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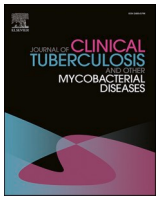

In vitro evaluation of the binding activity of novel mouse IgG1 opsonic monoclonal antibodies to *Mycobacterium tuberculosis* and other selected mycobacterial species

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ABSTRACT

Antimicrobial resistance alongside other challenges in tuberculosis (TB) therapeutics have stirred renewed interest in host-directed interventions, including the role of antibodies as adjunct therapeutic agents. This study assessed the binding efficacy of two novel IgG1 opsonic monoclonal antibodies (MABs; GG9 & JG7) at 5, 10, and 25 μg/mL to live cultures of *Mycobacterium tuberculosis, M. avium*, *M. bovis*, *M. fortuitum*, *M. intracellulare*, and *M. smegmatis* American Type Culture Collection laboratory reference strains, as well as clinical susceptible, multi-drug resistant, and extensively drug resistant *M. tuberculosis* strains using indirect enzyme-linked immunosorbent assays. These three MAB concentrations were selected from a range of concentrations used in previous optimization (binding and functional) assays. Both MABs bound to all mycobacterial species and sub-types tested, albeit to varying degrees. Statistically significant differences in MAB binding activity were observed when comparing the highest and lowest MAB concentrations ($p < 0.05$) for both MABs GG9 and JG7, irrespective of the *M. tuberculosis* resistance profile. Binding affinity increased with an increase in MAB concentration, and optimal binding was observed at 25 μg/mL. JG7 showed better binding activity than GG9. Both MABs also bound to five MOTT species, albeit at varied levels. This non-selective binding to different mycobacterial species suggests a potential role for GG9 and JG7 as adjunctive agents in anti-TB chemotherapy with the aim to enhance bacterial killing.

1. Background

Mycobacterial infections pose major challenges to public health globally, with tuberculosis (TB) disease, caused by members of the *Mycobacterium tuberculosis* complex, prominently contributing to the high morbidity and mortality rates in low- and middle-income countries worldwide [1]. *M. tuberculosis* remains one of the single most successful infectious agents, responsible for approximately 1.3 million deaths in 2022 [1].

Significant progress in the fight against TB has been achieved over the years. However, there are several challenges that have decelerated the progression towards TB elimination, including the limited efficacy of the only licensed TB vaccine, Bacille Calmette-Guérin (BCG), which mainly prevents disease progression and severity, but does not protect against infection [1,2]; drug resistance [3]; the increased risk of susceptibility to mycobacterial infections in immunocompromised individuals [1,3,4], and an increased risk of TB/mycobacteria other than tuberculosis (MOTT) coinfections [5]. These outcomes may result in the misdiagnosis and inaccurate treatment of patients and further impede progress in the fight against TB [6,7].

Abbreviations: ATCC, American Type Culture Collection; BCG, Bacille Calmette-Guérin; ELISA, enzyme-linked immunosorbent assays; HDT, host-directed therapy; MAB, monoclonal antibody; MDR, multidrug-resistant; MGIT, mycobacteria growth indicator tubes; MOTT, mycobacteria other than tuberculosis; OADC, oleic acid, albumin, dextrose, and catalase; PANTA, polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin; PBS, phosphate buffered saline; TB, tuberculosis; XDR, extensively drug-resistant.

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In addition to *M. tuberculosis*, pulmonary MOTT infections have increasingly become a global health concern [8]. These MOTT infections are not notifiable in many countries and therefore, the exact burden is unknown [8,9]. In South Africa, there is very limited data on the prevalence of MOTT species; a 2014 study by Sookan and colleagues detected MOTT species in 66.5 % of 200 suspected MOTT cultures, of which *M. avium* and *M. intracellulare* comprised 57.1 % [10]. Some of the most common MOTT species involved in pulmonary disease are *M. avium*, *M. intracellulare* and less frequently, *M. fortuitum* [5,6]. The impact of these pulmonary MOTT diseases in an ongoing TB epidemic is unclear. Nevertheless, it is important to target pathogenic mycobacterial infections concurrently because of the significant morbidity and mortality associated with these bacteria [11,12].

Host-directed therapies (HDTs) have provided new avenues of combating mycobacterial infections. They modulate the immune response in favor of protection against or prevention of active disease [13,14] by regulating immune cell responses, enzyme pathways essential for *M. tuberculosis* pathogenesis, and cytokine and antibody production [14].

Studies have increasingly shown the protective role of antibodies produced during TB infection, and this has led to investigations of antibody-based HDTs [15–17], including the immunotherapeutic role of anti-TB antibodies in protection against TB disease [18]. Studies evaluating the therapeutic effects of monoclonal antibodies (MABs) against TB either through bacterial load reduction or bacterial clearance, have shown promising results [15,19–21]. Additionally, MABs have also been shown to reduce lung pathology and increase survival in animal models [19,22,23].

In 2014, Flores-Moreno and colleagues evaluated the binding activity of different MABs to clinically relevant mycobacteria including *M. tuberculosis* complex [23]. Cross-reactivity between the MABs tested and various mycobacterial species was observed; antibodies raised against a certain epitope from one mycobacterial species bound to other epitopes from other mycobacterial species, and the authors concluded that mycobacteria share certain antigenic determinants [23].

In a previous study, Sei and colleagues used enzyme-linked immunosorbent assays (ELISAs) to assess the binding activity of two novel mouse IgG1 anti-TB opsonic MABs, GG9 II G2 (GG9) and JG7 III D3 F9 (JG7), to inactivated and live clinical *M. tuberculosis* strains [24].They further demonstrated that these MABs at concentrations less than 25 μg/mL, significantly enhanced the phagocytic killing of *M. tuberculosis in vitro* and also significantly enhanced its clearance from blood in mice *in vivo* [24]. In addition, the results also indicated that the binding target of these MABs is peptidoglycan (PGN), a component of the mycobacterial cell wall. PGN is an essential mycobacterial virulence factor and designing *M. tuberculosis* surface antigen-specific antibodies may be beneficial for novel vaccine and drug development [17,25]. Differences in cell wall structure may influence MAB binding activity to different mycobacterial species.

We sought to assess whether differences in the cell wall structure of various mycobacterial species (*M. tuberculosis* and other mycobacterial species and sub-types) could influence the extent to which they bind to opsonic MABs GG9 and JG7.

2. Materials and methods

2.1. Study design and setting

This semi-quantitative study was conducted with three objectives in mind: (1) to increase the robustness (consistency) of a previously developed in-house ELISA for the detection of antibody binding to live mycobacterial strains by performing multiple runs with multiple strains; (2) to assess the binding effects of two selected novel MABs (GG9 and JG7) across the *Mycobacterium* genus using live reference strains; and (3) to assess the binding effects of novel MABs GG9 and JG7 to different resistance phenotypes using susceptible, MDR- and XDR clinical TB isolates. Ethical approval was obtained from the Human Research Ethics Committee at the Faculty of Health Sciences, University of Pretoria (Ethics Reference Number: 483/2017) before the study commenced. Laboratory work was performed at the National Health Laboratory Services (NHLS) - TB laboratory, Tshwane Academic Division and the Department of Microbiology, Faculty of Health Sciences, University of Pretoria.

2.2. Bacterial strains

Fifteen *M. tuberculosis* clinical isolates were collected based on their resistance profiles, from the NHLS and divided into three groups: drug susceptible, multidrug-resistant (MDR), and extensively drug-resistant (XDR). Furthermore, American Type Culture Collection (ATCC) strains of *M. tuberculosis* (H37Ra; ATCC® 25177™), *M. smegmatis* (ATCC® 19420™) (as a non-pathogenic control), *M. avium* subsp. *avium* (ATCC® 15769™), *M. bovis* (BCG; ATCC® 27290™), *M. fortuitum* subsp. *fortuitum* (ATCC® 6841™), and *M. intracellulare* (ATCC® 13950™), were obtained via a commercial supplier (Thermo Scientific, USA). Strain descriptions have been included in **Supplementary Table S1**.

2.3. Monoclonal antibodies

Two purified IgG1 MABs, GG9 and JG7, were obtained from Longhorn Vaccines and Diagnostics, USA. These MABs were produced by fusing single spleen cells from immunized BALB/c mice, with SP2/0 myeloma cells to form hybridoma cells which were then tested, selected, and subsequently cloned. The MABs produced were purified by chromatography, quantified by IgG capture ELISA, and characterized as opsonic monoclonal antibodies [24]. These MABs have been deposited at ATCC (designated as GG9-01 and JG7-01) and are available for research use.

2.4. Bacterial culture and identification

M. tuberculosis ATCC® 25177™, *M. smegmatis* ATCC® 19420™, *M. bovis* ATCC® 27290™, and the clinical *M. tuberculosis* isolates were cultured in mycobacteria growth indicator tubes (MGIT) by adding 500 μL of the isolate suspension into MGIT tubes containing 7 mL of Middlebrook 7H9 broth supplemented with 800 μL of a reconstituted antibiotic mixture composed of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) and BACTEC MGIT Growth Supplement (Becton Dickinson & Company, USA) consisting of oleic acid, albumin, dextrose, and catalase (OADC). The tubes were incubated in the BACTEC™ MGIT™ 960 instrument (Becton Dickinson & Company, USA) for 42 days until the bacteria reached early phase or mid-logarithmic phase. Early log phase was defined as the period immediately after MGIT flagged positive, and mid-log phase was defined as two days after MGIT flagged positive.

M. avium subsp. *avium* ATCC® 15769™, *M. fortuitum* subsp. *fortuitum* ATCC® 6841™, and *M. intracellulare* ATCC® 13950™ were cultured in MGIT tubes as described above, scanned out of the BACTEC™ MGIT™ 960 instrument, aliquoted into 2 mL cryovials, and frozen. However, a different approach (section 2.7) was followed in preparing these MOTT species for ELISA experiments.

The standard Ziehl-Neelsen acid-fast staining technique was carried out on MGIT culture-positive isolates which were viewed under the microscope for the presence of acid-fast bacilli. The MPT64 antigen test (Becton Dickinson & Company, USA) was carried out on the cultures for confirmation of *M. tuberculosis*. MOTT species were confirmed using the GenoType Mycobacterium CM VER 2.0 kit (Hain Lifesciences, Germany).

2.5. Bacterial cell enumeration

Ten-fold serial dilutions of the bacteria were performed by adding

100 μL of bacteria from culture-positive MGIT to 900 μL of phosphate buffered saline (PBS) (Sigma-Aldrich life science, Germany). The last five bacterial dilutions (10^{-6} – 10^{-10}) were plated on Middlebrook 7H10 or 7H11 agar, and incubated at 37 °C. The incubation times varied with the strain used, from 2 to 7 days (*M. fortuitum* and *M. smegmatis)* to up to 42 days (for the other strains). The viable plate count method was used to estimate the number of bacteria used in the ELISA experiments and results were recorded as colony forming units per milliliter (CFU/ mL).

2.6. Preparation of clinical M. tuberculosis strains for ELISA

Binding activity of MABs GG9 and JG7 to *M. tuberculosis* ATCC® 25177™ and bacteriologically susceptible, MDR, and XDR *M. tuberculosis* clinical isolates was assessed using stock cultures at mid-logarithmic phase from MGITs with growth units (GU) ranging between 1 800 and 2 900. A volume of 3 mL of each bacterial suspension from MGIT was pipetted into 25 mL of a 1:300 dilution of bovine serum albumin (BSA) in PBS and centrifuged (Beckman Coulter Inc., USA) at 2 600 rpm for 10 min at room temperature. The supernatant was discarded, and the pellet was re-suspended in 15 mL of PBS-BSA and centrifuged as previously described, in order to get rid of the 7H9 broth. The bacteria were re-suspended in a final volume of 5 mL of PBS-BSA.

2.7. Preparation of MOTT strains for ELISA

Frozen stock was thawed, and a 200 μL volume of each bacterial suspension inoculated onto appropriate solid Middlebrook agar (7H10 or 7H11). The agar plates were then incubated at 37 °C for up to 42 days. Approximately 5 mL of a 0.5 McFarland (range, 0.50—–0.55) was prepared from pure bacterial colonies on agar. The resulting suspension was vortexed for 20 – 30 s using a Vortex Genie 2 mixer (Scientific Industries, Inc., USA) to ensure colony separation. Measurements were read by a portable DensiCHEK™ Plus instrument (BioMérieux, France). The suspension was then used in the subsequent ELISA.

2.8. Dilution of MABs for ELISA

PBS plus Tween 20 (PBS-T; pH 7.4) was prepared by dissolving PBS-T powder (Sigma-Aldrich life science, Germany) in 1 L of distilled water as per the manufacturer's instructions. Working solutions of MABs GG9 and JG7 were prepared in PBS-T according to calculated dilutions from different stock concentrations to final concentrations of 5 μg/mL, 10 μg/mL, and 25 μg/mL. These concentrations were chosen based on previous titration and optimization assays [24]; concentrations > 25 μg/mL (up to 100 μg/mL were tested in our initial pilot study (Shey B, 2016; unpublished data), but did not yield discriminatory binding data.

2.9. Determination of ELISA target phase

M. smegmatis (ATCC® 19420™) and *M. tuberculosis* (ATCC® 25177™; coded as TB1 and TB2) were used in preliminary experiments to determine the target phase for the ELISAs. The two time-points investigated were early log phase and mid-log phase. TB1 and TB2 refer to aliquots of the same strain that were grown until early log phase and mid-log phase, respectively. Based on the results obtained, the ELISAs were run at mid-log phase. Analysis of binding activity was based on qualitative (color change) and semi-quantitative (absorbance values corresponding to color intensity) data.

2.10. Indirect live-cell ELISA binding experiments

A volume of 200 μL of the bacterial suspension (either from 5 mL PBS-BSA or 5 mL 0.5 McFarland) was dispensed into each well of the first, fifth, and ninth column of a 96-well polypropylene microplate (Thermo Scientific, USA) with the exception of the intersecting wells in the seventh row (row G; **Supplementary Figure S1**), which had the same volume of PBS-BSA to serve as an indicator of background signal. The cells in the eighth row (row H; **Supplementary Figure S1**) had bacteria but no MAB, to serve as a negative control. A volume of 100 μL of PBS-BSA was pipetted into the remaining wells and two-fold serial dilutions of the bacteria were done in the microplate resulting in four bacterial dilutions (undiluted, 1:2, 1:4 and 1:8) being tested.

Approximately 50 μL of the primary antibodies (GG9 and JG7) were transferred to corresponding wells of the 96-well plate containing the bacteria and mixed thrice. The plate was sealed and incubated for 1 h at 37 °C in a shaking incubator (BMG Labtech, Germany). After incubation, the plate was centrifuged at 25 °C for 5 min, washed with 200 μL of a 1:300 dilution of BSA in PBS-T, and centrifuged as described previously. This process was carried out twice to remove unbound MAB. A volume of 50 μL of isotype-specific peroxidase-labelled goat anti-mouse IgG (secondary antibody; Biocom, SA) was added to each well of the plate. The plate was sealed and incubated at room temperature for 30 min.

After incubation, the plates were washed twice with PBS-T/BSA as previously described. Tetramethylbenzidine (TMB) Substrate Solution (Fisher Scientific, USA) was added to each well in 100 μL aliquots and incubated in the dark for 15 min. A volume of 100 μL of TMB STOP solution (Fisher Scientific, USA) was then added to each well. Immediately, 180 μL of each sample was transferred to the wells of a 96-well polystyrene plate (Thermo Scientific, USA) and the ELISA ELX800 reader (Biotek, USA) was used to read absorbance at 450 nm. Each of the binding experiments was carried out at least three times per parameter or condition tested.

2.11. Data management and statistical analysis

Results obtained during experiments were printed out from the ELX800 reader and transferred onto a data capturing electronic spread sheet. Microsoft Excel software was used for initial data assessment. Background signal values were subtracted from the values of samples where MAB was incubated with live bacteria. The difference is indicative of MAB affinity to bacteria.

Dixon's Q test was used to identify and reject outliers in replicate data within a single run. Descriptive statistics using means with standard deviations and medians with ranges were computed in order to compare the binding effects of the two MABs to the different mycobacterial species and sub-types. Variance was also measured to assess inter-run reproducibility. Graphs were constructed using GraphPad Prism software and Microsoft Excel 2016. Data were analyzed using the SPSS Software version 24 statistical package (IBM, United States). Differences in binding capacity as measured in optical density (OD) between groups were analyzed using the Mann-Whitney (two groups) and the Kruskal-Wallis tests (multiple groups). Statistically significant differences were determined using a p-value < 0.05.

3. Results

3.1. Mycobacterial identification

A total of 15 live clinical *M. tuberculosis* isolates were collected, confirmed as culture-positive, and correctly identified as *M. tuberculosis* through microscopy (86.7 %) and antigen testing (100 %) before use in the binding ELISA experiments. The MOTT species grown on solid Middlebrook agar were identified by colony morphology and the GenoType Mycobacterium CM VER 2.0 assay.

3.2. Determination of M. tuberculosis target phase

Following, the binding activities of two novel mouse IgG1 MABs, GG9 and JG7, to *M. tuberculosis* strains with different drug resistance profiles and to a selected sample set of clinically important MOTT species were evaluated. MABs GG9 and JG7 demonstrated binding activity to *M. smegmatis* (ATCC® 19420™; data not shown) and *M. tuberculosis*

(ATCC® 25177™; presented as TB1 and TB2) at both early-log and mid-log phases, and enhanced MAB binding was evident at mid-log phase (Fig. 1). Subsequent ELISAs performed using mid-log phase bacteria at a starting concentration of 1800 – 2900 growth units (GU) as recorded by the BACTEC™ MGIT™ 960 instrument (approximately 1.0 x 10^7 CFU/mL –1.0 x 10^8 CFU/ mL) showed good binding activity at all three concentrations of MABs GG9 and JG7 (5, 10, and 25 μg/mL).

3.3. MAB binding activity to *M. tuberculosis* strains with different susceptibility profiles

Notably, MAB binding activity increased with an increase in MAB concentration, and optimal binding demonstrated at 25 μg/mL and at the 1:2 bacterial dilution (approximately 2 × 10^5 CFU/mL) (Fig. 2). Statistically significant differences in MAB binding activity were observed when comparing the highest MAB concentration of 25 μg/mL and the lowest concentration of 5 μg/mL ($p < 0.05$) for both GG9 and JG7, irrespective of the *M. tuberculosis* resistance profile (Table 1). However, differences in binding activities of MABs GG9 and JG7 at the low (5 μg/mL) and medium (10 μg/mL) concentrations were not statistically significant at serial bacterial dilutions (1:2, 1:4, and 1:8) with approximately 5 × 10^4 –2 × 10^5 CFU/mL.

MABs GG9 and JG7 bound strongly to live susceptible and resistant *M. tuberculosis* strains (XDR-TB and MDR-TB) and showed similar binding trends across different bacterial dilutions. MAB JG7 performed better than MAB GG9 in all binding experiments. Notably, the highest MAB binding activity was observed with the XDR-TB strains.

3.4. MAB binding activity to MOTT strains

MABs GG9 and JG7 bound non-selectively to both *M. tuberculosis* and MOTT species at varying degrees (Fig. 3); however, both MABs had stronger binding activity to *M. bovis* and *M. fortuitum* (Fig. 3**a & 3c**) and *M. avium* and *M. intracellulare* (Fig. 3**b & 3d**), in comparison to *M. tuberculosis,* regardless of MAB concentration or bacterial dilution. Similar to the MAB/clinical isolate's data, a higher quantity of antibody resulted in better binding for most species except *M. avium* and *M. fortuitum*.

4. Discussion

Our study evaluated the binding capacity of two novel IgG1 MABs to susceptible and resistant *M. tuberculosis* strains as well as ATCC laboratory strains of selected MOTT species. First, we determined the target bacterial growth phase for our ELISA experiments and observed better binding at mid-log phase compared to early log phase. This may be due to an increased abundance of free antigen available for the formation of an antigen–antibody complex [26]. This determination was important because with further optimization experiments, we could extrapolate this *in vitro* binding data and hypothesize the approximate timepoint to add these MABs during *in vivo* experiments as timing and delivery of therapeutic agents is exceptionally important [2].

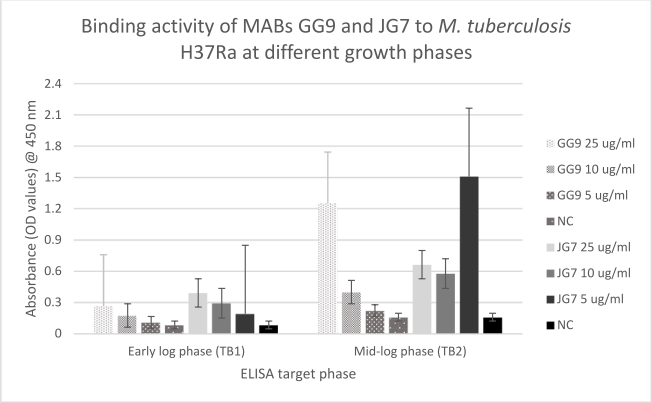

Fig. 1. Absorbance at 450 nm showing binding activity of MABs GG9 and JG7 at 5, 10, and 25 μg/mL to TB1 and TB2 at early log phase (day 1 of MGIT positivity; 1.0 × 10^6 CFU/mL) and mid log phase (day 3 of MGIT positivity; 1.0 × 10^8 CFU/mL), respectively. Bacterial suspension without MAB served as the negative control. MABs, monoclonal antibodies; MGIT, mycobacterial growth indicator tube; CFU/mL, colony-forming unit per milliliter.

MABs GG9 and JG7 bound to live *M. tuberculosis* strains regardless of the resistance profile (susceptible, MDR, and XDR). These results are consistent with, and confirm preliminary reports by Sei et al, (2019) who published data showing MABs GG9 and JG7 enhanced binding activity to both live and killed susceptible *M. tuberculosis* strains.

MAB binding activity increased with increasing MAB concentration. These results are consistent with other reports which show that higher antigen concentration promotes antibody-antigen complex formation [26]. Increasing the antibody concentration further increases the binding activity until an equilibrium is reached, and a balance between the bacteria (antigen) and the MABs must be obtained to reach an optimal reaction [26].

Of note, the highest MAB binding activity was observed with XDR strains followed by susceptible and MDR strains, in that order. This might partially be explained by a previous study which reported that the cell wall thickness increases as strains become more resistant [27]. Therefore, the thicker the cell wall, the higher the binding activity could be hypothesized. However, the anomaly of MDR-TB strains showing the lowest binding activity instead of the susceptible strains may be due to differences in ultra-structures [27] and warrants further investigation.

Importantly, our results indicate that IgG1 MABs GG9 and JG7 bind to *M. tuberculosis* strains with different resistance profiles. Furthermore, our data suggests increased binding activity of these MABs as the number of mutations in *M. tuberculosis* increases, i.e., better binding was observed with the resistant strains than with susceptible ones. Due to the lipid-rich cell wall of mycobacteria, drug resistance is a major challenge [12,28], but our findings indicate that potential adjunctive immunotherapeutic agents, such as our MABs, may overcome this challenge.

The incidence of MOTT disease and mortality is increasing globally, and this is a public health concern because these diseases are difficult to diagnose and treat. The most common MOTT infections in humans are caused by *M. avium* and *M intracellulare*, and to a lesser extent, *M. fortuitum* [28]. Therefore, we evaluated the binding activity of MABs GG9 and JG7 to a few selected clinically relevant MOTT species.

Our results indicate that GG9 and JG7 bound to all ATCC laboratory MOTT strains. These results align with a previous Mexican study which indicated non-selective binding of IgG2 MABs to different mycobacterial species [22]. The study also reported increased binding activity between IgG MABs and the epitope from the species against which the MABs had been produced and suggests shared antigens within the genus *Mycobacterium* [22]. Interestingly, saturation occurs only with *M. fortuitum* and *M. avium*, indicating that the other MOTT species may require more antibody reach saturation.

In addition, Alvarez and colleagues (2013) reported a similar phenomenon and observed relatively higher binding activity to *M. bovis* BCG in comparison to *M. tuberculosis*, although they investigated human secretory anti-TB IgA [21]. Similarly, we observed that *M. bovis* exhibited better binding than the *M. tuberculosis* H37Ra strain. This may be attributed to epitope configurations, but further investigations would be required to support this claim.

These mycobacterial species are known to have specific, conserved, unique cell envelope components as well as shared components, such as mycolic acids, arabinogalactan, and PGN [29]. Data from a study by Sei et al. (2019) indicated that the target of the MABs used in this study is most likely an epitope on PGN. PGN is a large polymer that makes up the rigid cell wall of bacteria, and in mycobacteria, it anchors a waxy, lipid-rich coat which has a major role in preventing drugs from entering the

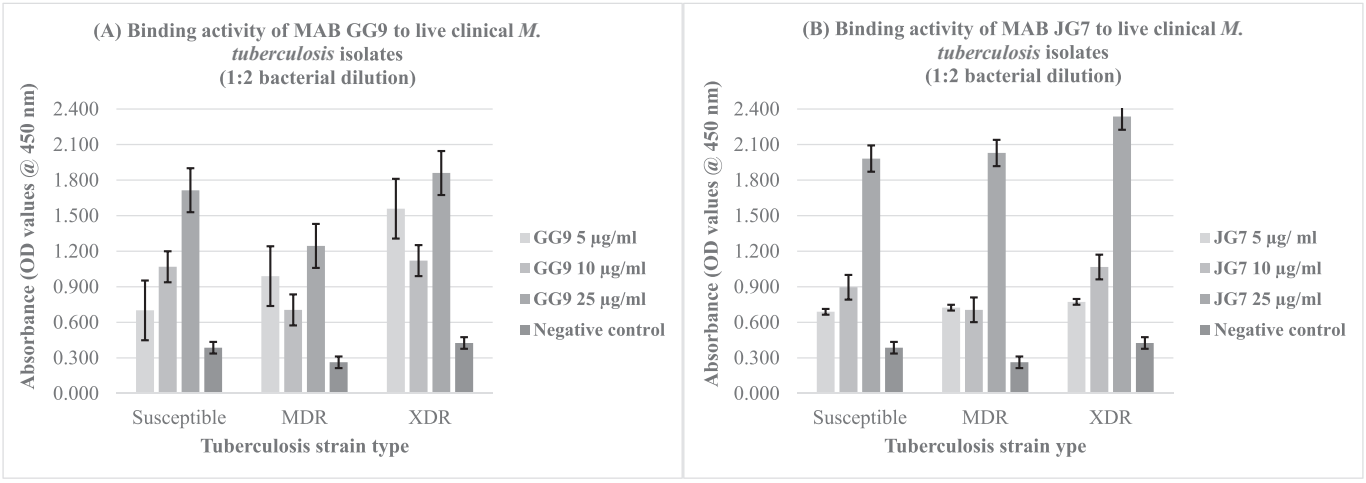

Fig. 2. Binding activity of (A) MABs GG9 and (B) JG7 at 5, 10, and 25 µg/mL to a 1:2 bacterial dilution (approximately 2×10^5 CFU/mL) of live clinical *M. tuberculosis* strains with different resistance profiles. MDR, multidrug resistant; XDR, extremely drug resistant. MABs, monoclonal antibodies; CFU/mL, colony-forming unit per milliliter.

Table 1
Comparison of binding activity of MABs GG9 and JG7 to different resistant groups. Statistical significance was set at $p < 0.05$.

MAB GG9						MAB JG7					
SUSCEPTIBLE	**P value**	**MDR**	**P value**	**XDR**	**P value**	**SUSCEPTIBLE**	**P value**	**MDR**	**P value**	**XDR**	**P value**
Overall	<0.001	Overall	0.012	Overall	0.054	Overall	<0.001	Overall	0.004	Overall	0.007
5 vs 10	0.056	5 vs 10	0.44	5 vs 10	0.65	5 vs 10	0.067	5 vs 10	0.49	5 vs 10	0.41
5 vs 25	<0.001	5 vs 25	0.023	5 vs 25	0.026	5 vs 25	<0.001	5 vs 25	0.001	5 vs 25	0.002
10 vs 25	0.011	10 vs 25	0.006	10 vs 25	0.067	10 vs 25	0.005	10 vs 25	0.010	10 vs 25	0.019

MAB, monoclonal antibody; MDR, multidrug resistant; XDR, extensively drug resistant.

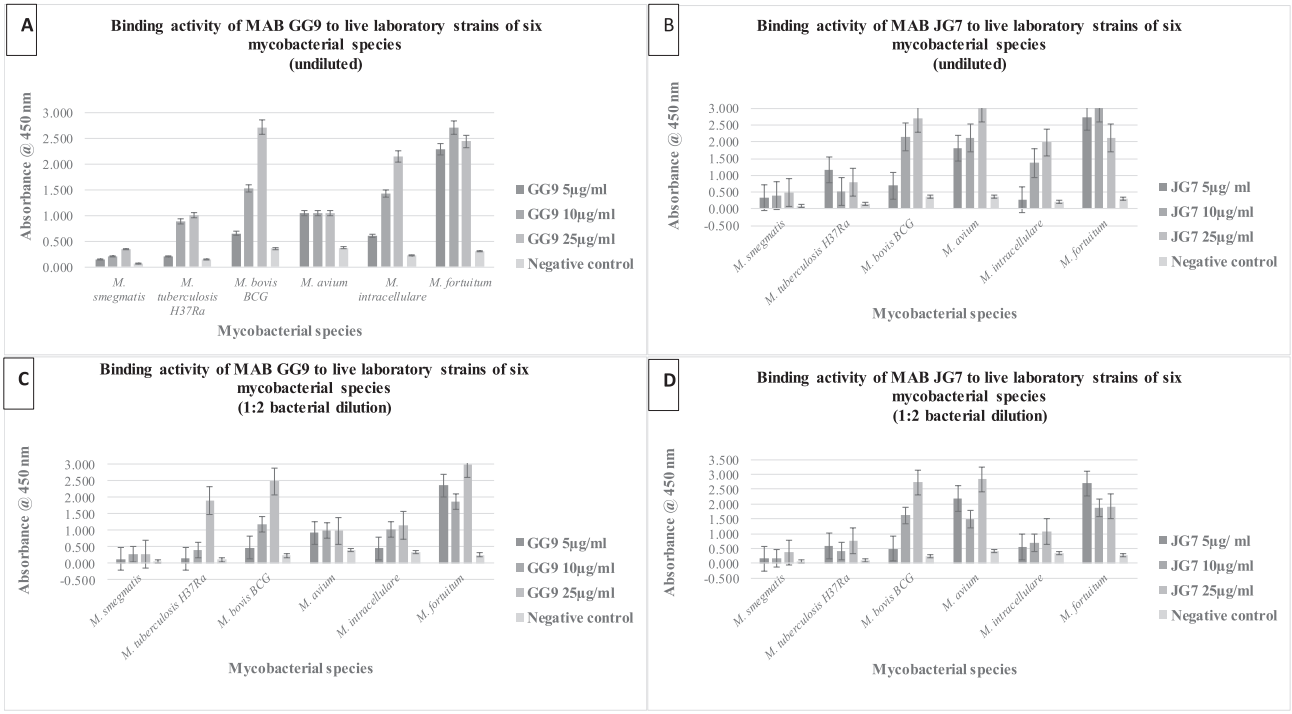

Fig. 3. Live ELISA showing binding activity of MABs GG9 (graphs A and C) and JG7 (graphs B and D) at concentrations 5, 10, and 25 µg/mL to *M. tuberculosis* (H37Ra; ATCC® 25177™), *M. smegmatis* (ATCC® 19420™; as a non-pathogenic control), *M. avium* subsp. *avium* (ATCC® 15769™), *M. bovis* (BCG; ATCC® 27290™), *M. fortuitum* subsp. *fortuitum* (ATCC® 6841™), and *M. intracellulare* (ATCC® 13950™) at bacterial dilutions of 4×10^5 CFU/mL (neat/undiluted) and 2×10^5 CFU/mL (1:2). ELISA, enzyme-linked immunosorbent assay; MABs, monoclonal antibodies; CFU/mL, colony-forming unit per millimeter.

bacilli [30], particularly β-lactams [31]. Thus, these anti-TB MABs could theoretically bind to different mycobacterial species due to the presence of PGN in mycobacteria; however, differences in binding affinity may occur due to stereochemical reactions and these variations can be manipulated for development of therapeutic agents [32]. Different cross linkages between PGN monomers may be investigated for further manipulation in the treatment of mycobacterial infections [32]. Therefore, the binding of MABs GG9 and JG7 to different mycobacterial species although to varying degrees is a promising result. This recognition of different mycobacterial species may suggest a potential role for MABs GG9 and JG7 as adjunct therapeutic agents with the aim to enhance bacterial killing.

The variance in binding activity may be attributable to the species-specific cell wall components. For example, glycopeptidolipids are unique to the *M. avium-M. intracellulare* complex [33] and phenolic glycolipids are present in *M. bovis* [33]. Another unique example is *M. fortuitum* with phosphoinositol caps that bind to lipoarabinomannan [33]. These variations could have influenced the MAB binding dynamics and specificity to cell wall components of mycobacteria [34]. It would be beneficial to perform mass spectrometry and bioinformatic analyses to elucidate the epitopes recognized by our MABs as done in a similar study [23]; this would also shed light on the degree of similarity of these epitopes and explain the correlation between this similarity and the binding activity observed, as well as expected results for similar *in vivo* experiments.

5. Conclusions

This study evaluated the binding capabilities of novel IgG1 MABs GG9 and JG7 to live resistant *M. tuberculosis* strains and clinically relevant MOTT species. Both MABs demonstrated binding activity across various live mycobacteria strains. One limitation of this study was that we assessed the binding activities of IgG1 MABs GG9 and JG7 to *M. tuberculosis* and MOTT isolates separately; therefore, it was difficult to extrapolate the data for *M. tuberculosis*/MOTT co-infections without an appropriate model. Despite this limitation, we successfully demonstrated that two novel IgG1 MABs, namely GG9 and JG7, bind to mycobacteria regardless of species or sub-type (resistance profile), and showed the highest binding profile to XDR-TB. Further investigations to assess whether these MABs, or other IgG1 MABs, may be useful as adjunctive therapeutic agents through enhanced bacterial killing with widespread use in the management/treatment of mycobacterium infections are warranted. Additionally, it would be beneficial to investigate whether GG9 and JG7 restrict the growth of MOTT in macrophages or whole blood as this points to a shortened treatment duration due to reduced bacterial loads if the MABs are used in conjunction with the readily available treatment for the MOTT species.

Ethical statement

Ethical approval was obtained from the Human Research Ethics Committee at the Faculty of Health Sciences, University of Pretoria (Ethics Reference Number: 483/2017) before the study commenced.

CRediT authorship contribution statement

Kudzai B. Nyazema: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **Bong-Akee Shey:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Clara J. Sei:** Formal analysis, Resources, Writing – review & editing. **Remco P.H. Peters:** Conceptualization, Formal analysis, Writing – review & editing. **Nontuthuko E. Maningi:** Supervision. **Gerald W. Fischer:** Resources, Writing – review & editing. **P. Bernard Fourie:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jctube.2024.100435.

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