 10^5 CFU/mL. For isoniazid-containing media at cell concentration C) 10^6 CFU/mL and D) 10^5 CFU/mL when observed for 10 days.

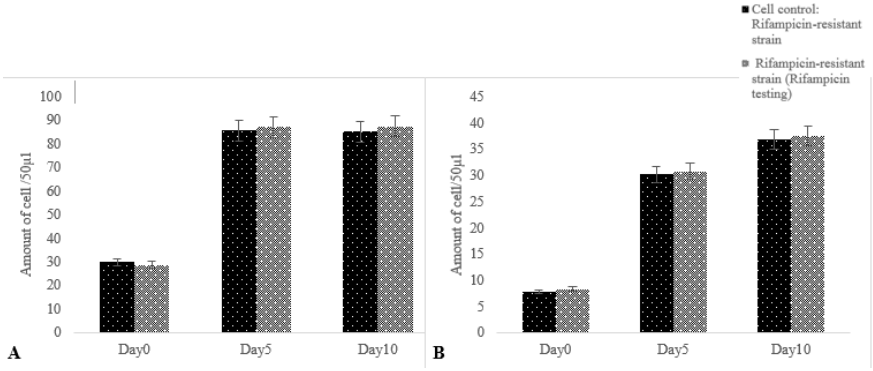

Figure 3 Antimycobacterial susceptibility testing of rifampicin-resistant strain in rifampicin-containing media compared with cell control which has only media at cell concentration A) 10^6 CFU/mL and B) 10^5 CFU/mL when observed for 10 days.

Discussion and Conclusions

This study presented the appropriate condition that type of magnetic beads are crucial for such image processing detection system. By this showed that Dynabeads®-conA is an appropriate magnetic bead isolation method. In part of preparation of Dynabeads®-conA, 5 mM Tris pH 7.0 is chosen because this solution did not cause precipitation when dissolved with divalent cations including calcium ion and manganese ion which are required for conA binding to the cell (Porter *et al*., 1998). In addition, the amount of magnetic beads and cell densities affected to the binding efficacy. By the ratio of *Mtb* H37Ra concentration was significantly increase when used 10^7 beads/mL with 10^4 CFU/mL of cells which showed greatest ability in capturing. Thus, our study showed Dynabeads®-conA is suitable magnetic bead technique because of characteristic of bead which thin polymer shell and translucent bead not only provide a specific and defined surface for binding but also allow the strong fluorescent signaling for detection. Conversely, characteristic of TB-Beads™ showed opaque surface that might overlay the cell resulting in hard to detect the fluorescent signaling. Although it has a low cost and can keep under room temperature when compared with Dynabeads®-conA.

In part of mycobacterial growth detection, it was performed in 96-well plate for simulation the condition that will use in microfluidic chip. This study presented that the image processing detection system can report the result within 10 days that all cell died. Normally, the method which used to report is culture, conventional method, which time consuming require about 3-4 weeks and cumbersome process. Thus, these optimization are essential information for testing the mycobacterial growth detection and antimycobacterial susceptibility testing for further use with the microfluidic chip that use image processing detection system to report the result when pathogen was detected.

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