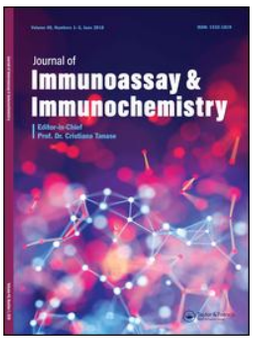

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ABSTRACT

Mycobacterial infection, leading to pulmonary disease, remains a world health problem. Clinical symptoms of pulmonary disease caused by *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM) are very similar. A rapid method for the differentiation of MTBC and NTM infection is essential for appropriate therapy. In this study, we aim to establish an antibody-based biosensor system for the identification of MTBC and NTM infection. Monoclonal antibodies (mAbs) specific for Ag85B proteins of mycobacteria were generated and characterized. The generated anti-Ag85B mAb clones AM85B-5 and AM85B-8 reacted to Ag85B of *Mycobacterium* spp.; in contrast, clone AM85B-9 specifically reacted to Ag85B of MTBC. By employing the produced mAbs, single and sandwich antibody-based biosensors using bio-layer interferometry were established for determination of Ag85B proteins. The sandwich antibody-based biosensor system was demonstrated to be suitable for detection of Ag85B protein and identification of MTBC and NTM. Using anti-Ag85B mAbs AM85B-8 and AM85B-9 as immobilized antibodies on sensor chips and using mAb AM85B-5 as secondary antibody, the established sandwich antibody-based biosensor could discriminate MTBC and NTM. The developed biosensor system can be used for culture confirmation of mycobacteria and speciation to MTBC and NTM.

KEYWORDS

Tuberculosis; Ag85B; *Mycobacterium tuberculosis* complex; nontuberculous mycobacteria; antibody-based biosensor

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Introduction

Tuberculosis (TB) is an airborne disease caused by *Mycobacterium tuberculosis* complex (MTBC), particularly *Mycobacterium tuberculosis* (MTB). Currently, TB is a world health problem and is ranked second on the world's deadliest infectious diseases.[1,2] In addition to MTBC, the genus of *Mycobacterium* comprises over 150 other species, some of which can also cause diseases in humans.[3,4] The nontuberculous mycobacteria (NTM), especially in immunocompromised persons, causes pulmonary disease that has symptoms very similar to MTBC infection.[5,6] Treatment of diseases caused by MTBC and NTM, however, is different.[5,6,7]

Currently, diagnosis of TB is still a major concern for mankind. From the 4.9 million pulmonary TB patients in 2013, only 58% of them were correctly diagnosed.[8] Identification and differentiation of MTBC and NTM in the clinical specimens of patients with pulmonary TB-like symptom is also very important as NTM is resistant to most first-line antibiotics used in TB therapy.[5,7] The gold standard method for TB diagnosis is mycobacterial culture method.[1] This method can identify and differentiate MTBC and NTM, however, the results are obtained in 2 months. Consequently, the delayed results procurement delays the starting of treatment and leads to disease spreading. Recently, PCR-based diagnostic methods were developed and endorsed by the World Health Organization (WHO) for using in TB endemic countries.[9] By this technique, identification of mycobacteria from cultures or clinical samples can be achieved.[10,11] Although the results of PCR-based methods can be obtained within 2 hr[10,11,12], its high cost and need for highly skilled technicians are the main barriers for the application of this technology in the endemic areas.[13] Rapid diagnosis of pulmonary disease that can identify MTBC and NTM is, hence, necessary and will be one of the crucial steps for the effective treatment of patients. Recently, immunochromatographic (IC) assays have been developed for the discrimination between MTBC and NTM. These methods detect the MPT64 proteins which are secreted during growth by MTBC.[14] By the IC assays, the positive reactivity indicates the presence of MTBC and negative results assume the presence of NTM. However, some false-negative results can occur for some MTBC or *M. bovis* BCG strains.[15,16]

The antigen 85 (Ag85) complex is the most abundant secretory protein of mycobacteria.[17,18] The Ag85 protein possesses mycolyltransferase activity and is involved in the formation of the mycobacterial cell wall.[19,20] This complex is comprised of three related proteins, including Ag85A (31 kDa), Ag85B (30 kDa) and Ag85C (31.5 kDa). The Ag85B proteins are the major Ag85 proteins expressed among other Ag85 proteins. Differences in amino acid sequences of Ag85B proteins produced by various mycobacteria have been reported.[21,22] The Ag85B, therefore, was purposed to be a marker for the identification of mycobacteria in bacterial culture and for TB diagnosis.[18,23]

In our research center, several mAbs specific for Ag85B were generated. Interestingly, the generated anti-Ag85B mAbs could discriminate between Ag85B produced by MTBC and NTM. By employing the generated anti-Ag85B mAbs, in this study, a novel sandwich-type antibody-based biosensor platform was developed. The developed biosensor can be used for the detection of Ag85B proteins and identification of MTBC and NTM.

Methods

Antibodies and reagents

The anti-Ag85B monoclonal antibodies (mAbs) clones AM85B-5 (Isotype IgG2b), AM85B-8 (IgG1)[23,24], AM85B-9 (IgG1), rabbit anti-Ag85 polyclonal antibody[23] and the anti-CD14 mAb clone MT14/2 (IgG2b)[25] were generated in our laboratory. Horseradish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulin antibody (Dako, Glostrup, Denmark), HRP-conjugated rabbit anti-mouse immunoglobulin antibody (Dako), EZ-Link™ Sulfo-NHS-Biotin (Pierce, Rockford, IL, USA), The BLItz bio-layer interferometry biosensor[26] and the streptavidin sensor chip (FortéBio, Pall Life Sciences, Menlo Park, CA, USA) were purchased from source as noted. Recombinant Ag85B and recombinant CD147 proteins linked with biotin carboxyl carrier protein (BCCP) were produced from the *E. coli* Origami B strain harboring vector encoding Ag85-BCCP or CD147-BCCP, respectively.[23,27] The culture filtrates (Middlebrook 7H9 broth with OADC) of various *Mycobacterium* species were obtained from Dr. Ponrut Phunpae, Faculty of Associated Medical Sciences, Chiang Mai University.[23]

Indirect ELISA for determination of anti-Ag85B monoclonal antibody activity

The ELISA plates were coated with recombinant Ag85B or CD147 protein in ELISA coating buffer (carbonate/bicarbonate buffer, pH 9.6). The plates were washed 4 times with ELISA washing buffer (0.05% tween 20 in PBS, pH 7.2) and blocked with blocking buffer (2% skimmed milk in PBS). Next, 50 µl of 10 µg/ml of anti-Ag85B mAbs or isotype-matched control mAbs were added and incubated at 37°C for 1 h. The plates were washed and the antigen-antibody complexes were monitored by adding HRP-conjugated anti-mouse immunoglobulin antibody. After washing 4 times, tetramethylbenzidine (TMB) substrate was added and the reaction was stopped with HCl. The absorbance was determined by an ELISA reader at 450 nm.

Sandwich ELISA for detection of native Ag85 protein produced by various mycobacterium species

Anti-Ag85B mAbs (AM85B-5, AM85B-8 or AM85B-9) were coated on the ELISA plates at a concentration of 10 μg/ml and the plates were blocked with 2% skimmed milk in PBS. The culture filtrates of various *Mycobacterium* species were added and incubated at 37°C for 1 h. The plates were washed. Afterward, 20 μg/ml of anti-Ag85 pAb was added and incubated at 37°C for 1 h. The antigen-antibody complexes were monitored by adding HRP-conjugated anti-rabbit immunoglobulin antibody and incubated at 37°C for 1 h. TMB substrate was added and the reaction was stopped with HCl. The absorbance was determined at 450 nm.

Immobilization of biotinylated antibodies onto streptavidin biosensor chips

Anti-Ag85B mAbs or isotype-matched control mAbs were biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotin according to manufacturer instructions. The antibodies were labeled with biotin at a molar coupling ratio of 2:1 (2 moles of biotin to 1 mole of antibody). The biotinylated mAbs were prepared at various concentrations (12.5, 25 and 50 μg/ml) in sample diluent (0.1% BSA, 0.02% Tween 20, 0.05% NaN_3 in PBS, pH 7.2). The streptavidin sensors were hydrated for 15 min before immobilization with biotinylated mAbs.[26] BLItz bio-layer interferometry biosensor was used to monitor the binding of mAbs on the sensor chip as per the following steps. The initial baselines were achieved by immersing the streptavidin sensor in sample diluent for 30 s and then immersing into various concentrations of biotinylated mAbs solutions for 400 s as called the loading step. Then, the sensors were washed to remove excess antibodies with sample diluent for 30 s as called the dissociation step.

Antibody-based biosensors

The anti-Ag85B mAb-immobilized sensors were immersed in sample diluent, purified recombinant Ag85B protein, recombinant CD147 protein (control) or the mycobacterial culture filtrates containing native Ag85B proteins. The antigen–antibody interactions were observed for the binding signal by BLItz bio-layer interferometry biosensor for 120 s. Subsequently, the sensors were washed with sample diluent for 30 s. The antibody sensors were then immersed into the secondary anti-Ag85B mAb (secondary antibody; 50 μg/ml) for 90 s. The excess secondary antibodies were washed out with sample diluent for 30 s. The signals were measured using the BLItz bio-layer interferometry biosensor both before and after adding the secondary mAb.

Data analysis

The binding signals were measured and calculated for the delta binding signal ($\Delta Signal = Signal_{end\ point} - Signal_{starting\ point}$). Mean and standard deviation of the experiments were calculated.

Results

Specificity of anti-Ag85B monoclonal antibodies

By standard hybridoma techniques and using recombinant Ag85B protein as immunogen, three anti-Ag85B mAbs named AM85B-5, AM85B-8, and AM85B-9 were generated.[23] These mAbs reacted strongly to the recombinant Ag85B protein but did not react to recombinant CD147 protein, an irrelevant recombinant antigen (Figure 1a). We then investigated the specificity of the generated anti-Ag85B mAbs to native Ag85B protein. Culture filtrates of various *Mycobacterium* spp. were used as the sources of native Ag85B proteins. The mycobacterial culture filtrates used in this study could be divided into three groups. The first group was MTBC, including the virulent strain of *M. tuberculosis* H37Rv, non-virulent strain of *M. tuberculosis* H37Ra, and *M. Bovis* BCG. The second group was a slow-growing species of NTM: *M. avium, M. intracellulare*, and *M. kansasii*. The third group was rapid-growing species of NTM, *M. smegmatis*. The Ag85B proteins presented in the culture filtrates were determined by ELISA using the generated mAbs as the capture mAbs. As shown in Figure 1b, mAb AM85B-5 reacted to native Ag85B of MTB complexes and reacted weakly to *M. kansasii* and *M. smegmatis*. mAb AM85B-8 showed positive reactivity with Ag85B secreted by all *Mycobacterium* spp. tested. mAb AM85B-9 reacted only with Ag85B of MTBC. All tested culture filtrates showed strongly positive reactivity to mAb AM85B-8 suggesting that all culture filtrates contained Ag85B proteins. The data indicated that the generated anti-Ag85B mAbs could react to native Ag85B proteins; however, each anti-Ag85B mAb showed a different reactivity pattern with native Ag85B proteins produced by different *Mycobacterium* species.

Establishment of antibody-based biosensors for detection of Ag85B proteins

As we have anti-Ag85B mAbs that could detect native Ag85B proteins and differentiate MTBC and NTM, the mAbs were employed for the establishment of antibody-based biosensors for the identification and discrimination of MTBC and NTM. According to the high-affinity binding between avidin-biotin, streptavidin sensor was chosen to establish the antibody-based sensor system. To immobilize mAbs on sensor tips, anti-Ag85B mAbs clones AM85B-5, AM85B-8, and AM85B-9 and isotype-matched control mAb were first labeled with biotin.

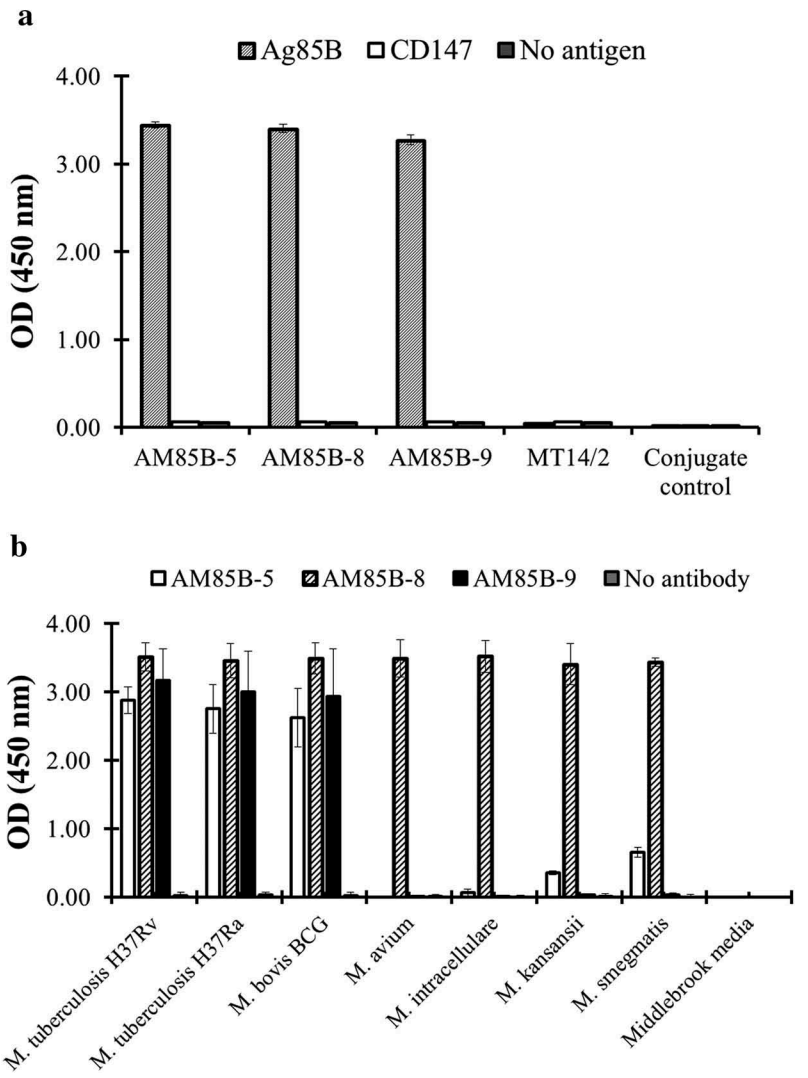

Figure 1. (a) The immunoreactivity of anti-Ag85B mAbs to recombinant Ag85B protein. Anti-Ag85B mAbs AM85B-5, AM85B-8, and AM85B-9 were tested for their reactivity with recombinant Ag85B and recombinant CD147 proteins by indirect ELISA. mAb MT14/2 was used as isotype-matched control antibody. (b) The immunoreactivity of anti-Ag85B mAbs to native Ag85B protein of various *Mycobacterium* spp. Anti-Ag85B mAbs AM85B-5, AM85B-8, and AM85B-9 were tested for their reactivity using culture filtrates obtained from the indicated *mycobacteria* or Middlebrook media. Mean ± SD of two independent experiments is presented.

The streptavidin sensor tips were then dipped into various concentrations of biotinylated mAbs. The mAb binding signal was real-time monitored by BLItz bio-layer interferometry biosensor. As shown in Figure 2a, the biotinylated mAbs displayed consistent binding signal rise and achievement of saturation point in the mAb immobilization step within 400 s. The binding signal remained

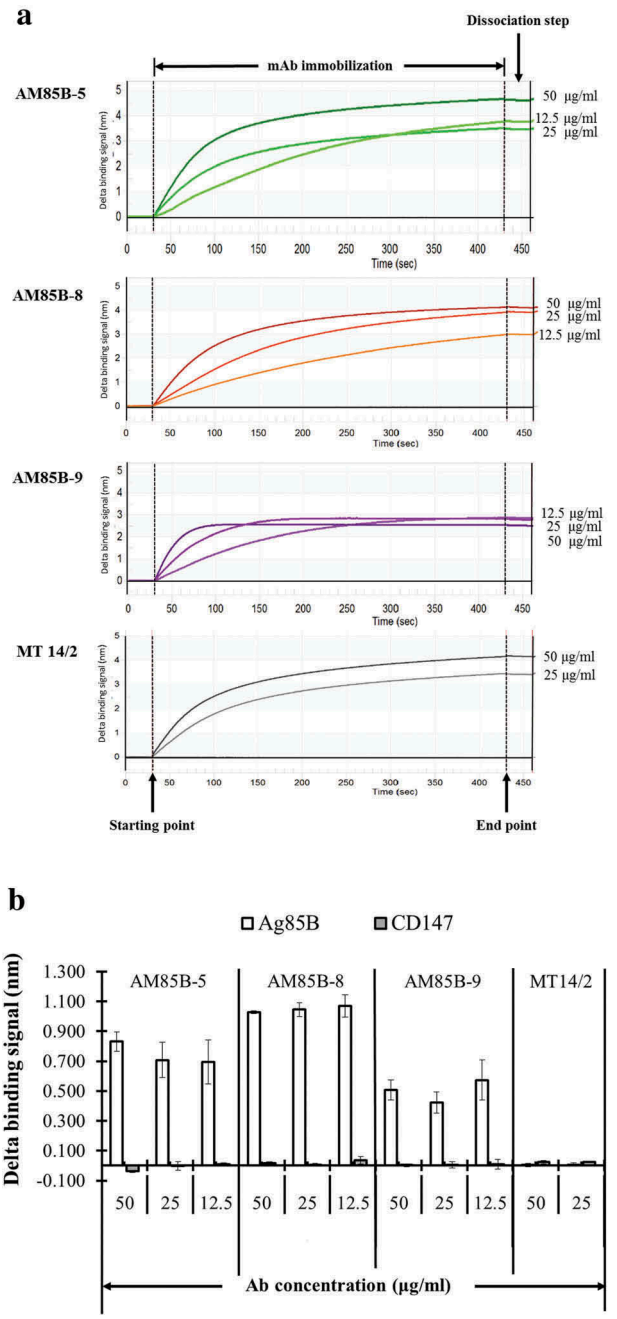

Figure 2. Sensograms of the biotinylated mAb immobilization on streptavidin sensor. (a) After initial baseline with sample diluent, various concentrations (12.5, 25, 50 µg/ml) of biotinylated mAbs were introduced to streptavidin sensor. The binding signals were monitored by BLItz biolayer interferometry biosensor. The concentrations of biotinylated mAbs are indicated on the right side of the figure. (b) After anti-Ag85B mAbs (AM85B-5, AM85B-8, or AM85B-9) or MT14/2 control mAb were immobilized on streptavidin sensors, the recombinant Ag85B or CD147 proteins (20 µg/ml) were introduced to antibody-immobilized sensors and antigen-binding signals were measured by BLItz biolayer interferometry biosensor. Mean ± SD of binding signal of two independent experiments is presented.

stable in the washing (dissociation) step, pointing to steady immobilization of biotinylated mAb onto the streptavidin sensor.

To examine the ability of the antibody-biosensor in detecting Ag85B, the antibody-streptavidin sensors were then immersed into recombinant Ag85B protein (20 μg/ml). The antigen-binding signals were observed in all anti-Ag85B mAb sensors, but no signal was observed in CD147 recombinant protein control or isotype-matched control sensor (Figure 2b). The concentrations of 25, 25 and 12.5 μg/ml for mAbs AM85B-5, AM85B-8, and AM85B-9, respectively, were chosen as optimal concentrations for immobilization on sensor chip in further studies.

Two types of antibody-based biosensor systems, single and sandwich antibody-based biosensors, were designed. For single antibody-based biosensor, the mAbs were coated on sensor chips and dipped into sample to capture Ag85B proteins. The binding of antigen at the sensor tips was real-time monitored by BLItz biosensor. For sandwich antibody-based biosensor, the mAb coated sensor chip was used to capture Ag85B proteins. Then, the secondary anti-Ag85 mAb was added to detect the Ag85B proteins bound on the sensor and monitored by BLItz biosensor.

As anti-Ag85B mAbs clones AM85B-8 and AM85B-9 could differentiate MTBC and NTM, in our designed, the mAbs AM85B-8 and AM85B-9 were immobilized on each sensor (via biotin–avidin system) to capture Ag85B proteins in the single antibody method. Then, a different clone of anti-Ag85B mAb was applied as secondary antibody in the sandwich antibody method.

As shown in Figure 3a,b, using mAb AM85B-8 or mAb AM85B-9 upon detection of 500 ng/ml recombinant Ag85B proteins, the sandwich antibody method showed higher binding signal than the single antibody method.

Focusing on the sandwich antibody-based sensor system, AM85B-8 and AM85B-9 immobilized sensors were dipped into recombinant Ag85B proteins, and the binding signals were observed after adding different clones of secondary mAb AM85B-5, AM85B-8 or AM85B-9. As shown in Figure 3a,b, using mAb AM85B-8 or AM85B-9 immobilized sensor in combination with any secondary mAbs, the recombinant Ag85B proteins could be detected. These data suggested that a sandwich antibody-based sensor using a different pair of mAbs could be developed. In fact, in our study, a sandwich antibody-based sensor was successfully established to detect Ag85B proteins.

Validation of sandwich antibody-based sensors for identification and differentiation of MTBC and NTM

We then investigated whether the developed sandwich antibody-based sensors could be used to detect native Ag85 proteins and differentiate MTBC and NTM. Culture filtrates of various mycobacteria were used as samples for this verification. Anti-Ag85B mAb-immobilized sensors (AM85B-8 and AM85B-9

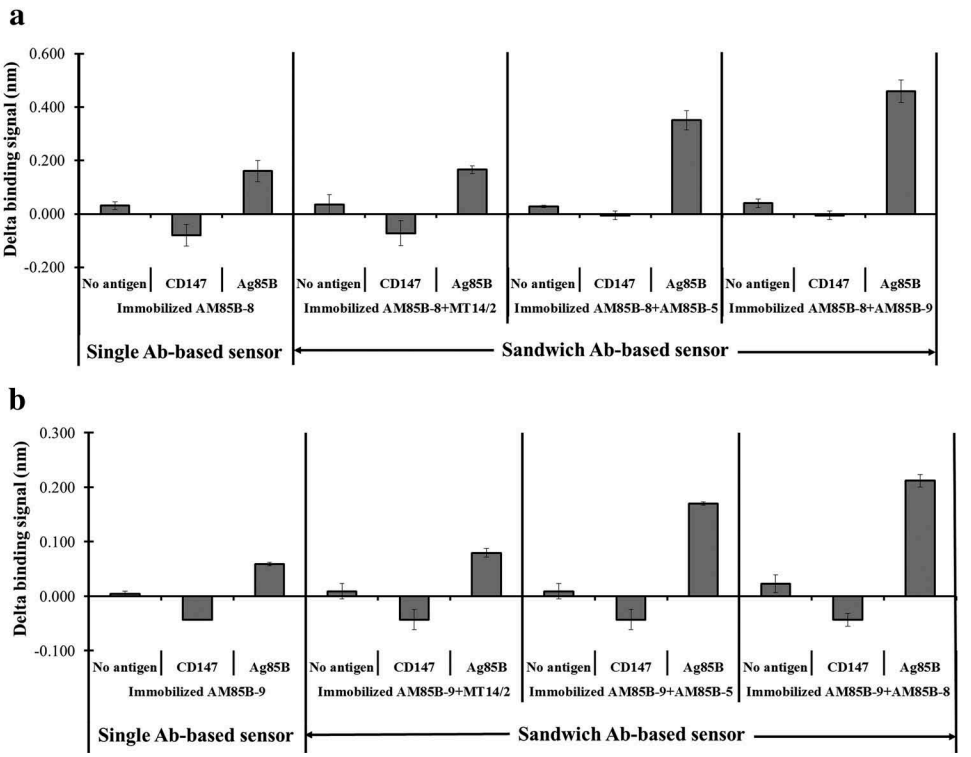

Figure 3. Binding signals of single and sandwich antibody-based biosensor systems. (a) mAb AM85B-8 immobilized sensors and (b) mAb AM85B-9 immobilized sensors were immersed into recombinant Ag85B proteins (Ag85) or recombinant CD147 control proteins (CD147) at 500 ng/ml or without antigen (no antigen) and followed by secondary anti-Ag85 mAbs, i.e. mAb AM85B-5, AM85B-8, AM85B-9 or MT14/2 as indicated. The antigen-binding signals were measured before (Single Ab-based sensor) and after adding the secondary antibody (Sandwich Ab-based sensor). The delta binding signals (mean ± SD) of two independent experiments are presented.

sensors) were dipped into culture filtrate containing Ag85 proteins followed by secondary mAb clone AM85B-5, AM85B-8 or AM85B-9. The binding signals were determined after the second mAb added and delta binding signals were calculated. Surprisingly, using mAb AM85B-5 as the secondary mAb was shown to be the best for differentiating *Mycobacterium* spp. in using with either mAb AM85B-8 or AM85B-9 immobilized sensor (Figure 4a,b).

It was found that when using AM85B-8 immobilized sensors followed by secondary mAb AM85B-5, Ag85B proteins produced by all tested *Mycobacterium* spp. could be detected (Figure 4a and Table 1). In contrast, when using AM85B-9 immobilized sensors followed by secondary mAb AM85B-5, only Ag85B proteins produced by MTBC could be detected (Figure 4b and Table 1).

Taken together, the results indicated that by using a proper pair of our generated anti-Ag85B mAbs, a sandwich antibody-based sensor platform was established and this biosensor was able to distinguish MTBC and NTM.

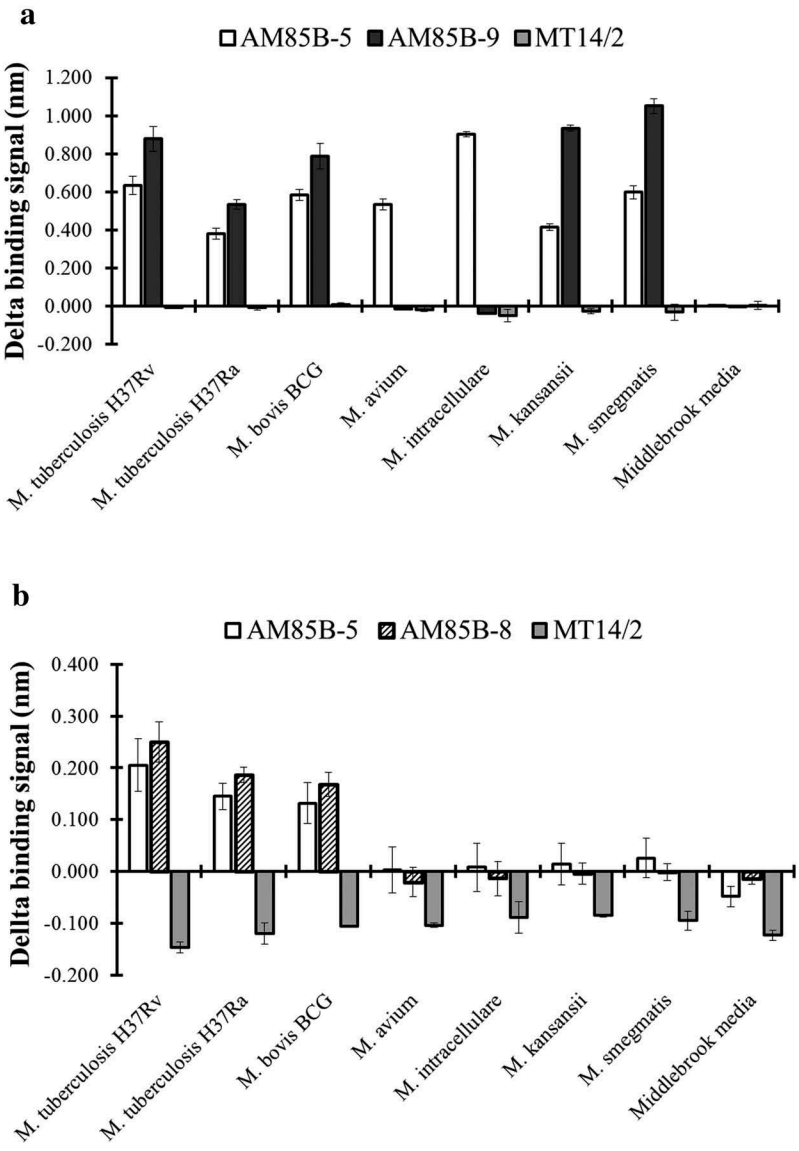

Figure 4. Detection of native Ag85 proteins in mycobacterial culture filtrates by sandwich antibody-based biosensor system. AM85B-8 (a) and AM85B-9 (b) immobilized sensors were immersed into culture filtrates from the indicated *Mycobacterium* species or Middlebrook media control followed by indicated secondary mAbs. The delta binding signals (mean ± SD) of two independent experiments are presented.

Discussion

The Ag85 complexes, including Ag85A, Ag85B, and Ag85C, are the proteins that secreted by actively replicating live mycobacteria.[17,28] Mycobacteria produces and secretes Ag85 complexes into liquid media during cultivation.[29] Detection of Ag85B proteins is beneficial for the determination of mycobacterial growth.[23,29,30]

Table 1. Differentiation of *Mycobacterium* spp. by sandwich antibody-based biosensor system.

	Immobilized mAb			
	AM85B-8		AM85B-9	
Mycobacterial culture filtrate	AM85B-5*	AM85B-9*	AM85B-5*	AM85B-8*
M. tuberculosis H37Rv	+**	+	+	+
M. tuberculosis H37Ra	+	+	+	+
M. bovis BCG	+	+	+	+
M. avium	+	-	-**	-
M. intracellulare	+	-	-	-
M. kansasii	+	+	-	-
M. smegmatis	+	+	-	-

*The secondary antibody used

**Mean of isotype-matched control mAb + 3SD was used as a cutoff for determination of positive and negative.

We have successfully generated three hybridoma clones producing anti-Ag85B mAbs. Interestingly, the generated mAbs reacted differently to Ag85B proteins produced from different *Mycobacterium* spp. Variables in amino acid sequences of Ag85B proteins produced by various mycobacteria have been reported.[21,22] Ag85B proteins produced by different *Mycobacterium* spp. contain both common epitopes and specific epitopes. While the anti-Ag85B mAbs react to different epitopes on the Ag85B molecule, some mAbs might bind to common epitopes and some might bind to specific epitopes. Therefore, different clones of anti-Ag85B mAbs display different interaction patterns when reacting to Ag85B produced by different *Mycobacterium* spp. Moreover, the Ag85B proteins produced by different bacteria strains may have a different conformational structure causing different bindings even by the same mAb.[28]

In the characterization of our generated anti-Ag85B mAbs, the ELISA results indicated that anti-Ag85B mAb clone AM85B-8 broadly reacted to Ag85B proteins produced by various *Mycobacterium* spp., whereas clone AM85B-9 specifically reacted to Ag85B proteins produced by MTBC. Clone AM85B-5 reacted to Ag85B of MTBC and reacted weakly to some NTM. By the dissimilar reactivity patterns, the produced anti-Ag85B mAbs could be applied to differentiate MTBC and NTM. In this study, we then applied our generated anti-Ag85B mAbs in the development of immunoassay for the detection and discrimination of MTBC and NTM.

Several types of antibody-based sensors have been developed for determining antigens which can be real-time analyzed with high sensitivity.[31,32,33] We, in this study, employed our generated anti-Ag85B mAbs in combination with biosensor technology for the identification of MTBC and NTM. A non-flow dip-and-read bio-layer interferometry was employed in our design.[26] The BLI is an optical interferometry, having white light down the biosensor and collecting the light reflected back.[26] The interference patterns of the light reflected from two surfaces, an internal reference layer and a layer of molecules bound to its partner on the biosensor tip, are determined. By the antibody-biosensor technology, the interference pattern of reflected light can be used to real-time monitor the

interaction between antigen and specific antibody attached to the fiber-optic sensor surface. As a result, the biosensor system has a short processing time with minimal obstruction and interference from unbound molecules.

Using the produced anti-Ag85B mAbs as the sensor-immobilized mAbs together with BLI technology, antibody-based sensors (single antibody- and sandwich antibody-based sensors) were established. We demonstrated that the single antibody-based sensor had less sensitivity than the sandwich antibody-based biosensor. Addition of the secondary antibody in sandwich antibody-based sensor could generate higher binding signals. The sandwich antibody-based sensor system was, therefore, chosen for the development of the method for identification of MTBC and NTM.

In this study, anti-Ag85B mAb clone AM85B-8 and clone AM85B-9 were used as the sensor-immobilized mAbs and other different clones were used as the secondary mAb. The mAb AM85B-8 immobilized sensor would capture both Ag85B antigen produced by MTBC and NTM, while mAb AM85B-9 captured only Ag85B of MTBC on the sensor. The secondary anti-Ag85B mAb, then, recognized the Ag85B antigen bound on the sensor tips. The binding signal of the secondary anti-Ag85B mAb, therefore, could differentiate the group of mycobacteria. The biotin–streptavidin interaction, which is the strong type of non-covalent interaction, was employed for mAbs-sensor immobilization.[34] The binding mAb on the sensor tip via avidin–biotin interaction might improve the antigen-binding properties of the immobilized mAb by creating an appropriate orientation of antibody on the sensor surface. The concentration of biotinylated mAb used for immobilization on sensor tips was, then, optimized. The concentrations at 25 μg/ml for mAb AM85B-5 or AM85B-8 and 12.5 μg/ml for mAb AM85B-9 were demonstrated to be the optimal concentrations for antibody immobilization.

Using our designed sandwich antibody-based sensor, both mAbs AM85B-8- and AM85B-9-immobilized sensors in combination with mAb AM85B-5 as secondary antibody were demonstrated to be the appropriate pair of mAbs for the differentiation of MTBC and NTM. It was found that mAbs AM85B-8 and AM85B-9 immobilized sensors could capture Ag85B proteins in the samples and the mAb AM85B-5 was still able to amplify the Ag85B binding signals. Although mAb AM85B-5 showed low reactivity to Ag85B proteins of NTM in ELISA, it could react to Ag85B proteins of both MTBC and NTM after being captured on the sensors by both primary mAbs (mAbs AM85B-8 and AM85B-9). We speculated that the capturing of Ag85B on sensors by the first mAbs, either mAb AM85B-8 or AM85B-9, caused an appropriate orientation or opened up the hidden epitopes on Ag85 proteins that allowed the binding of mAb AM85B-5. In contrast to ELISA, the mAb AM85B-5 was coated on ELISA plate as capture antibody followed by second anti-Ag85B mAb. The captured Ag85B proteins by mAb AM85B-5 on ELISA plate may have orientation that hindering the secondary antibody binding.

The established sandwich antibody-based sensor was then determined for its ability in the identification and differentiation of *Mycobacterium* spp. We found that the positive patterns of the developed method using different primary mAbs could differentiate the group of mycobacteria. Using mAb AM85B-9 immobilized sensor, the positive reactivity was observed only with MTBC. In contrast, using mAb AM85B-8 immobilized sensor, the positive reactivity was observed with both MTBC and NTM. The established sandwich antibody-based biosensor, therefore, can be applied for the detection of the Ag85B protein present in mycobacterial culture filtrates and identification of MTBC and NTM.

In this study, we developed a sandwich-type antibody-based biosensor using BLI technology. The developed immunosensor could detect native Ag85B proteins produced by *Mycobacterium* spp. and could distinguish MTBC and NTM. This technique could be applied for the development of a rapid method for culture confirmation of mycobacteria and speciation to MTBC and NTM.

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Conflict of Interests

No conflict of interest declared.

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