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Regular Article

Expression and Characterization of Chandipura Virus Proteins

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Chandipura virus (CHPV) has recently emerged as an extremely lethal human pathogen in the family *Rhabdoviridae* and is linked to significant encephalitis outbreaks in different parts of India. The biology of CHPV remains less studied to date and the availability of reagents such as purified proteins can enhance research in this direction. In this study, we have overexpressed four of the CHPV proteins namely Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M) and Glycoprotein (G) using three distinct tags in bacterial system and with changes in inducer concentration, growth and solubilisation conditions successfully purified M and G proteins for the first time along with N and P. Furthermore, the interactions of CHPV M protein with other viral proteins (G, N and P) was investigated using ELISA and GST pull down assays to show the utility of solubilised proteins. The results of both the assays demonstrated that M protein interacts with both G and N proteins, while it does not interact with P protein, in a similar manner as reported for Vesicular Stomatitis Virus.

Keywords: Chandipura virus, Matrix protein, Glycoprotein, Protein purification, Solubilisation

Chandipura virus (CHPV), an emerging encephalitis-causing human pathogen endemic to India belongs to the genus *Vesiculovirus* of the family *Rhabdoviridae* (Chadha *et. al.,* 2005; Tandale *et. al.,* 2008). It is closely related to, but is phylogenetically distinct from Vesicular Stomatitis Virus (VSV) [the prototype virus of the genus *Vesiculovirus*]. CHPV has been found to infect children below the age of 14 years with significantly high mortality rate which vary from 55–75% (Kumar and Arankalle, 2010). The genome of CHPV is made of a negative sense single stranded RNA enwrapped by nucleocapsid protein (N protein) in such a way that the genomic RNA lies in the lumen which is formed between the N and C terminal lobes of the N protein. The 11 kb genome codes for five different proteins namely nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large protein (L) in the form of five monocistronic mRNAs (Basak *et. al.,* 2007). The N-RNA (Nucleocapsid-RNA) complex is associated with the viral RNA dependent RNA polymerase (RdRp) which is composed of L protein (the catalytic subunit) and phosphorylated form of P protein (the transcriptional activator) (Basak *et. al.,* 2007; Mire *et. al.,* 2009). The L, P and N proteins together with the RNA form the

ribonucleoprotein (RNP) particle. During assembly the M protein associates with the RNP and condenses it into a tightly packed helix. This condensation results in the characteristic bullet shape morphology of the virus (Mire *et. al.,* 2009). The M protein enclosed RNP is then surrounded by an envelope of host origin during budding. The envelope contains approximately 400 trimers of G protein spikes that protrude from the virion surface (Mire *et. al.,* 2009). G protein is the sole spike protein of CHPV that is involved in virus assembly and budding. It is a typical type I transmembrane protein that is embedded into the viral envelope with a short cytoplasmic tail, a transmembrane domain and a large antigenic ectodomain (Rose and Welch, 1980; Rose and Doolittle, 1982). Viral entry, a critical step in the life cycle of the virus, is mediated by G protein.

Apart from functioning as a structural component, M protein controls the switch between viral replication and transcription (Finke *et. al.,* 2003; Connor *et. al.,* 2006) by condensing the nucleocapsid into a tightly coiled helical structure (Newcomb *et. al.,* 1982). It also promotes viral budding (Harty *et. al.,* 1999; Bieniasz, 2006) and modulates host transcription (Komarova *et. al.,* 2007) and translation (von Kobbe *et. al.,* 2000); thus responsible for the cytopathic effects (CPE) exhibited by the virus. M protein regulates the translation of host mRNA by binding to and/or modulating the phosphorylation state of translation initiation factors (Connor *et. al.,* 2004; Komarova *et. al.,* 2007) as well as inducing the production of viral proteins through an unknown mechanism (Connor *et. al.,* 2006).

The information related to functions of CHPV proteins is hypothesized from studies on VSV. Keeping in mind the difference in target hosts, lethality of the virus and phylogenetic difference between CHPV and VSV, there is a requirement to get purified proteins from CHPV itself to understand its specific biological mechanisms. In the current study, we have successfully solubilised and purified M and G proteins of CHPV using bacterial system along with N and P proteins. Significantly, purification of G protein using a bacterial system has never been reported for any of the rhabdoviruses. The proteins discussed here are useful in detection and confirmation of protein-protein interactions and could find usage for antibody generation, structural and other biological studies. Using ELISA and GST pull down based methods, the interactions of M protein with other CHPV proteins i.e., G, N and P have been probed which highlight the utility of reagents described.

MATERIALS AND METHODS

1. Reagents and Plasmids:

Clones carrying N, P, M and G genes of CHPV were kindly provided by Prof. Dhrubajyoti Chattopadhyay of B.C. Guha Centre, Kolkatta.

2. PCR amplification and Cloning:

Genes encoding N, P, M and G proteins of CHPV were PCR amplified using gene specific primers designed for cloning in pGEX4T3 (GST tag; Novagen Biosciences, USA), pLTA (His tag), and pCAK (Strep tag) vectors. pLTA and pCAK vectors have been designed and described earlier by one of the authors (Gupta, 2009). pGEX4T3 is a high copy number plasmid with ampicillin resistance while pLTA and pCAK are low copy vectors carrying ampicillin and kanamycin resistance genes as selectable markers, respectively.

The primer sequences with characteristic restriction site incorporated to facilitate cloning are listed in Table 1. PCR reaction was performed in a final volume of 100 µl using 10 pmol of each primer, 1 mM dNTPs (Sigma Aldrich, USA), 3.0 U of Taq DNA polymerase (Sigma Aldrich, USA), Pfu DNA polymerase (Promega, USA) and 1 ng of template DNA. The products were purified using PCR purification kit (Sigma Aldrich, USA). For cloning in pLTA and pCAK vectors, PCR purified products were treated with T4 DNA polymerase and dTTP to generate *Bsa*I compatible ends. Bacterial

vectors (pCAK and pLTA) were digested with *Bsa*I enzyme and gel purified. Following ligation, the transformants were selected on appropriate antibiotic. For cloning in pGEX4T3 vector, the restriction enzyme combinations *Sma*I-*Xho*I for N and P genes and *EcoR*I-*Not*I for M and G genes were used. The genes and vector were digested with the respective enzyme combinations and finally ligated to get recombinant pGEX4T3 vectors carrying viral genes.

Table 1: Primers of CHPV genes for cloning in pGEX4T3, pLTA and pCAK vectors

S.no	Gene ID	Primer Sequence
1	N F (pLTA & pCAK)	5′ CGGCAGCAGTTCTCAAGTATTCTGCATTT 3′
2	N R (pLTA & pCAK)	5′ CTCCACCTCATGCAAAGAGTTTCCTGG 3′
3	P F (pLTA & pCAK)	5′ CGGCAGCGAAGACTCGCAACTGTATCAA 3′
4	P R (pLTA & pCAK)	5′ CTCCACCTCAATTGAACTGGGGCTCAAG 3′
5	M F (pLTA & pCAK)	5′ CGGCAGCCAACGTCTGAAGAAGTTTATAG 3′
6	M R (pLTA & pCAK)	5′ CTCCACCTCAATGACTCTTAGAAATCAGC 3′
7	G F (pLTA & pCAK)	5′ CGGCAGCACTTCTTCAGTGACAATTAGTGT 3′
8	G R (pLTA & pCAK)	5′ CTCCACCTCATACTCTGGCTCTCATGTT 3′
9	N F (pGEX4T3)	5′AATCCCCGGGGCAGTTCTCAAGTATTCTGCATTTC3′
10	N R (pGEX4T3)	5′ CATCTCGAGTCATGCAAAGAGTTTCCTGGC 3′
11	P F (pGEX4T3)	5′ AGAGCCCGGGGCGAAGACTCGCAACTGTATCAA3'
12	P R (pGEX4T3)	5′ TGCCTCGAGTCAATTGAACTGGGGCTCAAG 3′
13	M F (pGEX4T3)	5′ CTCGAATTCCCAACGTCTGAAGAAGTTTATAG 3′
14	M R (pGEX4T3)	5′ TGGGCGGCCGCTCAATGACTCTTAGAAATCAGC 3′
15	G F (pGEX4T3)	5′ CAGGAATTCCACTTCTTCAGTGACAATTAGTG 3′
16	G R (pGEX4T3)	5′ CACGCGGCCGCTCATACTCTGGCTCTCATGTT 3′

3. Protein Expression and solubilisation:

All the positive recombinant clones (in pGEX4T3, pCAK and pLTA) for each gene of CHPV were transformed in BL21 (DE3) strain of *Escherichia coli* to carry out expression studies. Transformed cells were grown in 30 ml Luria Broth medium supplemented with appropriate antibiotic (30 µg/ml kanamycin and 100 µg/ml ampicillin). The cultures were induced at different conditions of temperature, induction time and suitable inducer at the OD_{600} value of 0.3. At desired expression levels, the cells were harvested by centrifugation at 6000 rpm for 6 min at 4 ^{o}C. This was followed by cell lysis to obtain soluble fractions of fusion proteins by IBA Lysis buffer (IBA-GmbH, Germany) according to the manufacturer's protocol. Protease inhibitor cocktail (Clontech, USA), lysozyme (1µg/ml) and nucleases [DNase (20 ng/ml), RNase (6 ng/ml)] were also added along with the lysis buffer. The soluble and insoluble fractions were collected by centrifugation at 13,000 rpm for 20 min at 4 ^{o}C. The fractions were analysed by 10% SDS-PAGE and western blot.

4. Solubilisation of Glycoprotein using sarkosyl:

G protein, which was insoluble after cell lysis was solubilised using sarkosyl, a detergent used to solubilise the protein from inclusion bodies. Lysis buffer containing 50 mM Tris, 5 mM $ZnCl_2$, 300 mM NaCl, 10 mM β-mercaptoethanol and 10% sarkosyl was mixed with the pellet obtained after lysis and incubated overnight at 16 °C. The solubilised protein was then recovered as supernatant by centrifugation at 13000 rpm for 10 min. The sarkosyl concentration was reduced by dialysis in

PBS (Phosphate Buffer Saline) at 4 °C. The dialysed fraction was then checked for the presence of G protein by SDS PAGE followed by western blotting using anti-GST antibody (Sigma Aldrich, USA).

5. Purification of M and G proteins as GST fusions and immunoblotting:

The soluble fraction for M protein and the dialyzed sample of G protein were loaded on Glutathione sepharose beads (Clontech, USA) and incubated at 4 ^{o}C for 2 hours. The samples were centrifuged at 2100 rpm for 5 min at 4 ^{o}C to remove the unbound proteins. The beads were washed with PBS thrice and subsequently the target proteins were eluted from the beads using 10 mM reduced Glutathione (Sigma Aldrich, USA) at room temperature for 15 min. The eluted samples were electrophoresed by 10% SDS-PAGE and transferred on to the charged PVDF (Polyvinylideneflouride) membrane (Millipore, USA). The membrane was incubated with monoclonal anti-GST antibody (Sigma Aldrich, USA); 1:10,000 dilution for 1 h and subsequently with secondary antibody, horseradish peroxidase conjugated goat anti-mouse IgG (G Biosciences, India); 1:2000 dilution, for 1 h at room temperature. The reaction bands were developed by 0.05% DAB (Diamino Benzidine) and H_2O_2. The reaction was stopped in distilled H_2O.

6. ELISA and GST Pull down assays for M protein interaction studies:

For ELISA, Streptactin coated microtiter plate (IBA-GmbH, Germany) was incubated overnight with M Strep lyaste at 4 ^{o}C. After washing with PBST (PBS containing 0.05% Tween-20), second lysates of G His, N His and P His were loaded on plate for 2 hours at 4 ^{o}C. As primary and secondary antibodies, anti-His monoclonal antibody at 1:6000 dilution (Sigma Aldrich, USA) and anti-mouse IgG HRP conjugate at 1:4000 dilution (G Biosciences, India) were used. Plate was incubated with both antibodies for 1 hr each at room temperature. After each incubation, wells were washed thrice with PBST. Finally, 100 µl of substrate (TMB, 3,3,5,5-tetramethylbenzidine; Sigma Aldrich, USA) was added and reaction stopped with 2N HCl.

For GST pull down assay, M-G, M-N and M-P interactions were analysed. The lysates of solubilised samples of M GST and G His, M GST and N His and M GST and P His were mixed and incubated for 2 hours at 4 ^{o}C. The mixed lysates were then loaded on to the Glutathione resin (Clontech, USA) and incubated at 4 ^{o}C for 2 hours. Unbound protein fraction (flowthrough) was collected by centrifugation at 4 ^{o}C/2100 rpm/5 min. After 3 washes with 1X PBS, the bound proteins were eluted in 10 mM reduced Glutathione (Sigma Aldrich, USA) at room temperature. Eluted fractions were checked for interacting partners by western blot analysis with anti-His monoclonal antibody. For control, only GST lysate was mixed with G His, N His and P His lysates respectively and analysed in similar manner as tests.

RESULTS AND DISCUSSION

1. Cloning of CHPV genes in expression plasmids:

All the four genes (N, P, M and G) were observed at appropriate sizes (N-1.2 kb, P-0.8 kb, M-0.69 kb and G-1.5 kb) after PCR amplification on 1.2% agarose gel. The amplicons were ligated with the digested vectors and recombinant plasmids were confirmed for cloning by restriction enzyme digestion.

2. Overexpression and Solubilisation of fusion proteins:

For solubilisation analysis, we took three different tags of varying sizes and properties (i.e. GST tag- 220 aa, His tag- 6 aa and Strep tag- 8 aa) to check their effect on solubilisation of proteins. Conditions such as induction temperature, inducer concentration and induction time were standardized with M protein for every tag to increase the solubility (Table 2). For *Ara* promoter of pCAK vector, two different concentrations of arabinose 0.2% and 0.5%

were checked. At 0.5% arabinose, all the four proteins showed suitable expression for solubilisation. Similarly, for *Tet* promoter of pLTA vector, three different concentrations of anhydrotetracycline checked were 20 ng/ml, 50 ng/ml and 100 ng/ml. Anhydrotetracycline at 20 ng/ml was found to be optimum for solubilisation experiments. For *Tac* promoter of pGEX4T3 vector, 1 mM IPTG (Isopropyl β-D-thiogalactopyranoside) was used for expression and purification of GST fusion proteins. Further these concentrations were analyzed at different temperatures (16 °C, 25 °C and 37 °C) for increasing the soluble fraction of proteins. At 37 °C only N and P were found to be soluble. However at 25 °C for 4 hours with all three vector systems, N, P and M proteins were soluble i.e., present in the supernatant after cell lysis as indicated in fig. 1 (Lanes 2, 7 and 9, respectively). Importantly G protein, irrespective of all variations and tags, was found insoluble (Fig. 1, Lane 4) [data not shown for solubilisation of viral proteins with pCAK and pLTA vectors].

A recently described protocol was adopted for G protein solubilisation using sarkosyl (Tao *et. al.*, 2010). The insoluble pellet of G GST after cell lysis was treated with sarkosyl and the solubilised protein was purified. The protocol was modified by introducing a dialysis step to lower down the sarkosyl concentration before purification. Using this protocol we were able to solubilise all three G GST, G His and G Strep fusion proteins (Fig. 3, Lane 2; data not shown for G His and G strep).

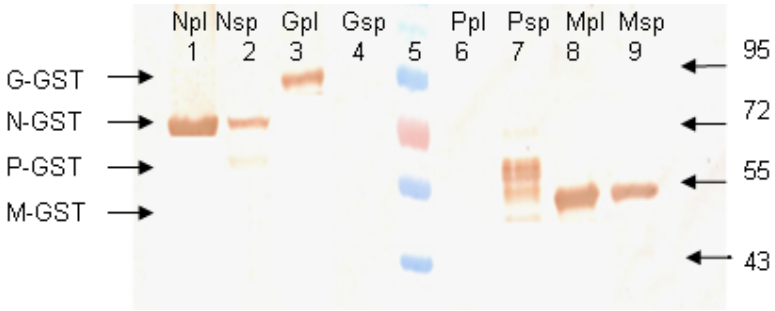

Fig. 1: Solubilisation profile of CHPV proteins: CHPV proteins expressed with GST tag in *E. coli* BL-21 (DE3) cells were checked for solubilisation after cell lysis using IBA lysis buffer. The soluble (supernatant, sp) and insoluble (pellet, pl) fractions collected by centrifugation were analysed by western blotting using anti-GST monoclonal antibodies. N, P and M proteins were found to be partially soluble as they were present partly in the supernatant [lane 2: Nsp, lane 7: Psp, lane: 9 Msp] and partly in the cell pellet [lane 1: Npl, lane 6: Ppl, lane: 8 Mpl]. G protein however was found to be completely insoluble as it was detected only in the cell pellet (lane 3: Gpl) and not in the supernatant (lane 4: Gsp).

Table 2: Different parameters considered for solubilisation analysis of viral proteins.

Parameters	**GST Tag**		**Strep Tag**		**His Tag**		
Inducer	IPTG		Arabinose		Tetracycline		
Inducer Conc.	1 mM		0.20% 0.50%		20 ng/ml 50 ng/ml 100 ng/ml		
Induction Time	4 hours	2 hours	4 hours	2 hours	Overnight	4 hours	2 hours
Induction Temp	25° C	37° C	25° C	37° C	16° C	25° C	37° C

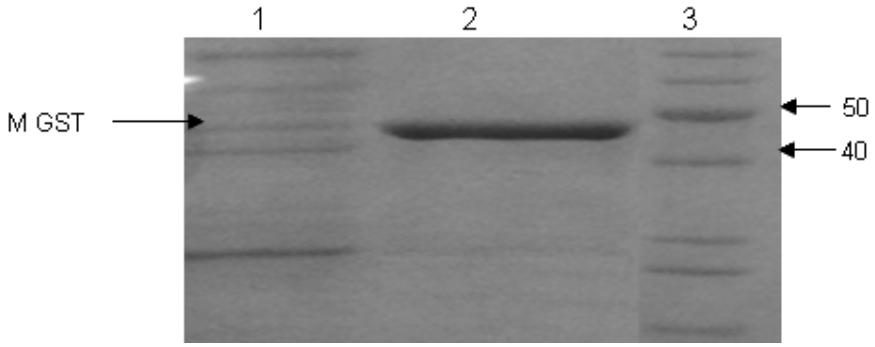

Fig. 2: Purification of CHPV Matrix protein: *E. coli* BL-21 (DE3) cells transformed with M GST were induced with 1mM IPTG for protein expression. Cells were lysed and the soluble fraction (supernatant/ cell lysate) was loaded onto the glutathione sepharose beads. After elution of the bound protein, samples were then analysed on 10% SDS-PAGE followed by Coomassie Brilliant Blue staining. Purified M GST fusion protein was present in the eluted fraction. (lane 2). The unbound proteins were present in the flowthrough (lane 1). Lane 3 is the protein ladder (molecular sizes are indicated in kDa).

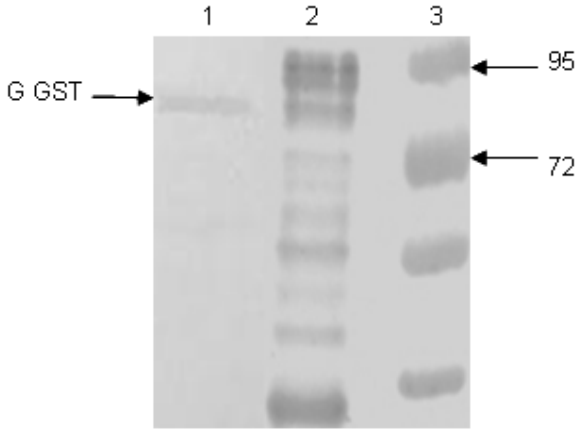

Fig. 3: Purification of CHPV Glycoprotein: BL-21 (DE3) cells expressing G GST fusion protein were lysed and the soluble (supernatant) and insoluble (cell pellet) fractions were collected after centrifugation. G GST protein was detected in the insoluble fraction and was solubilised using sarkosyl. The solubilised fraction was dialysed and loaded on glutathione sepharose beads. Following elution, the samples were analysed by SDS-PAGE. Purified G GST protein was present in the eluate (lane 1) and Lane 2 represents the solubilised fraction loaded on glutathione beads for purification. Lane 3 is the protein ladder (molecular sizes are indicated in kDa).

3. Purification of M GST and G GST fusion protein:

Having achieved solubilisation of all CHPV proteins with different tags, GST fusions of M and G were purified using Glutathione sepharose beads. The purified (eluted) samples were analysed by SDS PAGE followed by Coomassie brilliant blue staining. Eluted fractions showed a prominent band at the size of 52 kDa for M GST (Fig. 2, Lane 2) and 92 kDa for G GST (Fig. 3, Lane 1).

Unsuccessful attempt has been reported earlier for VSV G protein solubilisation by removal of transmembrane domain and by using *Bacillus subtilis* secretion vector system (Lundstrom, 1984). Recently G protein purification of CHPV has been reported from baculovirus expression system (Venkateswarlu *et. al.*, 2009) but this is for the first time that M and G proteins of

CHPV have been purified successfully using bacterial system. M protein of VSV, however, has been purified earlier as GST and His Tag fusion (Glodowski *et. al.,* 2002; Connor *et. al.,* 2006).

4. Detection of viral protein interactions by ELISA:

For the detection of interactions between viral proteins, solubilised proteins were used in ELISA as well as GST pull down assays. ELISA was performed to study the interactions of M (strep tag) protein with other viral proteins i.e., G (His), N (His) and P (His). Only M Strep lysate was taken as negative control (Fig. 4A, Lane 2). Following the incubation of lysates with anti His antibodies, the His tagged fusion proteins (G, N and P) interacting with Strep tagged M protein were detected by the addition of the substrate. Development of blue colour after substrate addition indicated a positive interaction as observed for M-G (Fig. 4A, Lane 5) and M-N (Fig. 4A, Lane 4) interactions while absence of colour for M-P (Fig. 4A, Lane 3) proved them as non-interactors. The OD_{450} values were determined after stopping the reaction with 2N HCl. The OD_{450} values observed for M-G, M-N and M-P interactions were 1.6, 1.34 and 0.06 respectively (Fig. 4B). The experiment was performed in duplicate.

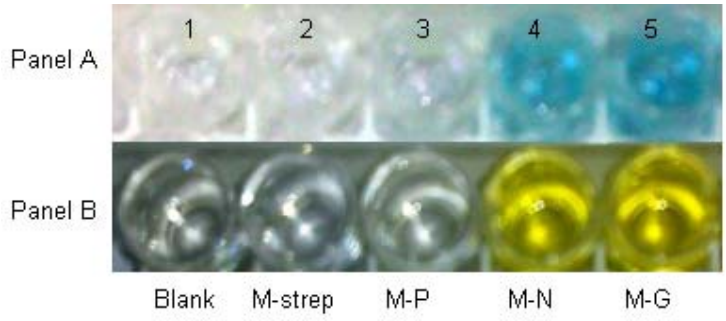

Fig. 4: Validation of CHPV M protein interactions by ELISA: Streptactin coated microtiter plate was incubated with M Strep lysate overnight followed by incubation with His fusion proteins (G, N and P). After washing with PBST, the interacting His fusion protein bound to M Strep was detected using anti His monoclonal antibody The lysate of only M Strep (without any His fusion protein lysate) was taken as negative control (Panel A, B: lane 2) while M-P constituted as a negative interaction control (Panel A, B: lane 3). Blue colour production after addition of substrate (TMB) indicated a positive interaction as observed for M-N (Panel A, B:lane 4) and M-G (Panel A, B:lane 5) interactions. Panel A, lane 1 was air blank. Panel B shows the final product after stopping the reaction with HCl in corresponding lanes.

5. Confirmation of protein interactions by GST pull down:

GST pull down was performed to confirm the interactions detected by ELISA. The lysates taken for the study were G His, N His and P His with M GST as test and only GST as control. Column containing M GST (test) or only GST (control) was prepared and allowed to bind with lysates of His tagged G, N and P. The interacting proteins remained bound to the column while non interacting proteins came out in the flowthrough (unbound fraction). The column was washed and interacting proteins were eluted with reduced glutathione. The samples were analysed by western blotting using anti GST (M GST: 52 kDa) and anti His (G His: 70 kDa, N His: 48 kDa, P His: 35 kDa) monoclonal antibodies.

For M-G interaction, the presence of G His (Fig. 5A, Lane 2) band in eluted fraction confirmed the interaction among M and G proteins. In column containing only GST, the G His band was not observed in the eluate (Fig. 5A, Lane 1) showing the specificity of M-G interaction. The binding

of GST and M GST fusions with beads was also detected for control purpose (Fig. 5B). Similarly, M-N interaction was confirmed by presence of N His band in eluted fraction (Fig. 5A, Lane 5). In M-P pull down, however, P His was not observed in the eluted fraction (Fig. 5A, Lane 7) while M GST was present in the same eluted fraction (Fig. 5B, Lane 7) indicating that M and P proteins do not interact. ELISA and pull down results confirm M-G and M-N interactions and prove M-P to be a non interacting pair.

The observed interactions have also been shown in VSV earlier. Interaction of M and G proteins in VSV and its indispensable role in virus assembly has been demonstrated by Lyles and co-workers (Lyles *et. al.*, 1992). Later in 1994, Chong and his colleagues proved the interaction of VSV matrix protein and N protein (Chong and Rose 1994). Recently, the disruption of this interaction was shown to be responsible for impaired viral assembly (D'agostino *et. al.*, 2009).

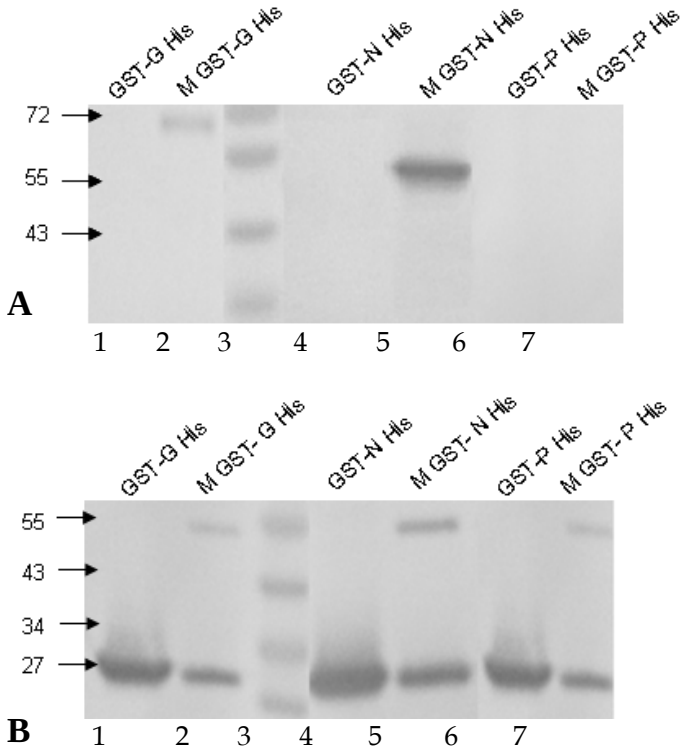

Fig. 5: Validation of CHPV M protein interactions by GST Pull down: For GST pull down assay, M GST was used as a bait to identify the interacting His tagged protein partners i.e., G, N and P. Following incubation, the lysates (M GST with His fusion proteins) were loaded on glutathione sepharose beads. The interacting His fusion proteins remained bound to the beads while the non-interacting partner came out in the flowthrough (unbound fraction). The bound proteins were analysed by western blotting using anti His (A) and anti GST (B) monoclonal antibodies. Binding of GST with His tagged proteins was taken as negative control (A: lanes 1, 4 and 6). The assay shows that M protein interacts with G and N proteins (A: lanes 2 and 5 respectively) but not with P protein (A: lane 7). Eluted fractions of pull down were also analysed for the presence of GST (B: lanes 1, 4 and 6) and M GST (B: lanes 2, 5 and 7) proteins. Molecular sizes of the protein ladder are indicated in kDa (A, B: lane 3).

CONCLUSION

Our work provides the first insight with the help of different tags, temperature and inducer concentration on the property of solubility of CHPV proteins. The benefits of having all proteins with different tags along with successful solubilisation protocols can be utilized for pull down studies, Co-IP assays etc., to check and validate protein-protein interactions in virus–virus and virus–host context. The purified fusion proteins with small tags can also be used for generation of antibodies, creation of diagnostics and carrying out other functional studies. We have shown the utility of solubilized proteins by checking the interaction of CHPV matrix protein with other viral proteins for the first time using pull down and ELISA based methods. Furthermore the methods described here can be utilized for expression and purification of M and G proteins of other major viruses of rhabdoviridae family.

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