REPORT

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

1.	Name and designation of ICMR- IF	: Dr Ajay Kumar, Scientist
2.	Address	: Biochemistry Division, IVRI, Izatnagar, Bareilly, U.P.-243122
3.	Frontline area of research in which training/research was carried out	:

Introduction

Primary infection with herpes simplex virus-1 (HSV-1) results in productive replication of the virus at the site of infection. During this initial phase, virus enters trigeminal ganglia and virus replication occurs there but within a few days no virus can be detected. The genome, however, persists in neurons in a latent state from which it reactivates periodically to resume replication and produce infectious virus. In mice, latency can be established efficiently after inoculation with HSV-1 in the cornea. The steps of the latency-reactivation cycle have been operationally divided into three major steps: establishment, maintenance, and reactivation. Establishment of latency includes entry of the viral genome into a sensory neuron and acute infection. Viral gene expression is then extinguished, with the exception of the latency-associated transcript (LAT). Maintenance of latency is a phase that lasts for the life of the host. In general, abundant expression of viral genes that are required for productive infection does not occur. LAT is abundantly expressed during this stage of latency. The promoter that directs expression of the latency-associated transcript (LAT) is activated in sensory neurons.

Therefore, I started my work with purification of available HSV-1 virus by picking single plaque through Agarose overlay method and through multiple passages in Vero cells (Fig.1), titer of virus was increased to $10^{8.5}$. Since latency associated transcripts (LAT) play role in establishment, maintenance and reactivation of virus from latency and is also involved in antisense suppression mechanism because it overlaps the 3′ end of infected cell protein 0 (ICP0) mRNA, an immediate early gene of virus lytic cycle responsible for productive infection therefore, proposed work was planned on understanding the mechanism of latency. Virus genomic DNA was isolated by standard kit. Primers were designed for amplification of LAT gene. For development of latency, mouse neuroblastoma cell line was used and infected with 0.1 MOI (Multiplicity of Infection) and cells were subcultured three times and titer of virus was calculated in pfu and there was no cytopathic effect seen in this cell line unlike Vero cells since virus donot replicate in neuronal cells. US3, One of the important gene of HSV-1, a serine/threonine protein kinase gene, is responsible for neuroinvasiveness and reactivation from latency. It phosphorylates UL47, a major virion protein responsible for HSV-1 nuclear egress. UL47 enhance the efficiency of alpha TIF (VP16)-mediated alpha gene expression through an unknown mechanism of action and exhibits altered viral thymidine kinase gene expression and thus affects the virulence and pathogenesis.

Therefore, present study was targeted to delete US3 gene from genome of HSV-1 and see its role in latency reactivation cycle of virus in mice model.

Experimental plan:

US3 gene was deleted by overlap PCR using Gibson assembly master mix of NEB. For that 1.5 kb upstream to the US3 gene and 1.4kb downstream to the US3 gene was amplified (Fig. 2 and 3.) and for selection of US3 mutant virus, red fluorescent protein along with CMV IE promoter region from PDS red Max N1 vector was also amplified (Fig. 2). For all three amplified products, primers were designed as per the protocol of NEB Gibson assembly. All three amplified products were assembled. Following primers were used.

HSVUS3-5FFP: 5'GATCGAAGCTTGCCGATACCAGCTCCGTGGAACG 3'

HSVUS3-5FRP: 5'CTCAGTGCTATCCATTCGCCGCACCGTGAGTGCCA 3'

PDSREDMXFP: 5'CGAATGGATAGCACTGAGAACGTCATCAAG 3'

PDSREDMXRP: 5'CTGTTGAAACAGCGGCGCCTTAAGATACATTGATGAGTTTGG 3'

HSVUS3-3FFP: 5'GCGCCGCTGTTTCAACAGAAATGACC 3'

HSVUS3-3FRP: 5'GATCGGAATTCGGCGCACCGGTGATTTATACC 3'

Restriction enzyme HindIII and EcoRI was kept in forward primer of upstream flanking region of US3 gene and reverse primer of downstream flanking region of US3 gene respectively for cloning in pUC19 vector.

Chemically competent E. coli cells were transformed with whole of the assembled product and Plated on LB agar plates containing 100 µg/mL ampicillin. Plate was incubated overnight at 37 °C. Individual colonies were picked from plates and transferred to tubes containing LB broth with 100 µg/mL ampicillin. Tubes were incubated overnight at 37 °C while shaking at 200 rpm.
Plasmids were isolated from the overnight culture (Fig. 4) using a plasmid miniprep kit. Concentration and purity of the DNA was checked using a spectrophotometer. Recombinant plasmid was checked for presence of all three amplified insert by restriction digestion by HindIII and EcoRI as shown in Fig. 5 and confirmed plasmid was stored at -20 °C.
This recombinant pUC19 vector was used for transfection in Vero cells along with co infection of cells with HSV-1 virus. This was done for homologous recombination and RFP reporter gene from recombinant pUC19 vector will be introduced in place of US3 gene

Generating the recombinant virus (ΔUS3 HSV-1)

Confluent monolayer of Vero cells was infected with the HSV-1 virus to be recombined at a multiplicity of infection of 1.0 (MOI = 1.0) in a 6-well plate and incubated at 37 °C and 5% CO2 for 1 h. Then media was aspirated and replaced with fresh DMEM without serum. Infected cells were transfected with 500 ng of the recombinant pUC19 vector using a commercially available Lipofectamine 3000 transfection reagent following the manufacturer's protocol and incubated at 37 °C and 5% CO2 for 36 h. One well was used as negative control only having infection of HSV-1 virus and second well was kept as a positive control and transfected with PDS Red Max N1 plasmid. 36 hours post-infection, plates were visualised in fluorescent microscope and infected monolayer transfected with recombinant pUC19 vector (Fig. 6) was harvested by three time freeze-thaw method. Harvested cells lysate were serially 10-fold diluted from 10^{-1} to 10^{-6} by

adding 120 µL of the lysate to 1080 µL of DMEM (10-1), and then adding 120 µL of this dilution to 1080 µL of DMEM and repeated this process four more times. 1 mL of each dilution was added to an individual, confluent well of Vero cells cells and incubated at 37 °C and 5% CO2 for 1 h. Then medium was aspirated and replaced with fresh DMEM and again incubated the infected cells at 37 °C and 5% CO2. 24 to 48 hours post-infection, recombinant viruses were again seen by fluorescence microscopy for expression of RFP protein (Fig. 7). Plaques from recombinant viruses expressing red fluorescence due to integration the RFP gene was picked. Plaque purification of recombinant viruses were followed three times on Vero cells (Fig. 8). After the final round of plaque purification, all plaques were expressing red fluorescence. One of the RFP expressing plaques was dissolved in 200ul DMEM media without serum and whole of this media with plaque was added to confluent Vero cells and visualized after 36 hrs in fluorescent microscope and infected cells started showing RFP expression as shown in Fig. 9. This is the way of generating scarless mutant virus lacking US3 gene. This mutant virus will be used for intraocular inoculation in mice for latency reactivation study.

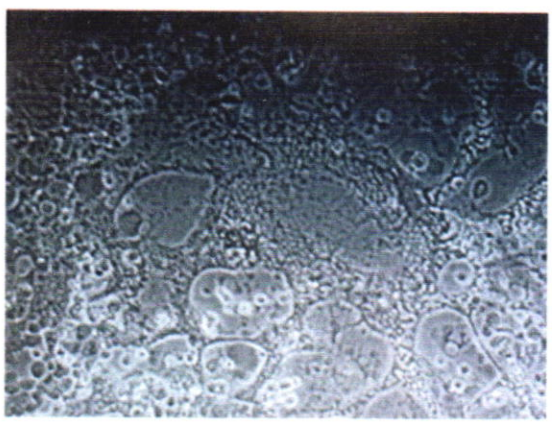

Fig. 1: Cytopathic effect in Vero cells infected with HSV-1

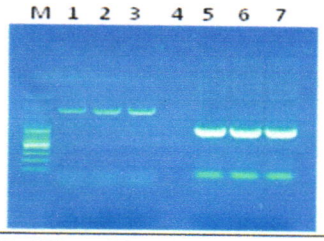

Fig.2: PCR amplification of upstream of US3 gene and RFP
M: 1kb DNA ladder
1,2 and 3: 1.5 kb amplified product of upstream flanking region of US3 gene
5,6 and 7: 750bp amplified product of RFP gene

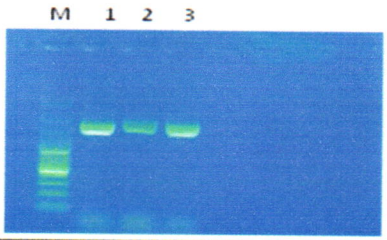

Fig.3: PCR amplification of downstream of US3 gene
M: 1kb DNA ladder
1: 1.4 kb amplified product of downstream flanking region of US3 gene

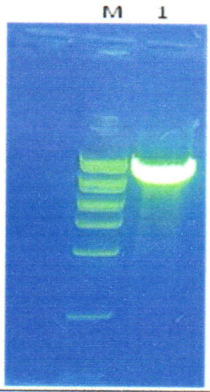

Fig.4: Recombinant pUC19 vector ligated with all three amplified product
M: 1kb DNA ladder
1: 6.1kb Recombinant pUC19 vector

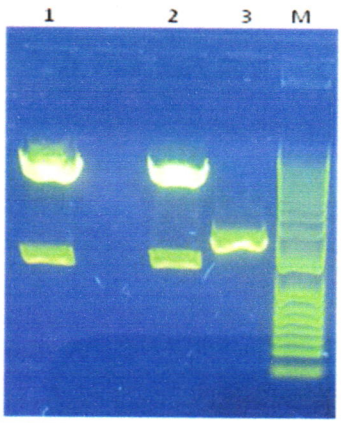

Fig.5: Restriction enzyme digestion of recombinant pUC19 vector for release of 6.1kbinsert
1: Plasmid digestion from colony 1
2: Plasmid digestion from colony 2
3: Plasmid digestion from colony 3(false product)
M: 100b DNA ladder

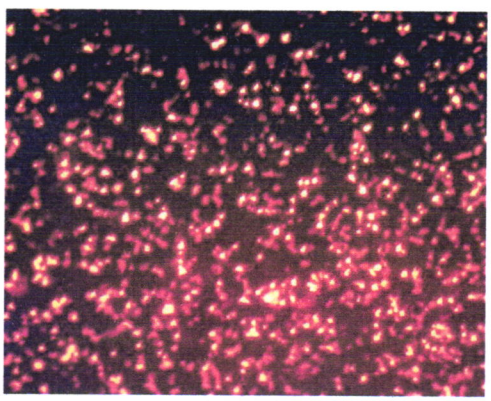

Fig.6: Vero cells infected with 1 MOI HSV-1 virus and cotransfected with rpUC19vector seen at 36 hrs in fluorescent microscope

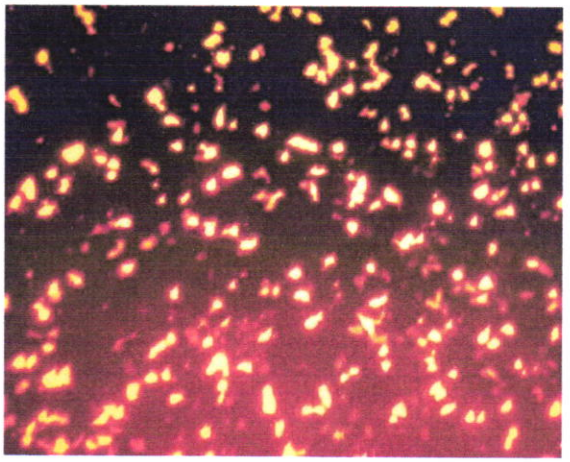

Fig.7: Vero cells infected first freeze thawed cells (infected with HSV-1 and cotransfected with rpUC19 vector at 10^{-3} dilution as taken from fig 6.)

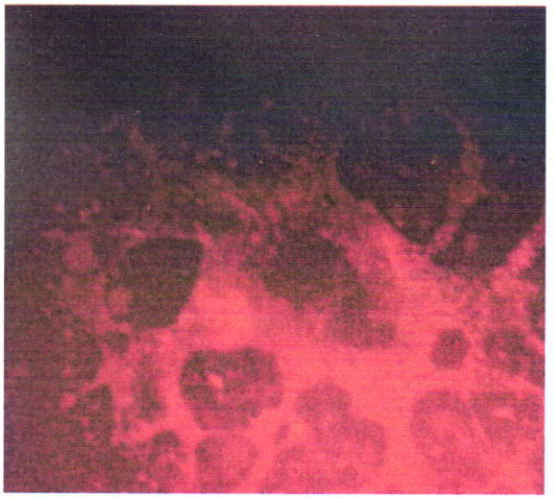

Fig.8: Vero cells infected with 10^{-3} dilution third passage of mutant virus and picked from agarose overlay cells showing expression of RFP replacing US3 gene

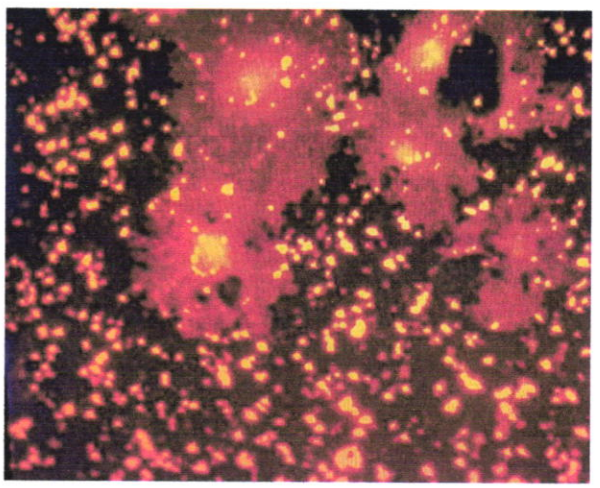

Fig. 9: Infection of Vero cells with individually picked RFP expressed HSV-1 plaque from Agarose overlaid well

During my stay there, I have also learned a novel area: nanobody generation against a particular antigen. Here, a nanobody library of yeast cells was procured from Kruse lab, Department of Biological chemistry and molecular pharmacology, Harvard Medical School, Boston, USA.
Nanobodies are a novel class of therapeutic proteins based on single-domain antibody fragments that contain the unique structural and functional properties of naturally occurring heavy chain only antibodies. Ablynx's proprietary Nanobody platform allows for the rapid generation and large-scale production of novel biological therapeutics that have potential in a wide range of human diseases. The Nanobody technology was originally developed following the discovery and identification that Camelidae (e.g. camels and llamas) possess fully functional antibodies that consist of heavy chains only and lack light chains. These heavy-chain only antibodies contain a single variable domain (VHH) and two constant domains (CH2, CH3). The cloned and isolated single variable domains have full antigen binding capacity and are very stable. These single variable domains, with their unique structural and functional properties, form the basis of a new generation of therapeutic molecules which Ablynx has named "Nanobodies". Its stability, smaller size (12-15kDa) and hydrophilic nature made it a novel class and better choice from monoclonal antibody (Fig.1). We have got yeast library of nanobody from Kruse lab and library was made in such a way that it has all combination of binding any kind of antigen. Yeast cells display nanobody (with HA tag) on its surface as shown in Fig.2 and billions of yeast cells are there displaying different types of nanobody. Our lab was working on herpes stromal keratitis (HSK) developed by intraocular inoculation of HSV-1 in mice and main reason of HSK is due to infiltration of a subset of CD4+ T cells called TH17 cells producing IL17 cytokine. This is a proinflammatory cytokine responsible for vascularisation of cornea and finally leading to blindness or loss of vision. Therefore, we have targeted generation of nanobody against IL-17 cytokine and it will be used for neutralizing the effect of IL-17 in HSK. Following the protocol of Kruse lab, anti HA antibody conjugated with Alexa Flour 488 and anti IL-17 antibody conjugated with Alexa Flour 647 (two different fluorophores) was added to yeast library and with the help of magnetic assisted cell sorting, positive selection was done to remove those yeast cells not producing nanobody and those not bound with antibody. Thereafter, flow cytometry was done to find out double positive yeast cells (positive for anti HA-AF 488 and anti IL-17 AF 647). Double positive cells were individually sorted in 96 well plates and grown and stained with anti IL-17 AF 647 and analysed in flow cytometer (Fig.3). After enrichment, few yeast cells producing nanobody against IL-17 and were high binders with IL-17 protein were again tested in flow cytometer and found that nanobody numbered 6,9 and 10 showed high affinity with IL-17 (Fig.4) and finally nanobody no 10 were selected and was grown in large amount and pelleted and plasmid was extracted and gene along with HA tag was removed from plasmid and ligated with pET22b vector for periplasmic expression of nanobody in soluble and native form in bacterial cells in large amount. Sequencing of nanobody was done to find out the CDR3 region responsible for binding to antigen. Expressed protein was identified at 15kDa in SDS-PAGE and confirmed by western blotting with the help of anti His antibody (Fig.5). Nanobody was quantified by BCA method. Optimization of recombinant IL-17A coated on ELISA plate was done and 100ng of IL-17A was coated on each well for overnight and followed with washing and blocking with skimmed milk powder and 200ng of purified nanobody (after optimization and keeping proper control) was added to well and incubated for 1 hr and washed. Anti HA antibody was added and then HRPO labelled secondary antibody was added and colour development was done by addition of H2O2 and OPD substrate. This way nanobody showed its binding with IL-17A cytokine and

this nanobody can be used for its therapeutic application for neutralization of IL-17 and thus severity of disease can be mitigated.

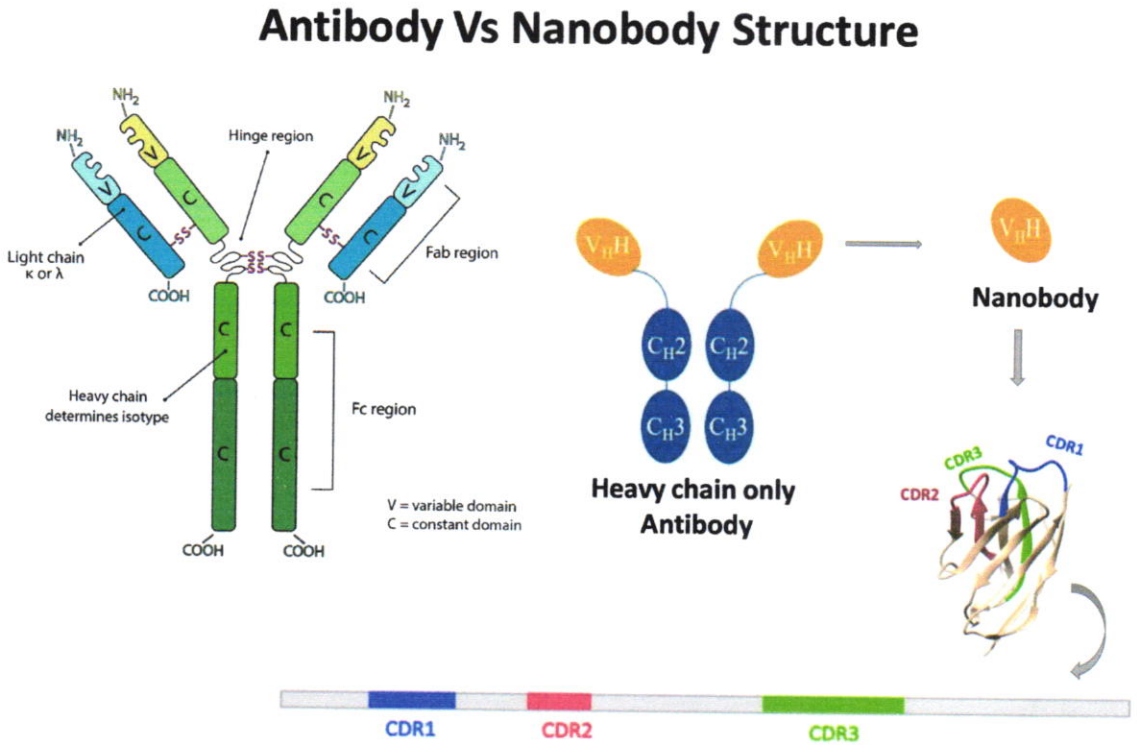

Fig. 1: Structural difference between Antibody and Nanobody

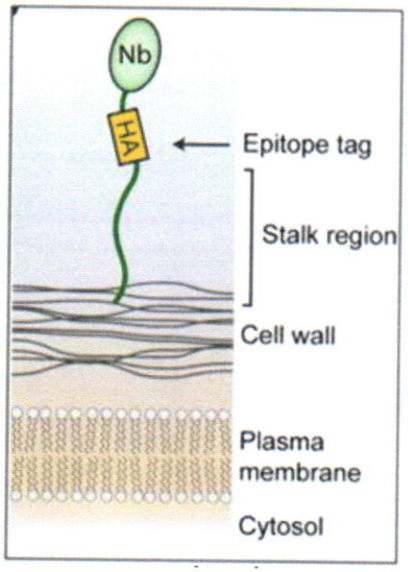

Fig.2: Yeast display of Nanobody at its surface with epitope HA tag for identification

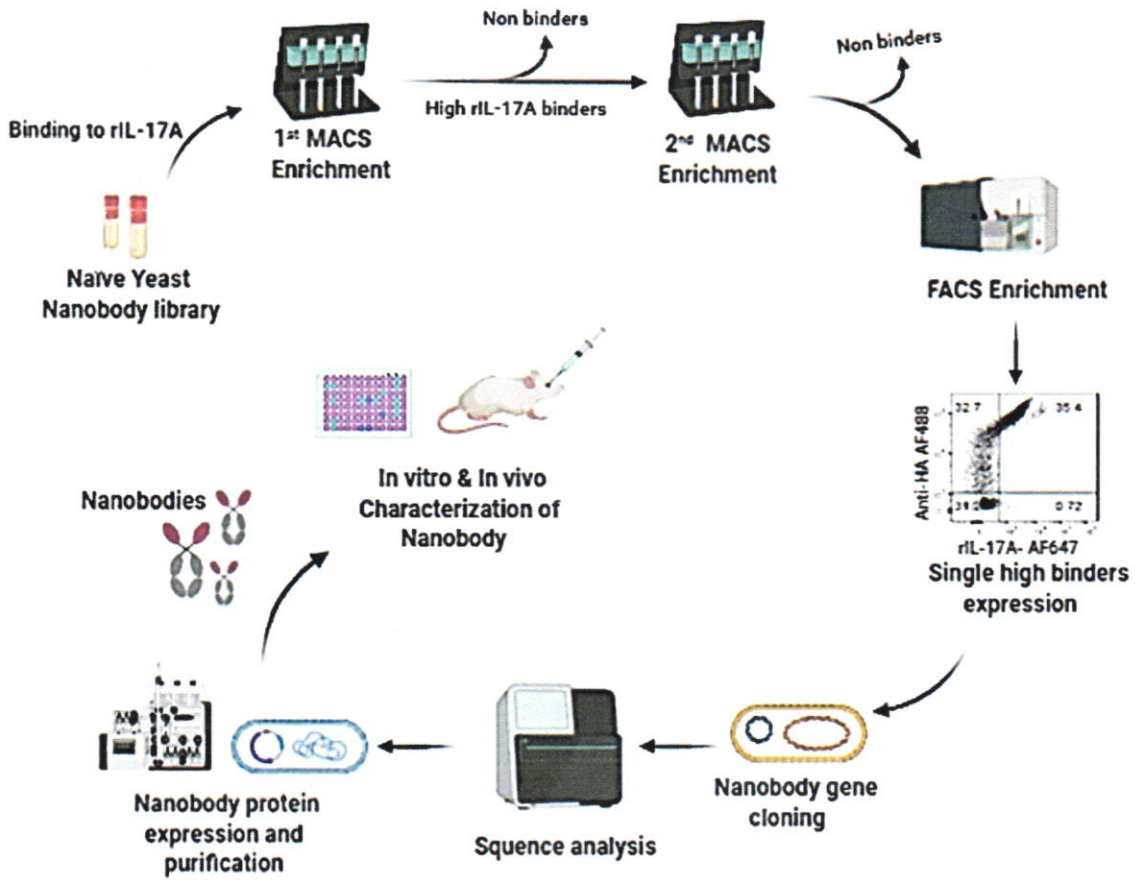

Fig.3: Schematic diagram showing identification of rIL-17 nanobody displaying yeast cells, its enrichment, sorting from flow cytometer, plasmid isolation and cloning in bacterial plasmid for high level expression of nanobody and its *in vitro* and *in vivo* characterization

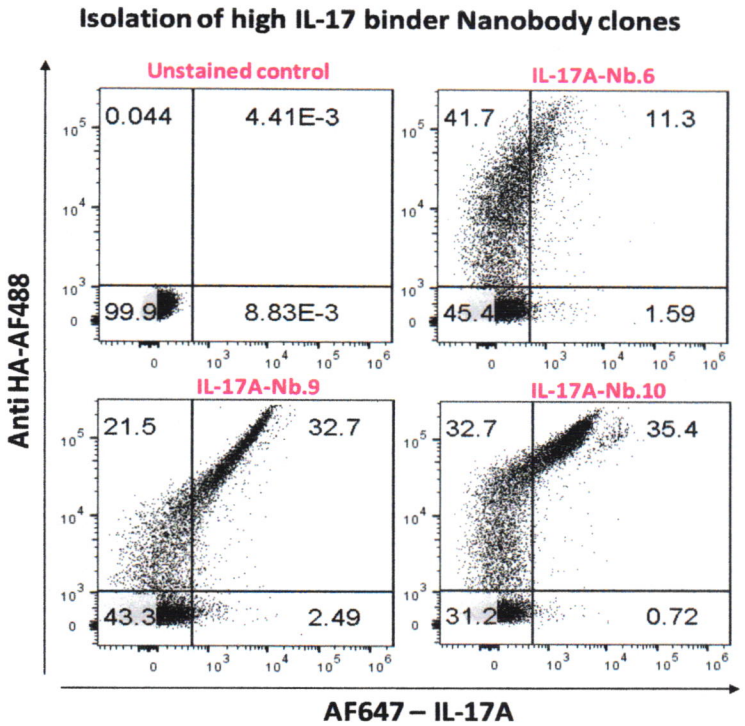

Fig.4: Nanobody 6,9 and 10 showing binding with rIL-17A cytokine and nanobody 10 relatively high affinity selected for high level protein expression

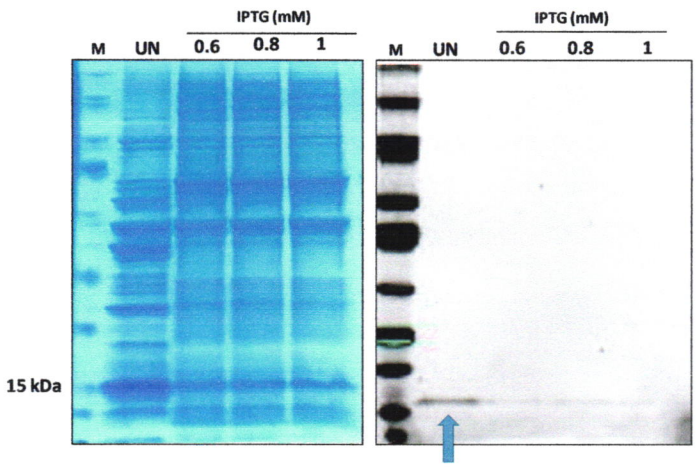

Fig.5: SDS-PAGE and western blot analysis of IL-17 nanobody 10 with anti His antibody at different mM IPTG concentration for optimization of expression
Lane M: Protein marker
Un: Uninduced cells
Lane 0.6,0.8 and 1.0: mM concentration of IPTG for induction of nanobody protein expression

4. Name & address of Professor and host institute : Dr Amol Suryawanshi,
Department of Pathobiology,
College of Veterinary Medicine,
Auburn University, Auburn,
Alabama, USA

5. Duration of fellowship with exact date :2nd February 2020 to 14th January2021

6. Highlights of work conducted :

i) Technique/expertise acquired :

1. Finding promoter region of a gene through online tools
2. Characterization of this promoter by its cloning in a promoterless vector
3. Ligation of three or more PCR amplified product through designing overlap primers
4. Plaque purification of virus
5. Transfection of vector in cell line
6. Generating mutant virus through homologous recombination
7. Selection of nanobody against IL-17 cytokine from yeast library
8. Enrichment of selected yeast cells displaying anti IL-17 nanobody on its surface through MACS positive selection method
9. Sorting of high binder anti IL-17 nanobody displaying on single yeast cell through FACS
10. Cloning and expression of IL-17 nanobody in prokaryotes
11. Functional characterization of nanobody through ELISA.

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

1. Promoter region of latency associated transcript (LAT) of HSV-1 was characterized.
2. US3, a serine/threonine kinase gene causing phosphorylation of many proteins responsible for latency reactivation of HSV-1 in pathogenesis of disease, was successfully deleted from genome of virus through homologous recombination
3. Identification of mutant virus was done through RFP reporter gene and US3 was replaced with RFP. This will help in tracking virus in different organs in lab animal model
4. I learned generating a nanobody against IL-17 pro-inflammatory cytokine.
5. MACS selection of anti IL-17 nanobody on yeast cells was done.
6. Single yeast cells displaying anti IL-17 nanobody was sorted by FACS.
7. Gene responsible for nanobody was cloned in bacterial system for high level expression.
8. Anti IL-17 nanobody was expressed and purified through Ni-NTA affinity chromatography.
9. Anti IL-17 nanobody protein was characterized by SDS-PAGE and western blotting.

10. Functional activity of generated nanobody was done by its binding with rIL-17 cytokine in ELISA.

iii) Proposed utilization of the experience in India :

This training provided me sufficient information and knowledge to understand the basic biology of herpes virus and I learned many techniques during my research work while deleting one of the gene of HSV-1 from its genome and this gene was responsible for latency reactivation and involved in pathogenesis. This helped me in understanding the latency of virus and since latency is the hallmark of herpes virus and HSV-1 is the prototype of bovine herpes virus-1 (BHV-1). I am working on BHV-1 from last ten years, the techniques I learned, can be applied to BHV-1, so my main focus in near future will be to target latency reactivation cycle of BHV-1 and if this cycle is compromised, virus should not undergo latency and developed mutant virus can be used as a vaccine candidate for protection of animals from BHV-1 infection. This training can be applied in deleting gene from virus/bacteria to reduce its virulence. One new technique, I learned, was generating nanobody, a single domain variable region of heavy chain only antibody (VHH) against pro-inflammatory cytokine IL-17. This cytokine is responsible for vascularization in cornea and reduces its transparency in HSV-1 infection and causes herpes stromal keratitis (HSK) and leads to loss of vision. Nanobody is used now a days for its therapeutic application in diseases or cancer because of ease of production, cost effective and small size, soluble and hydrophilic molecules and can enter cells easily as compare to monoclonal antibodies.

03/03/2021
Signature of ICMR-IF