GOVERNMENT DEGREE COLLEGE RAMPACHODAVARAM

DEPARTMENT OF ZOOLOGY

REPORT ON CSIR SUMMER RESERCH FELLOWSHIP PROGRAMME -2025

The Department of Zoology encouraged the students to participate in work shops, seminars, PG Entrance exams. M. Yedukondalu (III B.Sc B.Z.F.) applied for the **CSIR Summer Research Fellowship Programme 2025.** Principal Dr. K.Vasudha encouraged the students in all aspects. She gave her valuable suggestions to the student. Later the student was selected for two months summer research programme. Officials from CSIR Institute IGIB contacted the student through E-mail. M. Yedukondalu was selected and the confirmation mail was received. Then immediately principal and staff members were very happy and they congratulated the student and they sent the student to New Delhi for **2 months CSIR Summer Research Fellowship Programme**. M. Yedukondalu reported to the allotted Guide **Dr. Sheetal Gandotra Senior Principal Scientist and Infectious Disease Biology Unit (CSIR – IGIB New Delhi)**. Under the Guidance of Dr. Sheetal Gandotra he completed his project on **Using Macrophage Infection Models to Understand Host-Pathogen Interaction in Mycobacterial Infections.**

NAME OF SRF : M. Yedukondalu

REGISTRATION NO : LFS1831

INSTITUTION WHERE WORKING : CSIR – IGIB, NEW DELHI

DATE OF JOINING : 15/04/2025

DATE OF COMPLETION OF THE PROJECT : 15/06/2025

NAME OF THE GUIDE : Dr. SHEETAL GANDOTRA

PROJECT NAME : Using Macrophage Infection Models to Understand Host-Pathogen Interaction in Mycobacterial Infections.

सी.एस.आई.आर.-जीनोमिकी और समवेत जीवविज्ञान संस्थान
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Head, HR Division
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CSIR-IGIB/HR/T-GR/AL#1591 **Date: 15-04-2025**

Mr Yedukondlu Marmam

Sub: Trainee at CSIR-IGIB - Delhi

I am happy to inform you that your application for **Trainee** has been accepted and you will be supervised by

Dr. Sheetal Gandotra for the period from **15-Apr-25 to 15-Jun-25** Extension will be granted only in exceptional

cases.

The work carried out by the trainee shall belong to CSIR-IGIB only and CSIR-IGIB shall publish it, if so required. It is
mandatory to get signature on NDA (Non Disclosure Agreement) from your supervisor at CSIR-IGIB, prior to joining the institute for training.

At the time of joining, please report to your guide (contact details on website), fill the form attached and get it signed by supervisor and submit to HR at IGIB. Please note that change of guide is not permissible.

After completion of training, certificate will be issued upon submission of report for above mentioned duration.

A soft copy of the progress report along with your NDA (scan copy) is required to be submitted in HRD with clear mention of title of your report.

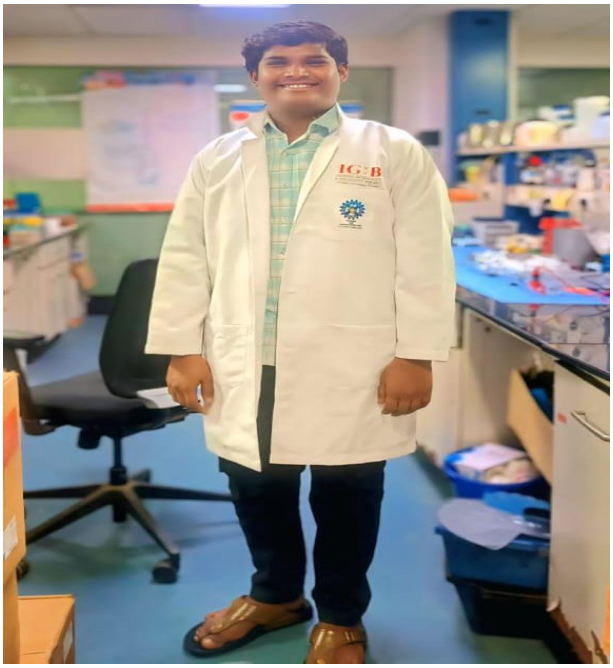
Note: . **Trainee fee is waived off.**

Sincerely yours,

Head, HR Division

This is computer generated letter, no signature required

PHOTOGRAPHS WHILE DOING WORK IN IGIB



PHOTOGRAPH WITH Ph.D SCHLOARS AND IGIB TEAM IN NEW DELHI

PROJECT REPORT

IAS-NAS-INSA

Summer Research Fellowship Programme-2025

USING MACROPHAGE INFECTION MODELS TO UNDERSTAND HOST-PATHOGEN INTERACTION IN MYCOBACTERIAL INFECTIONS

M. Yedukondalu
Government Degree College, Rampachodavaram
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ISO9001 : 2015, ISO14001 : 2015, ISO50001 : 2018, Certified Organization
Accredited by NAAC with "B" Grade

Under the guidance of:

Dr. Sheetal Gandotra
Senior Principal Scientist and
Infectious Disease Biology Unit
CSIR- Institute of Genomics and Integrative Biology
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Genomics Knowledge Partner

NAME OF SRF : M.Yedukondalu

REGISTRATION NO : LFS1831

INSTITUTE WHERE WORKING : CSIR-IGIB, NEW DELHI

DATE OF JOINING : 15/04/2025

DATE OF COMPLETION OF THE PROJECT : 15/06/2025

NAME OF THE GUIDE : Dr. SHEETAL GANDOTRA

PROJECT NAME : **Using Macrophage infection models to understand Host–pathogen interaction in Mycobacterial infection**

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जीनोमिकी और समवेत जीव विज्ञान संस्थान
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
विश्वविद्यालय परिसर, माल रोड, दिल्ली–११०००७
Institute of Genomics & Integrative Biology
(COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH)
DELHI UNIVERSITY CAMPUS
MALL ROAD, DELHI-110007, INDIA

CERTIFICATE

This is to certify that **M. Yedukondalu** bearing Registration No. **LFS1831** has completed a research internship under the supervision of **Dr. Sheetal Gandotra**, Senior Principal Scientist, at the **Infectious Disease Biology Unit**, **CSIR-Institute of Genomics and Integrative Biology (CSIR-IGIB)**. The internship was carried out as part of the project entitled: **"Using macrophage infection models to understand host-pathogen interaction in mycobacterial infections."** During the tenure, the student actively participated in experimental work, data analysis, and discussions relevant to the project and demonstrated scientific rigor and enthusiasm. The work exhibited a commendable dedication, curiosity, and collaborative spirit.

Duration: April 15th, 2025 –June 15th, 2025.
Place: New Delhi
Date:

Dr. Sheetal Gandotra
Senior Principal Scientist
CSIR-IGIB

(Official Seal/Signature)

DECLARATION

I, M.Yedukondalu, declare that the work presented in this report entitled: "Using macrophage infection models to understand host-pathogen interaction in mycobacterial infections. I have carried it out as part of my research internship under the supervision of **Dr. Sheetal Gandotra, Senior Principal Scientist and Infectious Disease Biology Unit, CSIR-Institute of Genomics and Integrative Biology (CSIR-IGIB).** I further declare that this work is original and has not been submitted elsewhere for any degree, diploma, or fellowship award. All sources of information have been duly acknowledged.

Place: Rampachodavaram. M.Yedukondalu.

Date : Signature of the Student

ACKNOWLEDGMENT

The journey of writing this Report has been my first profound experience with scientific research. While I have strived to put forth my best efforts, I am deeply humbled and immensely grateful for the invaluable guidance, support, and encouragement I have received from so many incredible individuals. Their generosity, patience, and belief in me have played a crucial role in the successful completion of this work.

I would like to express my profound gratitude to **Dr. Sheetal Gandotra**, Senior Principal Scientist and Infectious Disease Biology Unit , CSIR-Institute of Genomics and Integrative Biology (CSIR-IGIB), for her exceptional mentorship, invaluable guidance, and constant support throughout this research. Her scientific insight and encouragement have played a pivotal role in shaping this work.

I extend my sincere gratitude to **Ms. Muskan Goel** for her dedicated supervision, patience, and expert guidance, which significantly contributed to my technical and conceptual development during this project.

I am also thankful to the members of the laboratory—**Neetu, Vaishnavi, Priya, Manya, Anwesha, Milin, Arpita, Rakesh, Sachin, Rabia, Adithya**, **Amit, and Ritika** for their assistance, collaboration, and for fostering a positive and intellectually stimulating research environment.

I gratefully acknowledge the **CSIR-Institute of Genomics and Integrative Biology (CSIR-IGIB)** for providing the necessary infrastructure, resources, and institutional support essential for the successful completion of this work.

Lastly, I am deeply indebted to my **family and friends** for their unwavering support, understanding, and encouragement throughout this journey. Their presence has been a constant source of strength and motivation.

With immense gratitude and respect,

M.Yedukondalu.

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Key words:

Mycobacterium tuberculosis

M. marinum

THP – 1 cell line

Monocytes

Macrophages

RPMI-1640 - Roswell Park Memorial Institute 1640

Tween 80

Amikacin

SYTOX Green

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease that mainly affects the lungs, termed as pulmonary tuberculosis, but can also affect other organs like the skin, lymph nodes, heart, spine, Central Nervous System, etc, termed as extra-pulmonary tuberculosis. TB remains one of the most serious health threats worldwide, with 10.8 million active cases and 1.15 million deaths in 2023 due to TB. According to the Global TB Report 2024 by WHO, TB has become the leading cause of death from a single infectious agent, replacing COVID-19 worldwide (Global TB Report 2024, WHO). The causative agent of tuberculosis is *Mycobacterium tuberculosis* (*Mtb*). When an actively infected person coughs, sneezes, or talks, the water droplets containing the bacteria are released into the environment. These droplets can enter any healthy person's body through the nose or mouth and can reach the alveoli. Alveolar macrophages engulf the bacteria and simultaneously secrete cytokines to recruit other immune cells like neutrophils, dendritic cells, natural killer (NK) cells, T lymphocytes, and B lymphocytes. All these cells aggregate to form a solid structure called a granuloma, which is the hallmark characteristic of TB. As the disease progresses, the granuloma ruptures and thousands of bacteria are released into the airway for further transmission (Rahlwes *et. al.,* 2023).

Studying host–pathogen interactions is essential for developing effective therapeutic and preventive strategies. In humans, immune cells, particularly macrophages, not only serve as the first line of defence but also represent the primary intracellular niche exploited by mycobacteria. In recent years, in vitro macrophage infection models have become essential for investigating the molecular and cellular dynamics of mycobacterial infections. Cell lines such as THP-1, RAW 264.7, or primary macrophages provide controlled systems to study bacterial entry, survival, replication, and host responses.

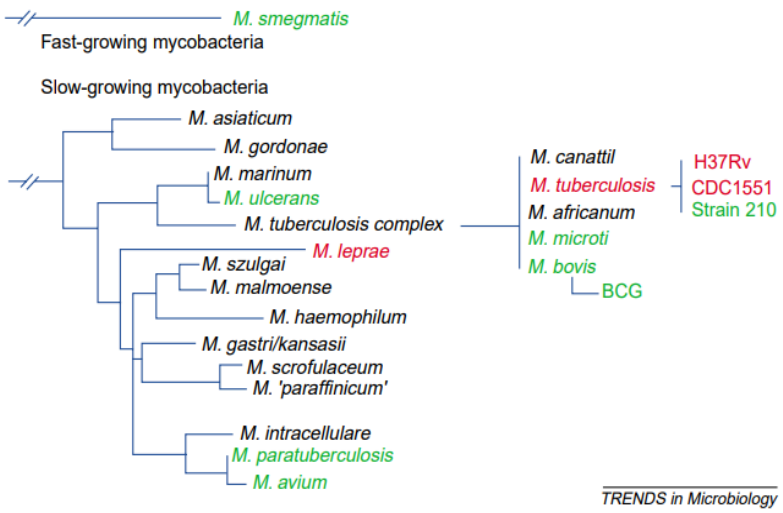

Figure 1: Evolution of Mycobacterial pathogenicity (Brosch *et. al.,* 2001)

MYCOBACTERIA

Mycobacterium tuberculosis belongs to the genus *Mycobacterium,* characterized by a thick, lipid-rich cell wall containing mycolic acids, contributing to their resistance. Over 190 species have been identified, most of which are free-living and inhabit diverse niches. Slow-growing species include *M. asiaticum*, *M. gordonae*, *M. marinum*, and *M. tuberculosis*, while *M. smegmatis* is a fast-growing species (J.B.Payeur, 2014, Encyclopedia of food microbiology). *M. marinum* and *Mtb* have more than 99.3% genome similarity and share major virulence factors. *M. marinum* has a faster doubling time (6-8 hours) as compared to *Mtb,* which has a doubling time of 24-30 hours. Where a BSL3 facility and special training are needed to handle *M. tuberculosis*, *M. marinum can* be handled at a BSL2 safety level. *M marinum* is a non-tuberculous mycobacterium. It causes a tuberculosis-like illness in fish. In humans, when injured skin is exposed to an aqueous environment contaminated with *M. marinum*, infection occurs (Chen *et. al.,* 2017; Brosch *et. al.,* 2001).

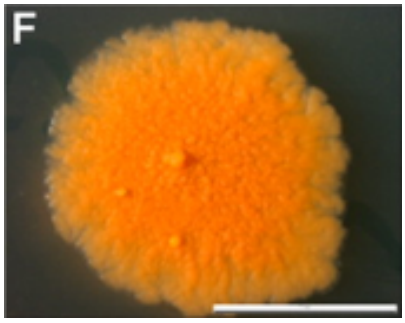

Figure 2: *Mycobacterium marinum* (Julian *et. al.,* 2010)

THP-1 CELLS AS A MODEL OF INFECTION:

Monocytes, derived from myeloid progenitors during haematopoiesis, are the largest type of white blood cells and differentiate into macrophages upon entering tissues. Macrophages, including resting M0, pro-inflammatory M1, and anti-inflammatory M2 subsets, play essential roles in immune defence, tissue monitoring, and homeostasis. M0 macrophages express markers like CD14 and CD68 and polarize into M1 or M2 in response to cytokines. M1 macrophages, activated by IFN-γ and LPS, produce pro-inflammatory cytokines such as TNF-α and IL-1β, while M2 macrophages, induced by IL-4, IL-10, or IL-13, promote tissue repair and secrete IL-10 and TGF-β, expressing CD163 and CD206 (Clare Brown, 2020, macrophages in microbiology).

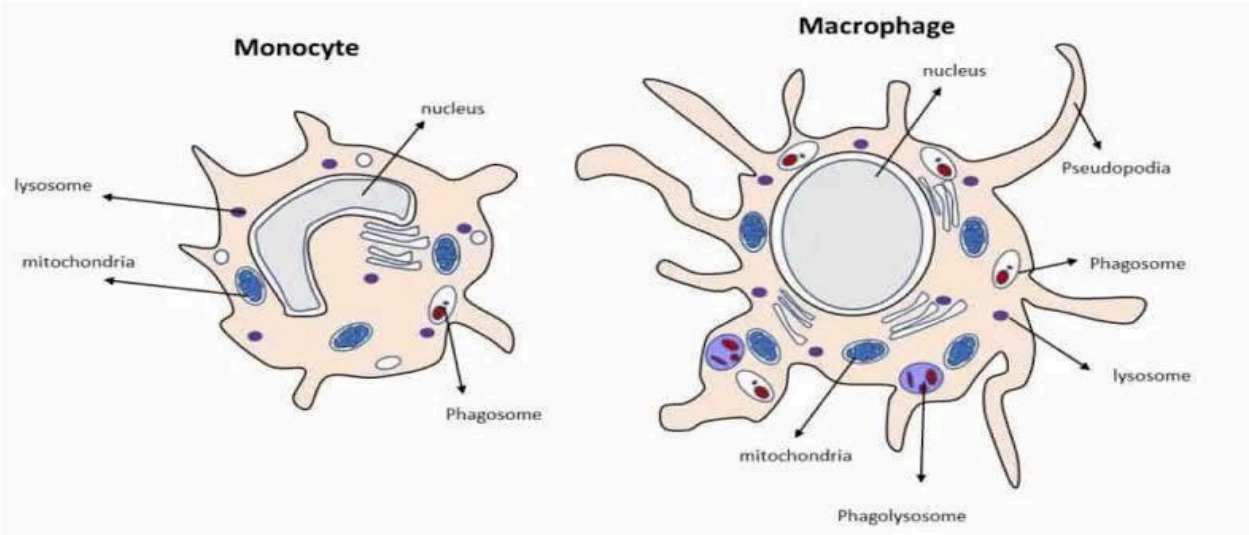

Figure 3: Showing the Monocytic stage and Macrophage stage (Clare Brown, 2020)

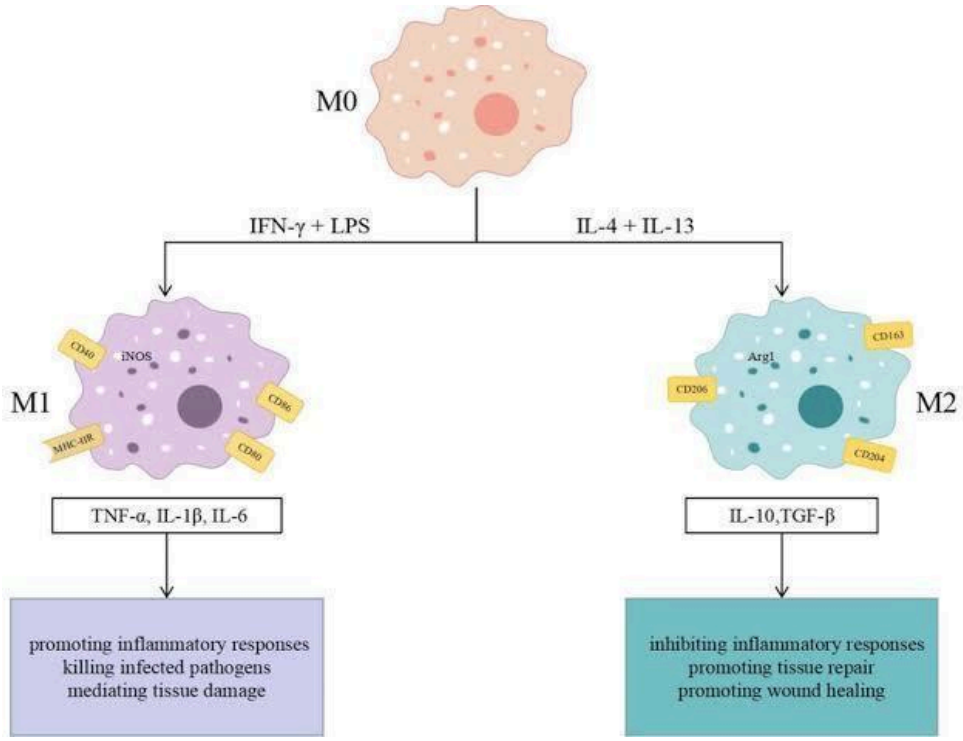

Figure-4 : Differentiation of M0 macrophage into M1 and M2 Macrophages (Xia., et al, 2023)

The human leukemic cell line THP-1 is widely used as an in vitro model to study host–pathogen interactions. Established in 1980, THP-1 cells were derived from the peripheral blood of a patient with acute monocytic leukaemia. THP-1 cells can be differentiated into macrophage-like cells using phorbol 12-myristate 13-acetate (PMA), with optimal differentiation typically achieved after 48 hours of incubation at minimal PMA concentrations. Following differentiation, a 24-hour resting period in PMA-free media is recommended to enhance macrophage marker expression. Differentiated THP-1 macrophages exhibit increased adherence, phagocytic capacity, and expression of surface markers such as CD14, CD36, and TLR-2 (Xia et al., 2023).

Aim of the study:

Establishing a Macrophage model system to perform various screens to investigate host-pathogen interactions during mycobacterial infection.

Objectives:

1) Generation of Teal and Blue fluorescent protein expressing *M.marinum* cells.
   a) Isolation of pTEC17 (mTFP1) and pTEC18 (EBFP2) plasmids using the Qiagen Mini Prep kit.
   b) Preparation of *M. marinum* electrocompetent cells.
   c) Electroporation of pTEC17 and pTEC18 plasmids in *M. marinum* electrocompetent cells.
2) Growth curve analysis of Teal and Blue fluorescent protein expressing *M. marinum.*

Materials Required: Glycerol stocks of pTEC17, pTEC18 plasmids, and *M. marinum* (Wild type).

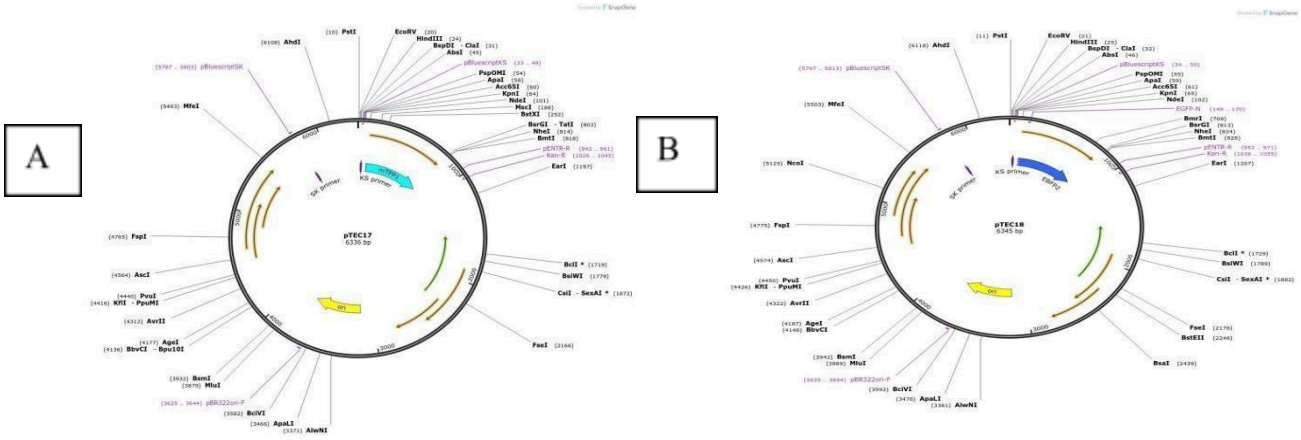

Figure 5: Plasmid map of pTEC17 plasmid (A) and pTEC18 (B)

Methodology:

1. **Plasmids, Bacterial Strains, and Culture**

Mycobacterium marinum (WT) was grown in Middlebrook 7H9 broth (BD Biosciences, USA) supplemented with 0.5% glycerol and 0.05% TWEEN 80. 7H10 agar media supplemented with 0.5% glycerol and 10% OADC was used for growing mycobacterial culture on solid agar plates. The media was supplemented with hygromycin (50µg/ml) wherever required. *E. coli* strains were cultured in LB broth (Himedia). The media was supplemented with hygromycin (150 µg/ml).

2. **Isolation of plasmid DNA**

Steps:

i. Pelleted down 10 ml bacterial culture overnight by centrifugation at 3500 rpm for 15 mins at 4°C, and the supernatant was discarded.
ii. Resuspend pelleted bacterial cells in 300µl buffer P1 (Resuspension Buffer) and transfer to a microcentrifuge tube.
iii. Add 300µl Buffer P2 (Lysis Buffer) and mix thoroughly by inverting the tube 4 to 6 times until the solution becomes clear. (Do not allow the lysis reaction to proceed more than 2 – 3 times)
iv. Add 400µlBuffer N3 (Neutralization Buffer) and mix immediately and thoroughly by inverting the tube 4-6 times.
v. And we centrifuged for 10 min at 13000 rpm (~ 17900 × g) in a tabletop microcentrifuge at RT.
vi. Applied 800 µl of supernatant from Step 5 to the Qia prep 2.0 spin column by pipetting and centrifuging for 60 seconds and discarding the flow through, this was done for 2 times.
vii. **Recommend:** Washed the Qiaprep 2.0 spin column by adding 500µlBuffer PB, centrifuged for 60s, and discarded the flow through, this was done 2 times.
viii. **Reason:** This step is only required when using end A + strains or other bacterial strains with high nuclease activity (or carbohydrate content).
ix. Washed the Qia prep column with diluted PE Buffer 1: 4 (100% ethanol) and centrifuged at 1300 rpm for 2 minutes at Room temperature, and centrifuged for 2 minutes to remove residual wash Buffer.

x. Placed the Qia prep 2.0 column in a clean 1.5 ml microcentrifuge tube.
xi. Finally, DNA was eluted by adding 40µl warm NFW to the centre of the Qia prep 2.0 column, after incubating the column for 5 minutes and then centrifuging for 1 minute at 13000 rpm, at RT.
xii. Eluted DNA was stored at −20°C.

3. Preparation of Electro-competent cells of *Mycobacterium marinum* (Sahar H El-Etr *et. al.,* 2004)

i. A 50 mL culture of *Mycobacterium marinum* at an optical density (OD_{600}) of approximately 0.4–0.8 was harvested.
ii. Before preparing electrocompetent cells, both the 50 mL culture and the 10% glycerol solution were chilled on ice for 20 minutes.
iii. The culture was centrifuged at 3,440 rpm for 20 minutes at 4°C using a swinging-bucket rotor.
iv. The supernatant was carefully decanted, and the cell pellet was resuspended in 5 mL of ice-cold 10% glycerol. The final volume was adjusted to 40 mL with 10% glycerol.
v. The cell suspension was washed twice with ice-cold 10% glycerol by centrifugation at 3,440 rpm for 20 minutes at 4°C.
vi. Aliquots of 400 µL electrocompetent cells were prepared on ice and afterwards stored in -80°C.

4. Electroporation of plasmid DNA into competent cells (Sahar H El-Etr *et. al.,* 2004)

i. For Electroporation, 400 ng of plasmid DNA were added to respective microcentrifuge tubes containing 400 µL of electrocompetent *M. marinum* cells.
ii. Electroporation was performed using 2 mm gap cuvettes with the following parameters: 2.5 kV, 1000 Ω resistance, and 25 µF capacitance on Bio-Rad Gene Pulser Xcell.
iii. The cells were then transferred to 1 mL of media and incubated for 5 hours at 30°C in a shaking incubator to revive.
iv. Next, it was centrifuged at 3000 rpm for 5 minutes at RT.
v. Pellet was formed, and the supernatant was discarded. Pellet was resuspended with the remaining media and then plated on antibiotic plates.

5. Growth Curve Analysis of *M.marinum* cells having pTEC17 and pTEC18 plasmids.

i. **Initial Inoculation:**
   - Two colonies each from pTEC17-Neat and pTEC18-Neat plates were inoculated in 7H9 broth supplemented with Hygromycin B at a final concentration of 50 µg/mL.

ii. **Subculturing:**
   - Subcultures were prepared when the optical density (OD600) of the parent culture reached approximately 0.5–1.0.
   - The subcultures were inoculated at an initial OD600 of 0.05 using the parent culture.

iii. **Growth Conditions:**
   - Each subculture was grown in 10 mL of 7H9 broth containing 10 µL of Hygromycin (50 µg/mL stock).
   - Cultures were incubated at 30°C with shaking at 180 rpm**.**

iv. **Time Point Measurement:**
   - The start of incubation was marked as 0 hours.
   - OD600 measurements were taken at 5-hour intervals thereafter.

v. **Sample Preparation for OD600 Measurement:**
   - At each time point, 500 µL of culture was mixed with 500 µL of sterile 7H9 broth to prepare a 1:1 dilution.
   - A blank cuvette was prepared using 1 mL of plain 7H9 broth to calibrate the spectrophotometer (Eppendorf) at 600 nm.
   - The 1 mL diluted sample was then transferred into a clean cuvette for OD600 measurement.

vi. **OD Calculation:**
   - The observed OD600 value of the diluted sample was multiplied by 2 to obtain the actual OD600 of the original culture.

vii. **Data Recording:**
   - Both raw (diluted) and corrected (actual) OD600 values were recorded at each time point.

6. Single Cell Suspension (SCS) of Bacterial Culture for Infection;

A. Subculture of Mycobacteria:

- On the day THP-1 cells are seeded, subculture *Mycobacterium marinum* (pTEC17 and pTEC18) into fresh 7H9 media at a 1:7 dilution ratio.
- Incubate at 30°C, shaking at 180 rpm for 4 days.

B. OD Measurement:

- After 4 days, measure the optical density (OD600) using a spectrophotometer.
- Use sterile 7H9 media as the blank.
- Acceptable OD600 range for infection: 0.5–1.0

C. Harvesting Bacteria:

- Centrifuge the cultures at 3500 rpm for 4 minutes at room temperature (RT).
- Discard the supernatant.
  Note: This high-speed spin helps remove dead bacteria and pellet down viable cells.

D. PBST Washes (De-clumping and Cleaning):

- Wash 1:
  - Resuspend the bacterial pellet in PBST (0.05% Tween-80 in PBS) using the same volume as the original culture (e.g., 10 mL).
  - Mix thoroughly to break clumps.
- Centrifuge at 3500 rpm for 4 minutes at RT and discard the supernatant.
- Wash 2:
  - Resuspend the pellet again in 10 mL PBST and mix well.
  - Centrifuge at 3500 rpm for 4 minutes at RT and discard the supernatant.
- Wash 3:
  - Repeat the wash with 10 mL PBST.
  - Centrifuge at 3500 rpm for 4 minutes at RT and discard the supernatant.

Note: The second and third washes are critical for breaking bacterial clumps due to the action of Tween-80.

1 OD = 500 million bacteria

E. PBS Wash for SCS Preparation:

- Resuspend the pellet in 5 mL of tissue culture-grade PBS.
- Centrifuge at 800 rpm for 10 minutes at RT.
  Note: The low-speed spin helps remove remaining clumps while retaining a single-cell bacterial suspension in the supernatant.
- Carefully collect the supernatant, which contains the Single Cell Suspension (SCS) of *M. marinum*.

Amikacin Treatment of Bacterial Culture (Pre-infection):

- To prevent extracellular growth and maintain MOI (Multiplicity of Infection), treat the SCS with Amikacin.
- Use:
  - **Stock concentration:** 10 mg/mL
  - **Working concentration:** 40 µg/mL
- Add an appropriate volume of Amikacin to the bacterial SCS and incubate before infection.

Infection of THP-1 Macrophages:

A. Infection Step:

- Remove the existing media from THP-1 macrophage wells.
- Add the prepared infection media containing bacteria to each well.
- Incubate at 37°C for 3 hours to allow infection.

B. Post-Infection Amikacin Treatment:

- After 3 hours of incubation, remove the infection media from the wells.
- Add Amikacin-containing media at 40 µg/mL to each well to kill extracellular bacteria.
- Incubate at 37°C for 1 hour.

C. Final Wash:

- Remove the Amikacin media after 1 hour.
- Replace with fresh complete RPMI media for further incubation or analysis.

Result and Discussion:

1) THP-1 cells were seeded in 48-well tissue culture-treated plates following the standard protocol. The optical density (OD600) of the mycobacterial culture was measured at 0.558, and the corresponding single-cell suspension (SCS) had an OD600 of 0.156, equivalent to approximately 78 million bacteria per mL.

   To achieve the desired multiplicity of infection (MOI), a total of 7.8 million bacteria were required per well. Accordingly, 100 µL of the SCS was calculated and used per well. The SCS volume was mixed with complete RPMI media such that the final volume in each well supported proper infection while maintaining the intended MOI.

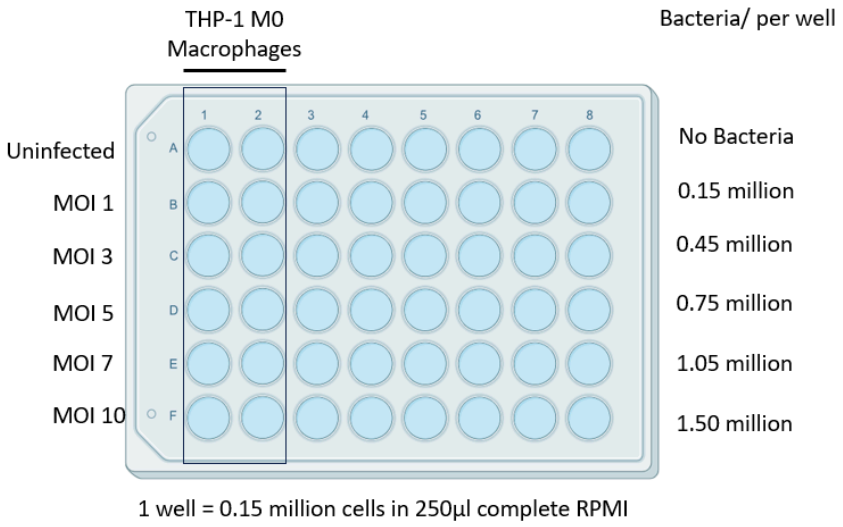

Fig: Plate layout showing THP-1 macrophages infected with *Mycobacterium marinum* at varying MOIs (1–10).

After completion of the Amikacin treatment, the THP-1 macrophages were replenished with fresh complete RPMI (cRPMI) supplemented with SYTOX Green, a fluorescent dye that selectively stains dead cells.

Fluorescence images were captured at 0 hours (immediately after Amikacin treatment was stopped) and again at 5 hours post-treatment. These time points were selected to assess the extent of infection and cell viability across different MOIs,

allowing visualization of the MOI at which the majority of cells remained infected yet viable.

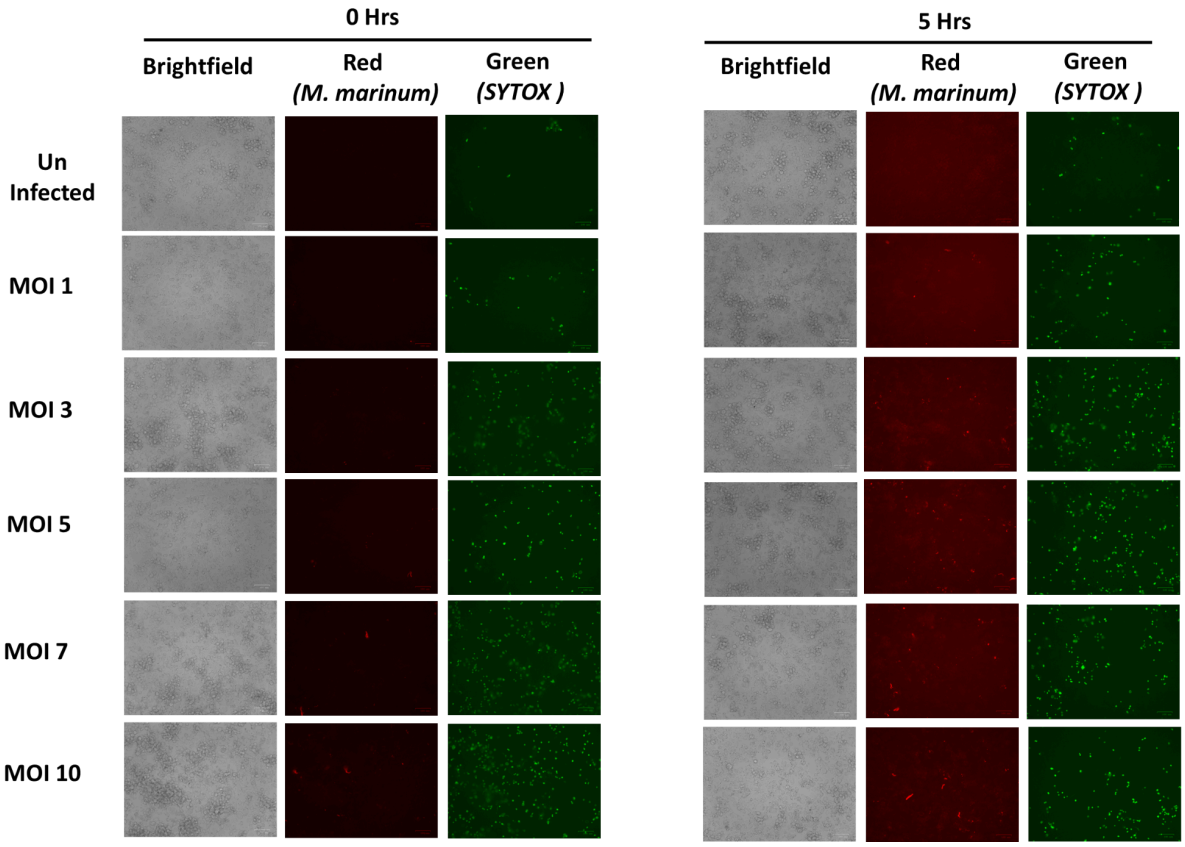

Fig: Zoe images of THP-1 Macrophages infected with *M. marinum* (red) at different MOI and stained with SYTOX Green (Green) at time 0 hrs and 5 hrs post Amikacin treatment.

mCherry Channel (Red Fluorescence): Red fluorescence intensity increases proportionally with MOI, confirming that higher MOIs correspond to a higher number of intracellular bacteria per cell.

SYTOX Green Channel (Green Fluorescence):

- At MOI 1–5, most cells remain SYTOX-negative (non-fluorescent), indicating that the majority of infected cells are viable and healthy at both 0 and 5 hours.
- At MOIs 7 and 10, there is a marked increase in SYTOX Green fluorescence by 5 hours, suggesting significant cell death at these higher MOIs.

- The increase in dead cells over time at high MOIs implies MOI-dependent cytotoxicity.

Phase Contrast Images: No major changes in overall cell morphology are observed between time points at lower MOIs, but higher MOIs (particularly MOI 10) show some signs of cellular disruption and reduced cell density by 5 hours.

2) Previously isolated pTEC17 and pTEC18 plasmids were quantified using Nanodrop, and size was determined by running a 0.7% agarose gel.

Nanodrop reading:

Plasmid	ng/µl	A260/280
pTEC17	125ng/µl	1.91
pTEC18	90 ng/µl	1.92

The extracted DNA was analysed on a gel, and 1 volume of orange G was added to 5 volumes of purified DNA. We mixed the solution by pipetting up and down before loading the gel.

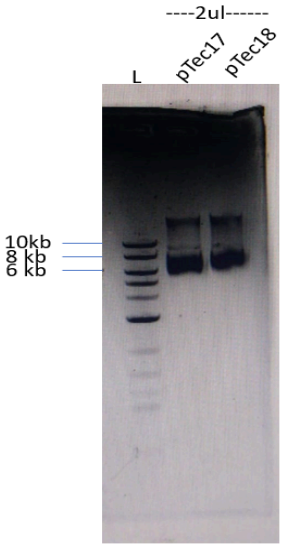

Figure 6: 0.7% Agarose gel picture of pTEC17 and pTEC18 plasmids

During Electroporation, the Time constant (ms) and Voltage values were as follows:

Sample	TC (ms)	Voltage
No DNA control	21.6	2482
pTEC17 (400ng)	21.0	2482
pTEC18 (400ng)	21.3	2483

A no-DNA control was also included. Post-electroporation recovery was carried out in Middlebrook 7H9 broth for 5 hours at 30°C with shaking. We plated neat and 1/10 dilution of pTEC17 and pTEC18 on 7H10 Hyg50 plates, also no DNA control on 7H10 and 7H10 Hyg50 plates. Then we keep these plates at 30° C. After 6 days, we get the transformation on the plates. We get the lawn on 7H10 plate (No DNA control) and no Colonies on 7H10 Hyg50 (No DNA control).

The following table shows the Number of colony counts:

Sample	Plate type	Colonies count
No DNA control	7H10	Lawn
No DNA control	7H10 Hyg50	00
pTEC17 (neat)	7H10 Hyg50	28
pTEC18 (neat)	7H10 Hyg50	32
pTEC17 (1/10 dilution)	7H10 Hyg50	08
pTEC18 (1/10 dilution)	7H10 Hyg50	02

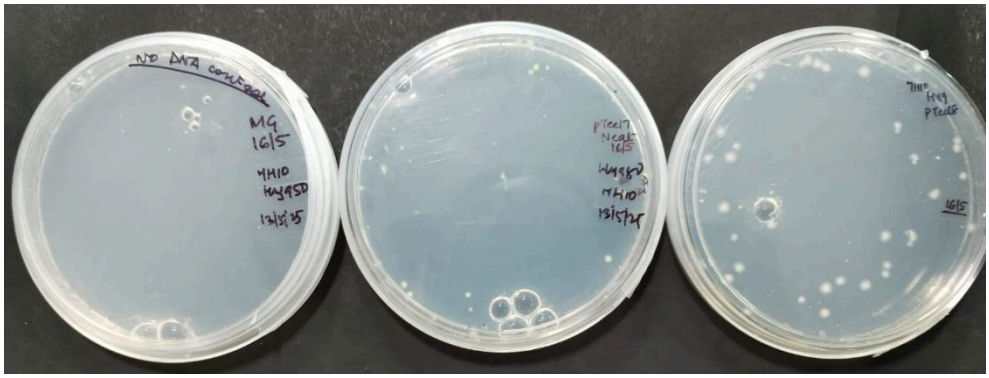

Fig 7: Images of 7H10 hyg plates having Transformants A) No DNA Control, B) *M. marinum* (pTEC17 -mTFP1), and C) *M. marinum* (pTEC18 -EBFP2)

pTEC17 harbouring *M. marinum* cells appear to be neon green in colour, whereas pTEC18 is white.

3) Growth curve of *Mycobacterium marinum:*

The growth curve of *M. marinum* includes four phases: **lag, log, stationary**, and **death**. In the **lag phase**, *M. marinum* adapts to the environment with minimal cell division. During the **log (exponential) phase**, it divides actively with a generation time of approximately **4-8 hours** under optimal conditions (25–35°C). In this phase, population doubling is consistent. In the **stationary phase**, nutrient limitation and waste accumulation slow growth, balancing cell division and death. Eventually, in the **death phase**, cell death exceeds division due to unfavorable conditions.

Time points and OD at 600nm were as follows:

Sl No	Time Point (hrs)	OD of pTEC17 *M.marinum*	OD of pTEC18 *M.marinum*
1	0	0.006	0.03
2	12	0.112	0.086
3	17	0.132	0.092
4	22	0.158	0.148
5	36	0.35	0274
6	41	0.314	0.316
7	46	0.434	0.378
8	60	0.596	0.626
9	65	0.726	0.73
10	70	0.772	0.8
11	84	0.906	1.108
12	89	1.08	0.996
13	94	1.026	1.018
14	99	1.172	1.16

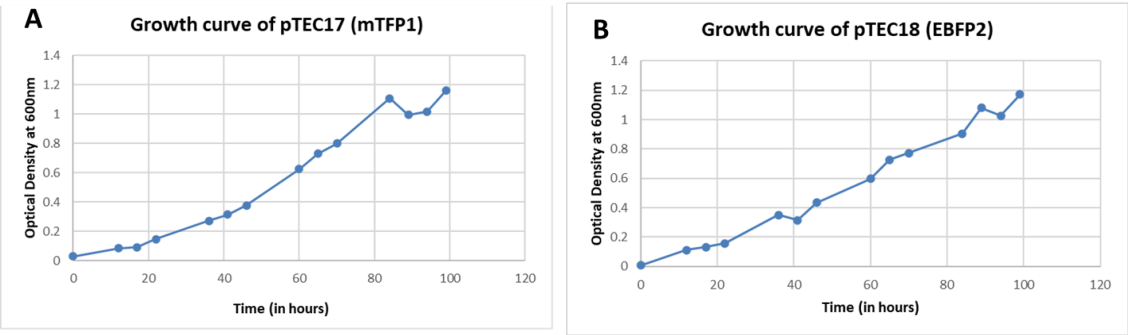

Fig 8: Growth curves of A) *M. marinum* (pTEC17 -mTFP1), and B) *M. marinum* (pTEC18 -EBFP2)

Doubling time is the time required for a bacterial population to double in number during the **exponential (log) phase** of growth. It is a key parameter that reflects how quickly a bacterial culture is growing under given conditions.

Doubling Time: (Time2-Time1)/log2(B/A)

OD at Time 2 - B, and Time 1 - A

Type of Plasmid	Time2-Time1	B/A	Log2(B/A)	Doubling Time
pTEC18	14	2.215189873	1.14743036365523	12.20117616
pTEC17	14	1.851351351	0.888578717331577	15.75549777

Doubling time was found to be 12 hours and 15 hours for *M. marinum* harbouring pTEC18 and pTEC17 plasmids, respectively.

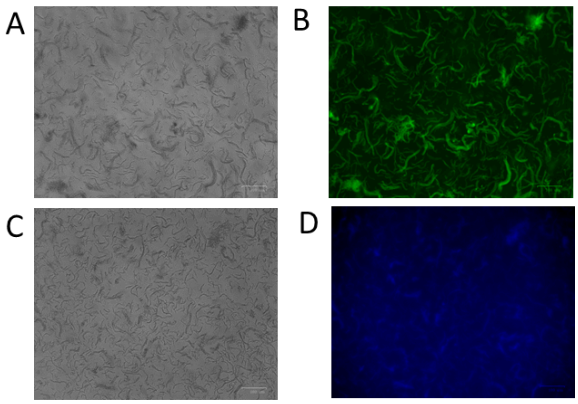

Figure: Zoe-images of *M. marinum* expressing Teal fluorescent (A-B) and blue fluorescent protein (C-D)

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