# REPORT

**Report on participation of the ICMR International Fellow (ICMR-IF) Year 2018-19 in Training/Research abroad.**

1. **Name and designation of ICMR- IF** :**Vivek Kumar Gupta, Scientist-D**

2. **Address** :Department of Biochemistry, ICMR-National JALMA Institute for Leprosy& Other Mycobacterial Diseases, Tajganj, Agra

3. Frontline area of research in which training/research was carried out :Mycobacterial Resuscitation Promoting factors

4. Name & address of Professor and host institute : Dr. Galina Mukamolova,

   Associate Professor, Department of Respiratory Sciences (previous name- Department of Infection, Immunity and Inflammation), University of Leicester LE1 7RH, UK

5. Duration of fellowship : Six months (2nd October2018 to 30th March 2019)

**6. Highlights of work conducted :**

**i) Technique/expertise acquired :**

ICMR-IF has acquired sufficient technical skills in Detection and characterization of resuscitation promoting factors using different techniques- Ammonium sulfate protein precipitation, Protein purification by Ion-exchange chromatography, SDS-PAGE, Western blotting, Colony PCR, Real Time PCR, purification of Rpf raised polyclonal antibody from animal sera, MPN counting for characterization of dormant bacilli population in sample etc.

**ii) Research results, including any papers, prepared/submitted for publication**

Tuberculosis (TB) is a curable disease but still remains one of the biggest killers in the world. It kills nearly 2 million people worldwide every year and 98% of tuberculosis deaths are in the developing world affecting mostly young adults in their productive years. One important characteristics of this disease is that the bacterium has an unusual ability to grow and survive for extended periods of time in human body. Therefore, it has been estimated that 2 billion people, equal to one third of the world's total population, are infected with the bacterium in whom it causes unnoticeable latent infection that gives rise to a 5-10% lifetime risk of active tuberculosis. These persistent bacteria cannot be cultured using standard microbiological methods and are not killed by the current tuberculosis drugs. Therefore, tuberculosis treatment needs at least 6 months with four drugs to cure the patients.

The persistent bacteria will be "woken" by the addition of resuscitation promoting factors (RPFs) which are proteins produced by *M. tuberculosis* (*Mtb*) to restart growth. The Rpf proteins, which are believed to have muralytic activity, are widely distributed throughout the actinobacteria, including *M. tuberculosis*, and they are implicated in the resuscitation of dormant forms of these organisms. Rpf proteins have clinical implications as they increase the recovery of *Mtb* from clinical samples of the patient with active TB and improved the sensitivity of culture-based *Mtb* tests (Mukamolova, 2010; Huang et al., 2014; Chengalroyen et al., 2016; Rosser, 2017). Moreover, Rpf-dependent *Mtb* are more resistant to antimicrobial agents and are extremely difficult to eradicate from patients (Turapov, 2016). Therefore, drugs specifically targeting non replicating persistence and Rpf-dependent *Mtb* are urgently needed (Kaprelyants et al., 2012).

Rpfs are widespread among Actinobacteria species with five homologues existing in *Mtb* (RpfA, RpfB, RpfC, RpfD, RpfE) and four (RpfA, RpfB, RpfC, RpfE) having been annotated in the closely related *Mycobacterium marinum. M. marinum* is increasingly being used to model TB infection. With a faster generation time of 12 hours, *M. marinum* is a close genetic relative of *M. tuberculosis* sharing 85 % nucleotide identity, and 3000 orthologs with an average amino acid identity of 85 % (Stinear et al., 2008). Key genes encoding proteins essential for the virulence and pathogenicity of *Mtb* are also conserved in *M. marinum* including the PE/PPE, ESX and Resuscitation promoting factor (Rpf) families.

**Research Results:** Considering the above facts, detection and expression of Rpf A was performed using *M. marinum* as model organism for this study. The detection of rpfA was carried out in the secretory proteins using different media. The culture conditions were standardized for secretory protein secretion. Sauton's Medium was found to be best medium for excretory protein yields. The two techniques TCA and ammonium sulfate precipitation were implemented for precipitation of proteins from culture filtrates.

Proteins was precipitated using 60 to 80% saturation of ammonium sulfate and purified through membrane dialysis but yields and amount were less. Ion exchange chromatography was used for protein purification with DEAE Sepharose. Ion exchange chromatography was found to be better technique for detection of secretory proteins through SDS-PAGE.

The RpfA was detected in *M. marinum* (WT) through immunoblotting. Purified proteins were analysed by SDS–PAGE. Coomassie brilliant blue was used to stain protein bands. Proteins were electroblotted onto nitrocellulose blotting membranes for western blot analysis. The polyclonal antibodies generated in rabbit against recombinant Rpf (1:10,000 dilution) was used as primary antibody for detection of RpfA. Secondary antibody was horseradish-peroxidase (HRP)-conjugated anti-rabbit IgG (1:10,000 dilution). The blot was detected under chemoluminance using substrate signal FireTM Elite ECL Reagent ((Fig. 1 & Fig. 2). RpfA secretory protein which was detected in culture filtrates of *M. marinum* (WT). The protocol for detection of RpfA from culture filtrates was developed during the study.

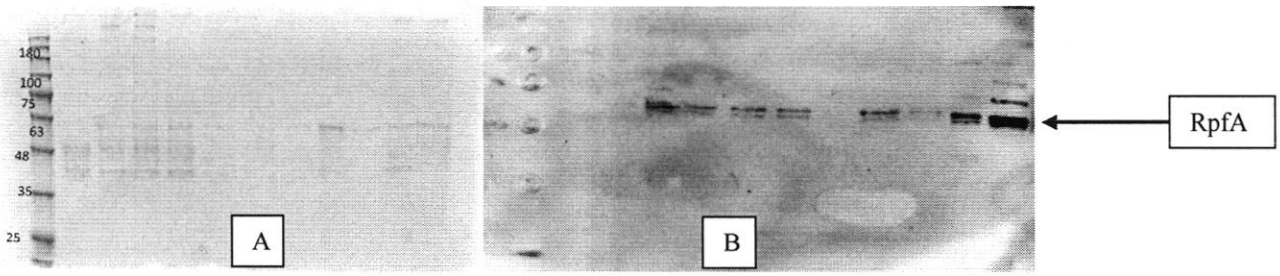



**Fig 1** : (A) SDS-PAGE of secretory proteins ; (B) their western blot showing band of RpfA

We had used different rpf A deletion mutants of *M. marinum* and confirmed that the band appeared on western blot on the blot membrane was RpfA. The rpf AB E deletion strain of *M. marinum* ΔrpfABE (triple mutant) was used to confirm the RpfA in secretory proteins. When the triple mutant complemented with rpfA-ΔrpfABE::rpfA, and chimeric strain ΔrpfABE::rpfA+N termin ActA, the complemented strains retain phenotype for RpfA (Fig. 2)

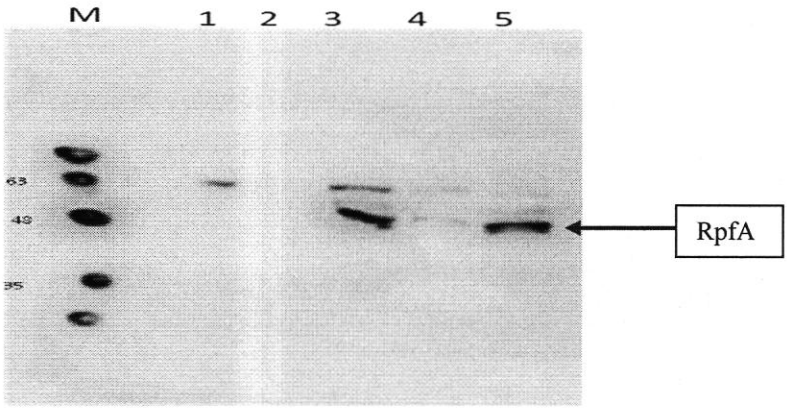



**Fig 2.** Western Blot showing lane 1&2 - ΔrpfABE (triple deletion mutant); lane 3&4- ΔrpfABE::rpfA (rpfA cpmplemented strain); lane 5- chimeric strain ΔrpfABE::rpfA+N termin ActA

I have also demonstrated that various regulatory elements loacated in the upstream of *rpfA* region from *Mycobacterium.tuberculosis* differentially impacted on production of RpfA. It was carried out using the different plasmid pMV306 constructs of *rpfA* of *M. tuberculosis* complemented in ΔrpfA deletion mutant of *M. marinum*.

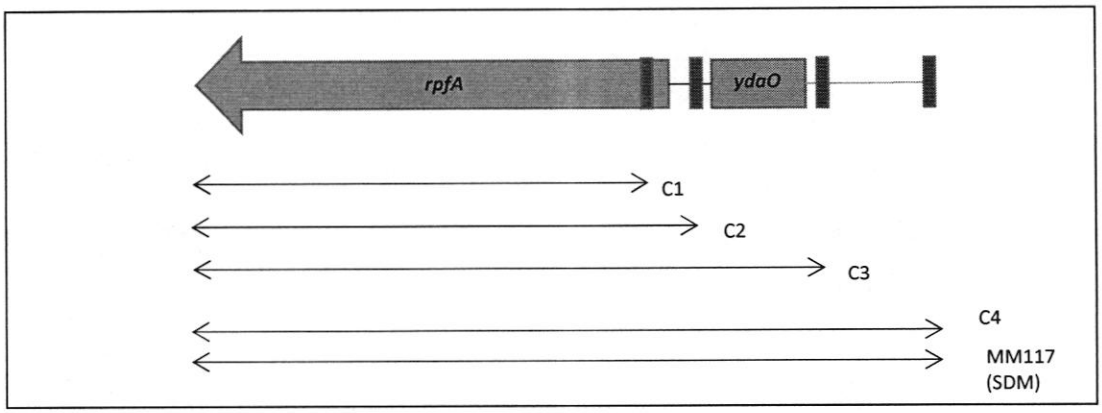



Fig 3. Schematic representation of *rpfA, ydaO* and the promoter regions (red boxes) upstream and internal to *rpfA (Mycobacterium tuberculosis).* Construct C1 amplified the region from the intragenic promoter to the end of the gene (965486-964315), C2 amplified from the promoter region before the transcriptional start site (965555-964315), construct C3 amplified from the promoter before the riboswitch (965832-964315) and construct C4 amplified the entire intragenic region (965985-964315).

- Δ*rpfA::C1*-MM127
- Δ*rpfA::C2*-MM128
- Δ*rpfA::C3*-MM129
- Δ*rpfA::C4*-MM126
- Δ*rpfA::C4 RM* (or SDM) MM117 - Δ*rpfA::C4* with mutation in the riboswitch.

The constructs (Fig. 3) were characterized for the expression of RpfA through western blot analysis. It was observed that constructs Δ*rpfA::C1*; Δ*rpfA::C2* and Δ*rpfA::C3* containing different region of rpfA not retained Rpf phenotype while construct C4 amplified the entire intragenic region retained Rpf phenotype in this experiment. Construct Δ*rpfA::C4* (SDM) with mutation in the riboswitch was able to produce RpfA at very low level (Fig. 4).

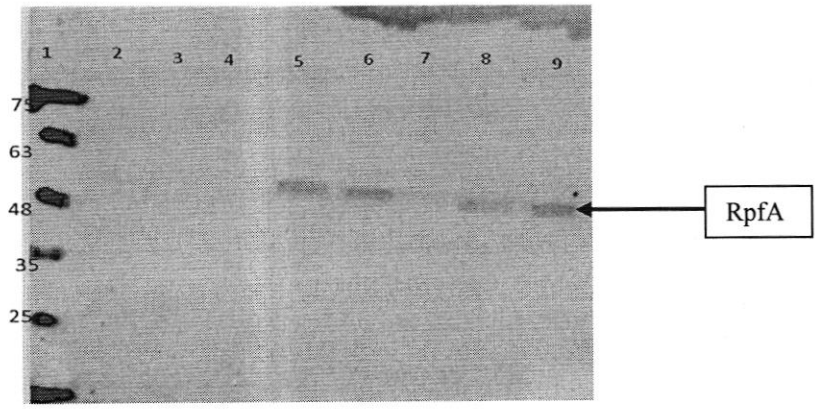



**Fig4:** Western Blot: Lane 1-marker; lane 2- Δ*rpfA::C1;* lane3- Δ*rpfA::C2*, lane 4- Δ*rpfA::C3*; lane 5 & 6-Δ*rpfA::C4*; lane 7-Δ*rpfA::C4*(SDM); lane 8 & 9- WT

Further, the rpf deletion strains of *M. marinum* was checked for deletion in rpfA through diagnostic colony (Fig. 5).

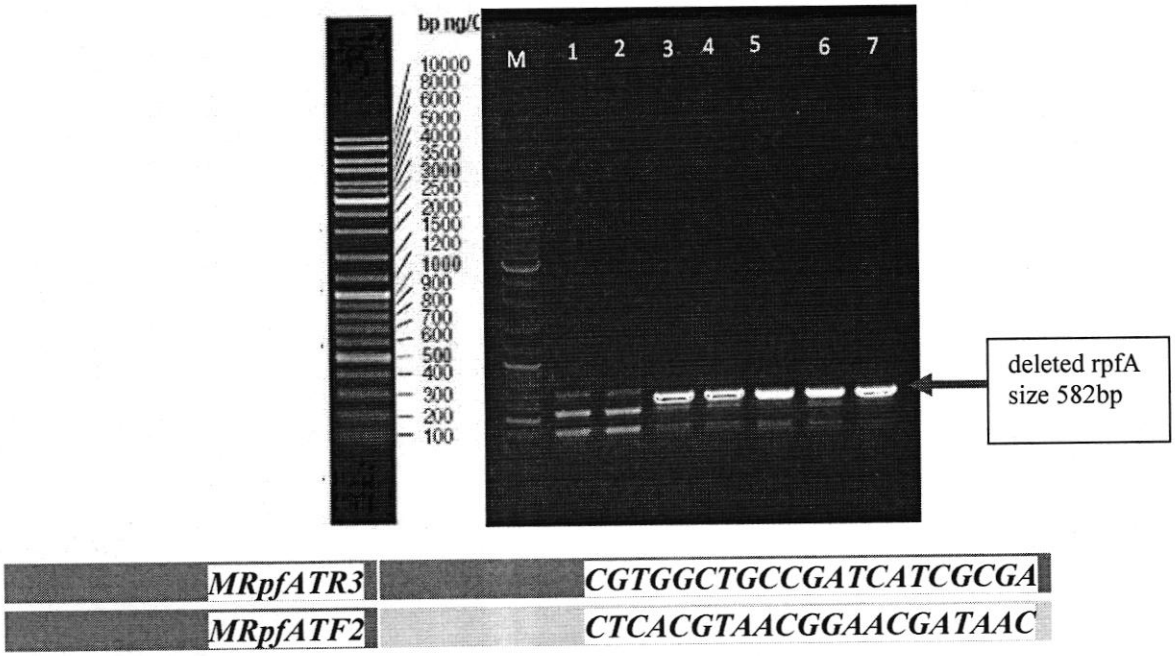
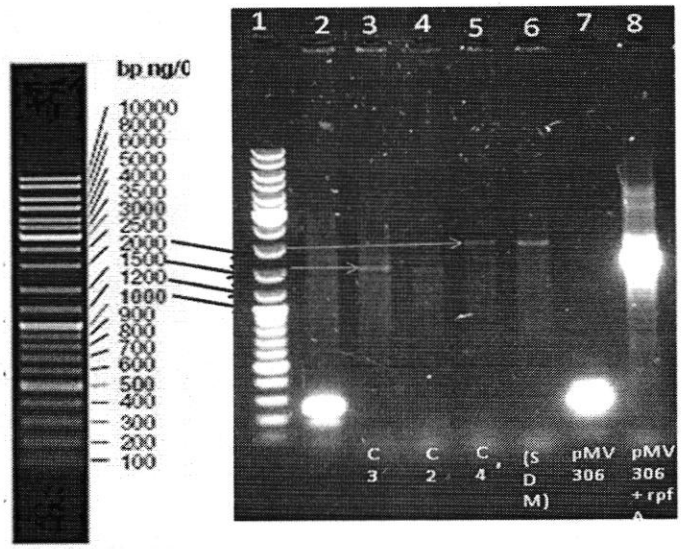



**Fig5:** Diagnostic PCR to confirm gene deletion in *M. marinum*. Deletion of the gene results in a PCR product that is significantly smaller (The expected band size is 582bp for deletion of rpfA deletion mutants lane 3 to 7) and deletion absent in WT (WT- lane1& 2)

The pMV306 plasmid construct of rpfA of *M. tuberculosis* from different regions was further confirmed through colony PCR. The different rpfA with different fragment length was amplified and detected in agarose gel electrophoresis (Fig 6).




| | |
|---|---|
| PR_1219 pMV306F | CCT TTG AGT GAG CTG ATA C |
| PR_1220 pMV306R | CGT TCG CCC TGT CGT TCA |

Fig 6. Detection of different fragment of rpfA of *M. tuberculosis* in plasmid insert in different constructs by colony PCR

Expression of rpfA was also detected through RT-PCR. Resuscitation of dormant bacteria was also observed using most probable number (MPN) assays, which was conducted in the presence of Rpf-containing and Rpf deficient culture filtrates.

**Research paper:** The results will be included in research paper of host laboratory and ICMR will be acknowledged.

**Attended meeting during Fellowship in UK:**

- **The Acid Fast Club, UK** -Annual Winter Meeting 2019, Friday 4th of January 2019 at University College London (UCL), London, UK

- Sleeping with The Enemy - Leicester TB Research Group event on Wednesday, 13 March 2019 at University of Leicester, UK

**References:**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- Kaprelyants AS, Mukamolova GV, Ruggiero A et al. (2012). Resuscitation-promoting factors (Rpf): in search of inhibitors. *Protein Pept Lett.* 19(10), 1026-34.
- Mukamolova GV, Turapov O, Malkin J, et al. (2010). Resuscitation-promoting factors reveal an occult population of tubercle bacilli in sputum. *Am J Respir Crit Care Med.* 181(2):174-180.
- Rosser A, Pareek M, Turapov O et al. (2017). Differentially culturable tubercule bacilli are generated during non-pulmonary tuberculosis infection. *Am J Respir Crit Care Med.* 2017 Sep 7. doi: 10.1164/rccm.201705-1048LE.
- Stinear, T.P., Seemann, T., Harrison, P.F et al. (2008). Insights from the complete genome sequence of *Mycobacterium marinum* on the evolution of *Mycobacterium tuberculosis*. *Genome Research* 18(5): 729-741.
- Turapov O, O'Connor BD, Sarybaeva AA et al. (2016). Phenotypically adapted *Mycobacterium tuberculosis* populations from sputum are tolerant to first-line drugs. *Antimicrob Agents Chemother* 60(4), 2476-83.

**iii) Proposed utilization of the experience in India :**

The training and research received in the Department of Respiratory Sciences at University of Leicester, UK has given sufficient hands on technical exposure to the fellow. The interaction with research scientists, faculty members of Leicester tuberculosis group (LTBG) helped me to generate new research ideas, laboratory setup etc.

The work done at host institution in the area of Resuscitation Promoting Factors may be explored for identification and quantification of Rpf dependent persistent bacilli during tuberculosis infection; targeting dormant bacteria; to reduce the treatment duration with reduced relapsed rate in tuberculosis; new drug target, to enhance the culturability of culture negative tubercle bacilli in sputum etc. The expertise acquired during training and research period will be utilized and implemented for further progress of my tuberculosis research at ICMR-NJIL& OMD, Agra, India. Furthermore, the fellowship has given an excellent opportunity for the ICMR-IF in building an international collaborative research team for future research in the field of TB and will be helpful in writing a collaborative project with the host UK laboratory in near future.

Date
22/04/19.

Vivek Kumar Gupta

Signature of ICMR-IF 22/04/19

(Dr. Vivek Kumar Gupta)

Dr. VIVEK KUMAR GUPTA
SCIENTIST-D
National JALMA Instt. for Leprosy &
Other Mycobacterial Diseases
Taj Ganj, AGRA-282004

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