RANDOM MUTAGENESIS FOR IDENTIFICATION OF GENE(S) RESPONSIBLE FOR BIOFILM FORMATION IN *Mycobacterium fortuitum*

Dissertation submitted in partial fulfillment of the requirement for the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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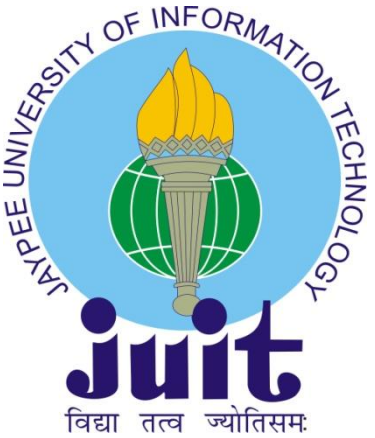
UNDER THE GUIDANCE OF

DR. RAHUL SHRIVASTAVA



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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the B. Tech. thesis entitled **"Random Mutagenesis for Identification of Gene(s) responsible for Biofilm formation in *Mycobacterium fortuitum*"** submitted at **Jaypee University of Information Technology, Waknaghat, India**, is an authentic record of my work carried out under the supervision of **Dr. Rahul Shrivastava** (Associate Professor) Department of Biotechnology and Bioinformatics. I have not submitted this work elsewhere for any other degree or diploma.

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Date:

SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the B. Tech. thesis entitled **"Random Mutagenesis for Identification of Gene(s) responsible for Biofilm formation in *Mycobacterium fortuitum*",** submitted by **Rahul Pramjeet (141808) at Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of his original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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Rahul Pramjeet (141808)

LIST OF SYMBOLS AND ACRONYMS

AFM	Atomic Force Microscopy
ALS	Alkaline Lysis Buffer
°C	Degree Celsius
ECM	Extra Cellular Matrix
EPS	Extracellular Polymeric Substances
GTE	Glucose Tris EDTA
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani Broth
LBGT	Luria Bertani Broth Glycerol Tween
µF	Micro-farad
µl	Micro-liter
µg/ml	Microgram per Milliliter
mg/ml	Milligram per Milliliter
Nm	Nanometer

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ABSTRACT

Mycobacterium fortuitum is rapid growing non tuberculous mycobacteria which is present ubiquitously in our environment. The bacteria have become a pathogen of interest because of increasing cases of human infections caused by them. They are reported to cause opportunistic infections like ocular infections, surgical infections and healthcare associated disseminated infections. The increase in incidence of nosocomial infections can be related to the ability of the bacteria to form biofilms which allow it to survive in adverse condition while being attached to biotic or abiotic surfaces. Biofilm also provides resistance against currently prescribed drugs, thus obstructing its treatment. Biofilm plays a very important role in the pathogenesis of *M. fortuitum* and thus, the present study aims at identifying genes responsible for biofilm formation.

It has been reported by various studies that membrane proteins are involved in formation of biofilm. So, random mutagenesis of wild type *M. fortuitum* (ATCC 6841) was done using the transposon TnphoA having kanamycin marker and alkaline phosphatase gene. *M. fortuitum* mutants having disrupted membrane gene were screened on NAT having kanamycin and X-gal. Blue coloured colonies were formed by mutants that have transposon inserted in the membrane genes and these mutants were stored to replenish the library.

10 mutants from the borrowed library were screened for biofilm formation using crystal violet assay and carbol fuchsin staining using two set of protocols. In the first protocol, biofilm formation is assayed in intervals for 28 days and in the second protocol biofilm formation is assayed after 4 days. Both of the protocol yielded the same result and out of all mutants, 3 mutants i.e. M02, M05 and M08 showed attenuated biofilm formation. The disrupted gene in these mutants can be identified by DNA sequencing followed by bioinformatics analysis. Identified gene can be used as a potential drug target or for other novel intervention strategies to treat infections caused by *M. fortuitum*.

CHAPTER 1

INTRODUCTION

Non tuberculous mycobacteria (NTM) are a group of opportunistic, environmental infection causing bacteria. These are gaining relevance as important human pathogens because they are responsible for several respiratory, skin, and extra-respiratory infections in both immunocompetent and immunocompromised individuals [1]. NTM are found ubiquitously in nature and has been isolated from natural resources like soils, water, animals, and food products [2].

M. fortuitum belongs to the group of NTM and are classified as rapid growing NTM as visible colony of it can be seen within 7 days of culturing. It is an opportunistic infection known to cause various respiratory, disseminated and healthcare associated infections. It has been reported to cause infections like keratitis, folliculitis, therapy associated infections, and infections due to use of catheter, prosthetic devices, artificial knees, lens implants, and metal rods for stabilising fracture [3].

Biofilms are microbial communities clustered in extracellular polymeric matrix secreted by them. The matrix also known as slime consists of polysaccharides, proteins and extracellular DNA. Biofilm provide the ability to bacteria to adhere to biotic as well as abiotic surfaces [4]. Biofilm is a successful survival strategy used by bacteria to live in adverse environmental conditions. Bacteria suffer several changes during biofilm formation, thus leading to formation of a dynamic heterogeneous community. The ubiquitous presence and the ability to form biofilm play an important role in pathogenesis of the NTM. Biofilm also confers resistance to antibiotics in bacteria through various strategies like delayed entry, and presence of differentiated population [1][2][4].

Through various studies, it has been established that membrane proteins have a very important role in biofilm formation. In a study, it was found that most of the proteins found in biofilm formed by *Haemophilus influenza* are associated with bacterial membrane [5]. It has been also reported that outer membrane proteins are important for biofilm formation in marine bacterium *Pseudoalteromonas* sp. D41 [6]. A study

reported that targeting the outer membrane protein A (OmpA) of *Pasteurella multocida* has reduced its ability to form biofilms [7]. These all studies validate the role of membrane proteins in biofilm formation. The role of membrane protein in biofilm formation in NTM can also be deduced by looking into these studies.

Identification of a gene can be achieved by mutagenesis and gene silencing. Gene silencing requires the sequence of the gene to be silenced whereas in random mutagenesis, the sequence of the gene is not required. Random mutagenesis can be achieved by using transposon i.e. jumping gene that goes and integrate at random locations in genome. By specifically looking into the disruption of membrane gene by blue white screening, mutants having defective membrane gene can be selected. Such mutants can further be screened for its ability to form biofilm. Mutants showing less biofilm formation can be further processed for identification of disrupted gene. Such gene can be targeted as a novel mechanism for intervention in case of *M. fortuitum* infections.

CHAPTER 2

REVIEW OF LITERATURE

2.1. *Mycobacterium*

Mycobacterium is a genus that belongs to the family Mycobacteriaceae, order Actinobacteria. The genus contains over 190 species [8]. It gets its name myco- which means fungus due to its mold like growth. These are called acid fast bacteria due to their special staining characteristics under the microscope, which is because of presence of mycolic acid in the cell wall of the bacteria. The genus mycobacteria can be broadly classified into *M. tuberculosis* complex (MTC) and a large group of Non tuberculous mycobacteria.

MTC includes pathogenic mycobacteria like *M. tuberculosis, M. canetti*, and *M. africanum.* These are responsible for tuberculosis in humans and other living beings. Nontuberculous mycobacteria (NTM) are group atypical mycobacteria that are non-motile slender bacilli unable to form spores. These are opportunistic aerobic free living microorganisms found ubiquitously in environment. They are present in water sources, soil, dust, foods, aerosols, and animals. They usually exhibit symbiotic, commensally and saprophytic behaviour[2]. Their ability to form biofilm make them resistance to routine disinfectants, chlorine in water, low pH, salinity, oxygen tension, range of temperature and low nutrient conditions [4].

NTM are group of environmental microorganisms consisting of approximately 186 unique mycobacterium species [9]. NTM are broadly classified into fast growing mycobacteria and slow growing mycobacteria. Fast growing mycobacteria produces mature growth on media plate within 7 days and comprises of approximately 50% of the validated mycobacterial species and are further divided into six major groups viz. *M. chelonae/M. abscessus* complex, *Mycobacterium fortuitum* group, *M. mucogenicum* group, *M. smegmatis* group, *M. mageritense/M. wolinskyi*, and the pigmented fast growing mycobacteria [3]. Slow growing mycobacteria requires more than 7 days to

reach mature growth include *M. avium, M. xenopi, M. kansasii, M. intracellulare,* and *M. marinum* [10].

The rate of NTM infection was estimated to be 1.8 cases per 100,000 during 1970-1980 in United States according to non-comprehensive national survey data of mycobacterial isolates. It was also found that 74% of all MTB isolated were NTM during 1991-1992 according to a survey done by Centers for Disease Control and Prevention [11]. NTM are responsible for healthcare associated infections, infections related to medical procedures, disseminated infections and increased risk of pulmonary infections in immunocompetent individuals [2][1]. *Mycobacterium kansasii, M. marinum, M. gordonae, M. scrofulaceum, M. avium-intracellulare (MAI), M. ulcerans, M. fortuitum, M. chelonae,* and *M. abscessus* are the most clinically important pathogenic NTM [3][11]. The distribution of NTM is affected due to geographical difference highlighting its environmental nature. The most predominant NTM isolated in European Union and USA belongs to Mycobacterium avium complex (MAC). In Saudi Arabia, most frequent isolated species were M. abscessus, *M. fortuitum*, and M. intracellulare and so in India where *M. fortuitum* is frequently isolated [2].

2.2. *Mycobacterium fortuitum*

Mycobacterium fortuitum (earlier known as *Mycobacterium ranae*), was originally isolated from frogs in 1905. In 1938, a new mycobacterial species (named *M. fortuitum* by da Costa Cruz) was thought to be isolated from a patient with a skin abscess caused after he received local vitamin injections. With time both the organisms were proven to be same and the species retained the name *M. fortuitum*[3]. It belongs to the fast growing NTM having an ability to form biofilm that helps in its pathogenesis as well as resistance to currently prescribed drugs. It has been found that *M. fortuitum* has greater affinity for biofilm formation in stainless steel, polyvinyl chloride, and polycarbonate. Therefore, it can easily form biofilm in pipelines contaminating water and medical equipments like catheter leading to health care associated and disseminated infections [2]. It is an opportunistic pathogen generally affecting immunocompetent patients and also causes disseminated infection in person receiving glucocorticoid therapy and impaired cellular immunity. In immunocompromised patients particularly HIV/AIDS,

it causes lymphadenopathy and skin lesions [10]. A study found that *M. fortuitum* is resistant to disinfectants like chlorine, ozone and ultraviolet making it more difficult to kill [12].

2.3. Infections caused by *Mycobacterium fortuitum*

A 194.2% increase in occurrence of *M. fortuitum* from year 1994 to 2014 has been reported in USA [9]. Following are the list of infections reported to be caused by *M. fortuitum*:

Keratitis: The inflammation of cornea of the eye due to infection of bacteria or fungi is termed as keratitis. Keratitis consists of 36.6% of all ocular disease followed by sclera buckle infection (14.8%) and socket implant infections (14.8%) [13]. *M. fortuitum* has been recognised as an important cause of ocular morbidity. It can cause periocular, cutaneous, orbital, sclera and corneal infections. Ocular trauma, recent ocular surgery, and systemic immunosuppression are some of the risk factors for the infections [4].

Granulomatous Lobular Mastitis: *M. fortuitum* has been reported for the first time to be causing granulomatus lobular mastitis which is a rare inflammatory disease of the breast. Besides general symptoms of the disease like swelling and firmness, multiple breast abscesses is observed in *M. fortuitum* infections [14].

Nail Salon Footbath-Associated Folliculitis: Salon's customers were identified with persistent skin infections below the knee. Often, the infections involved furunculosis of the lower leg hair follicles. The disease was caused because of the contamination footbath water by *M. fortuitum* due to irregular cleaning of filters [15].

Anti-TNF-α Therapy-Associated Infections: Biological therapy to inhibit tumour necrosis factor (TNF-a) have been associated to different clinical manifestation of *M. fortuitum*. Immunosuppressive therapies using infliximab, etanercept, and adalimumab were identified to cause *M. fortuitum* infection [16].

IFN-γ/IL-12-Associated Infections: Intracellular killing of mycobacteria is caused by immunological pathway of Interferon gamma/interleukin 12 (IFN-γ/IL-12). Infections caused by *M. fortuitum* affected lymph nodes, osteoarticular tissue, lungs, skin, and/or soft tissues [17].

Health care associated disease: Health care associated infections can be associated with various contaminated water sources. Biofilm forming ability of *M. fortuitum* allows it to be present in water pipes. Water based solutions can also be responsible for sporadic health care associated infections such as catheter related infections, wound infections and post surgical infections. There has been report of infections caused because of insertion of prosthetic devices like prosthetic heart valves, lens implants, artificial knees and hips, and metal rods to support bone after fractures. Cosmetic surgical procedures like liposuction, breast augmentation and mesotherapy can also cause *M. fortuitum* infections [18].

Localized Posttraumatic Wound Infections: *M. fortuitum* is widely responsible for wound infections caused due to accidental penetrating trauma. Local redness, swelling and osteomyelitis are observed as a result of infection. There is absence of systemic symptoms like fever, chills and fatigue [19].

Surgical Wound Infections: *M. fortuitum* has been reported to cause to post surgical wound infections which include cataract excision, laser surgery, prosthetic hip and knees, coronary artery bypass and cosmetic surgeries. Symptoms are usually observed after incubation of 2 to 8 weeks, redness and serious drainage is seen in healing wound [20].

Catheter Related Infections: The use of catheter has been responsible for *M. fortuitum* infection showing clinical manifestation like occult bacteremia, granulomatous hepatitis, septic lung infiltrates, tunnel infections, or exit site infections. Use of catheters for long term as in case of chronic peritoneal dialysis catheters, haemodialysis catheters, nasolacrimal duct catheters, and ventri-culoperitoneal shunts are also responsible for *M. fortuitum* infections [21].

Disseminated Cutaneous Infections: *M. fortuitum* has been reported to cause disseminated systemic skin infection in immunocompromised patients with fatal disorders, especially in case of uncontrolled lymphomas and leukemias [22].

Chronic Pulmonary Infections: Patients with achalasia and other forms of gastrointestinal disturbances are at risk of pulmonary infections caused *M. fortuitum*. The bacteria is a rare cause of lung disease in healthy individuals and generally do not

require any treatment [3]. *M. fortuitum* has also been reported to cause thoracic empyema in a 61 year old man [23].

CNS Infections: *M. fortuitum* is responsible for central nervous system (CNS) infections in cases where patients has been associated to foreign body infections as a result of motor vehicle accident, lumbar discectomy, brain abscess, chronic mastoiditis , chronic otitis media, deep wound infection, and infection of a ventriculoatrial shunt [24].

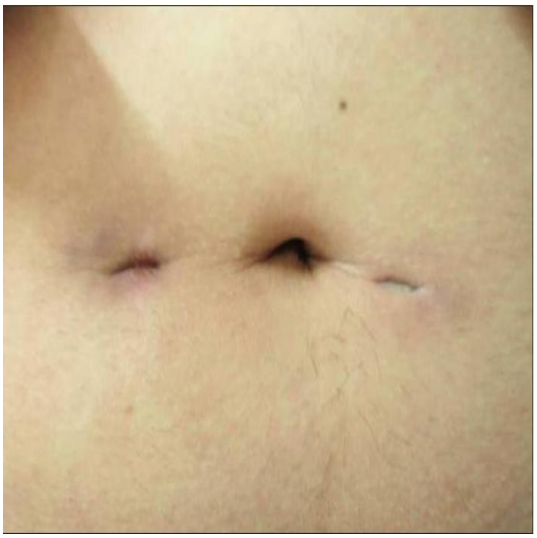

Figure 2.1: Discharging sinus in right due to *M. fortuitum* and the healing incision wound in left paraumbilical region [41].

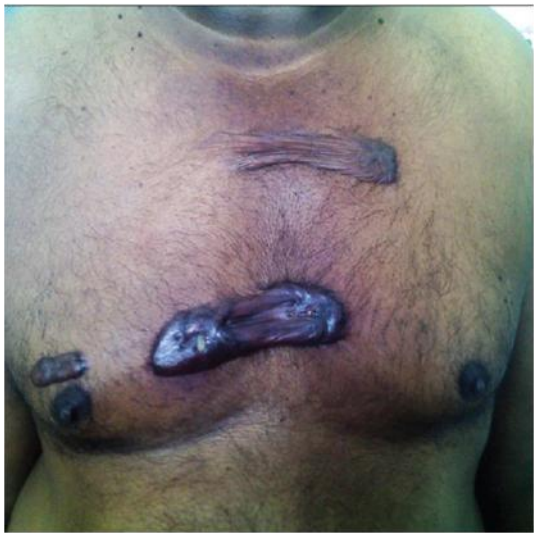

Figure 2.2: Multiple keloids lesions on chest due to *M. fortuitum* infection [42].

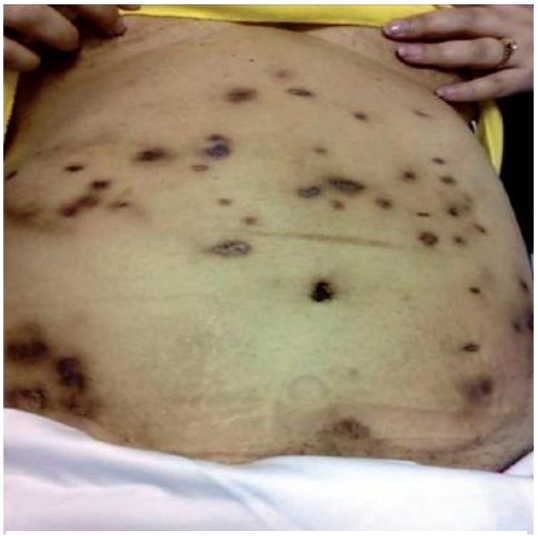

Figure 2.3: Abdominal wall abscesses after liposuction due to *M. fortuitum* [43].

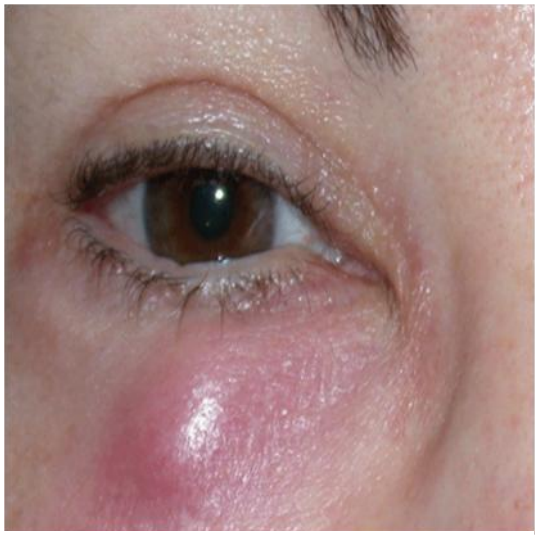

Figure 2.4: Edema and erythema below eye after oculoplastic due to *M. fortuitum* infection [44].

2.3.1 Treatment: The therapeutic regime for treatment of *M. fortuitum* infections include use of amikacin, cefoxitin, imipenem, tigecycline (Parenteral); ciprofloxacin, levofloxacin, trimethoprim- sulfamethoxazole, moxifloxacin, clarithromycin (80%), doxycycline (50%), and linezolid (86%)(Oral) [3].

M. fortuitum has been showing increased intrinsic resistance towards large number of antimicrobials utilized among them. Some of new drugs having better efficacy than older drugs are tabulated below [25]:

Table 2.1: New therapeutic drugs for *M. fortuitum* treatment

Drug	Mode of action	Minimum Inhibitory Concentration (MIC) (ug/ml)
DC- 159a	Inhibits supercoiling of DNA gyrase	0.25
Delamanid	Inhibits synthesis of mycolic acid	>100
Pretomanid (PA-824)	Inhibits cell wall formation and respiratory poisoning	>100
Tigecycline	Protein synthesis inhibitors	=<0.03–0.5
Bedaquiline	Targets ATP synthase	0.13-0.25
TP-271	Inhibit transcription/translation	0.06

2.4. Biofilms

2.4.1. Introduction

Biofilms are sessile cell communities embedded in self produced extracellular polymeric substances (EPS). Major components of EPS include proteins, polysaccharides and extracellular DNA that allows cells to attach to a substratum. The ability to form biofilm has been adapted roughly 3.25 billion years ago by microorganisms and it plays an important role in bacteria survival in adverse environmental conditions. The cells present in biofilm are different

from planktonic cells in terms of gene transcription and growth rate. The structural unit of biofilm is known as microcolony. Bacteria are present in microcolonies having a network water channels that provides nutrients and oxygen to cells. In spite of presence of these channels, there exists decreased availability of oxygen and nutrients in deeper layers of the biofilm. Biofilm forming bacteria become recalcitrant to adverse environmental stress such as UV damage, metal toxicity, salinity, desiccation, bacteriophages, and phagocytosis (host immune response) [26].

2.4.2. Steps involved in biofilm formation

Quorum sensing is a unique way of communication in bacteria that depends on signalling molecules called auto inducers. The production, detection and response to these auto inducers allow quorum sensing. The role of quorum sensing in biofilm formation was first confirmed in *Pseudomonas aeruginosa* by Greenberg and his colleagues. Biofilms and QS are linked together as they represent behaviour of bacterial community [26]. Biofilm formation process can be divided into following phases viz. reversible attachment, irreversible attachment, mature biofilm formation and dispersion [1]. The process is explained below [27]:

1. Attachment of bacteria to the surface: Biofilm formation is most preferred at a solid-liquid interface (e.g. water, blood). The interface provides an ideal environment for the bacteria to attach and form microcolonies. The binding of cells is driven by cell hydrophobicity and bacteria are more likely to attach if it has high cell surface hydrophobicity. Biofilm formation is favoured on rough surfaces as compared to the smooth one. Once a bacteria attaches to a surface, other microorganism might attach to it forming a mixed community.
2. Formation of microcolony: Bacterial attachment to the surface is followed by formation of a stable microcolony. After attachment, bacteria releases chemical signals for intercommunication among themselves. Once the signal crosses the threshold level, genes responsible for exopolysaccharides are activated and the bacteria starts to multiply while embedded in the matrix.

3. Maturation and three-dimensional structure formation: After the microcolony establishment in the matrix, bacteria starts to create water filled channels inside the matrix. These channels are required for providing nutrition and removing waste materials.
4. Dispersion of biofilm: Mature biofilm can be dispersed by either of the following ways: (a) shedding of daughter cells from actively dividing cells, (b) biofilm detachment because of quorum sensing or nutrient levels, and (c) shearing leading to continuous removal of small portions of the biofilm because of the flow effects.

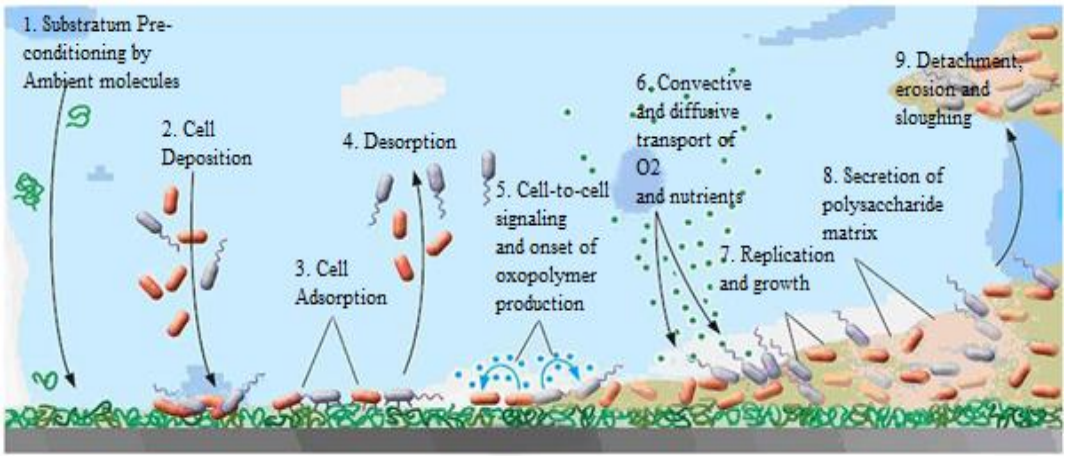

Figure 2.5: Process of biofilm formation [27].

2.4.3. Composition of biofilm

Biofilms are made of two components: microbial cells and EPS which account for 50-90% of the total organic carbon of biofilms. The EPS in biofilm is highly hydrated as it can integrate large amount of water into its structure by hydrogen bonding. The structure (physical as well as chemical) and amount of EPS varied among different bacteria[27]. Biofilms formed by mycobacteria are different from biofilms formed by other pathogens as it contains some unique components viz. free mycolic acids, lipid containing molecules and gycopeptidolipids [28].

2.4.4. Importance of biofilm in bacterial pathogenesis

Biofilms are naturally present in human pathogenic systems and more than 65% of chronic infections are caused by biofilm forming bacteria. It has been found that 60-70% of nosocomial infections are caused because of presence of biofilm

on implanted medical devices like endotracheal, catheters, heart valves and cardiac pacemakers[27]. Biofilms have been found to have a role in persistent infections like cystic fibrosis, diabetic foot ulcers, chronic wounds, osteomyelitis, meloidosis and nosocomial diseases. The multicellular organisation and heterogeneity in biofilm plays an important role in conferring resistance in bacteria against antibiotics (10-1000 times more resistant than planktonic cells) through different strategies: (1) the ECM matrix delays the permeation of antibiotics as a result antibiotic stress is induced in bacteria due to exposure to sub-inhibitory concentrations of antibiotic, (2) heterogeneous population of bacteria are formed as a result of differential gradients of nutrients, oxygen and waste, thus bacteria are not susceptible to one antibiotic, (3) the matrix accumulates antibiotic degrading enzymes like B-lactamases that renders antibiotics inactive, (4) the presence of persister cell subpopulation which are responsible for re-establishment of colony in case biofilm is eradicated, (5) slow growth leading to slow metabolism of drug[29], (6) changes in quorum sensing, and (7) it enhances horizontal gene transfer of antibiotic resistance from plasmid and promotes mutability [26].

2.4.5. Methodology to study biofilms

1. Microtiter plate test: In this test, bacteria are allowed to adhere to abiotic surface i.e. the surface of plate. The planktonic cells are discarded and the biofilm is visualised using crystal violet stain. Quantification is done through spectrophotometry. It is the cheapest and easiest way to detect biofilm formation by bacteria [2].
2. Microfermentor test: In this method, abundant biomass is generated and has an advantage of extracting nucleic acids and proteins which in turn provides more information on the assembly of biofilm [2].
3. Atomic force microscopy (AFM): It is a sensitive tool that allows study of bacterial morphology and surfaces with high resolution. The advantage of this technique lies in minimal sample preparation and acquiring 3D images of the surface [2].
4. Fluorescence microscopy looks for biofilm on basis of its reactivity to an antibiotic.

5. Confocal laser scanning microscopy is useful in study of thick samples.
6. Scanning electron microscopy (SEM) can also be used for identification of biofilm formation by bacteria.

2.4.6. Genes involved in biofilm

Rv0024 in *Mycobacterium tuberculosis* has been reported to form biofilm and establishing initial infection and drug resistance. It contains NlpC/P60 domain which is highly conserved in mycobacteria genus. The gene may be responsible for altering mycobacterial cell wall lipids that is associated with cell wall assembly [30]. Besides these there are at least three genetic loci, pks16, helY, and pks1, that has role in biofilm formation [31]. Lsr2 (Nucleoid associated protein) regulates mycolyldiacylglycerol (MDAG) synthesis and free mycolic acids (FM) is induced through GroEL1-dependent modulation of type II fatty acid synthases in Mycobacterium biofilm synthesis [31].

CsgD in *Escherichia coli* represses flagellar synthesis and motility and promotes biofilm formation by inducing cyclic diGMP which is a secondary messenger for EPS synthesis. Several other genes have also been identified to have a role in biofilm formation in *E. Coli*, some of which are flgA for assembly of protein for flagellar basal body, motA for proton conductor component of flagella motor, csG for outermembrane lipoprotein, nifU for scaffold protein, and tolB for periplasmic protein [32].

In *Bacillus subtilis*, the genes for biofilm formation are under regulation of SinR (positive regulation) and SinI (negative regulation) [31]. Similarly, many genes were identified to have a role in biofilm formation in *Staphylococcus aureus* such as icaA (intercellular adhesion), fnbA (fibronectin binding protein), clfA (clumping factor), eno (laminin binding protein), and cna (collagen binding protein) [33].

2.5. Techniques for gene identification

2.5.1. Transposon mutagenesis

In this method of gene identification, a vector having a transposon is used. The host organism in which the gene is to be identified is made competent and vector is introduced in the host cell. Upon insertion, the transposon excise out and integrate itself in the genome of the organism at random locations. The insertion is purely random in nature and can lead to inactivation of gene of interest. The transformed cells are screened for presence of antibiotic resistance (integrated along with transposon) and absence of the gene of interest. Then, using sequencing the nearby sequences where the transposon has integrated in the genome is identified and by the use of bioinformatics tools, gene is finally identified. Similar work has been reported, where a transposon mutant library of 13,536 MAP K-10 Tn5367 mutants of *Mycobacterium avium subsp. paratuberculosis* was constructed. The library was followed by in vitro screening for phenotypes related to virulence. The transposon insertion sites were identified in mutants having desired phenotype using PCR, Southern blotting and DNA sequencing [34].

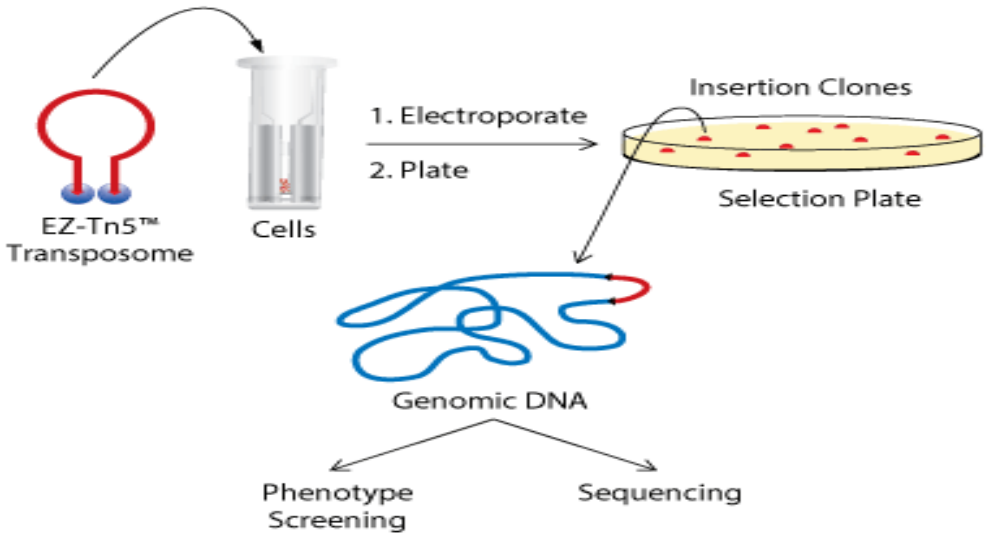

Figure 2.6: Schematic representation of use of transposon mutagenesis for gene identification [45].

2.5.2. Gene Knockout

The complexity of metabolic network makes it difficult to predict the effects of gene modification. So, as to predict the function of a gene, various gene knockout strategies are used to deactivate the specific genes. The gene deactivation leads to inactivation of certain proteins which leads to loss of function in organism. The function of the deactivated gene is interpreted as difference between the knockout organism and the normal organism. Various wet lab gene knockout strategies include, CRISPR/Cas9, PCR based strategy, Lambda Red recombination strategy, and Zinc finger nucleases [35].

In recombination strategy, gene is deactivated in the organism through combination of various techniques: First of all, the vector is cultured and it is introduced into the stem cells of the embryo. The vector is designed in such a way that it goes and recombine to the target gene. This recombination is facilitated by incorporating gene sequences in the vector itself. This leads to the recombination and insertion of the foreign sequence in the gene, thus rendering the gene nonfunctional. As the sequence has been disrupted, the protein translated from it is also nonfunctional. It is preferred that the foreign sequence is a reporter sequence as recombination is a rare event. This allows easy identification of the successful knockout of the gene [36].

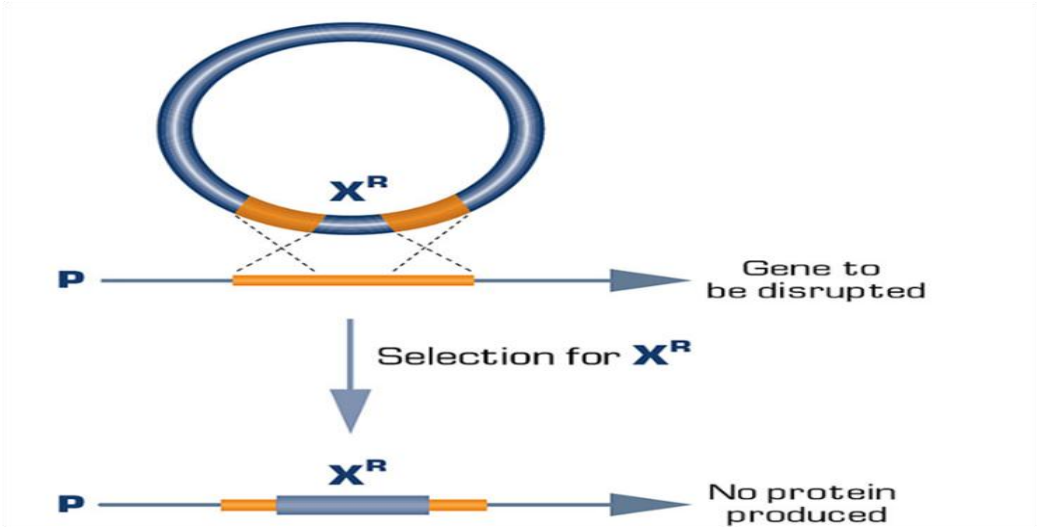

Figure 2.7: Schematic representation of recombination strategy to knock out a gene [46].

2.5.3 Gene silencing by antisense technology

Gene silencing is defined as an epigenetic regulation of a gene that prevents gene expression of the previously active genes. The repression of the gene can be done either at transcriptional or translational level. It is similar in approach as of gene knockout but gene silencing do not inactivate the gene completely rather it reduces the expression by at least 70% [37]. A gene can be silenced at transcriptional level by using following strategies: genomic imprinting, paramutation, transposon silencing, transgene silencing, position effect, and RNA-directed DNA methylation. Similarly, silencing at translational level can be achieved by: RNA interference, RNA silencing, and Nonsense mediated decay. The most common approach for gene silencing includes use of antisense RNA to bock the translation of messenger RNA (mRNA). mRNA is formed as result of transcription and it carries genetic information from the DNA to cellular machinery involved in protein production. The gene silencing is achieved by using an antisense strand i.e. a complementary or mirror strand to the sense strand (5' to 3'). The introduction of antisense strand leads to formation of double stranded mRNA or RNA dimer. Presence of RNA dimer halts the translation process as mRNA is not available for protein synthesis. Besides stopping the translation process, the double stranded mRNA is naturally degraded by the cell as it is identified as a foreign element and as result gene is turned off [38]. Antisense technology can also be applied in case of DNA by building triplex formation. The exact mechanism for antisense strand is not known but it is hypothesized that it leads to the silencing of the gene by: blocking RNA splicing, accelerating the degradation of RNA molecule, preventing introns from being spliced out, impeding the exportation of mRNA into the cytoplasm, hindering translation and resulting in triplex formation in DNA [39].

The technology is useful in regulating the gene expression of essential genes as their expression in required for the cells to live. The mutants lacking the expression of these essential genes will be unable to survive. It also offers advantage in cases where deactivation of gene is difficult. Antisense approach

has been used to decrease the level of sigA, and Rv3303c in *M. tuberculosis* [40].

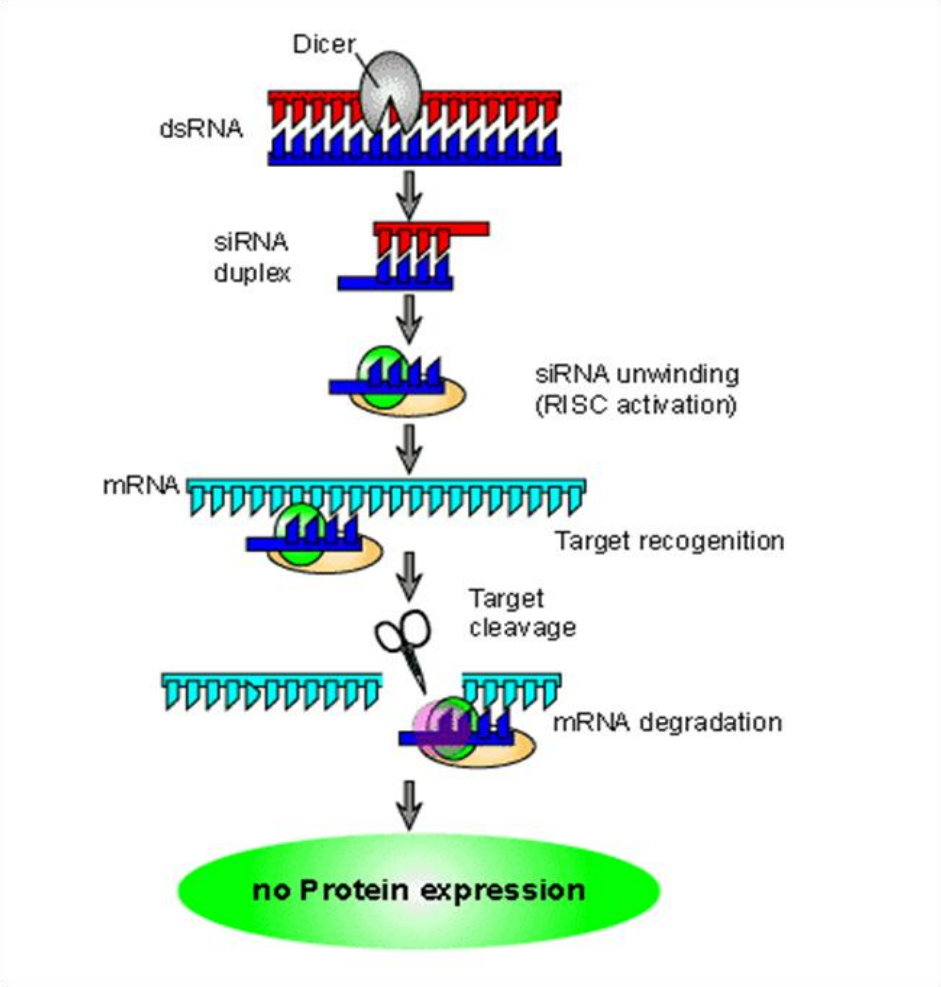

Figure 2.8: Schematic representation of Antisense technology to silence gene [47].

CHAPTER 3

MATERIALS AND METHODS

3.1. Construction of *M. fortuitum* library by random mutagenesis

3.1.1. Isolation of plasmid pRT291

Chemicals required: Glucose tris EDTA (GTE) - ALS 1 (Alkaline lysis buffer 1), SDS-NaOH solution, acetate solution, absolute ethanol, 96% ethanol, 70% ethanol, autoclaved distilled water.

Equipments used: Centrifuge, Centrifuge tubes, Mini centrifuge tubes, Pipette and Tips.

Procedure:

1. The culture was centrifuged at 7000 rpm for 10 minutes.
2. Then pellet was dried properly.
3. 300µl of GTE (Glucose tris EDTA- ALS 1) was added and vortexed.
4. 600µl of SDS-NaOH solution was added and mixed gently.
5. It was then incubated at 37°C for 5 minutes.
6. 450µl of acetate solution was added.
7. It was gently mixed for 5-6 times.
8. It was then incubated in ice for 30 minutes and centrifuged at 7000 rpm for 25 minutes.
9. Supernatant was transferred to fresh centrifuge tube and 2700µl of absolute ethanol was added.
10. The mixture was then incubated overnight at -20°C.
11. Centrifuged at 12000 rpm for 15 minutes and supernatant was removed.
12. 96% ethanol was added and centrifuged at 10,000 rpm for 12 minutes.
13. Supernatant was removed and 70% ethanol was added and centrifuged at 10,000 rpm for 3 minutes.
14. Pellet was dried properly until ethanol evaporated.
15. 40µl of autoclaved distilled water was added and tube was placed at -20°C.

3.1.2. Preparation of electrocompetent cells

Chemicals required: NBGT, 5% glycerol, Cycloheximide.

Equipments used: Centrifuge, Ice box, Spectrophotometer, Flask, Pipette and Tips.

Procedure:

1. Seed culture was prepared by inoculating 10µl of culture in 10 ml NBGT with 50µg/ml cycloheximide.
2. Seed culture was inoculated into 100 ml NBGT with 50µg/ml cycloheximide and incubated with shaking at 37^0C overnight.
3. Absorbance at 600 nm was taken till it reached to 0.6-0.8.
4. The cells were kept on ice before harvesting for about 40-60 minutes.
5. Then, cells were centrifuged at 4000 rpm for 10 minutes at 4°C.
6. Supernatant was discarded and the pellet was dried properly.
7. 20 ml of 5% glycerol was added to cells followed by centrifugation at 7000 rpm for 15 minutes.
8. Supernatant was discarded and 10 ml of 5% glycerol was added to the pellet.
9. Centrifuged at 7000 rpm for 15 minutes and supernatant was discarded.
10. Pellet was air dried properly.
11. 2 ml of 5% glycerol was added to pellet to make a uniform suspension.
12. Cells were aliquoted to microcentrifuge tubes and stored at -80°C.

3.1.3. Protocol for electroporation

Chemicals required: Electrocompetent cells, plasmid (pRT291), LBGT, X-gal, IPTG, Cycloheximide, Kanamycin and 5% Glucose.

Equipments used: Cuvettes, Electroporation machine, Ice box, Pipettes and Tips.

Procedure:

1. 400 µl of electrocompetent cells with 25µg/ml of plasmid (pRT291) were taken in a microcentrifuge tube.
2. Microcentrifuge tube was incubated at room temperature for 10 minutes.
3. Sample from microcentrifuge tube was transferred into electroporation cuvette.
4. Cuvette was kept in ice for 5-10 minutes.

5. Electroporation conditions were set to capacitance-25 µF, resistance-1000Ω, voltage-2500 volts, and cuvette-2 mm.
6. Pulse was given twice and cuvette was again kept in ice.
7. 2ml of LBGT was added and incubated at 37 ^{0}C for 6 hours with shaking.
8. Then, sample was spreaded on selection plate containing X-gal, IPTG, 50 µg/ml Cycloheximide, 30µg/ml Kanamycin, and 5% Glucose.
9. After incubation, plates were observed for blue colonies.

3.2. Culture Preparation for Biofilm assay

3.2.1. Preparation of Seed Culture of Wild-type and Mutants

Chemicals Required: MB7H9 medium, Cultures (Wild-type, M01, M02, M03, M04, M05, M06, M07, M08, M09, and M10), Kanamycin [30µg/ml] and Cycloheximide [50µg/ml].

Equipments Used: Pipette, Tips and Shaker.

Procedure:

1. 30 µg/ml of Kanamycin and 50 µg/ml of Cycloheximide were added to 10ml of MB7H9 medium.
2. A colony of mutant M01 was picked from the plate and inoculated into the test tube containing medium.
3. Same procedure was repeated for other 9 mutants (M02, M03, M04, M05, M06, M07, M08, M09, and M10).
4. 50 µg/ml of Cycloheximide was added to 10 ml of MB7H9 medium.
5. A colony of wild-type was picked from plate and inoculated into the test tube containing medium.
6. All the test tubes were incubated in shaker at 37 ^{0}C for 3 days.

3.2.2. Acid fast staining to check purity of culture:

Chemicals required: 70% ethanol, *M. fortuitum* wild type and mutants, Carbol fuchsin, 20% Acid alcohol, Malachite green, distilled water.

Equipments used: Slides, Loop, Hot plate, Dropper and Microscope.

Procedure:

1. The slides were made grease free by washing with detergent.

2. Smear was prepared by adding 50µL of culture (*M. fortuitum* wild type and mutants) on the slide and heat fixing it.
3. Slides were flooded with carbol fuchsin and kept on hot plate at 80°C for 5-7 minutes.
4. After 5 minutes, the slides were left to cool down and then washed with distilled H_2O.
5. Then, few drops of 20% acid alcohol were added to decolorize the slides and washed after 20-30 seconds.
6. Few drops of malachite green were added as a counter stain, kept for 45 seconds and then washed off with distilled H_2O.
7. The slides were air dried and then observed under the microscope at various magnifications of 100X, 400X and 1000X.

3.2.3. Preparation of Secondary Culture

Chemicals Required: MB7H9 medium, Cultures (Wild-type, M01, M02, M03, M04, M05, M06, M07, M08, M09, and M10), Kanamycin [30µg/ml] and Cycloheximide [50µg/ml].

Equipments Used: Pipette, Tips and Shaker.

Procedure:

1. 500 µL of seed culture of mutants were inoculated in different medium flasks containing 30 µg/mL of Kanamycin and 50 µg/mL of Cycloheximide in 100 mL of MB7H9 medium.
2. 500 µL of seed culture of wild type was inoculated in medium flask containing 50 µg/mL of Cycloheximide in 100 mL of MB7H9 medium.
3. All flasks were incubated in shaker at 37 ^{0}C for 1 day.

3.3. Biofilm Assay (Standardised protocol -1)

3.3.1. Plating of Wild-type and mutants cultures in 96-well plate

Chemicals required: MB7H9 medium, Cultures (Wild-type, M01, M02, M03, M04, M05, M06, M07, M08, M09, and M10), Kanamycin [30µg/ml], Cycloheximide [50µg/ml] and Tween normal saline.

Equipments used: Centrifuge tubes, Centrifuge, Vortex, Spectrophotometer, 96-well plate, Pipette, Tips and Incubator with shaker.

Procedure:

1. Cultures were transferred to 50 ml centrifuge tubes.
2. Cultures were centrifuged at 5000 rpm for 5 minutes.
3. Supernatant was discarded and pellet was dissolved in 5 ml of tween normal saline.
4. Culture was vortexed to dissolve the pellet.
5. OD of the culture was set to 0.6 at 600 nm using tween normal saline.
6. Culture was diluted 10-fold in MB7H9 medium.
7. 200 µl of culture was added in the wells of microtiter plate in triplicates.
8. 4 such plates were prepared.
9. Plates were incubated in orbital shaker at 37°C at 200 rpm for 28 days and processed at different time intervals for biofilm formation.

3.3.2. Crystal Violet Assay

Chemicals required: Autoclaved distilled water, Methanol, Crystal violet and 33% Glacial acetic acid.

Equipments used: Pipette, Tips and Spectrophotometer.

Procedure:

1. Plate was taken out of the shaker.
2. Media of each well was discarded.
3. Each well was washed thrice with autoclaved water vigorously.
4. Methanol was added to each well to fix the cells and plate was left undisturbed for 10 minutes.
5. Methanol was discarded and 200 µl of crystal violet was added to each well.
6. Plate was left undisturbed for 15 minutes.
7. Then crystal violet was discarded and extra stain was washed off.
8. Plate was air dried and biofilm was dissolved in 200 µl of 33% glacial acetic acid.
9. After 10 minutes, absorbance was taken at 570 nm in spectrophotometer.

3.3.3. Carbol Fuchsin Staining

Chemicals required: Autoclaved distilled water, Carbol fuchsin stain and Absolute ethanol.

Equipments Used: Microscope, Pipette and Tips.

Procedure:

1. Plate was taken out of the shaker.
2. Media of each well was discarded.
3. Each well was washed thrice with autoclaved water vigorously.
4. 200 µl of Carbol fuchsin stain was added to each well and plate was left undisturbed for 30 minutes.
5. Stain was discarded and extra stain was washed off.
6. Wells were decoloured with 200 µl of absolute ethanol was for 10 seconds.
7. Wells were washed off with water.
8. Plate was air dried.
9. Wells were observed under inverted microscope for biofilm formation.

3.4. Biofilm assay (Standardised protocol II)

3.4.1. Plating of Wild-type and mutants cultures in 96-well plate

Chemicals required: MB7H9 medium, Cultures (Wild-type, M01, M02, M03, M04, M05, M06, M07, M08, M09, and M10), Kanamycin [30µg/ml], Cycloheximide [50µg/ml] and Tween normal saline.

Equipments used: Centrifuge tubes, Centrifuge, Vortex, Spectrophotometer, 96-well plate, Pipette, Tips and Incubator with shaker.

Procedure:

1. Cultures were transferred to 50 ml centrifuge tubes.
2. Cultures were centrifuged at 5000 rpm for 5 minutes.
3. Supernatant was discarded and pellet was dissolved in 5 ml of tween normal saline.
4. Culture was vortexed to dissolve the pellet.
5. OD of the culture was set to 0.4 at 600 nm using MB7H9 medium.
6. Culture was diluted 100-fold in MB7H9 medium.

7. 200 µl of culture was added in the wells of microtiter plate in triplicates.
8. Plates were incubated in orbital shaker at 37°C for 4 days in static condition and analysed for biofilm formation.

3.4.2. Crystal Violet Assay

Chemicals required: Autoclaved distilled water, Methanol, Crystal violet and 33% Glacial acetic acid.

Equipments used: Pipette, Tips and Spectrophotometer.

Procedure:

1. Plate was taken out of the shaker.
2. Media of each well was discarded.
3. Each well was washed thrice with autoclaved water vigorously.
4. 225 µl of crystal violet was added to each well.
5. Plate was left undisturbed for 15 minutes.
6. Then crystal violet was discarded and extra stain was washed off.
7. Plate was air dried and biofilm was dissolved in 200 µl of 95% ethanol.
8. After 10 minutes, absorbance was taken at 570 nm in spectrophotometer.

3.4.3. Carbol Fuchsin Staining

Chemicals required: Autoclaved distilled water, Carbol fuchsin stain and Absolute ethanol.

Equipments Used: Microscope, Pipette and Tips.

Procedure:

10. Plate was taken out of the shaker.
11. Media of each well was discarded.
12. Each well was washed thrice with autoclaved water vigorously.
13. 225 µl of Carbol fuchsin stain was added to each well and plate was left undisturbed for 15 minutes.
14. Stain was discarded and extra stain was washed off.
15. Wells were decoloured with 200 µl of 95% ethanol was for 10 seconds.
16. Wells were washed off with water.
17. Plate was air dried.
18. Wells were observed under inverted microscope for biofilm formation.

CHAPTER 4

RESULTS

4.1 Acid Fast Staining Results

Acid fast staining of mutants present in the borrowed library was done to check the purity of the cultures.

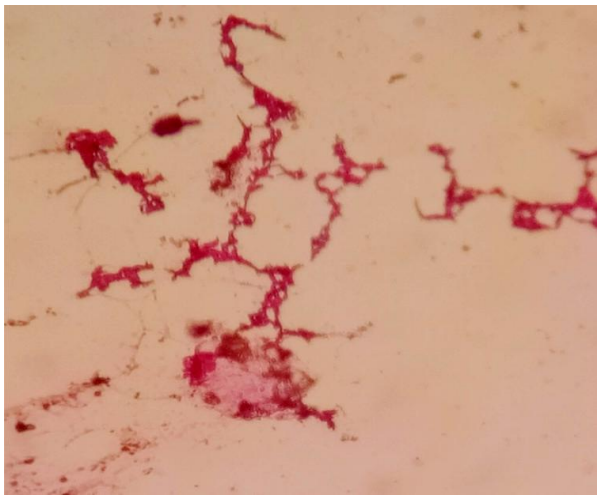

Figure 4.1: Acid fast staining of *M. fortuitum*mutant showing pure culture of red colored and rod shaped mycobacteria

4.2 Growth Observation

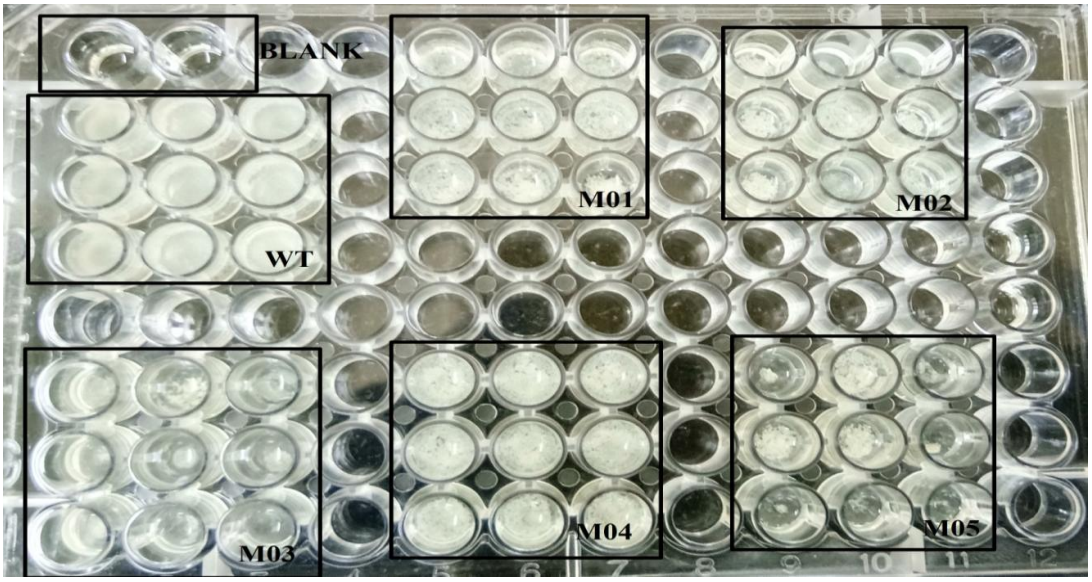

Figure 4.2: Growth in wild type and mutants of *M. fortuitum*

4.3 Crystal Violet Assay Results

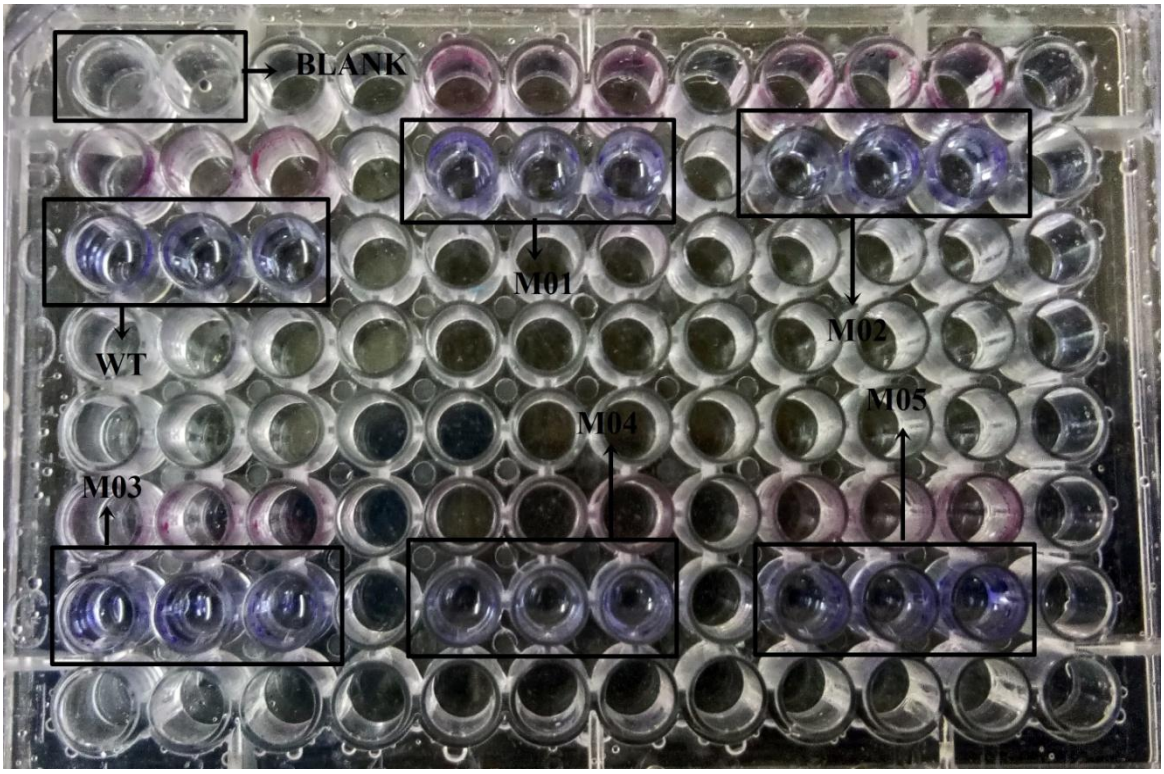

Figure 4.3: Crystal violet assay showing color variation in wild-type and mutants.

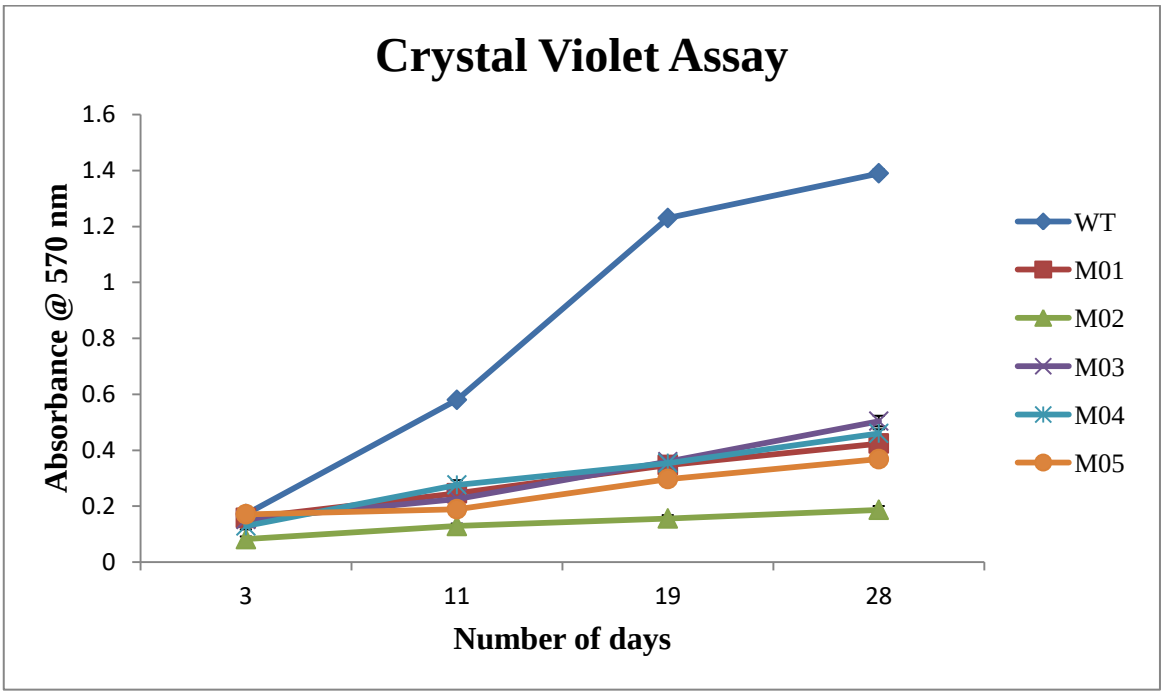

Figure 4.4: Biofilm formation curves of *M. fortuitum* wild- type and transposon mutants M01, M02, M03, M04, and M05.

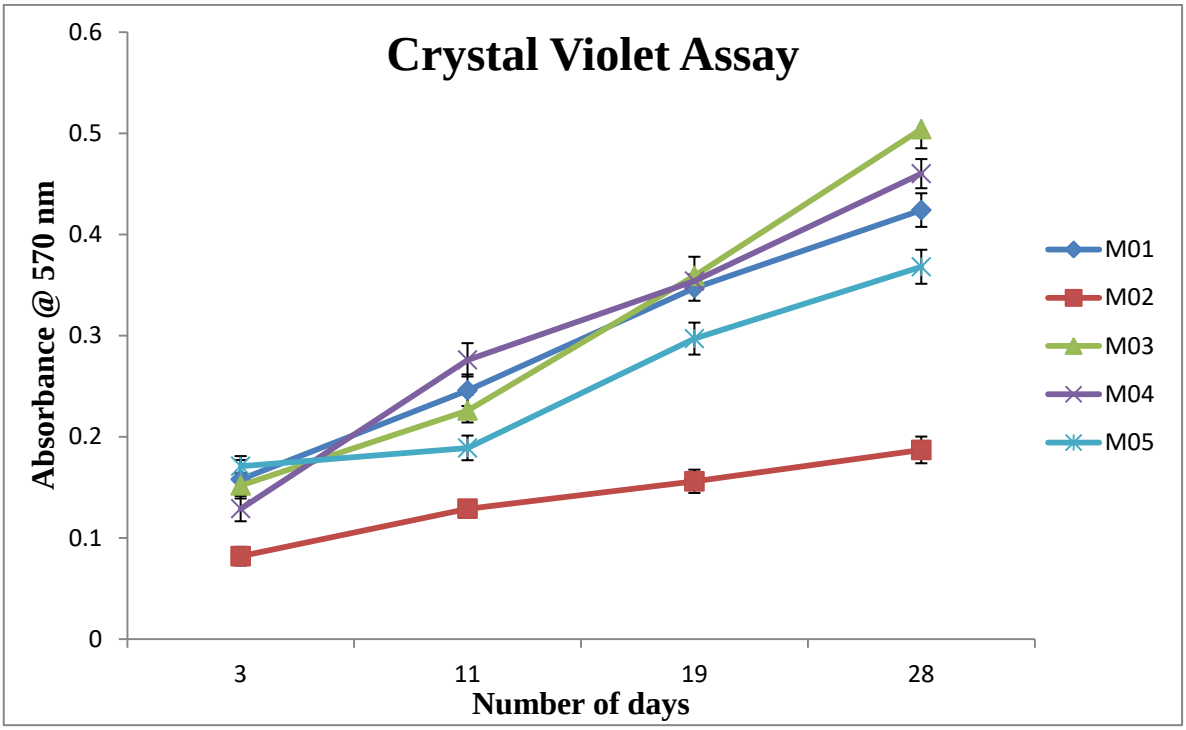

Figure 4.5: Biofilm formation curves of *M. fortuitum* transposon mutants M01, M02, M03, M04, and M05. Mutants M02 and M05 showed attenuated biofilm formation.

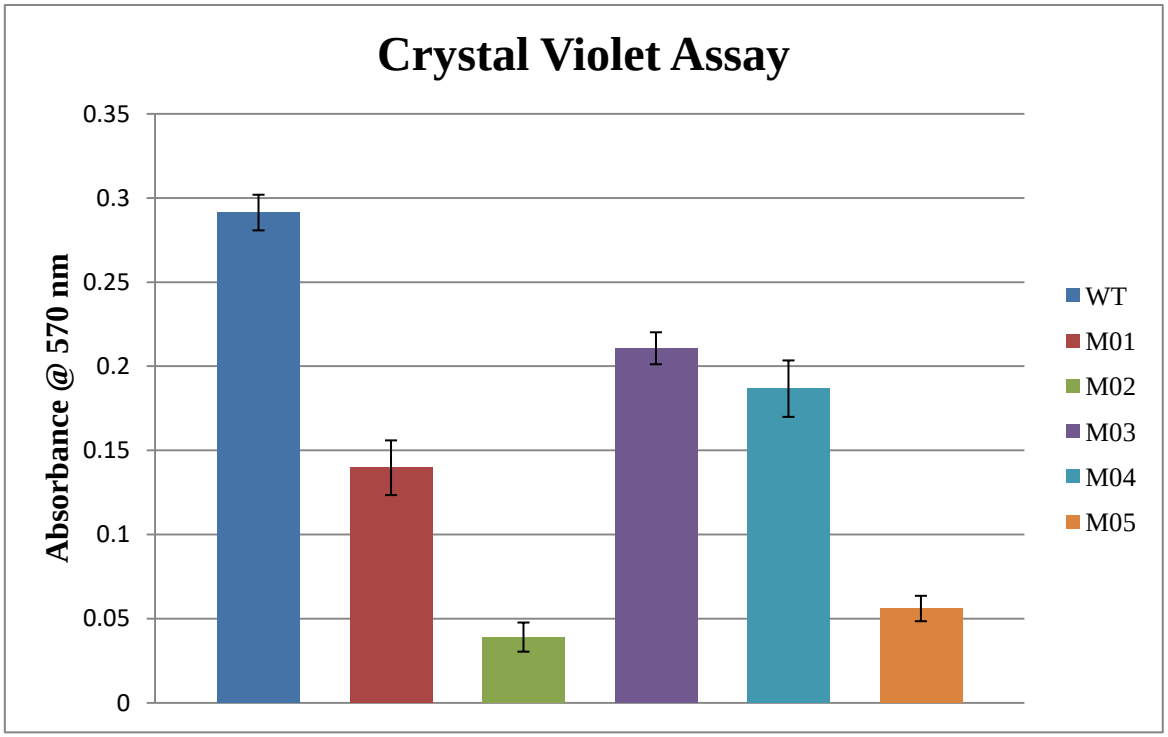

Figure 4.6: Biofilm formation curves of *M. fortuitum* wild- type and transposon mutants M01, M02, M03, M04, and M05. Mutants M02 and M05 showed attenuated biofilm formation.

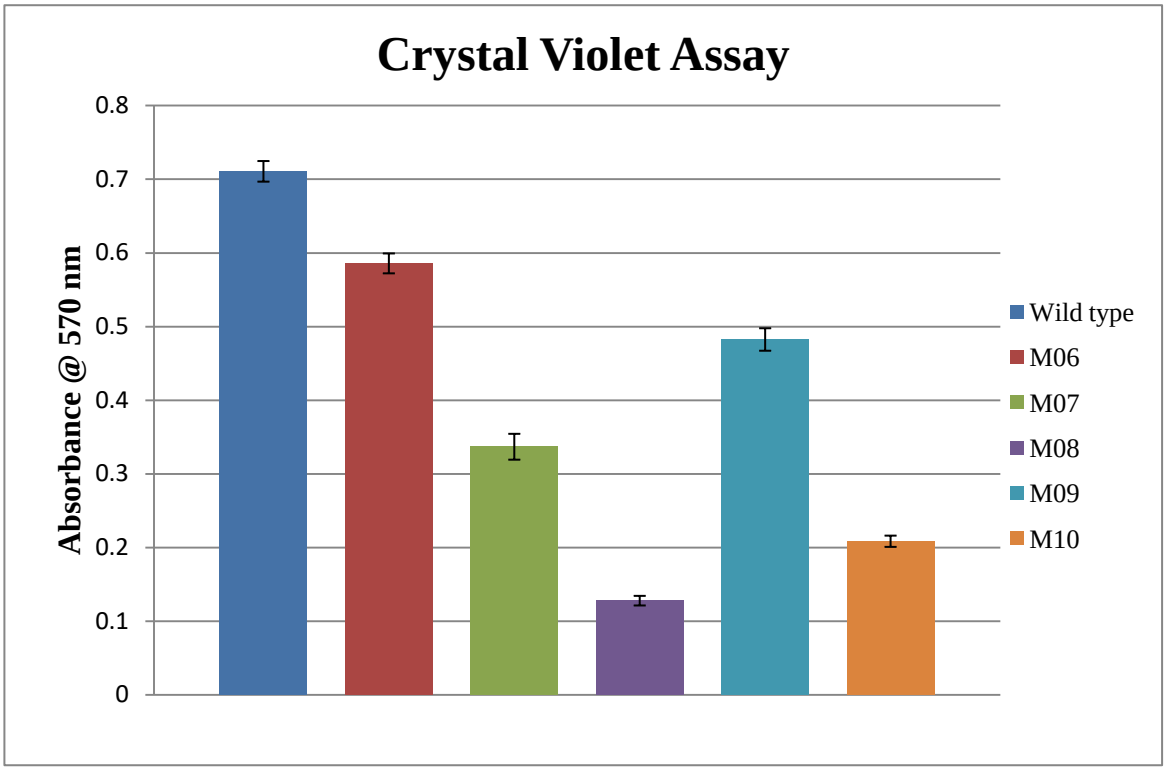

Figure 4.7: Biofilm formation curves of *M. fortuitum* wild- type and transposon mutants M06, M07, M08, M09, and M10. Mutant M08 showed attenuated biofilm formation.

Table 4.1: Percentage attenuation in biofilm formation in *M. fortuitum* mutants compared to wild type.

***M. fortuitum* mutants**	**Percentage (%) attenuation in biofilm formation**
M01	52.23
M02	**86.59**
M03	27.83
M04	36.08
M05	**80.75**
M06	17.6
M07	52.67
M08	**82.11**
M09	32.11
M10	70.7

4.4 Carbol Fuschin Staining Results

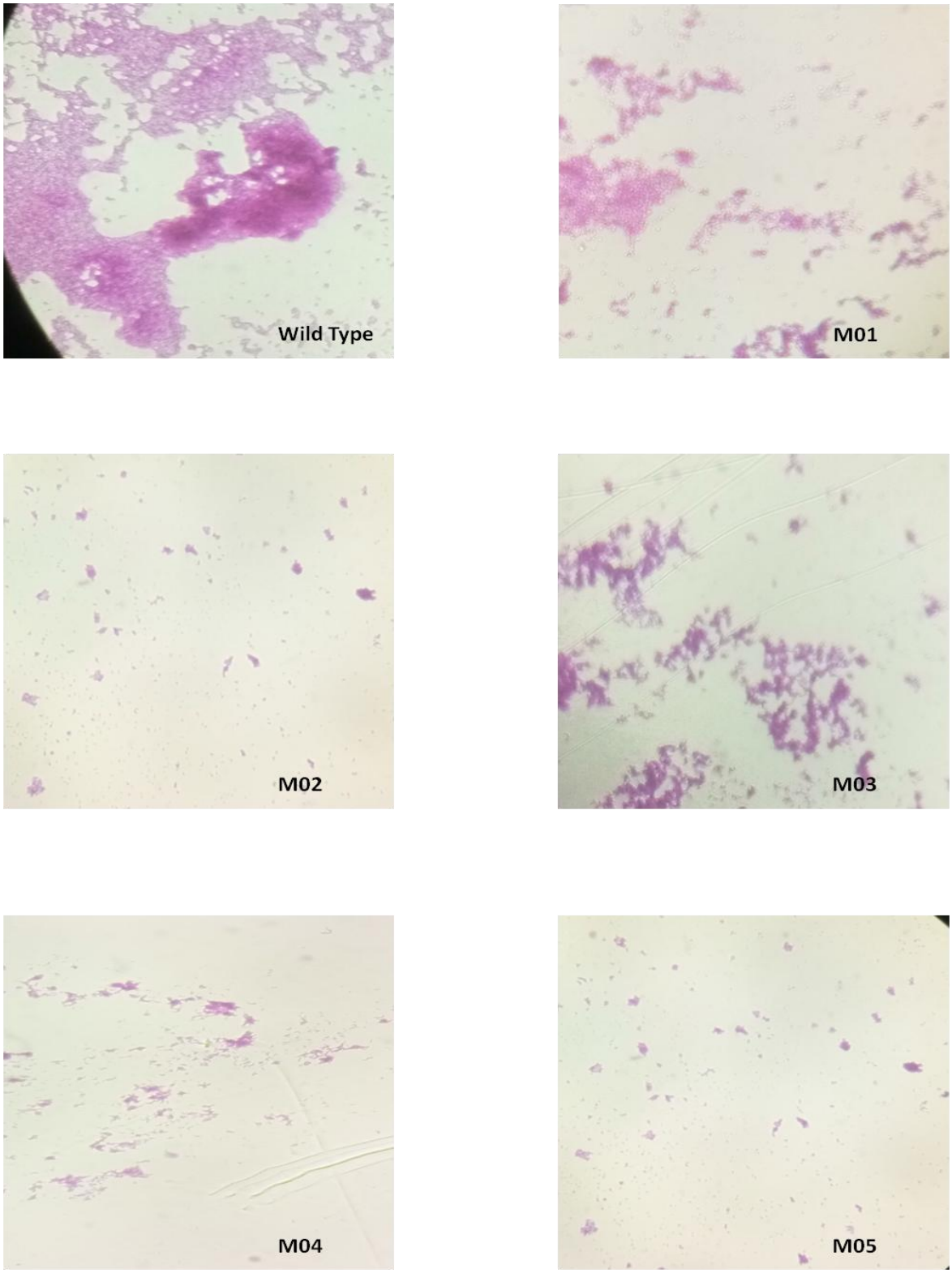

Figure 4.8: Day 3: Biofilm formed by *M. fortuitum* wild type and mutants stained with carbol fuchsin stain

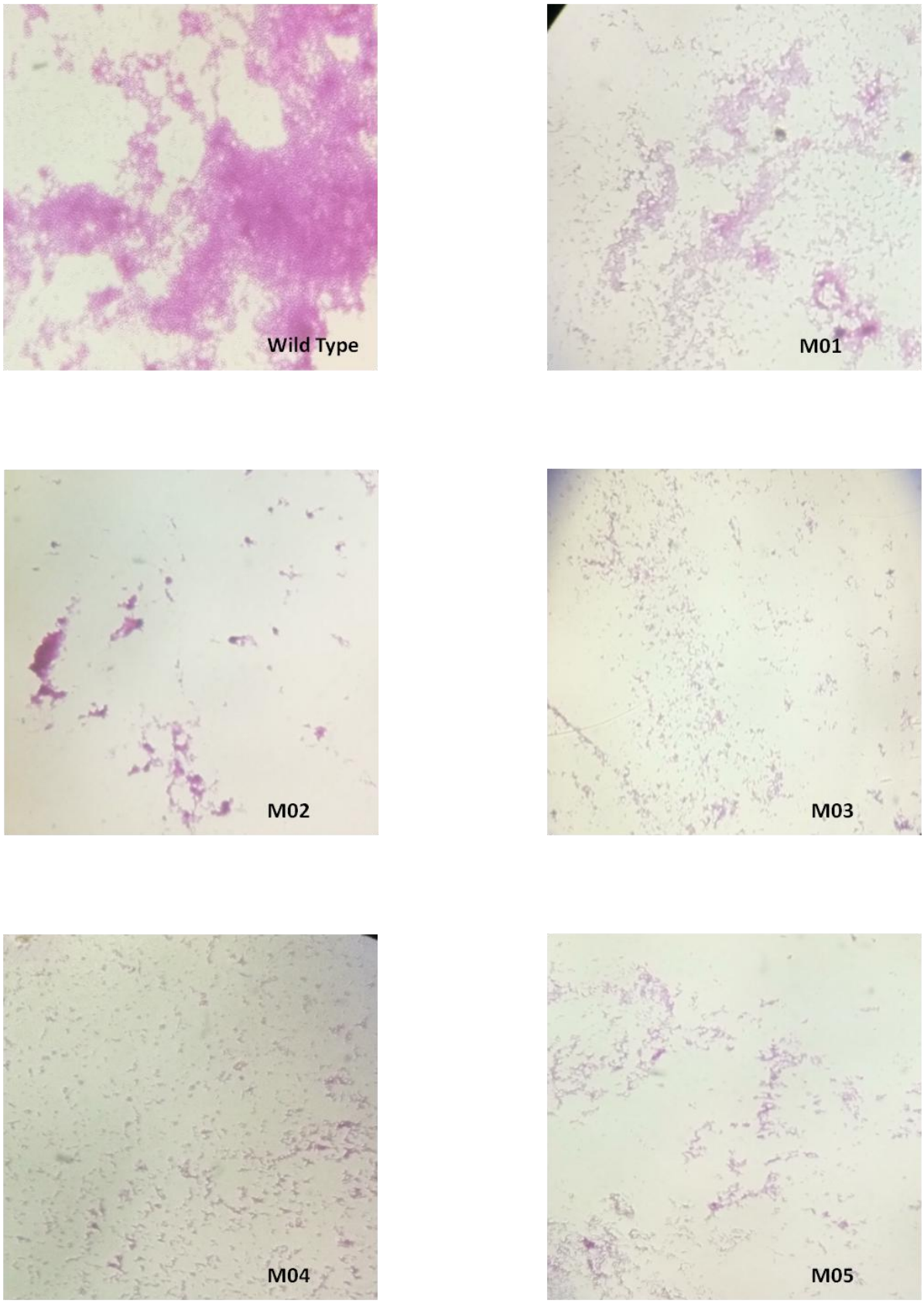

Figure 4.9: Day 11: Biofilm formed by *M. fortuitum* wild type and mutants stained with carbol fuchsin stain

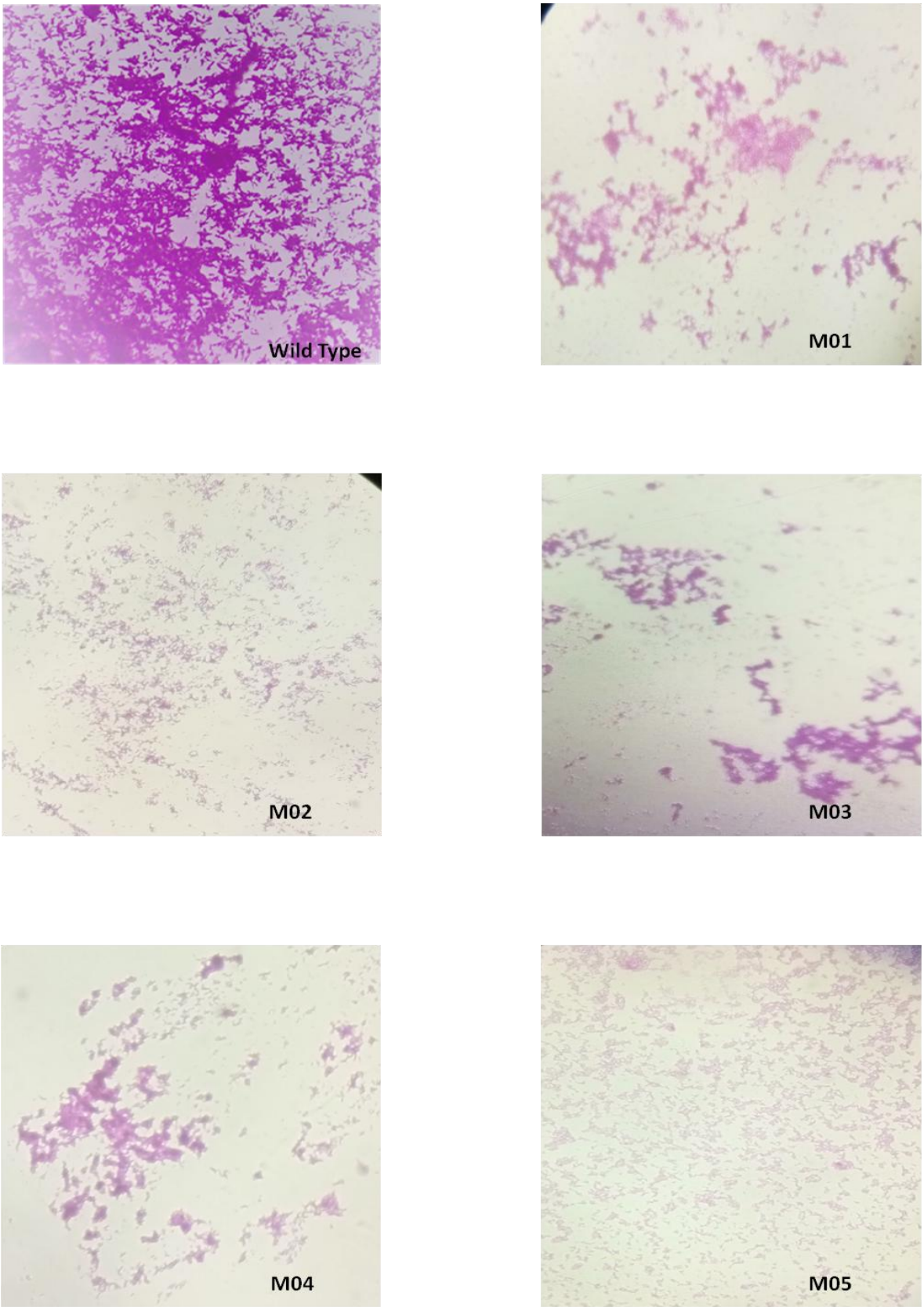

Figure 4.10: Day 19: Biofilm formed by *M. fortuitum* wild type and mutants stained with carbol fuchsin stain.

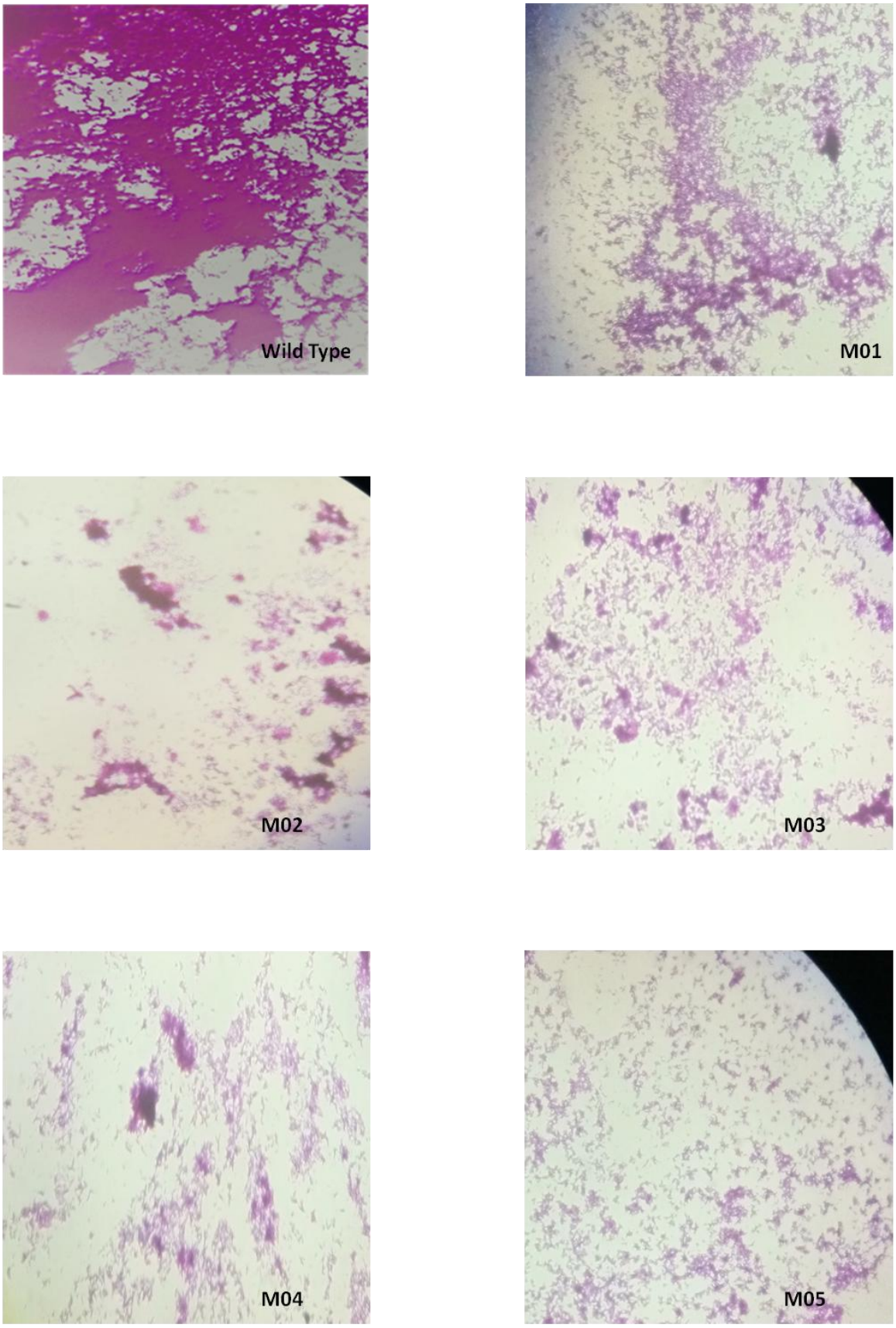

Figure 4.11: Day 28: Biofilm formed *M. fortuitum* wild type and mutants stained with carbol fuchsin stain.

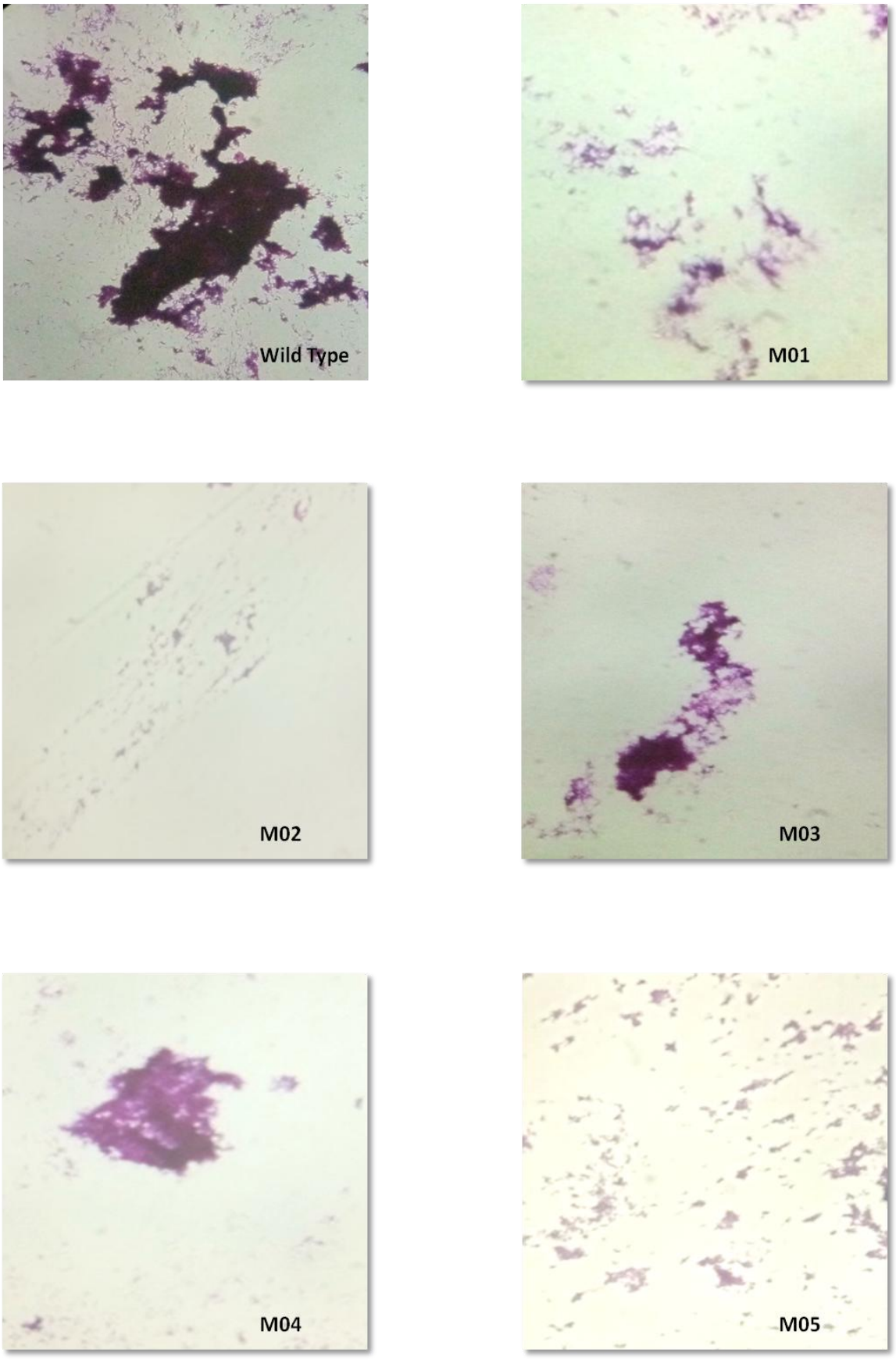

Figure 4.12: Day 04: Biofilm formed *M. fortuitum* wild type and mutants stained with carbol fuchsin stain.

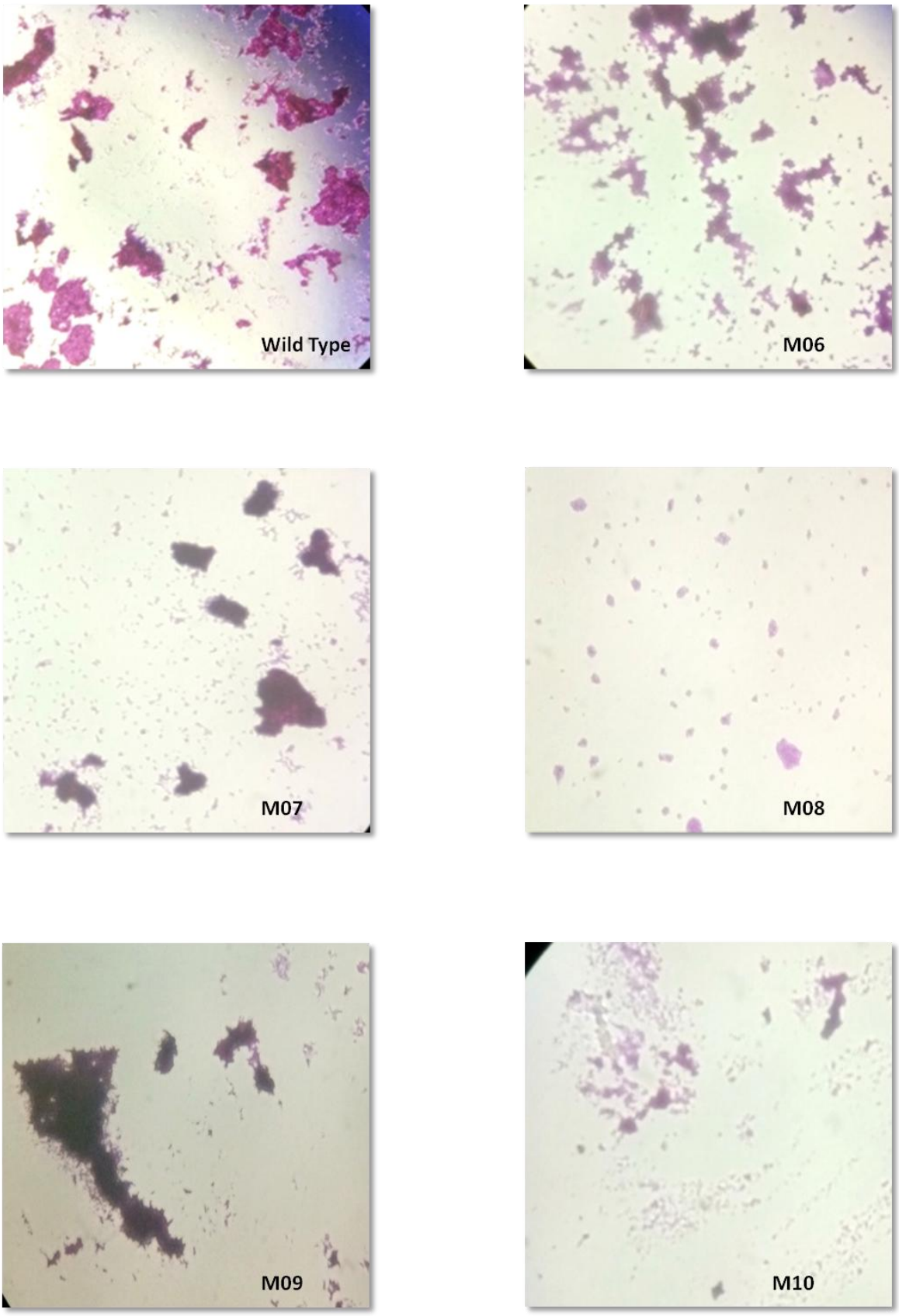

Figure 4.13: Day 04: Biofilm formed *M. fortuitum* wild type and mutants stained with carbol fuchsin stain.

CHAPTER 5

DISCUSSION AND CONCLUSION

M. fortuitum has been reported to cause multitude of infections like ocular morbidity, surgery related infections, infections due to use of catheter, hip implants, and other nosocomial infections. It has become an important microorganism because of its increasing pathogenesis towards human. Biofilm forming ability adds to its pathogeneticity as it provides resistance against antibiotics and allow them to grow in adverse environmental situation like water supply lines and so on. The increase in number of cases of *M. fortuitum* infections and its increasing resistance towards currently used antibiotics requires development of new intervention strategies for controlling infections.

The current study aims at finding the gene responsible for biofilm formation so, that it can be targeted for developing new intervention techniques. So as to identify the gene, a genome wide approach of random mutagenesis using transposon was used. This method has advantage over the gene silencing method as it allows identification of many genes, once the library has been created. The random nature of integration into the genome also increases the chance of finding some new gene, specific to the microorganism. Gene silencing approach requires the sequence of the gene as well as it only determines the function of the gene in study. Thus, transposon mutagenesis allows us to identify and determine the function of the gene at a wider scale. Transposon mutagenesis was achieved in my study by using the transposon TnphoA which has kanamycin marker and alkaline phosphatase enzyme. The transformed cells having TnphoA transposon can easily be identified using kanamycin as a marker along with X-gal to give blue colonies for visual selection. These blue colonies contain transposon mutants that have disrupted membrane gene. These mutants are screened for ability to form biofilm so as to identify the role of the gene in biofilm formation.

The screening test for determining the biofilm forming ability of the mutants is done through microtiter plate test as it offers following advantages: cost effectiveness, feasibility and less chance of contamination as compared to microfermentor test. The

screening test involves crystal violet assay and carbol fuchsin staining. Crystal violet assay allows the quantification of the biofilm formed in term of stain it can absorb. Carbol fuchsin staining allows visual microscopic examination of the biofilm formed. Both tests in tandem can help to deduce the amount of biofilm formed by the bacteria.

Based on previous study done on biofilm formation by mycobacteria, it has been reported that 28 days are required to have complete biofilm formation. So, in my study the first screening was done by assaying biofilm formation by *M. fortuitum* in course of 28 days. Out of the 5 mutants screened, 2 mutants i.e. M02 and M05 showed attenuation in biofilm formation. The screening condition yielded result but it require a time of 28 days which is not feasible for screening large number of mutants in short duration. Moreover, incubating for that long duration lead to increased chances of contamination.

So, as to address the problem of time a new protocol was standardised which includes incubation for just 4 days at static condition. Further screening of the earlier screened 5 mutants resulted in the same result i.e. attenuation of biofilm formation in M02 and M03. Thus, it was validated that the new protocol is at par with the old one with the advantage of having a short experimental time. The new protocol was used to screen 5 more mutants, out which one, M08 showed attenuated biofilm formation.

Overall out of 10 mutants screened, 3 showed attenuation in biofilm formation. The sequence of the disrupted gene in these mutants can be identified using DNA sequencing. Followed by bioinformatics analysis, the role of the gene can be identified. These genes or their products can be used as a target for drugs or any new intervention strategies to tackle the increasing infection problems.

The molecular mechanism of biofilm development in mycobacteria has not been fully understood till now. The molecular signalling pathways causing initiation and development of biofilm and molecules involved in quorum sensing has not been intensively studied. The onset of new high throughput technologies, advanced microscopy and biochemical tools can be used to understand the biological complexities involved in biofilm formation and their likely role in recalcitrance of mycobacterial infections.

CHAPTER 6

APPENDIX A

6.1 Bacteriological media

All the media were prepared in distilled water and autoclaved at 15 pounds per square inch for 15 min., unless otherwise indicated.

LB Broth (Luria Bertani Broth)

Table 6.1: Composition of LB broth

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

The components were dissolved in 950mL distilled water and the pH was adjusted to 7.5 with 5N NaOH and finally, the volume was made to 1000 mL with distilled water. Agar at a concentration of 1.5 % was added whenever solid medium was required.

Nutrient Broth

Table 6.2: Composition of Nutrient broth

Peptone	5 g
Yeast extract	1.5 g
Beef extract	1.5 g
NaCl	5 g

The components were dissolved in 950mL distilled water and the pH was adjusted to 7.5 with 5N NaOH and finally, the volume was made to 1000 mL with distilled water.

Nutrient Agar Tween80 (NAT)

Table 6.3: Composition of NAT

Nutrient Broth	13 g
Tween 80	500 µL (0.05%)

The components were dissolved in 950mL distilled water and the pH was adjusted to 7.5 with 5N NaOH and finally, the volume was made to 1000 mL with distilled water. Agar at a concentration of 1.5 % was added whenever solid medium was required.

Middle brook (MB)7H9 broth

Table 6.4: Composition of MB7H9 broth

MB7H9 broth base	4.7 g
Tween 80	1.5 mL (0.15%)
Glycerol	5 mL (0.5%)

The components were dissolved in 950mL distilled water and the pH was adjusted to 7.2 with 5N NaOH and finally, the volume was made to 1000 mL with distilled water.

6.2 Reagents for Acid Fast Staining

i. Carbol fuchsin (Primary stain)

Table 6.5: Composition of carbol fuchsin stain

Basic fuchsin	3 g
Phenol	5%
Ethanol (96%)	10 ml

10 mL of Basic fuchsin prepared in 96% ethanol was mixed with 90 mL of phenol and the solution was filtered through Whatman filter paper.

ii. Acid alcohol (Decolorizer)

Table 6.6: Composition of acid alcohol

HCL (conc.)	3 mL
Ethanol (96%)	97 mL

iii. Malachite green solution (Counter stain)

Table 6.7: Composition of malachite green

Malachite green	0.5 g
Distilled water	100 mL

6.3 Antibiotics and Substrates

All antibiotic solutions were filter sterilized by a 0.22 µm filter (Millipore) and stock solutions were stored at -20 ^{0}C for long-term use.

Table 6.8: Concentration of antibiotics and substrates

Reagent	Stock Solution	Final Concentration (in E. coli)	Final Concentration (in Mycobacterium)
Kanamycin	10 mg/ml in H_2O	50 µg/ml	30 µg/ml
Cycloheximide	5 mg/ml in H_2O	100 µg/ml	50 µg/ml
X-gal	20 mg/ml in DMSO	40 µg/ml	40 µg/ml

6.4 Reagents and Buffers

All the reagents and buffers for DNA and protein protocols were prepared in Milli Q grade water and sterilized by autoclaving for at 15-psi pressure 15 minutes unless otherwise indicated.

i. Tris HCL buffer

Tris-HCL buffer of desired strength was prepared by dissolving appropriate amount of Tris in distilled water and adjusting the pH with concentrated HCl. For bacteriological work 10 mM Tris-HCl (pH 8.0) was prepared.

ii. Ethylene diamine tetra acetic acid (EDTA)

0.5 M solution of disodium salt of EDTA was prepared in distilled water, pH was adjusted to 8.0 with NaOH pellets and stored at 4 ^{0}C.

iii. Normal Saline

Table 6.9: Composition of normal saline

NaCl	8.9 g
Distilled water	1000 ml (final volume)

iv. Tween Normal Saline

Table 6.10: Composition of tween normal saline

NaCl	0.9%
Tween 80	0.1%
Distilled water	100 ml

v. Alkaline Lysis Solution I

Table 6.11: Composition of ALS I

Tris-HCL (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM
Glucose	50mM

vi. Alkaline Lysis Solution II

Table 6.12: Composition of ALS II

NaOH	0.2N
SDS	1.0%

vii. Alkaline Lysis Solution III

Table 6.13: Composition of ALS III

5M Potassium acetate	60 ml
Glacial Acetic acid	11.5 ml
Distilled water	28.5 ml

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PUBLICATIONS

1. **Rahul Pramjeet, Poonam, Arpita Prasad, Rahul Shrivastava** (2017). Random Mutagenesis for Identification of potential drug targets for Non tubercular Mycobacteria. 2nd Himachal Pradesh Science Congress (HPSC-2017) [Hotel Peterhoff, Shimla, Himachal Pradesh, India: 20-21 November, 2017]

Poster

Random Mutagenesis for Identification of potential drug targets for Non-tubercular Mycobacteria

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ABSTRACT

Non-tuberculous mycobacteria (NTM) are a heterogeneous group of environmental bacteria with distinct virulence features. NTM includes *M. abscessus, M. fortuitum, M. avium,* etc. responsible for several opportunistic nosocomial infections causing disseminated healthcare associated diseases. NTM has been gradually evolving resistance against the currently prescribed drugs, obstructing treatment of diseases caused. Biofilm formation plays an important role in the pathogenesis of most NTM. The present study aims to identify genes responsible for biofilm formation in *M. fortuitum, a* representative of NTM, by random mutagenesis. Random mutagenesis of *M. fortuitum* was done by electroporation of vector *pRT291* containing transposon Tn5. *M. fortuitum* mutants were screened on NAT plates containing Kanamycin, Cycloheximide, X-gal, and IPTG. A library of about 5000 mutants was constructed, of which 127 blue mutants were confirmed for insertion of transposon in membrane gene by alkaline phosphatase activity. The mutants having highest alkaline phosphatase activity were individually subjected to the standardised biofilm forming protocol, to check for any deviation in the amount of biofilm formed. Crystal violet assay and carbol fuchsin staining of biofilm revealed reduced biofilm formation in one mutant. Genomic analysis followed by homology study of the mutant was done, which lead to identification of a hypothetical membrane protein showing 82% homology with *Rv3174* gene of *Mycobacterium tuberculosis,* involved in intermediary metabolism and respiration. Genes identified by the study would serve as potential drug targets for development of novel drugs or other intervention strategies applicable to NTM species with similar mechanism of biofilm formation during infection.

INTRODUCTION

- Mycobacteria are ubiquitous in nature and can be found in soil, dust, rocks, bioaerosols, and water.
- NTM are etiological agents of several respiratory and extra-respiratory infections.
- Presence of a mycolic acids in cell wall.
- Long generation time.
- Biofilm makes it resistant to high concentrations of antimicrobial drugs.
- Difficult to eradicate as compared to their planktonic counterparts.
- Membrane genes play important role in the biofilm formation.
- Identification of genes responsible for biofilm formation can help in development of novel drugs against Mycobacterium.

Fig.1 *M. fortuitum* under microscope (100X).

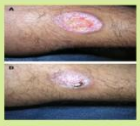


Fig.2. Patient with *M. fortuitum* skin infection before (A) and after (B) 2 months of treatment with clarithromycin and ciprofloxacin

Fig.3. Nasolacrimal obstruction and granulomatous eyelid disease caused by *M. fortuitum.*

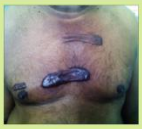


Fig.4. Multifocal keloids associated with M. fortuitum following intralesional steroid therapy

MATERIALS & METHOD

Construction of transposon mutant library of M. fortuitum using pRT291

↓

Selection of transposon mutants with X-gal, IPTG (Isopropyl β-D-1-thiogalactopyranoside) and glucose

↓

Determination of alkaline phosphatase activity by pNPP assay

↓

Attenuated mutants proceeded for cloning in pUC19 vector

↓

Selected mutants were subjected to standardized biofilm formation protocol

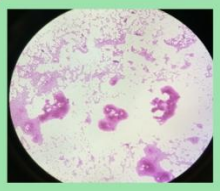
↓

Mutants ability to form biofilm was checked by staining with crystal violet and measuring optical density at 570nm

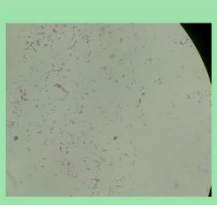
↓

Mutants were stained with carbol fuchsin to check biofilm formation

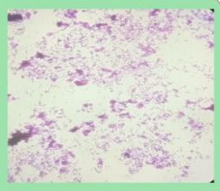
RESULTS



Wild type *M. fortuitum* showing biofilm formation on 11th day



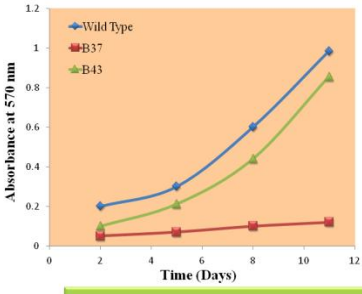
B37 mutant showing no biofilm formation on 11th day



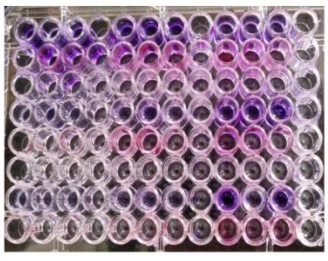
B43 mutant showing biofilm formation on 11th day

B37 TnphoA mutants of *M. fortuitum* showed attenuation in biofilm formation as compared to wild type *M. fortuitum*

Biofilm Solubilization



Crystal Violet Biofilm Assay



SIGNIFICANCE

B37 mutant showed attenuation in the biofilm formation as compared to the wild type. Genomic analysis of these mutants showed 82% homology with *Rv3174* gene of *Mycobacterium tuberculosis,* involved in intermediary metabolism and respiration. The present results indicate the probable role of this hypothetical membrane gene in the biofilm formation of *M. fortuitum* and thus, can be used as potential drug target against *M. fortuitum* and other non-tuberculous mycobacteria.

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