# IDENTIFICATION OF NOVEL GENE(S) RESPONSIBLE FOR DRUG RESISTANCE IN *MYCOBACTERIUM FORTUITUM*


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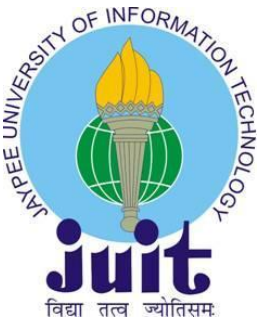


# CERTIFICATE

This is to certify that the work titled **"Identification of Novel Gene(s) Responsible for *Mycobacterium fortuitum* Drug Resistance"**, submitted by "**Monika Pradhan (121559) and Kanika Sharma (123816)"** in partial fulfillment for the award of degree of B. Tech in Biotechnology of Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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# ABSTRACT

*Mycobacterium fortuitum* is rapidly growing, nontuberculous mycobacteria (NTM) which is ubiquitously present in nature. It is one of the most important pathogenic species, representing more than 80% of clinical isolates of rapidly growing mycobacteria. To add up insult to injury, an obstruction is imposed on the way to its treatment as these organisms are evolving into a drug resistant species. Prolonged antibiotic therapy is now a necessity for *M. fortuitum* infection. Considering these crisis, our study aims to identify the resistant gene(s) and the mechanism associated, and the subsequent use of these findings in establishing effective drug targets.

As the genomic sequence of *M. fortuitum* is not known, random mutagenesis technology (using TnphoA) was employed. The plasmid (pRT291) containing transposon Tn5 was electroporated in *M. fortuitum* wild type strains which resulted in formation of 50 blue mutant colonies showing mutation. These mutants were further screened by β-galactosidase assay. 10 mutants (i.e. KM1 to KM10) having high β-galactosidase activity were shortlisted for drug profiling where Ciprofloxacin, Amikacin, Rifampicin, Isoniazid and Ethambutol were utilized as these are most commonly used for treatment of *M. fortuitum* related infections. The minimum inhibitory concentration (MIC) of mutant strain KM4 for Rifampicin was observed to increase by 4-fold whereas the strain KM5 was sensitive to it. All the mutants had same MIC as that of wild type when Isoniazid was used, but they were sensitive to Amikacin (4-fold decrease in MIC).

Genomic analysis of these mutants was done by restriction digestion of their genomic DNA, cloning of the insert containing transposon into pUC19, followed by sequencing. Comparative genomic analysis of mutants revealed two hypothetical genes of *M. fortuitum* i.e. "Antranilate synthase" and "NusA ribosome maturation factor RimP" which may be used as potential drug targets.

**Monika Pradhan (121559)** **Dr. Rahul Shrivastava**

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**Date:**

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# LIST OF SYMBOLS AND ACRONYMS

**ALS** - Alkaline Lysis Buffer

**$^0$C –** Degree Celsius

**GTE** - Glucose Tris EDTA

**IPTG –** Isopropyl β-D-1-thiogalactopyranoside

**KAN$^R$ –** Kanamycin Resistance

**LB –** Luria Bertani Broth

**MIC** – Minimum Inhibitory Concentration

**mg/mL –** milligram per milliliter

**nm –** Nanometer

**OD –** Optical Density

**ONPG –** Ortho-Nitrophenyl-β-Galactoside

**pNPP –** *p*-Nitrophenyl Phosphate

**RPM –** Revolution Per Minute

**SDS –** *Sodium Dodecyl Sulfate*

**sec. –** Seconds

**µF-** Micro-farad

**µL –** Micro-liter

**µg/mL –** Microgram per per milliliter

**X-GAL -**X –galactosidase

**% -** Percentage

# Chapter 1

# *Introduction*

# 1. Introduction

*Mycobacterium fortuitum* is categorized as nontuberculous mycobacteria (NTM) under the sub-division of rapid growers due to their ability to form colonies within 7 days. It is slender, nonmotile, acid-fast bacilli with a thick hydrophobic cell wall that assists in prevention of its dessication. NTM or environmental mycobacteria can be found in diverse environments across the world, and some species (including *M. fortuitum*) are capable of infecting humans and animals (WHO, 2004). Particularly, drinking water has been shown as a potential source of NTM infections due to the high resistance of NTM to disinfectants [1, 2]. A case of postoperative breast infection by *M. fortuitum* was also reported where in hospital water supply was seen as the definite source of infection. The route of infection was surgical site and it was deciphered that the patient had acquired the pathogen during postoperative care[3]. The recovery of NTMs in the hospital water supply has been widely documented, and the proportion of RGMs accounts for up to 50% of NTM isolates [4]. *M. fortuitum* is especially resistant to chlorination and is capable of biofilm formation. In chlorine and ozone disinfection, the Ct values for 3-log inactivation of *Mycobacterium avium* are at least 580- and 50-times greater, respectively, than those for *Escherichia coli* [5]. Mycobacterium fortuitum is 10-fold more resistant to ultraviolet (UV) than *E. coli* for 3-log inactivation. Accordingly, a study by Dodiuk-Gad et.al stated that the most common species in the United States and Europe are *Mycobacterium marinum* and the rapidly growing mycobacteria (RGM) *Mycobacterium abscessus, Mycobacterium fortuitum,* and *Mycobacterium chelonae* [6].

Launch of immunosuppressive therapies and the advent of the dreadful AIDS epidemic have increased the incidence of NTM-associated diseases dramatically and since then, NTMs have been acknowledged as important pathogens.

Human disease resulting from NTM infection is classified into distinct clinical syndromes which include; pulmonary infection, local non-tender lymphadenitis, skin and soft-tissue infections and chronic granulomatous infections of bursae, joints, tendon sheaths, and bones. In addition, infective complications caused by RGM after surgical procedures and catheter use, as well as

disseminated infections, especially in immunocompromised hosts, have been widely documented [7].

But sometimes, the isolation of NTM species from a respiratory sample is not a sufficient evidence to show the presence of NTM lung disease. Some patients are infected with NTM without any evidence of pulmonary disease. Such infection may indicate colonization (i.e., recovery of organisms more than once without demonstration of specific pulmonary disease) or transient infection (i.e., transient isolation of organisms with subsequent negative cultures and no evidence of disease progression).

For instance, in a study conducted by Sunghoon et.al to analyze clinical significance of *M. fortuitum* isolated from respiratory specimens; a chest radiograph and CT of a 53-year-old woman with a history of previous treatment for pulmonary tuberculosis 30 years earlier revealed multiple cavitary consolidation in the entire left lung. Numerous AFB was detected in multiple sputum specimens (4+).*M. fortuitum* was consistently isolated and so the patient was started on a three-drug regimen of clarithromycin, ciprofloxacin, and sulfamethoxazole. Her symptoms and radiographic abnormalities improved with continuing antibiotic treatment for 10 months and negative sputum smear and culture conversion was achieved and maintained after 1 month of treatment [8].

Several reports have also described *M. fortuitum* lung disease in patients with pre-disposing factors, such as malignancy, renal transplantation, chronic reflux disease, achalasia, bronchiectasis, and cystic fibrosis. Several centers around the world have reported the isolation of NTM in the respiratory tracts of CF patients. Nowadays, this prevalence varies between 5 and 20 %{ [9-14]}, with most of those cases (90%) caused by species of the *Mycobacterium avium* complex (MAC) and the *Mycobacterium abscessus* group. Besides, treatment of RGM infections is challenging; drug therapy normally comprises a multidrug regimen for a lengthy duration, is costly and is associated with drug-related toxicities. Moreover, response rates are highly variable, particularly in pulmonary RGM infections, with cure rates of only 30% to 50% [15].

S. S. Tang et.al reported in a study of rapidly growing mycobacteria in Singapore, (2006–2011) that, the two most common species of RGM identified were *M. abscessus* (73%) and *M.*

*fortuitum* group (22%), with amikacin and clarithromycin being most active against the former, and quinolones and trimethoprim-sulfamethoxazole against the latter. Increase in resistance of *M. abscessus* to linezolid by 8.8% per year (p 0.001) and *M. fortuitum* group by 9.5% per year (p 0.023) and 4.7% per year (p 0.033) to imipenem and clarithromycin were observed respectively. Incidence rates of 7.2 to 13.6 cases per 100 000 persons were recently reported [16]; [17]. The proportion of NTM contributed by RGM has increased more than 2-fold to 35% in 2001 vs. 14% for the period 1992 through 1996 [18].

As a supplement to this evidence; drug susceptibility testing of 31 antimicrobial agents on RGM isolates (from China) by Pang et.al revealed that the strains were highly resistant to the four first-line antituberculosis agents tested on them, especially *M. chelonae*, *M. abscessus*, and *M.fortuitum*. They showed that *M. abscessus* (75.34%) is the predominant RGM species in the 73 clinical isolates, followed by *M. fortuitum* (15.07%). Both organisms were susceptible to amikacin, linezolid, tigecycline, cefmetazole, capreomycin, moxifloxacin, macrolides, and carbapenems, but were highly resistant to the first line anti-tuberculous drugs, daps one, thioacetazone, and pasiniazid.

These studies indicate that an obstruction is imposed on the way to treatment of *M. fortuitum* related infections as these organisms are evolving into a drug resistant species. Prolonged antibiotic therapy is now a necessity in effective treatment but this strategy cannot be relied on perpetually. There is a dire need to have a profound knowledge about the gene(s) involved in resistivity to understand the virulence mechanism of this organism so that an effective drug target can be established to initiate a treatment with least possible adverse effect to its host.

# Chapter 2

# *Review of Literature*

# 2. Review of Literature

## 2.1 Introduction

Mycobacterium is found to be the best-studied bacterial genus and currently contains more than 100 species [19]. Rapidly growing mycobacteria (RGM) are categorized as Runyon group IV organisms that usually form colonies within 7 days of incubation as opposed to slow-growing mycobacteria, i.e., Runyon groups I, II, and III and the Mycobacterium tuberculosis complex which usually require more incubation time [20]. RGM has emerged as significant human pathogens, causing various infections mostly in immunocompromised hosts. Although general identification of RGM can be made with accuracy, further species identification is required which is generally a tedious biochemical process [21]. Genomics has defined an unsteady array of new nontuberculous mycobacterial species and, the modern taxonomic era has been defined. This more précised species-specific identification has become possible by sequencing a number of target genes that includes small ribosomal subunit (16S rRNA) gene which is accurate, and reproducible method that were in use for years .*Mycobacterium fortuitum* is known as a nontuberculous mycobacterium (NTM), a grouping that enclose all mycobacteria outside of the *Mycobacterium tuberculosis* complex. *M. fortuitum* is classified in the Runyon group IV category [21].

## 2.2 Epidemiology

*M. fortuitum* exceptionally tolerate and can survive in even the most hostile of environments, residing primarily in soil and water supplies. It has been found in both natural and processed water sources, as well as in sewage and dirt. Most of NTM infections are not reported [22]. Therefore, exact estimates of disease prevalence and incidence are impossible to control. The most recent estimates come from the reports presented by the Centers for Disease Control and Prevention (CDC). 4.65-5.99 cases per million persons were reported to the CDC, from 1993-1996. Sputum is a frequently reported site which is represented as most likely source to be cultured for mycobacteria [23].

## 2.3 Clinical Significance

Nontuberculous mycobacterial infections are becoming more common. Recently, *M. fortuitum* and other rapidly growing mycobacteria have been found to cause severe skin and soft-tissue infections in association with nail salon whirlpool footbaths. The first case was reported on 1990, When Sack (1990) described a patient with a history of intravenous drug abuse and AIDS, having cutaneous lesions from which *M. fortuitum* was isolated. It was recently investigated that a large outbreak of furunculosis among women who received pedicures at a single nail salon was due to *M. fortuitum*. The mean disease duration was 170 days (range, 41– 336 days). Forty-eight persons received antibiotic therapy for a median period of 4 months (range, 1–6 months), and 13 persons were untreated. Isolates were most susceptible to ciprofloxacin and Minocycline. Early administration of therapy was associated with shorter duration of disease only in persons with multiple boils. One untreated, healthy patient had lymphatic disease dissemination[24].

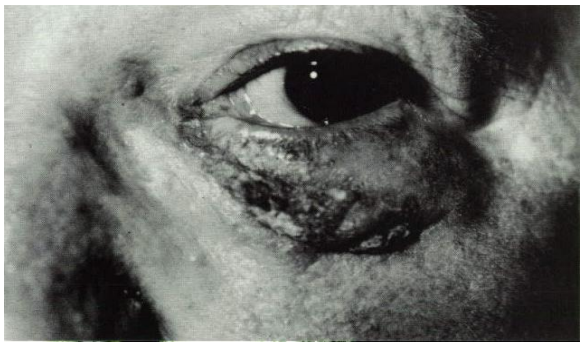


**Fig.2.** Patient with *M. fortuitum* skin infection before (A) and after (B) 2 months of treatment with clarithromycin and ciprofloxacin [25]

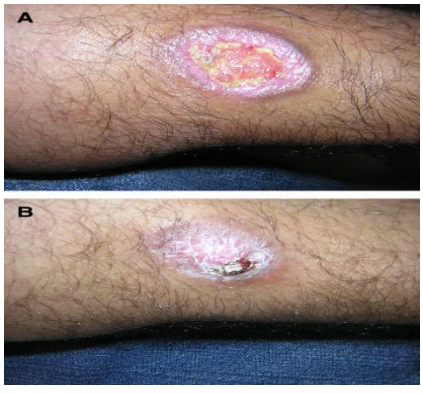



**Fig.3.** Patient with *M. fortuitum* skin infection before (A) and after (B) 2 months of treatment with clarithromycin and ciprofloxacin[6]

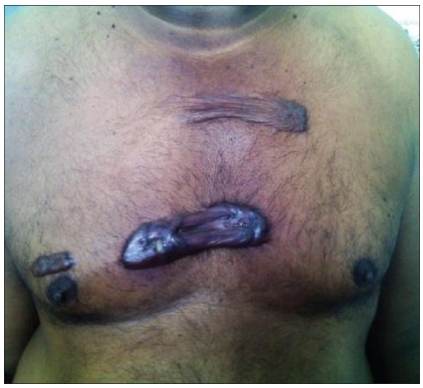


**Fig.4.** Multifocal keloids associated with *M. fortuitum* following intralesional steroid therapy[26]

*M. fortuitum* can also cause pneumonia, abscess and empyema in patients with predisposing lung diseases. However, pleurisy with effusion is rare. Fabbian et.al reported a case of a 74-year-old immunocompetent female patient (without apparent risk factors), who suffered haemorrhagic pleural effusion as the main clinical manifestation. Pleural nodules were detected by computed tomography scan, and microbiological analysis revealed *M. fortuitum* in the absence of other pathogens. The patient was treated with Ceftriaxone and Ciprofloxacin, and full recovery was ensued in 4 weeks. This was the first reported case of haemorrhagic pleural effusion in an immunocompetent patient without underlying diseases [19]. Hoy et.al states that usually, a non-tuberculous mycobacteria may not be accompanied by pleural effusion, however, *M. fortuitum* should be considered in such cases, especially when microbiology fails to detect the usual pathogens, and when the clinical picture is unclear [27].

### 2.4 Pathology

Human disease resulting from NTM infection is classified into distinct clinical syndromes which include; pulmonary infection, local non-tender lymphadenitis, skin and soft-tissue infections and chronic granulomatous infections of bursae, joints, tendon sheaths, and bones. In addition, infective complications caused by RGM after surgical procedures and catheter use, as well as disseminated infections, especially in immunocompromised hosts, have been widely documented [7].

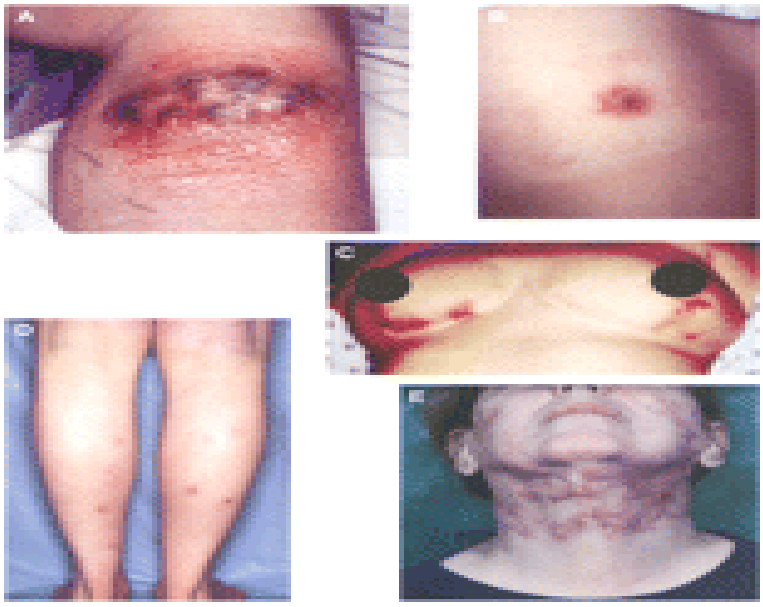


**Fig.5.** Infections due to rapidly growing mycobacterium. A, disseminated infection to soft tissues after systemic steroid used; B, abscess after infection with contaiminated adrenal cortex solution; C, breast reduction – associated infection; D, pedicure – associated infection; E, cosmetic surgery – associated facial infection.[28]

### 2.4 Treatment

Nonpulmonary infections due to *M. fortuitum* or *M. chelonei* were treated by wound debridement and with chemotherapy on the basis of in vitro susceptibilities of the organism. Non pulmonary infections due to *M. fortuitum* or *M. chelonei* were treated by wound debridement and with chemotherapy on the basis of in vitro susceptibilities of the organism [29]. For serious disease with *M fortuitum*, the aminoglycoside amikacin, combined with a β-lactam (cefoxitin or imipenem) or a quinolone (ciprofloxacin or Lofloxacin), has been used for initial therapy [30]. Surgical debridement of cutaneous or subcutaneous lesions, especially if the lesions are extensive, is usually required for cure [31].

Surgical debridement of ocular and bone lesions is almost always required. Surgical excision of pulmonary lesions may be considered if response to therapy is lacking or if the organism is relatively resistant to antibiotics. Surgical excision of lymphadenitis is the therapy of choice and is usually curative. If the infection involves an implanted device, removal of the device is usually necessary for cure [31].

In some cases antibiotics may not be effective, and the infection caused by *M. fortuitum* tends to be recurrent and resistant to treatment. Upon failure of the antibiotics, surgery is other option. Surgery entails removal of affected areas or implanted medical or cosmetic devices [32].

### 2.5 Antibiotics used for the treatment of *Mycobacterium fortuitum* infection

Resistance occurs naturally but it is greatly accelerated by patients who do not complete the prescribed treatment, generally when it lasts for months. Streptomycin (STR) resistance in *Mycobacterium,* a genus that includes important human pathogens, such as *Mycobacterium tuberculosis*, along with fast-growing opportunistic species, such as *M. fortuitum,* is due to several mechanisms. Mutations in either the S12 ribosomal protein or the 16S rRNA confer high and moderate levels of STR resistance. The drugs most commonly used for *M. fortuitum* treatment are Amikacin, Ciprofloxacin, Clarithromycin, and sulfamethoxazole.

The mode of action of ciprofloxacin is the inhibition of the enzyme topoisomerase II i.e. DNA gyrase and also topoisomerase IV that are the both type II topoisomerase that are essential for bacterial DNA replication, transcription, repair, and recombination.

A man-made aminoglycoside antibiotic i.e., Amikacin is similar to gentamicin and tobramycin. It mainly bind to some important bacterial proteins that are produced by the components of bacteria blocks the protein synthesis which lead to stopping further bacterial growth. Amikacin is used to treat bacterial infections that are resistant to tobramycin and gentamicin. It is also used to treat infections that are caused by different gram negative bacterial species such as *Escherichia coli*, Indole-positive and indole-negative *Proteus* species, *Pseudomona* species, *Providencia* species, *Klebsiella-Enterobacter-Serratia* species and Acinetobacter and also used to treat certain staphylococcus infection as well.

Clarithromycin binds to 23S rRNA that is a component of the 50S subunit of bacterial ribosome able to inhibit the translation of peptides and prevent bacteria by acting as protein synthesis inhibitor.

## 2.6 Transposon mutagenesis

Pathogenesis does not concern much unless it can be treated properly. But, the prokaryotic species have the ability to mutate at a much faster rate, for example, *M. fortuitum* has already started to develop a resistance against some of the drugs used for its treatment. And increasing the dosage may not be a good idea as it might be lethal for the patient also. So, the study of a particular gene involved in virulence and suppression of its expression might be a helpful tool in tackling problem due to drug resistance. One such method which can be utilized is, random mutagenesis of the gene coding for membrane protein of *M. fortuitum* by inserting a transposon (Tn5) which would blocks its expression. As *M. fortuitum* has not yet been fully sequenced, random mutagenesis would be most appropriate in the study of gene functions.

The principle of transposon mutagenesis involves insertion of foreign DNA, a transposable element, into many sites in the bacterial genome, ideally on a completely random basis. These events require a selectable phenotype, generally an antibiotic resistance marker carried within the transposon. The Tn5 based transposon inserts randomly (Meis, 2000) and can create knock-out in genes. Transposomes may be introduced into the chromosomes of a bacterial species by use of 'suicide vectors', or bacterial phages, carrying transposon and their electroporation (Goryshin *et al*., 2000; Hoffman *et al*., 2000).

Thus, to confirm the insertion of Tn*phoA* in the membrane spanning protein of the mutants obtained, alkaline phosphatase activity of all the mutants will be measured and compared with that of wild type (WT) *M. fortuitum*. About 2-3% of the insertion mutants within library are expected to show alkaline phosphatase activity and appear as 'blue' colonies on Nutrient Agar plates supplemented with 0.05% Tween 80 (NAT), due to a functional alkaline phosphatase gene. Such 'blue' mutants carry Tn*phoA* insertions inframe with genes encoding the secretory or membrane spanning proteins. **Figure 6** shows the map of Tn*phoA* and generation of fusion as a result of Tn*phoA* insertion. To confirm the inframe fusion, alkaline phosphatase activity of the blue mutants will be determined using p-NPP assay.

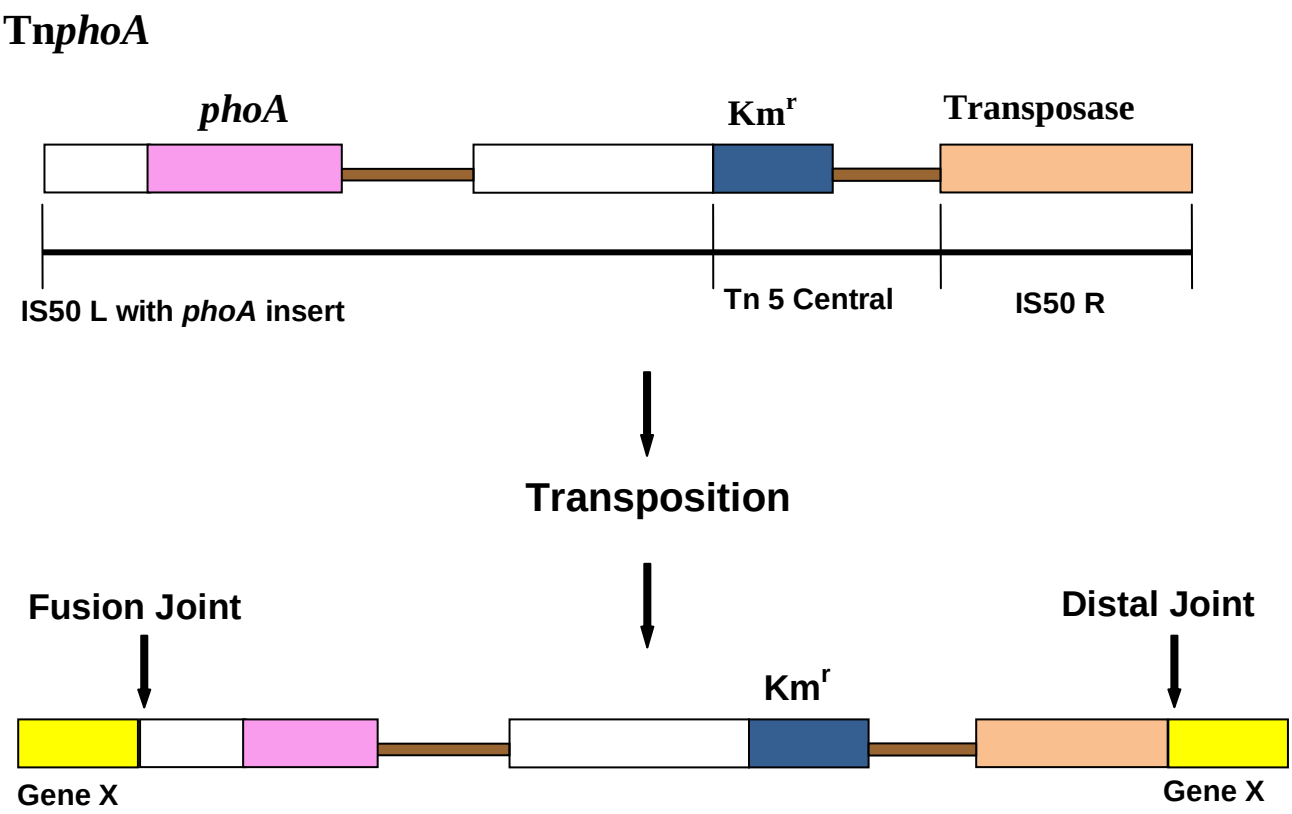



**Fig.6.** Schematic presentation of Tn*phoA* and Gene Fusion[33]. *Events leading to the formation of an active gene - transposon fusion employing use of TnphoA. The transposon is a derivative of Tn5 with a region encoding E. coli alkaline phosphatase, minus the signal sequence and expression signals, inserted into the left IS50 element. Active insertions into 'Gene X' interrupt the gene and result in the production of a hybrid protein from gene X - phoA fusion.*

# Chapter 3

# *Materials and Methods*

# 3. Materials and Methods

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

3.1.1 Isolation of plasmid pRT291:

*Chemical requirements*: 300µl of GTE (Glucose tris EDTA), ALS (Alkaline lysis buffer), 600µl of SDS-NaOH solution, 450µl of acetate solution, 2700µl of absolute ethanol, 96% ethanol, 70% ethanol, 40µl of autoclaved distilled water.

*Equipments used*: Centrifuge, Fresh tarson tubes, Eppendorf tubes, Discard box, Pipette, 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 $37^0$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 tarson tube and 2700µl of absolute ethanol was added.
10. The mixture was then incubated overnight at $-20^0$c.
11. The plasmid was then transferred into eppendorf tubes and centrifuged at 12000 rpm for 15 minutes and supernatant was removed.
12. 96% ethanol was added to eppendorf tubes and centrifuged at 10,000rpm 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 till ethanol was evaporated.
15. 40µl of autoclaved distilled water was added and placed at $-20^0$c.

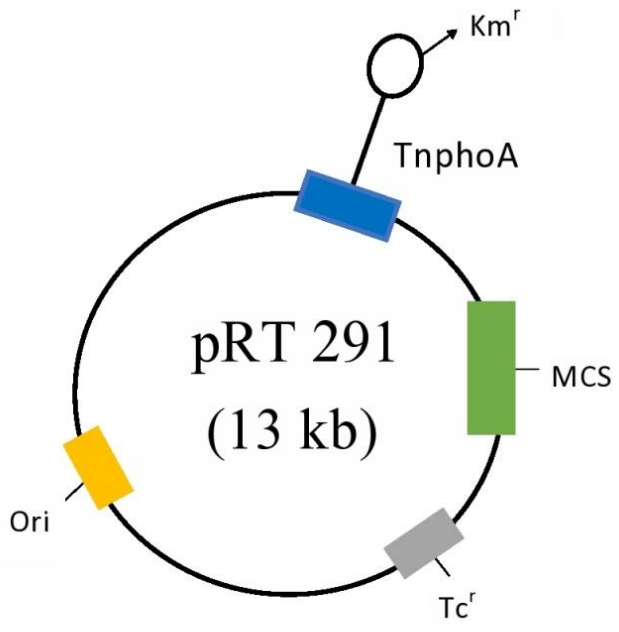



**Fig.7.** Vector map of pRT 291 having TnphoA, MCS, Tc$^r$, ORI (Origin of replication)

3.1.2 Preparation of electrocompetent cells:

*Chemical requirements*: 200 ml LBGT, 10% glycerol, seed culture.

*Equipments used*: Centrifuge, ice box, spectrophotometer, flask, pipette, tips.

Procedure:

1. Seed culture was inoculated into 200 ml LBGT and incubate it with shaking at $37^0$c overnight
2. O.D$_{600}$ was taken till it reached to 0.6-0.8.
3. The cells were kept on ice before harvesting for about 40-60 minutes.
4. Then centrifuged them at 4000 rpm for 10 minutes at $4^0$c.
5. The pellet was taken and washed with water or 10% glycerol. Again it was washed and reduced the volume of agent each time.
6. Then it was suspended in 1-2ml 10% glycerol.
7. Freezed them at $80^0$c.

3.1.3 Protocol for electroporation:

*Chemical requirements*: 400 micro liter of electrocompetent cells, 400 micro liter of electrocompetent cells, 2ml of LBGT, X-gal, IPTG, Ampicillin, Kanamycin.

*Equipments used*: Cuvettes, Electroporation machine, Ice box, pipettes, and tips.

Procedure:

1. 400 micro liter of electrocompetent cells were taken in which 400 micro liters of electrocompetent cells was added in a cuvette.
2. Cuvettes were kept on ice for 5-10 minutes.
3. Electroporation occur: capacitance-25 µF, resistance-1000Ω, voltage-2500 volts, and cuvette-2 mm.
4. Pulse was given twice and again kept them on ice.
5. 2ml of LBGT was added and incubated at $37^0$c for 3 hours with shaking.
6. Then it was spread on selection plate containing X-gal + IPTG + Ampicillin + kanamycin.

### 3.2 Screening and Selection of mutants by $Kan^r$ & pNPP assay to measure the amount of alkaline phosphatase activity

3.2.1 *p*-Nitrophenyl Phosphate (pNPP) assay:

*Chemical requirements*: Blue colonies of mutant strains , 10ml of LBGT media+ cyclohexemide [50µg/ml] + Kanamycin [30µg/ml], 5µl of lysozyme [2mg/ml], 50µl of toluene, 50µl of 1% SDS, 500µl Z-buffer, 200µl of ONPG [4mg/ml], 500µl of stopping solution i.e. 1M $Na_2CO_3$, distilled water.

*Equipments used*: Eppendorf, Centrifuge, Pipette, spectrophotometer, incubator, tips.

Procedure:

1. Blue colonies of mutant strains were picked up from the master plate prepared earlier and were inoculated in test tubes containing 10ml of LBGT media+ cyclohexemide [50µg/ml] + Kanamycin [30µg/ml].
2. The culture was incubated at $37^0$C until visible growth was observed.
3. 500µl of culture was pipette into 2ml eppendorf vials and 5µl of lysozyme [2mg/ml] was added to it. Then it was incubated at room temperature for 5minutes.
4. 50µl of toluene and 50µl of 1% SDS was added to vial above and vortexed.
5. Then the eppendorfs were placed in incubators with lids open for 15-20 minutes until toluene was evaporated completely.
6. Then 500µl of complete Z-buffer was added to it and incubated in room temperature for 2 minutes.
7. After that, 200µl of ONPG [4mg/ml] was added and incubated at $37^0$C with constant shaking until yellow color appeared.
8. 500µl of stopping solution i.e. 1M $Na_2CO_3$ was added and incubated at room temperature for 10 minutes.
9. The vials were the centrifuged at 10,000 rpm for 3 minutes and the supernatant was taken to dilute $1/4^{th}$ times with distilled water.
10. Then absorbance reading of diluted supernatant was taken in triplicates at wavelength of 420nm and 550nm each.

3.2.2 Acid fast staining to check purity of culture:

*Chemical requirements*: 70% ethanol, adding 50µl of culture (*M. fortuitum* wild type,B1, B2, B3, B4, B5), carbol fuchsin, acid alcohol, malachite green, distilled water.

*Equipments used*: slides, loop, hot plate, dropper, and microscope.

Procedure:

1. The slides were made grease free by cleaning with 70% ethanol.
2. Smear was prepared by adding 50µl of culture (*M. fortuitum* wild type, *KM1, KM2, KM3, KM4, KM5*) on the slide and heat fixing it.
3. Slides were flooded with carbol fuchsin and kept on hot plate of $80^0$C for 5-7 minutes.
4. After 5 minutes, the slides were left to cool down and then washed with $dH_2O$.
5. Then, few drops of acid alcohol was added to decolorize and washed after 20-30 seconds.
6. Few drops of malachite green was added as a counter stain, kept for 45 seconds and it was washed off with $dH_2O$.
7. The slides were then observed under the microscope at various magnifications of 10X, 40X and 100X.

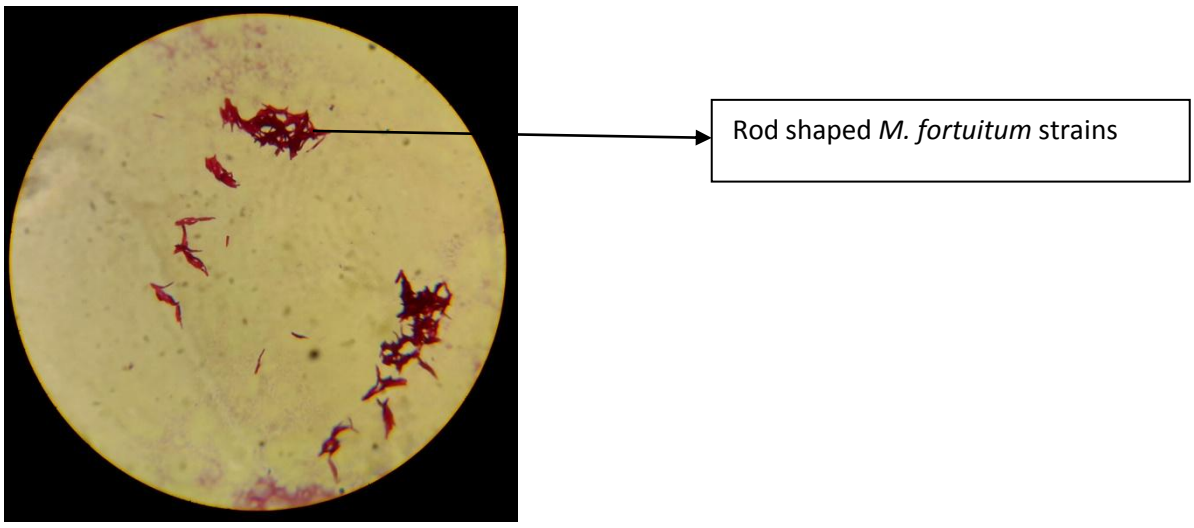



**Fig.8.** *M. fortuitum* (KM5) after acid fast staining at 100X

### 3.3 Drug Profiling of *Mycobacterium fortuitum* with current drugs in use

3.3.1 Antibiotic Susceptibility testing of wild type strain of *M. fortuitum* and its mutant strains:

*Chemical requirements*: Nutrient agar tween, antibiotic of different concentrations (Rifampicin, Isoniazid, Ethambutol, Amikacin, Ciprofloxacin), mutant strains including wild type of M. fortuitum, pipette, tips.

*Equipments used*: Flasks, Petri plates, incubator, micro-titer plate, and spectrophotometer.

Procedure:

1. Nutrient agar tween was prepared in different flasks and the antibiotic of different concentrations was added to it.
2. Then the media was poured into different plates and left to solidify.
3. Then, all the mutant strains including the wild type strain of *M. fortuitum* were streaked on the plate.
4. Plates were incubated at $37^0$C for 2 days and the susceptibility/resistance was observed.
5. Also the strains were cultured in a micro-titer plate containing various concentration of antibiotic to determine its minimum inhibitory concentration.

### 3.4 gDNA isolation of *M. fortuitum* and mutants strains containing the transposon

*Chemical requirements*: TES buffer, 2mg/ml of lysozyme, buffer phenol, chloroform: isoamylalcohol, isopropanol (chilled), Ethanol, autoclaved distilled water, culture of *M. fortuitum* and mutants.

*Equipments used*: centrifuge, viles, eppendorfs, incubator, discard box, gel doc, pipettes, and tips.

Procedure:

1. The culture was taken and pelleted in 50 ml tasson tube at 7000 rpm for 15 minutes.
2. The supernatant was discarded and TES buffer was added in 2ml pellet.
3. Then it was incubated at $80^0$c for about 1 hour. After that 2mg/ml of lysozyme was added to it i.e. 80 micro liter and incubated at $50^0$c for 1 hour.
4. Then buffer phenol was added in each tube and centrifuged them at 12000g for 15 minutes.
5. Then chloroform: isoamylalcohol was added in 24:1 ratio in each tube and centrifuged them at 12000g for 15 minutes.
6. Then the aqueous layer was transferred to fresh tubes. Again chloroform: isoamylalcohol was added and centrifuged at 12000g for 10 minutes.
7. The aqueous layer was transferred to fresh tubes and equal volume of isopropanol (chilled) was added.
8. It was incubated at $4^0$c for overnight and then centrifuged it at 12000g for 15 minutes. Supernatant was removed and 70% ethanol was added (1ml) and centrifuged it at 12000g for 15 minutes.
9. Ethanol was evaporated and 100 micro liter of autoclaved distilled water was added.
10. It was then placed in ice for about 2 hours for proper suspension of DNA.
11. Then electrophoresis was done to check the DNA.

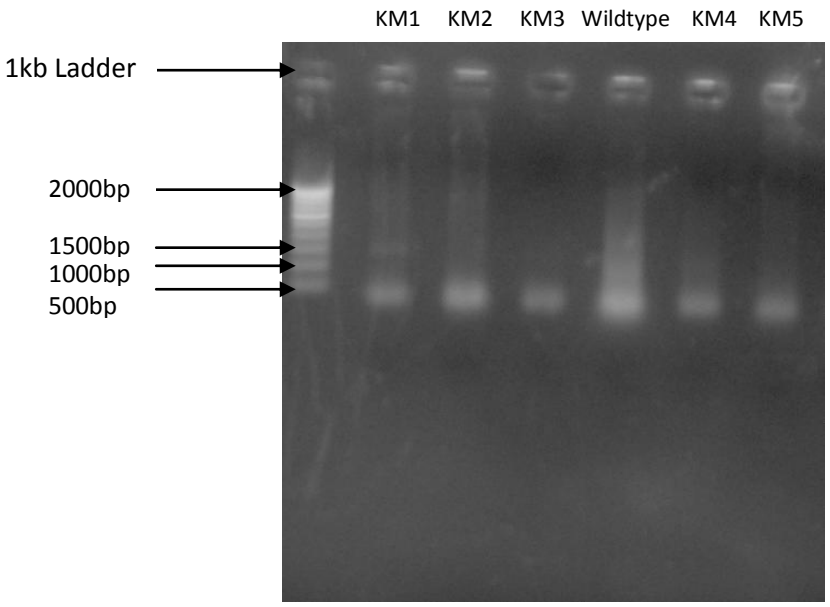



**Fig.9.** Electrophoresis of PCR amplicons of gDNA isolated from *M.fortuitum* wild type and mutant strains (KM1-KM5)

### 3.5 Restriction digestion of gDNA by EcoRV

*Chemical requirement*: BSA, distilled water, ECORV, T-buffer, Agrose gel, TAE buffer.

*Equipments used*: Thermo-cycler, incubator, vortex machine.

Procedure:

1. PCR reaction for restriction digestion:

| | |
|---|---|
| gDNA | - 25 µl |
| EcoRV | – 2 µl |
| T- Buffer | – 4 µl |
| BSA | – 0.4 µl |
| Distilled water | – 8.6 |
| Total | – 40 µl |

2. Reaction mixture was incubated at $30^0$c for 4 hours.
3. Agarose gel electrophoresis was done to visualize the amplicons on agarose gel (0.8%).
4. After that PUC19 plasmid was isolated.

### 3.6 Restriction Digestion of PUC19 with Sma I

*Chemical requirement*: BSA, distilled water, Sma I, T-buffer, Agrose gel, TAE buffer.

*Equipments used*: Thermo-cycler, incubator, vortex machine.

Procedure:

1. PCR reaction for restriction digestion:

| | |
|---|---|
| gDNA | - 25 µl |
| Sma I | – 2 µl |
| T- Buffer | – 4 µl |
| BSA | – 0.4 µl |
| Distilled water | – 8.6 µl |
| Total | – 40 µl |

2. Reaction mixture was incubated at $30^0$c for 4 hours.
3. Agarose gel electrophoresis was done to visualize the amplicons on agarose gel (0.8%).

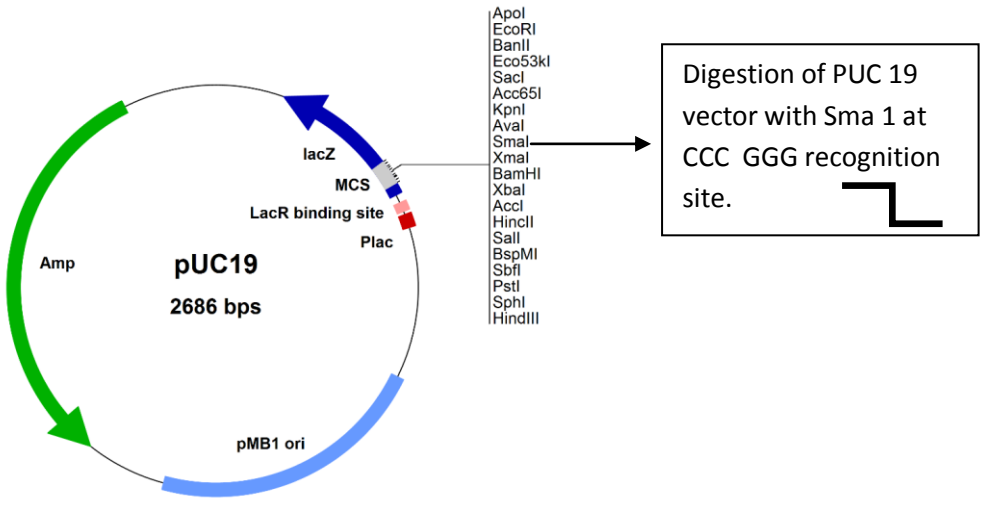



**Fig.10.** Circular map representation of PUC 19 vector[34]

**3.7 Ligation of EcoRV digested gDNA fragment with PUC19 digested with SmaI**

*Chemical requirement*: BSA, distilled water, DNA product, ligase product, T-buffer, Agrose gel, TAE buffer.

*Equipments used*: Thermo-cycler, incubator, vortex machine.

Procedure:

1. PCR reaction for restriction digestion:

| | |
|---|---|
| Vector | - 25 µl |
| DNA product | – 2 µl |
| T- Buffer | – 4 µl |
| Ligase product | – 0.4 µl |
| Distilled water | – 8.6 µl |
| Total | – 40 µl |

2. Reaction mixture was incubated at $30^0$c for 4 hours.
3. Agarose gel electrophoresis was done to visualize the amplicons on agarose gel (0.8%).

### 3.8 Transformation of ligated product into competent *E.coli* cells

3.8.1 Preparation of competent cells

*Chemical requirements*: transformation buffer-1 (100mM RbCl, 50mM $MnCl_2.4H_2O$, 3mM Potassium acetate, 10mM $CaCl_2.2H_2O$, 15% glycerol), transformation buffer-2 (10mM MoPs, 10mM RbCl, 75mM $CaCl_2.2H_2O$, 15% glycerol), 10ml LB.

*Equipments used*: centrifuge, viles, eppendorfs, tarson tubes, vortex, incubator, discard box, gel doc, pipettes and tips.

Procedure:

1. Single colony was picked and inoculated in 10 ml LB. It was incubated at $37^0$c for overnight.
2. 1 ml of overnight culture was added to 10 ml prewarmed LB medium and incubated with constant shaking at $37^0$c until $O.D_{600}$ reaches about 0.5.
3. The culture was cooled in ice for 5 minute and transferred to sterile round bottom centrifuged tube.
4. The cells were collected by centrifuged at low speed 4000g for 5 minute at $4^0$c. Supernatant was carefully discarded. The cells were always kept in ice.
5. The cells were gently resuspended in transformation buffer- 1 and kept on ice for an additional 90 minute.
6. Cells were collected by centrifuging for 5 minute at 4000 rpm at $4^0$c. The supernatant was discarded.
7. The cells were then carefully resuspended in 4 ml ice cold transformation buffer – 2.
8. Aliquots of 100-200 µl were prepared in sterile centrifuge tubes and freezed in liquid $N_2$. Then the cells were stored at $-80^0$C.

3.8.2 Transformation of Restriction digested gDNA and mutants

*Chemical requirements*: 15 µl ligated product, 200 µl competent cells, 800 µl of LB.

*Equipments used*: viles, eppendorfs, incubator, water bath, Petri plates.

Procedure:

1. Competent cells were thawed. 15 µl of ligated product was added in 200 µl of competent cells.
2. It was mixed by swirling and incubated in ice for 30 minutes.
3. After that, it was incubated in water bath set at $42^0$c for 90 minutes. Then, it was chilled on ice for about 5-15 minute.
4. Then, 800 µl of LB was added to the vials, and incubated for 45 minute a $37^0$c.
5. After 45 minutes of incubation, the cells were spread on selection plate with appropriate antibiotics.
6. White mutant colonies were selected from the selection plate and inoculated in LB- broth.
7. After an incubation of 2days at $37^0$c, the culture was centrifuged and plasmid was isolated.
8. After Isolation of plasmid, they were digested by EcoRV and further sent for genomic sequencing.
9. Then Comparative genomic analysis was done for sequences obtained and was aligned with the sequence showing most identical homology.

# Chapter 4

# *Results*

# 4. Results

When *M. fortuitum* wild strain was electroporated with plasmid pRT291 containing transposon Tn*pho*A (which lacks the promoter and translational start sequences along with the region encoding the amino-terminal leader peptide required for transmembrane transport) 50 blue mutant colonies showing mutation in membrane gene was formed. These mutants were selected and screen on agar plate containg Kanamycin,Cycloheximide, X-gal, IPTG and 5% glucose. The shortlisted mutants were further selected by measuring the strength of alkaline phosphatase.

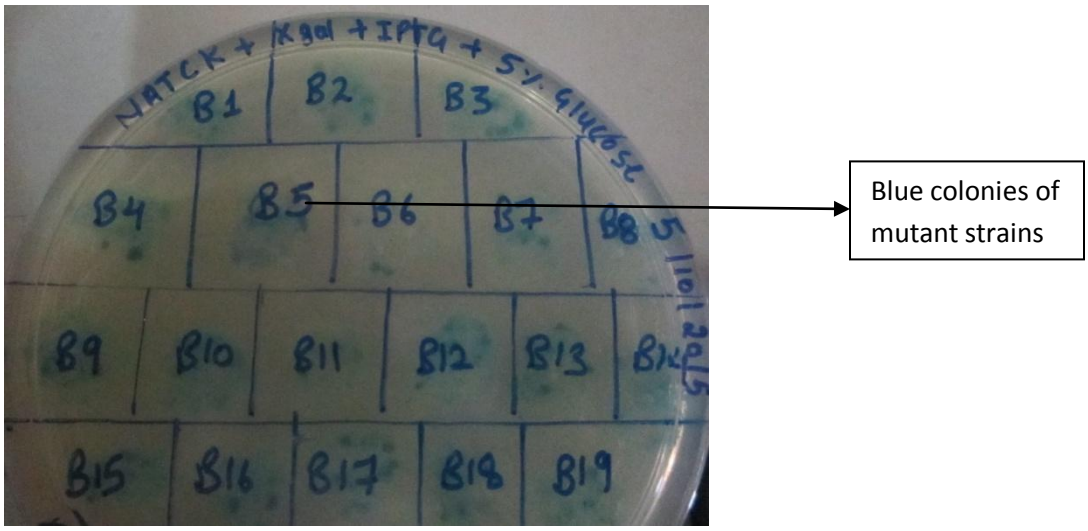



**Fig.11.** *Blue colonies of transposon mutant strains in nutrient agar tween cyclohexamide kanamycin (NATCK) plate containing X-gal + IPTG + 5% glucose.*

Quantitative assays for alkaline phosphatase (ALP) activity rely on the rate of hydrolysis of ρ-nitophenyl phosphate (PNPP) in permeabilized cells. ALP catalyzes the hydrolysis of ρ-Nitrophenyl phosphate (pNPP) to ρ-Nitrophenol. pNPP is colorless but ρ-Nitrophenol has a yellow color that can be used to check for enzyme activity by means of a colorimetric assay (at 420 nm wavelength). The spectrophotometric readings of pNPP assay are shown in table below which was calculated in miller units using the formula;

1 Miller Units (MU) = $$1000 * \frac{(Abs_{420} - (1.75 * Abs_{550}))}{(t * v * Abs_{600})}$$

Where;

- $Abs_{420}$ is the absorbance of the yellow o-nitrophenol
- $Abs_{550}$ is the scatter from cell debris, which, when multiplied by 1.75 approximates the scatter observed at 420nm
- $t$ = reaction time in minutes
- $v$ = volume of culture assayed in milliliters
- $Abs_{600}$ = reflects cell density

**Table.1. Quantitative estimation of alkaline phosphatase enzyme by pNPP assay (Absorbance at 420nm)**

| ***M. fortuitum* Mutant Strain** | **Miller units (pNPP assay)** |
|---|---|
| KM1 | 34.25333 |
| KM2 | 75.72414 |
| KM3 | 63.69128 |
| KM4 | 47.52169 |
| KM5 | 54.71751 |
| KM6 | 22.82245 |
| KM7 | 65.68837 |
| KM8 | 21.6443 |
| KM9 | 27.68396 |
| KM10 | 32.45235 |

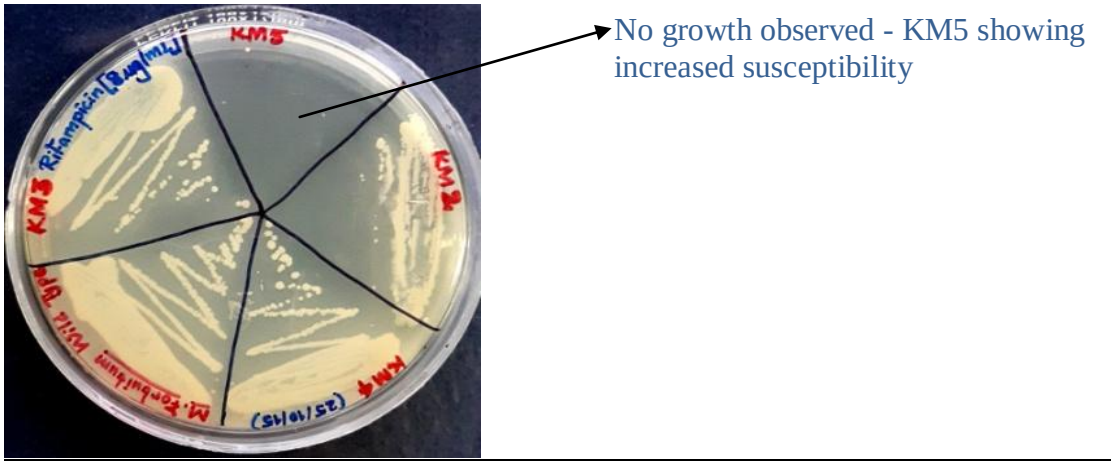



**Fig.12.** *Drug profiling result of mutant strains when Rifampicin [8µg/ml] was used*

**Table 2: MIC of mutants (KM1 – KM5) and *M. fortuitum* wild type with different antibiotics (Rifampicin, Ethambutol, Isoniazid, Amikacin)**

| MUTANTS | RIFAMPICIN (µg/ml) | ETHAMBUTOL (µg/ml) | ISONIAZID (µg/ml) | AMIKACIN (µg/ml) |
|---|---|---|---|---|
| **KM1** | 8 µg/ml | 5 µg/ml | 32 µg/ml | 15.625 µg/ml |
| **KM2** | 8 µg/ml | 5 µg/ml | 32 µg/ml | 15.625 µg/ml |
| **KM3** | 8 µg/ml | 5 µg/ml | 32 µg/ml | 15.625 µg/ml |
| **KM4** | 32 µg/ml | 5 µg/ml | 32 µg/ml | 15.625 µg/ml |
| **KM5** | 8 µg/ml | 5 µg/ml | 32 µg/ml | 15.625 µg/ml |
| ***M.fortuitum*(WT)** | 8 µg/ml | 5 µg/ml | 32 µg/ml | 62.5 µg/ml |

**Table 3: Shows the MIC of mutants (KM6 – KM10) and *M. fortuitum* wild type with different antibiotics (Rifampicin, Ethambutol, Isoniazid, Ciprofloxacin)**

| MUTANTS | RIFAMPICIN MIC in (µg/ml) | ETHAMBUTOL MIC in (µg/ml) | CIPROFLOXACIN MIC in (µg/ml) | ISONIAZID MIC in (µg/ml) |
|---|---|---|---|---|
| **KM6** | 16µg/ml | 10 µg/ml | 10 µg/ml | 32 µg/ml |
| **KM7** | 8 µg/ml | 5 µg/ml | 2.5 µg/ml | 32 µg/ml |
| **KM8** | 16 µg/ml | 20 µg/ml | 5 µg/ml | 32 µg/ml |
| **KM9** | 16 µg/ml | 20 µg/ml | 5 µg/ml | 4 µg/ml |
| **KM10** | 32 µg/ml | 20 µg/ml | 2.5 µg/ml | 8 µg/ml |
| ***M.fortuitum*(WT)** | 8 µg/ml | 5 µg/ml | 2.5 µg/ml | 32 µg/ml |

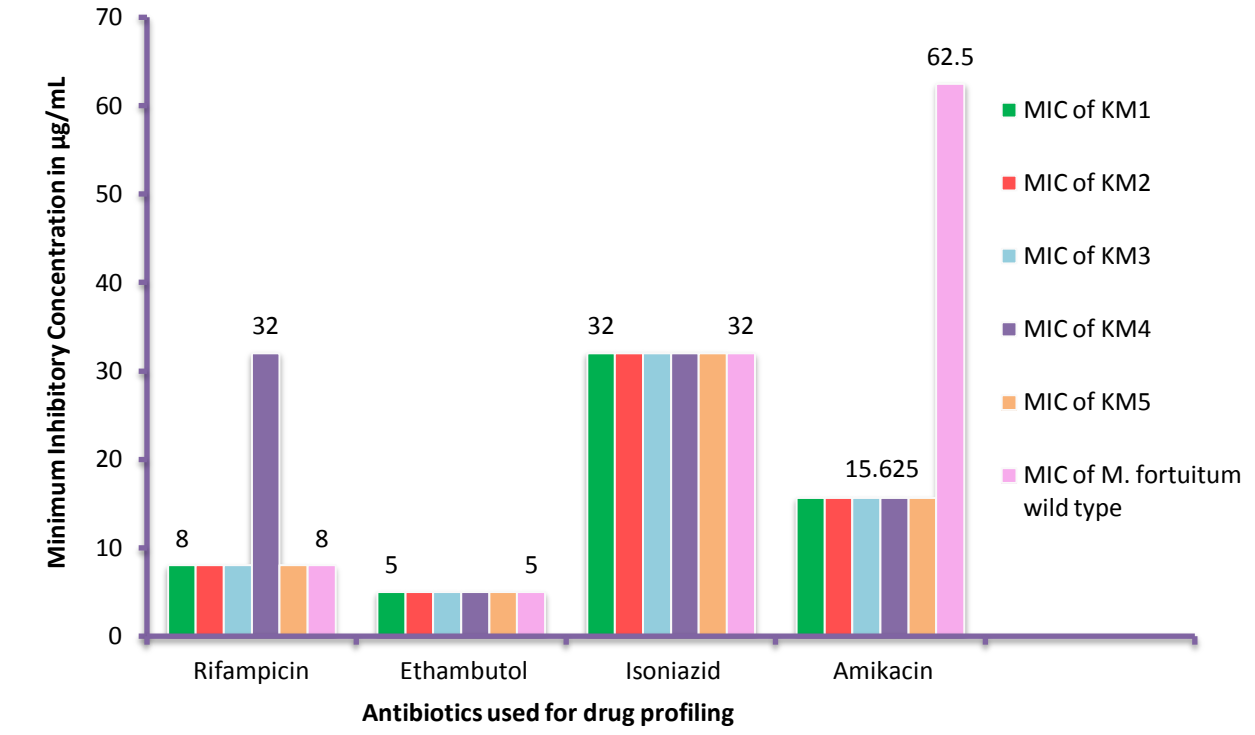



**Fig.13.** Column graph comparing MIC of mutants (KM1-KM2) with *M. fortuitum* wild type

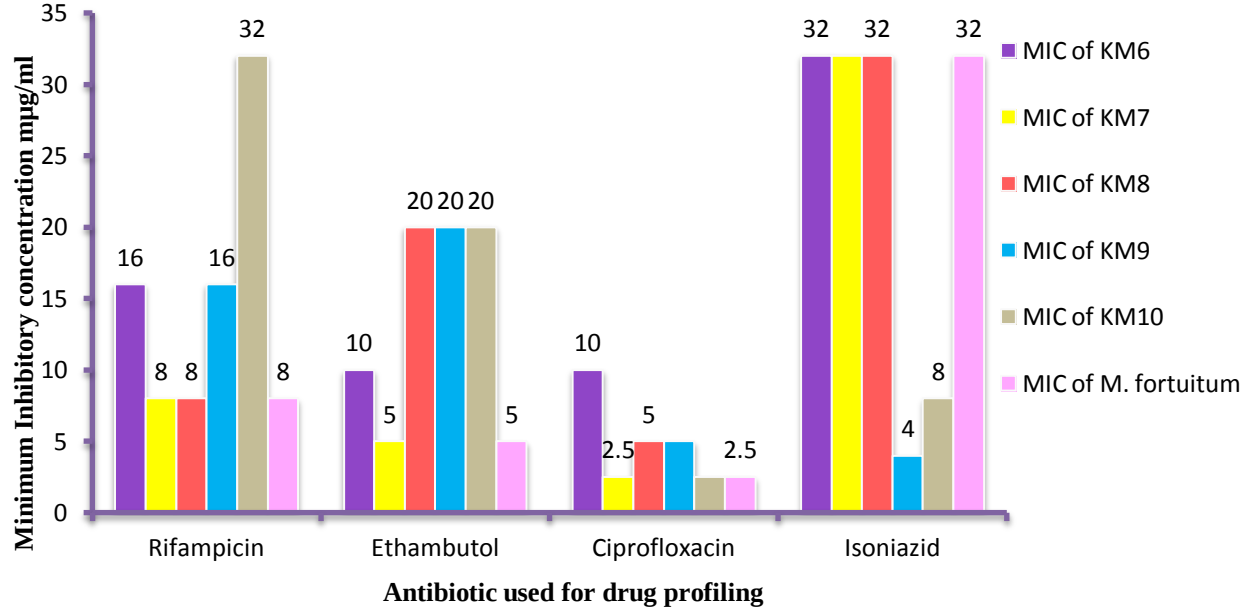
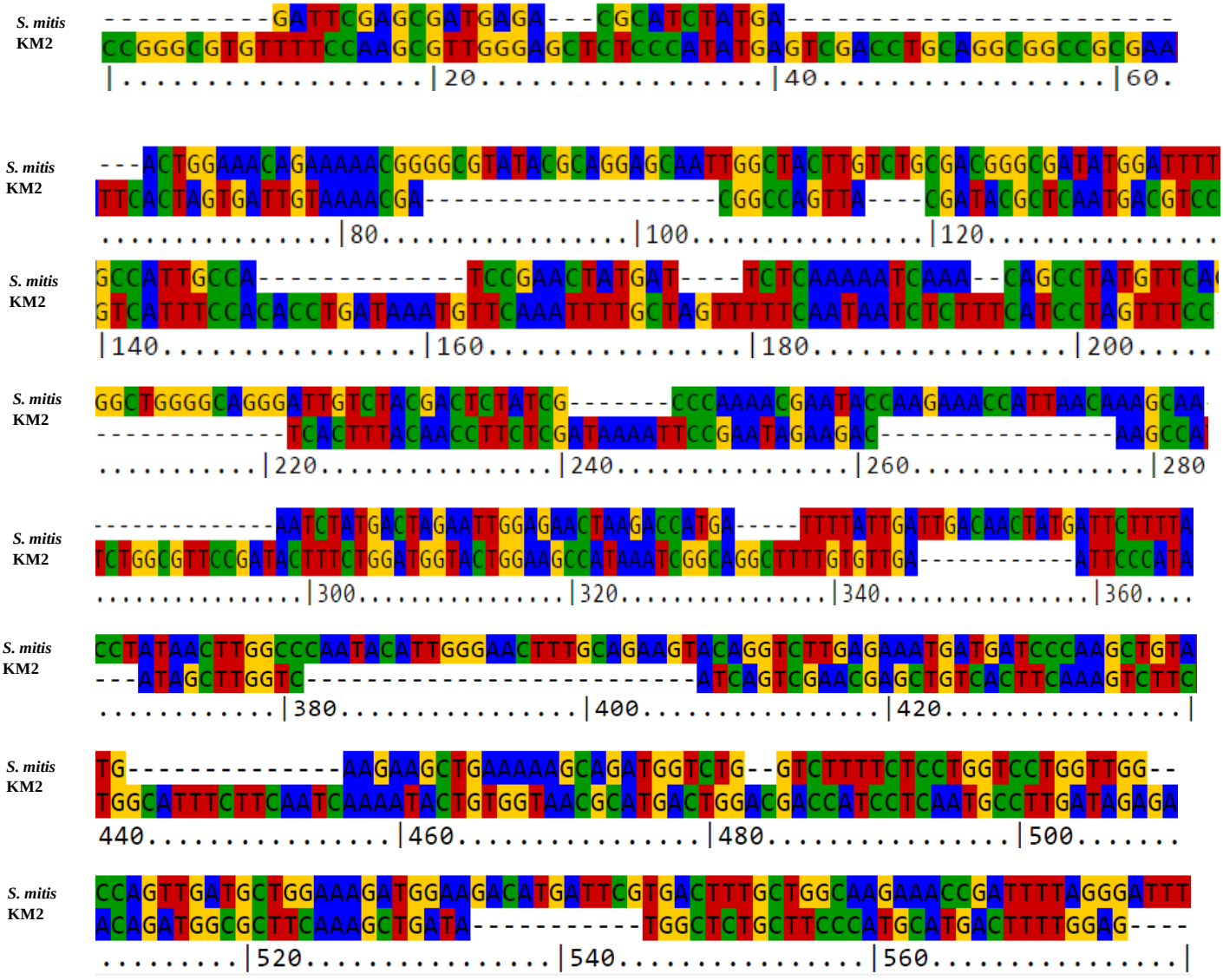



**Fig.14.** Column graph comparing MIC of mutants (KM6-KM10) with *M. fortuitum* wild type

Consequently, these mutants were employed in drug profiling studies using drugs commonly prescribed for treatment of *M. fortuitum* related infection. Results obtained showed a deviation in minimum inhibitory concentration (MIC) of mutants as compared to that of wild type. The MIC of mutant strain KM4 for Rifampicin was observed to increase by 4-fold whereas the strain KM5 was sensitive to it. All the mutants had same MIC as that of wild type when Isoniazid was used, but they were sensitive to Amikacin (4-fold decrease in MIC). Comparative genomic analysis of susceptible mutants revealed the presence of two hypothetical genes of *M. fortuitum* i.e. "*Antranilate synthase*" and "*NusA ribosome maturation factor RimP*" which may be used as potential drug target. Following figures depict the image of multiple sequence alignment of the above two genes showing maximum homology.



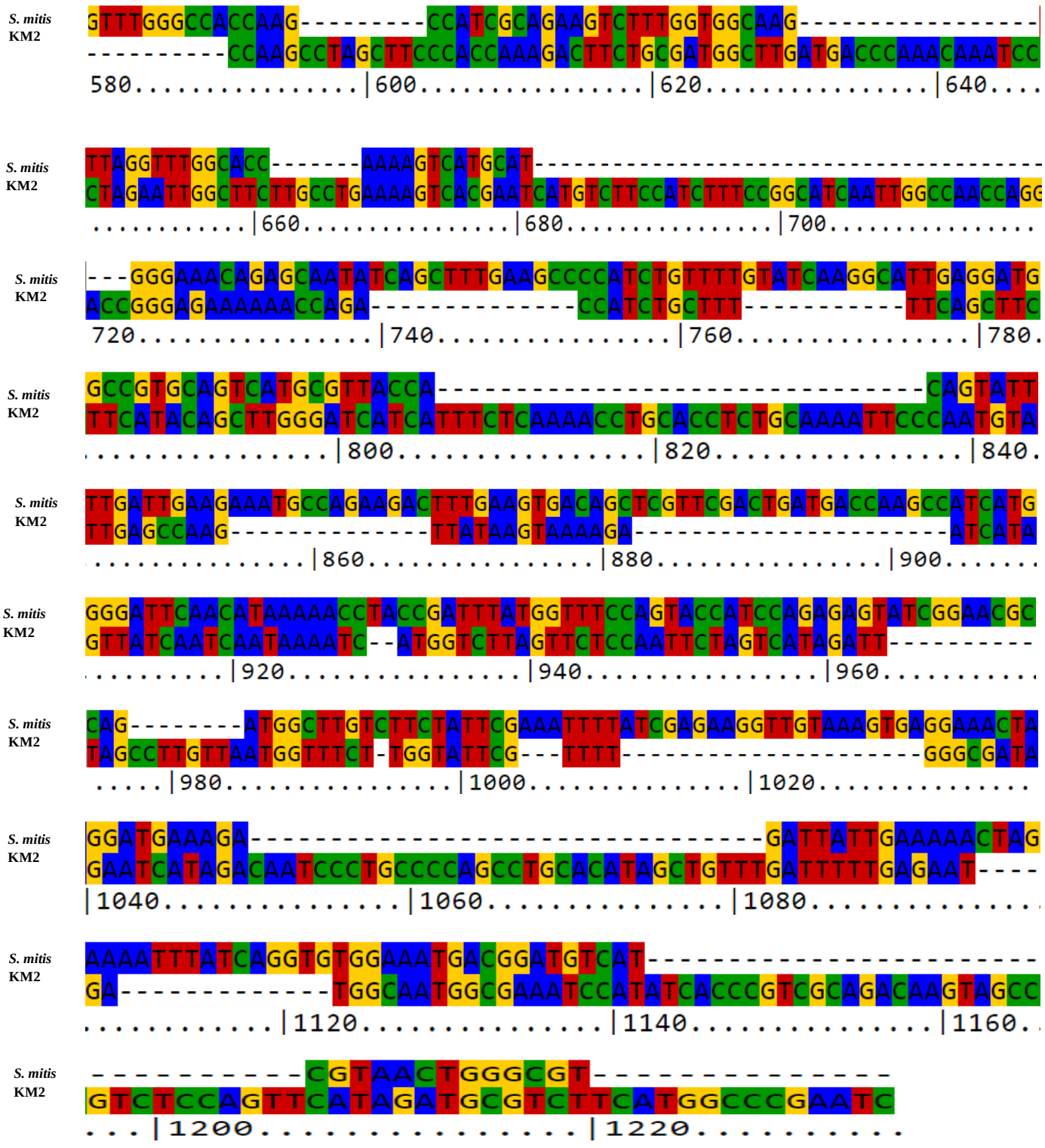
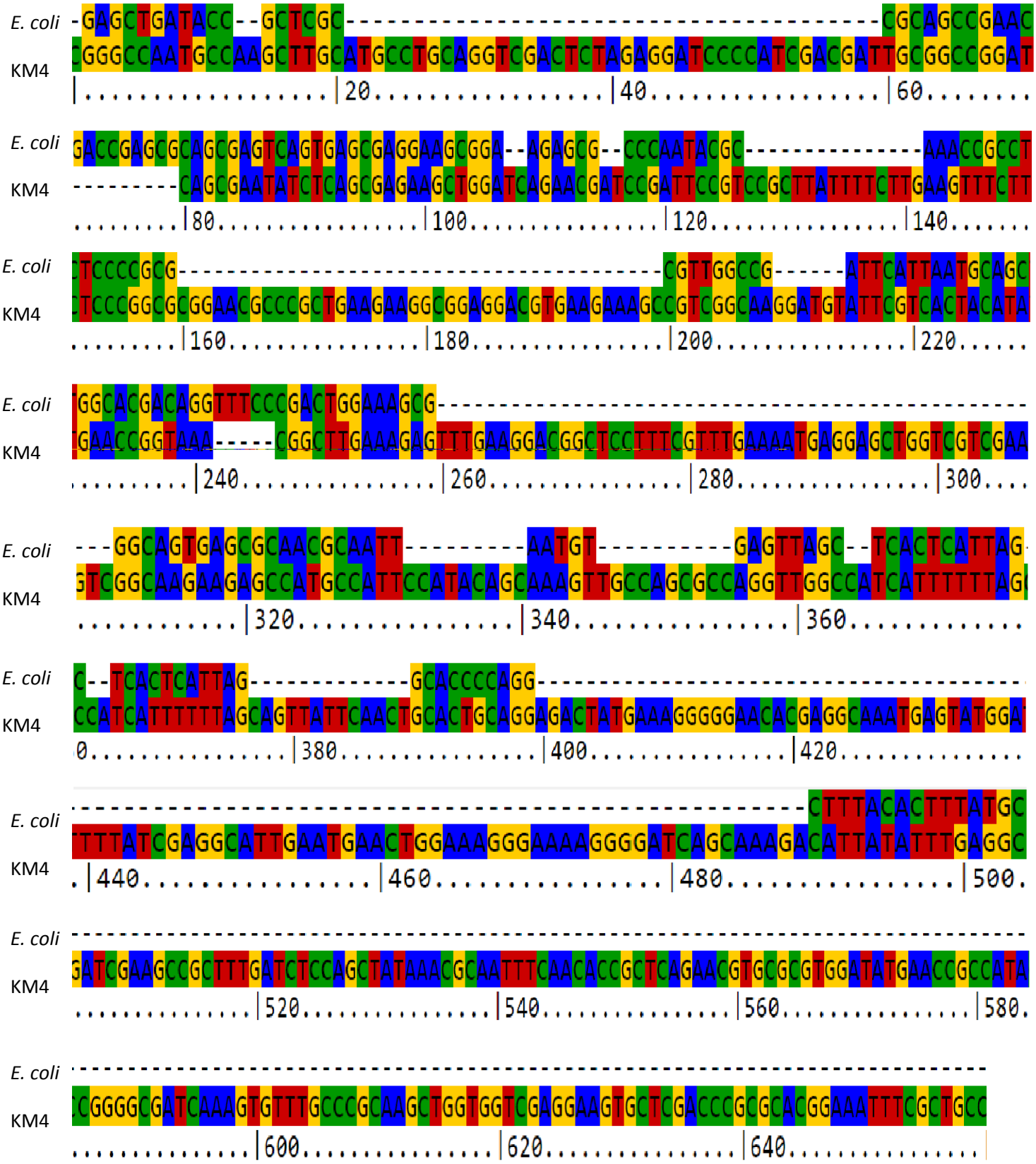



**Fig.15.** Nucleotide sequence alignment of KM2 gene fragment amplified using SP6 (primer) with *Streptococcus mitis* (*Anthramilate synthase* gene). Sequence showed 94 % homology.


E. coli
KM4
E. coli
KM4
E. coli
KM4
E. coli
KM4
E. coli
KM4
E. coli
KM4
E. coli
KM4
E. coli
KM4
E. coli
KM4

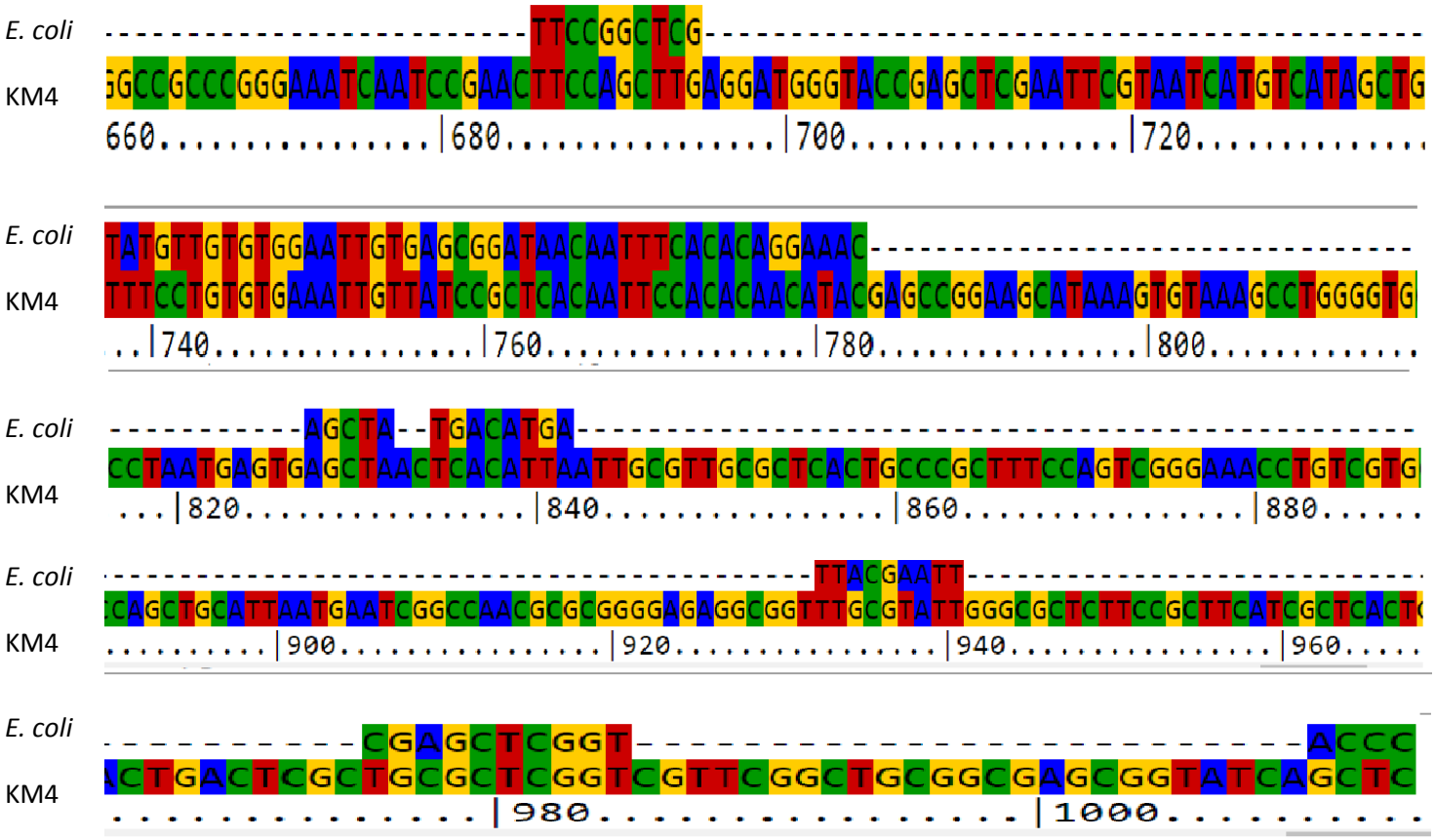



**Fig.16.** Nucleotide sequence alignment of KM4 gene fragment (amplified using M13F/R primers) with *NusA ribosome maturation factor RimP* gene of *E.coli.* The nucleotide sequence showed 99% homology.

# Chapter 5

# *Discussion & Conclusion*

## 5. Discussion & Conclusion

*Mycobacterium tuberculosis* was identified by Robert Koch in 1882, and *M. fortuitum* was identified shortly after. It was classified as a nontuberculous mycobacterium (NTM) and not much thought was given to it until 1954. Edward Runyon took it upon himself to categorize all the NTMs at this time. *M. fortuitum* is now classified as Runyon Group IV, which means, among other lab culture characteristics, it is a rapid grower.

Infection in healthy humans is rare, but exposure to large and repeated amounts of the organism can overwhelm the immune system and cause disease. Infections most likely occur in immunocompromised patients. Mortality is very rare, but death may come from extensive pulmonary or disseminated disease in immune-compromised patients. Although numerous reports have documented cases of successful therapy with one drug (eg, clarithromycin), reports also describe resistance to treatment. Therefore, antibiotic therapy with two drugs is preferable in most patients.

Amikacin is mostly used as a regimen for *M. fortuitum* related infections. However, we should not rely on it forever as the subsequent use of this drug in treating re-infection was reported to be ineffective in many cases [3]. Long term usage of this drug has led to evolved strains which are resilient and much challenging to get cured of. Positively, the mutants used in drug profiling were susceptible to Amikacin which may be a result of a helpful mutation which can further be used as a potential drug target.

As the MIC of mutants deviated from that of wild type, it indicates that the genes being intervened using transposon inserts do play a role in drug susceptibility/resistance and can further be used as an effective drug target. Furthermore, these genes are linked to membrane protein (confirmed by β-galactosidase assay), and any modification in such gene can be used as a weapon to combat drug resistance.

In our study of antibiotic susceptibility testing for drugs like amikacin, isoniazid, ethambutol, ciprofloxacin and rifampicin, the minimum inhibitory concentration was seen to decrease as compared to wild type. Amikacin is the aminoglycoside preferred for treatment of *M. fortuitum* infection, and almost all isolates are susceptible at a concentration of 31.25μg/ml and

above. Although first-line drugs used in treating tuberculosis was not being used until now, but our study showed that most mutants were susceptible to Ethambutol at a concentration above 5μg/ml. This might also mean that the insertional inactivation of the membrane protein due to transposon mutagenesis have resulted in the susceptibility of strain towards the particular antibiotic. And in further analyses two hypothetical genes of *M. fortuitum* were identified which can be further used as a potential drug target.

# Chapter 6

# *Appendices*

# 6. Appendices

### 6.1 Bacteriological media

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

**LB Broth (Luria Bertani Broth)**

| | |
|---|---|
| Tryptone | 10 g |
| Yeast extract | 5 g |
| NaCl | 10 g |

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

**Nutrient Broth**

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

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.5 with 5N NaOH and the volume adjusted to 1000 ml with Milli RO water.

**Nutrient Agar Tween80 (NAT)**

| | |
|---|---|
| Nutrient Broth | 13 g |
| Tween 80 | 2 ml |

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

**Middle brook (MB) 7H9 broth**

| | |
|---|---|
| MB7H9 broth base | 4.7 g |
| Tween 80 | 2 ml |

| | |
|---|---|
| Glycerol | 5 ml |
| Milli RO water | 900 ml |

The components were dissolved in 950ml Milli RO water and the pH adjusted to 7.2 with 5N NaOH and the volume adjusted to 1000 ml with Milli RO water.

## 6.2 Reagents for Acid Fast Staining

i. Carbol fuchsin (Primary stain)

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

Mixed 10 ml of Basic fuchsin to 90 ml of phenol and the solution was filtered through Whatman filter paper no. 1.

ii. Acid alcohol (Decolorizer)

| | |
|---|---|
| HCL (conc.) | 3 ml |
| Ethanol (96%) | 97 ml |

iii. Malachite green solution (Counter stain)

| | |
|---|---|
| Malachite green | 0.25 g in Milli RO water |

## 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.

| **Reagent** | **Stock solution** | **Final Conc.** (in *E. coli*) | **Final Conc.** (in Mycobacterium) |
|---|---|---|---|
| Ampicillin | 5 mg/ml in $H_2O$ | 50 µg/ml | - |
| Kanamycin | 5 mg/ml in $H_2O$ | 50 µg/ml | 25 µg/ml |
| Cycloheximide | 5 mg/ml in $H_2O$ | 100 µg/ml | 100 µg/ml |
| Streptomycin | 5 mg/ml in $H_2O$ | 50 µg/ml | 25 µg/ml |
| X-gal | 40 mg/ml in DMF | 40 µg/ml | 40 µg/ml |

### 6.4 Reagents and Buffers

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

#### 6.4.1 Commonly used Buffers

**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 used.

**ii. Ethylene diamine tetra acetic acid (EDTA)**

0.5 M solution of disodium salt of EDTA was prepared in Milli Q, pH adjusted to 8.0 with NaOH pellets and stored at $4^0$c.

**iii. Normal Saline**

| | |
|---|---|
| NaCl | 8.50 g |
| Milli RO water | 1000 ml (final volume) |

**iv. Tween Normal Saline**

0.02% Tween -80 was added to normal saline.

#### 6.4.2 Reagents for Genomic DNA isolation from Mycobacterium

**i. TE Buffer**

| | |
|---|---|
| Tris-HCl (pH 8.0) | 10 mM |
| EDTA | 1 mM |

**ii. Tris EDTA Saline (TES) Buffer**

| | |
|---|---|
| TRIS-HCL (pH 8.0) | 10 mM |
| EDTA | 1mM |
| NaCl | 150 mM |

**iii. Lysozyme**

| | |
|---|---|
| Lysozyme | 50 mg/ml in Milli Q |

**iv. Proteinase K**

| | |
|---|---|
| Proteinase k | 20 mg/ml in Milli Q |

**v. Buffer Phenol**

Molten phenol containing 0.1% 8-hydoxyquinoline was equilibrated with 1M Tris-HCl (pH 8.0) and twice with 0.1 Tris-HCl (pH 8.0) till the pH > 7.8 and then it is stored submerged in 10 mM Tris-HCl (pH 8.0) in dark bottle at $4^0$c away from direct light.

**vi. Chloroform: Isoamyl alcohol**

Solution contains 24 parts chloroform and 1 part Isoamyl alcohol. The solution is stored in dark bottle at $4^0$c.

### 6.4.3 Buffer for Plasmid Isolation from *E. coli*

**i. Glucose Tris EDTA Buffer (GTE)**

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

**ii. NaOH-SDS Mix**

| | |
|---|---|
| NaOH | 0.2 N |
| SDS | 1.0% |

**iii. Acetate Mix**

Solution contains 3 volumes of 3 M sodium acetate and 4 volume of 7.5 M ammonium acetate.

### 6.4.4 Buffers for Electrophoresis

**i. TAE Buffer (50 X)**

| | |
|---|---|
| Tris Base | 242 g |
| Glacial Acetic Acid | 57.1 ml |
| 0.5 M EDTA (pH 8.0) | 100 ml |
| Final Volume | 1000 ml |

**ii. TBE Buffer (50 X)**

| | |
|---|---|
| Tris Base | 54 g |
| Boric acid | 27.5 g |
| 0.5 M EDTA | 20 ml |
| Final Volume | 1000 ml |

**iii. Tris-glycine**

| | |
|---|---|
| Tris Base | 3.0 g |
| Glycine | 14.4 g |
| SDS | 2.0 g |
| Final Volume | 1000 ml |

### 6.4.5 Buffer for Transformation

#### i. Transformation Buffer 1 (TFB I)

| | |
|---|---|
| MOPS Buffer (pH 6.5) | 100 mM |
| RbCl | 10 mM |

#### ii. Transformation Buffer II (TFB II)

| | |
|---|---|
| MOPS Buffer (pH 6.5) | 100 mM |
| RbCl | 10 mM |
| $CaCl_2$ | 50 mM |

### 6.4.6 Buffer for Gel Loading

#### i. 6X dye for agarose gel electrophoresis

| | |
|---|---|
| Bromophenol Blue | 0.25% |
| Sucrose | 40% |

The volume was then made up to 10 ml with R Nase free water. For loading onto the gel the buffer was added to a final concentration of 1x to the sample, the mixture incubated at 650c for 3-5 min. and chilled on ice. Before loading 1 l of EtBr (1mg/ml) was added to the sample.

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## Publications

1. **Identification of macrophage invasion genes of *Mycobacterium fortuitum* by genome wide transposon mutant library**

   Poonam, **Monika Pradhan, Kanika Sharma**, Rahul Shrivastava

   **Poster presentation** at - 56th Annual Conference of Association of

   Microbiologists of India, organized by JNU, New Delhi from December 7-10, 2015

2. **Identification of Novel Gene(s) Responsible for Drug Resistance in *Mycobacterium fortuitum***

   **Monika Pradhan, Kanika Sharma**, Poonam, Rahul Shrivastava*

   **Poster presentation** at- SYSCON 2016, organized by AIIMS, New Delhi from May 26-27, 2016

**POSTER-1**

# Identification of *Mycobacterium fortuitum* virulence factors using transposon mutagenesis

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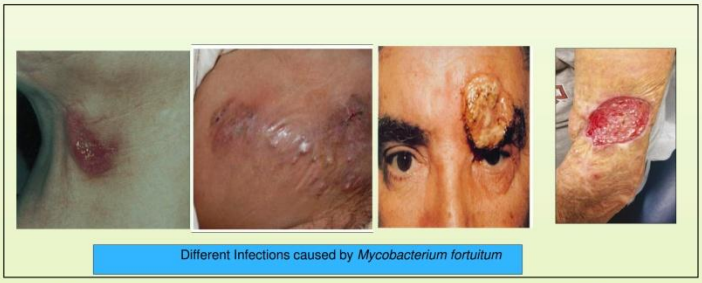
## ABSTRACT

*M. fortuitum* is rapidly growing, opportunistic, non-tuberculous mycobacteria causing infections ranging from localized lesions, pulmonary infections, iatrogenic infections to disseminated infections. The pathogenesis of M. fortuitum is not clearly known; hence, to make an insight into the pathogenesis of M. fortuitum and to identify genes responsible for its virulence, a transposon mutant library of about 5000 mutants was constructed using TnphoA transposon vector pRT291. 127 blue mutants were shortlisted to have mutation in membrane associated genes. Among 127 mutants, 30 transposon mutants with highest alkaline phosphatase activity were checked *in vivo* for any deviation in virulence and infection parameters in BALB/c mice model on the basis of symptoms, survival of the mice and bacillary load at different stages of infection. Four attenuated mutants were identified. Genomic analysis of the mutants was done by restriction digestion of their genomic DNA, cloning of the insert containing transposon into pUC19, followed by sequencing. Two genes have been identified, which may serve as potential drug targets for M. fortuitum. Homologues of these genes may also be important for virulence and pathogenesis of Mycobacterium tuberculosis, the causal organism of tuberculosis.



Different Infections caused by *Mycobacterium fortuitum*

## INTRODUCTION

- *M. fortuitum* is an ubiquitous, rapidly growing non-tuberculous mycobacteria
- Causes skin and soft tissue infections, post surgical infection, pulmonary infection and disseminated infection [1]
- Emergence of drug resistance leads to long therapy time [2]
- Bacilli resists phagocytosis by macrophages
- Membrane play significant role in pathogenesis
- Identification of these proteins/genes and their inhibitors can act/serve as a potential drug target

## MATERIALS AND 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

↓

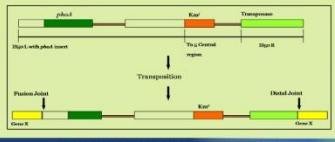
*in vivo* analysis of virulence characters of mutants

↓

attenuated mutants proceeded for cloning in pUC19 vector

↓

sequencing of cloned inserts and sequence analysis for homology



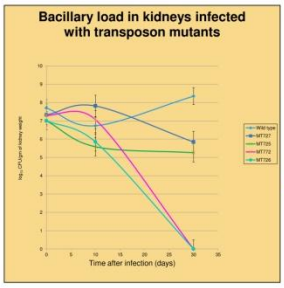

## RESULTS

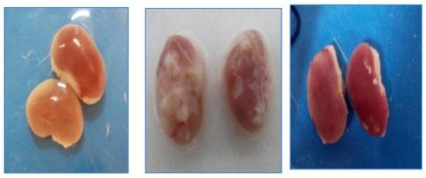
MT772 mutant showing less symptoms

Wild type *M. fortuitum* showing tilted neck

MT726 mutant showing less symptoms

**MT772 and MT726 TnphoA mutants of *M. fortuitum* showed virulence attenuation as compared to wild type *M. fortuitum***



Kidneys infected with MT772 mutant (no necrosis)

Kidneys infected with wild type *M. fortuitum* (necrosis)

Kidneys infected with MT726 mutant (no necrosis)

## SIGNIFICANCE:

*Invivo* and genomic analysis of TnphoA transposon mutants results in identification of two genes which may serve as potential drug target of *M. fortuitum*. Homologues of these genes may also act as important drug target for *M. tuberculosis*..

## ACKNOWLEDGEMENT

Authers are thankful to DST-SERB for providing financial assistance

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**POSTER-2**

# Identification of Novel Gene(s) Responsible for Drug Resistance in *Mycobacterium fortuitum*

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## ABSTRACT

Mycobacterium fortuitum is a rapidly growing, nontuberculous mycobacteria (NTM) which is ubiquitously present in nature. It is one of the most important pathogenic species, representing more than 80% of clinical isolates of rapidly growing mycobacteria.

It causes postsurgical infections, localized skin and soft tissue infections and pulmonary infections. To add up insult to injury, an obstruction is imposed on the way to its treatment as these organisms are evolving into a drug resistant species. Prolonged antibiotic therapy is now a necessity for M. fortuitum infection. Considering these crisis, our study aims to identify the resistant gene(s) and the mechanism associated, and the subsequent use of these findings in establishing effective drug targets.

As the genomic sequence of M. fortuitum is not known, random mutagenesis technology (using TnphoA) was employed. The plasmid (pRT291) containing transposon Tn5 was electroporated in M. fortuitum wild strains which resulted in formation of 50 blue mutant colonies showing mutation in membrane gene. Those mutants were further selected and screened by β-galactosidase assay. 10 mutants having high β-galactosidase activity were shortlisted for drug profiling where Ciprofloxacin, Amikacin, Rifampicin, Isoniazid and Ethambutol were utilized as these are most commonly used in treatment of M. fortuitum related infections. Genomic analysis of these mutants was done by restriction digestion of their gDNA, cloning of the insert containing transposon into pUC19, followed by sequencing.

## CONTACT

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## INTRODUCTION

M. fortuitum is slender, nonmotile, gram positive bacilli with a thick hydrophobic cell wall that assists in prevention of its desiccation. It is extremely hardy and can thrive even in most hostile conditions. Particularly, drinking water has been shown as a potential source of NTM infections due to high resistance of NTM to disinfectants[1]. M. fortuitum is 10-fold more resistant to ultraviolet (UV) than E. coli. It causes infections of the respiratory tract (most common), skin and soft tissue structures, bone and joint, lymphadenitis, ophthalmic infections, otitis media and infection of the central nervous system. In addition, infective complications after surgical procedures and catheter use, as well as disseminated infections, especially in immunocompromised hosts, have been widely documented. Treatment of RGM infections is challenging; drug therapy normally comprises a multidrug regimen for a lengthy duration, is costly and is associated with drug-related toxicities. Moreover, response rates are highly variable, particularly in pulmonary RGM infections, with cure rates of only 30% to 50% [2]. Virulence mechanisms and persistence of M. fortuitum is not discovered yet. Hence, identification of such proteins/genes and their inhibitors can function as a potential drug target.

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

Fig.2.Nasolacrimal obstruction and granulomatous eyelid disease caused by *M. fortuitum*.

Fig.3. Patient with *M. fortuitum* skin infection before (A) and after (B) 2 months of treatment with clarithromycin and ciprofloxacin

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

## METHODS AND MATERIALS

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

↓

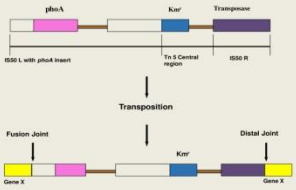
Antibiotic susceptibility test and calculation of minimum inhibitory concentration

↓

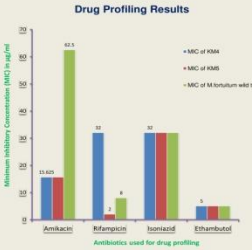
attenuated mutants proceeded for cloning in pUC19 vector

↓

sequencing of cloned inserts and sequence analysis for homology




## RESULTS




The minimum inhibitory concentration (MIC) of mutant strain KM4 for Rifampicin was observed to increase by 4-fold whereas the strain KM5 was sensitive to it. All the mutants had same MIC as that of wild type when Isoniazid was used, but they were sensitive to Amikacin (4-fold decrease in MIC). Comparative genomic analysis of susceptible mutants revealed the presence of two hypothetical genes of M. fortuitum i.e. "Antranilate synthase" and "NusA ribosome maturation factor RimP" which may be used as potential drug target.

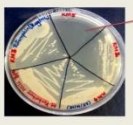



Fig.5. Antibiotic susceptibility test for Rifampicin [8µg/ml]

## DISCUSSION

As the MIC of mutants deviated from that of wild type, it indicates that the genes being intervened using transposon inserts do play a role in drug susceptibility/resistance and can further be used as an effective drug target. Furthermore, these genes are linked to membrane protein (confirmed by β-galactosidase assay), and any modification in such gene can be used as a weapon to combat drug resistance.

Amikacin is mostly used as a regimen for M. fortuitum related infections. However, we should not rely on it forever as the subsequent use of this drug in treating re-infection was reported to be ineffective in many cases [3]. Long term usage of this drug has led to evolved strains which are resilient and much challenging to get cured of. Positively, the mutants used in drug profiling were susceptible to Amikacin which may be a result of a helpful mutation which can further be used as a potential drug target.

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