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An optimized protocol for purification of virus-like particles of foot-and-mouth disease virus produced in the baculovirus expression system

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Abstract
Foot-and-mouth disease is a highly endemic, economically significant disease of livestock population across the world. Higher antigenic variation and short-lived immunity possess the threat of its recurrent incidence. Developments are underway to produce bio-safe and more stable vaccines using virus-like particles to enhance immunity duration without handling live infectious viruses. Besides this, VLPs possess an inherent property of differentiation of infected and vaccinated animals (DIVA) as they do not carry any non-structural proteins along with them. Here we demonstrate such a bio-safe platform to produce VLPs using a baculovirus expression system and its efficient purification. The purification protocol combines the process of filtration and isodensity centrifugation to separate the assembled VLPs from the host cellular components and demonstrated the presence of pure and concentrated VLPs of expected buoyant density for further structural and functional studies.

Keywords: foot-and-mouth disease, VLPs, Purification, isopycnic centrifugation

1. Introduction
Foot-and-mouth disease (FMD) is a vesicular disease of cloven-hoofed animals, including the domesticated and wild ruminants and pigs. FMD is caused by the foot-and-mouth disease virus (FMDV) that belongs to *the Picornaviridae* family in the genus *Aphthovirus*. FMDV genome consists of a single open reading frame (ORF), which codes for a long polyprotein that gets cleaved by viral coded proteases into individual mature viral proteins. A virus coded protease 3C performs the majority of cleavages that produce viral structural proteins and enable the assembly of FMDV capsid in the infected cells (Belsham, 1993) [2]. Each icosahedral capsid structure of FMDV comprises 60 copies of the subunit called the protomers. Each protomer is made up of single copies of structural proteins VP0 (VP4 and VP2), VP3 and VP1 and assembly of five such protomers results in a pentamer. Further assembly of 12 such pentamers forms the complete capsid and encases the nucleic acid within it (Grubman *et al*., 1995) [11]. Even though the combined efforts of vaccination and regulated culling had resulted in disease-free countries and zones, the virus shows its presence in these areas by sporadic severe outbreaks (Grubman and Baxt, 2004) [10].
The disease is endemic in most of the countries in the world, including India. An inactivated vaccine is currently used as a disease mitigation measure, and its manufacture require live virus handling and high containment infrastructure (Doel, 2003) [5]. This demerit of a chance of virus spillage resists the disease-free countries to produce the vaccine for emergency use. In order to address these issues, recombinant vaccines and virus-like particle (VLP) based vaccines have been attempted using various expression platforms (Bhat *et al*., 2013; Kumar *et al*., 2016; Xiao *et al*., 2016) [3, 12, 16], which eliminates live virus handling. Numerous researches are going to find the scope of these bio-safe VLPs in diagnostic and immunoprophylactic areas. In this context, detailed structural studies, stability assays, diagnostics, and immunological studies involving purified VLPs must be carried out. Analysis of structural and functional properties demands the need for highly purified VLPs excluded from the plasma membrane, baculoviruses and other cellular and viral proteins intermediates. Obtaining purified VLPs from the crude cell lysate is a cumbersome procedure usually involving the pelleting of VLPs, and in many instances, this causes structural deformations and loss in yield to the less stable VLPs (Fuscaldo *et al*., 1971) [8].

Here we demonstrate a comparatively less time-consuming purification method combining filtration and isopycnic ultracentrifugation to purify FMDV VLP from a baculovirus expression system.

2. Materials and Methods

2.1 Generation of recombinant bacmid DNA

The transfer plasmid vector pFBac-As1-P1-2A-3C142T carrying the capsid coding sequence of serotype Asia1/IND/63/72 available in the FMDV vaccine production laboratory, ICAR-IVRI regional unit Bengaluru (Basagoudanavar *et al*., 2015) [1] was used in this study. The transfer plasmid was transformed into DH10-BacTM competent cells (Life Technologies, USA), and positive colonies were selected by colony PCR after blue-white screening as described earlier by Ganji *et al*., 2018 [9]. The positive colonies with successful transposition were further gown in LB broth for overnight culture and isolated the recombinant bacmid DNA with Qiagen plasmid mini kit (Qiagen, Hilden, Germany) as per manufacturer's instruction.

2.2. Production of recombinant baculovirus

The isolated bacmid was transfected to Spodoptera frugiperda (Sf-21) insect cells maintained at 26°C in SF-900 II SFM serum-free medium (Invitrogen) in a six-well plate by Cellfectin II reagent (Life Technologies, USA). The plates were monitored for the next 72 -96 hr for visible cytopathic changes and harvested the supernatant containing recombinant baculovirus. The presence of FMDV capsid genes in the baculovirus was confirmed by PCR using gene-specific primers VP4F and 2B29R (Selvaraj *et al*., 2019) [15]. The confirmed baculovirus was amplified further and titrated before infection to Trichoplusia ni (Tn5) insect cells to achieve a high level of capsid protein expression (Wickham and Nemerow, 1993) [16].

2.3. Expression and confirmation of capsid proteins

The Tn-5 cells in a 175 cm2 having 20 ml media with a cell density of 0.8 million cells/ml were infected at a high moi of 5 with the recombinant baculovirus. Post three days of infection, the cells were recovered by brief centrifugation at 4000 rpm for 10 min. The cell pellet was freeze-thawed once at -80 ºC and incubated with 2 ml lysis buffer (50 mM Tris, 100 mM NaCl, containing 0.1% Triton X 100, 100 mM PMSF and protease inhibitor cocktail) for 30 min on ice. Following brief sonication for 20 sec, the suspension was centrifuged for 30 min at 10000g to separate the insoluble cell debris from the capsid protein-containing supernatant. The specificity of the expressed capsid protein in the supernatant was confirmed with serotype-specific sandwich ELISA (Bhattacharya *et al*., 1996) [4]. Further confirmation of the expressed proteins was ascertained in western blot with serotype-specific guinea pig serum raised against 146 S antigen of FMDV Asia1/IND/63/72. In brief, the expressed proteins in the infected and uninfected cell lysate were electrophoretically separated and transferred onto a PVDF membrane along with the protein ladder. Following blocking with 5% skim milk powder, the membrane was probed primarily with the specific guinea pig serum and further traced with polyclonal rabbit anti guinea pig Ig/HRP (Dako, Denmark). The development of specific bands upon exposure with West Pico Chemiluminescent Substrate (Thermo Scientific, RB231022) confirmed the expression of FMDV capsid proteins. Further assembly of these capsid proteins to form a VLP was ascertained with a 146 S specific monoclonal antibody (Mab) available in the lab, which detects only intact capsid (unpublished data). The assay was performed exactly as sandwich ELISA mentioned earlier except the use of the Mab instead of coating rabbit serum. The OD developed was measured at 492 nm with a plate reader (Tecan) after stopping the reaction. The mean OD of reactivity developed by the lysate was expressed as a bar diagram to indicate its reactivity with Mab.

2.4. Purification of expressed VLPs

The harvested Tn-5 cells after infection from each flask were lysed in 2ml lysis buffer as described earlier and collected the supernatant carefully after thorough centrifuge. The supernatant was filtered with a 0.45-micron syringe filter to clear the supernatant from coarse cellular debris before performing ultracentrifugation. Further loaded 12 ml of this supernatant to an 18 ml polyallomer tube and prepared a double sucrose cushion by underlaying 3ml of 15% sucrose prepared in sterile phosphate buffer (pH: 7.60) beneath the supernatant layer and 3 ml of 60% sucrose underneath the first sucrose layer using a long needle. The ultracentrifugation was carried out at 130000 g for 4 hr at 10 ˚C in AH-629 rotor of Thermo Scientific Sorwall ultracentrifuge, and the interface formed between the two cushions was collected. The collected interface was diluted to 10 ml with Tris NaCl buffer (pH: 7.60) and centrifuged again at 10000 g for 20 min to remove any coarse debris present. This clear supernatant collected was loaded over a continuous gradient cesium chloride bed made of 4 ml each of 1.2 and 1.4 g/ml of cesium chloride and performed ultracentrifugation for 16 hr at 130000 g at 4 ˚C. At the end of centrifugation removed the sample volume from the tube and collected the rest of the gradient volume as 0.5 ml aliquots, including the fine white layer formed slightly above the middle of the gradient. The total protein content in each fraction was assessed spectrometrically by measuring the absorbance at 280 nm and 230 nm. The specificity of capsid proteins and the presence of intact VLPs in each fraction were also ascertained with serotype-specific sandwich ELISA and 146S specific Mab ELISA as described earlier. The fractions showing higher reactivity in specific ELISA were pooled and dialyzed against Tris-NaCl buffer (pH: 7.60) to remove the cesium chloride and stored at -80 ˚C.

3. Results

3.1 Generation of recombinant baculoviruses.

The transformation of the transfer vector in DH10 BacTM competent cells resulted in the growth of white colonies, indicating the transposition and successful colonies were picked after observing the positive amplicons in colony PCR. The transfection of isolated bacmids in Sf-9 insect cells resulted in the development of evident cytopathic changes by 96 hr, indicating the generation of recombinant baculoviruses. PCR using the template from the culture supernatant showed an amplicon of 2.5 Kb corresponding to the P1-2A region, indicating the presence of the gene in the recombinant baculovirus.

3.2. Expression of capsid proteins and assembly of VLPs

Infection of Tn-5 cells with the rescued recombinant baculovirus at a high moi of 5 produced good CPE by 3 days, and analysis of the infected lysate in serotype-specific sandwich ELISA developed good immunoreactivity with

FMDV serotype Asia1 specific serum keeping minimum reactivity with the serum of other two serotypes (Fig. 1a) confirming the expression of serotype Asia1 capsid proteins. The development of Asia1 specific structural protein precursors in western blot also further confirmed the specificity of the expressed proteins. The lysate from the infected cell pellet produced 26 kDa (corresponding to VP1/VP3), 50 kDa (corresponding to VP3-VP1 complex) and 81 kDa (corresponding to P1-2A) proteins indicating the expression of various capsid proteins and their precursor polyproteins (Fig.1b). Reactivity of these proteins in the lysate with the 146 S specific Mab which specifically binds to the assembled or intact capsid confirmed the formation of VLPs (Fig. 2).

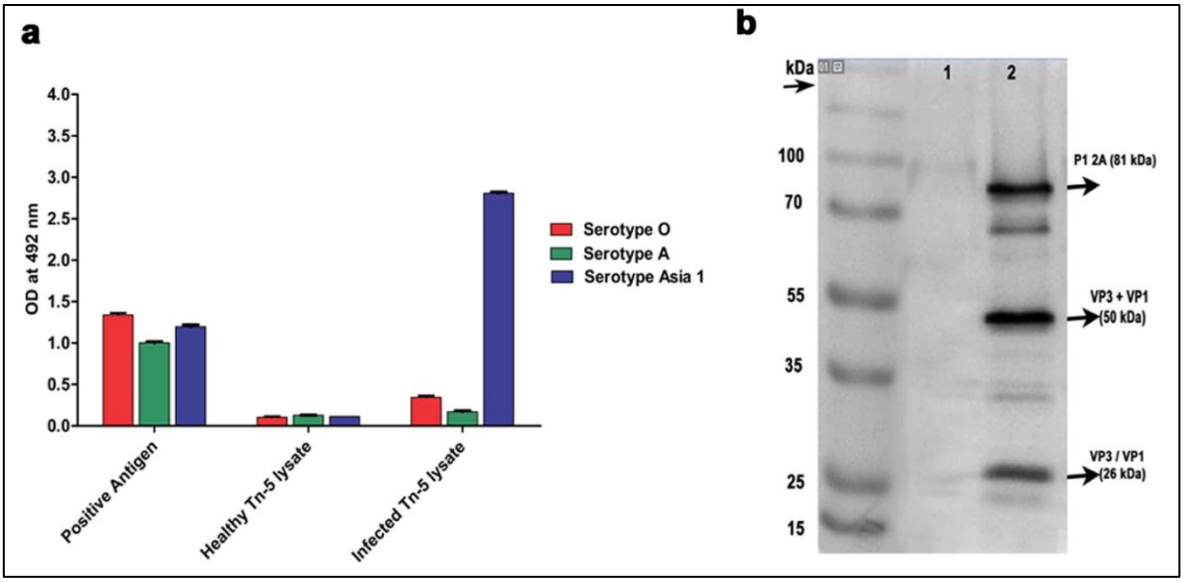

Fig 1: Confirmation of expression of FMDV capsid proteins in infected Tn-5 cell lysate. (1a) OD observed in serotype specific sandwich ELISA (1b) Specific reactivity of uninfected (Lane 1) and infected (Lane 2) in western blot.

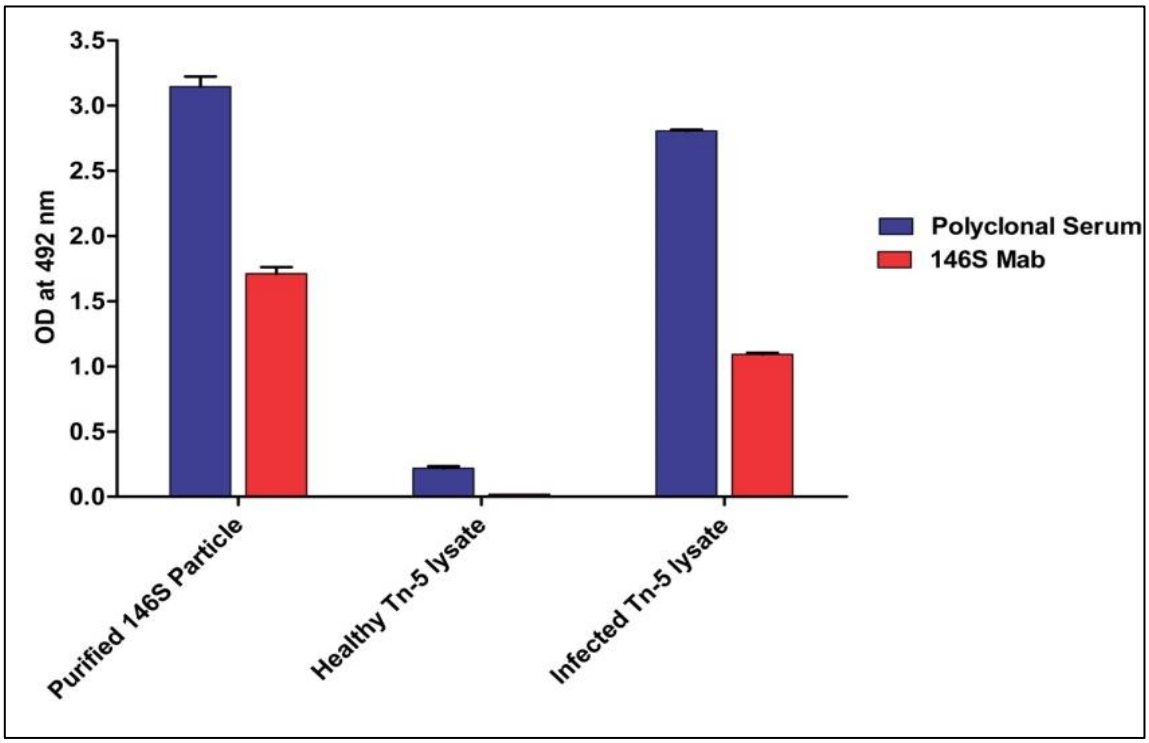

Fig 2: Confirmation of formation of VLPs by sandwich ELISA with 146S specific Mab.

3.3. Purification of the assembled VLPs.

The lysed cells from the infected flasks were centrifuged thoroughly to collect the supernatant and filtered with 0.45 µm filter before loading on a discontinuous gradient formed by 60% and 15% sucrose. A thick white band of expressed proteins and large host cell components were formed upon centrifugation at the interface between two gradients (Fig.3a). After a brief centrifugation, the collected band was diluted 1:10 in Tris NaCl buffer and loaded over a linear gradient of cesium chloride (1.2 to 1.4 g/ml). This isopycnic centrifugation resulted in the separation of VLPs from other large and small subunits with the formation of a clear white band slightly above the middle of the gradient (Fig. 3b). The spectroscopic assessment of protein content (Fig.4a) and specific protein assessment with polyclonal sera and Mab (Fig. 4b) with the different fractions also showed higher

absorbance and reactivity around this band region confirming the presence of concentrated and purified VLPs. These concentrated and purified VLPs were collected by pooling these fractions and stored after dialysis for further uses.

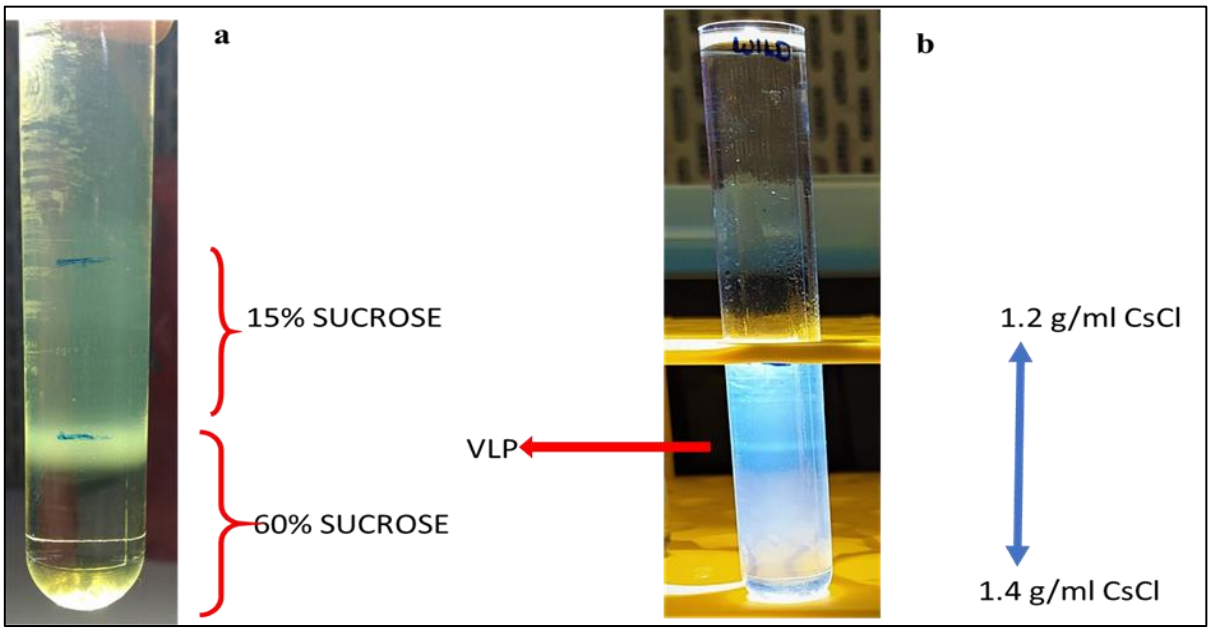

Fig 3: Purification of VLPs by ultracentrifugation. (a) The separation of VLPs on discontinuous sucrose gradient (b) Purification of VLPs by isopycnic centrifugation of continuous CsCl gradient.

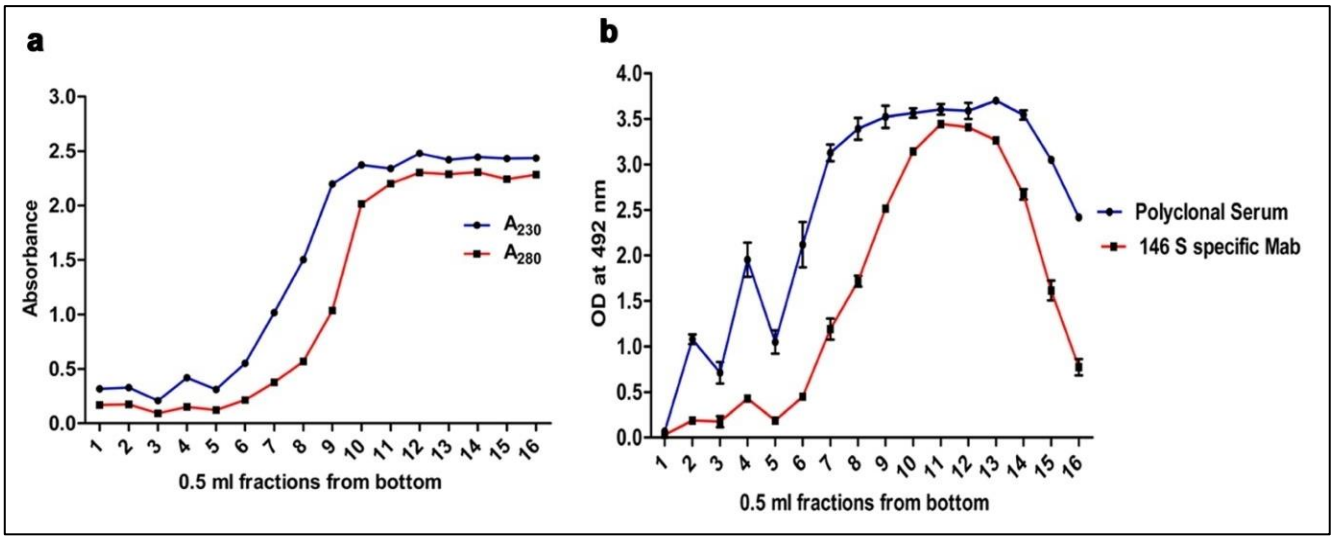

Fig 4: (a) The absorbance of the fractions at 280 and 230 nm in spectrophotometry (b) The specific reactivity of the fractions in sandwich ELISA with serotype specific polyclonal and 146S specific Mab

4. Discussion

The cloven-hoofed animals, including the wild ruminants and pigs worldwide, are still under the threat of the first discovered animal virus, foot-and-mouth disease virus (FMDV), despite the availability of a vaccine. The endemicity of this disease has a direct relationship with the economy and trade of the country. The possible chance of accidental virus spillage manufacturing unit will be detrimental and hence demands the development of alternate vaccine candidates like VLPs for FMDV. The development of VLP based vaccine requires further extensive studies on its structural and functional properties. Many expression platforms are being attempted to produce VLPs, but most of the system find difficulty in purification as the VLPs remains intracytoplasmic (Dong *et al*., 2014) [6]. This study had attempted to express FMDV VLPs in a baculovirus expression system and demonstrated a less time-consuming purification protocol combining filtration and isodensity ultracentrifugation. The infection with the rescued recombinant baculovirus carrying the P1-2A genome of serotype Asia1 resulted in the expression of capsid proteins. The results of serotype-specific sandwich ELISA confirmed the specificity of the expressed proteins. The reactivity of the Mab, which specifically binds with an intact capsid structure, confirmed the ordered assembly of the expressed capsid proteins to form VLPs. As this expression system allows the proper assembly of capsid proteins to form a three-dimensional capsid structure, the antigenic epitopes which are crucial for eliciting the neutralizing antibody response will be retained in the VLPs thus formed.

During the purification process of VLPs, the entire buffer conditions, especially the pH (around 7.60) and temperature (ice-cold condition) must be ensured throughout the process as they are very liable to dissociation with a mild change in

this ambient environment (Yuan *et al*., 2020). Further filtration of the lysed supernatant will help to remove a lot of insoluble debris, which are likely to hinder the proper sedimentation of the VLPs during ultracentrifugation (Fontana *et al*., 2019) [7]. The cells have to be lysed with a sufficient quantity of lysis buffer like 2 ml lysis buffer for each flask used in this study to avoid a thick viscous lysate that is likely to clog the filter. While handling a large volume of samples for filtration also can pose the same issue. This issue can be averted to a great extent by using syringe filters with glass fibre prefilters, as described earlier (Peyret *et al*., 2015) [14]. Further separation over a discontinuous gradient will help us to remove all small protein and cellular components from the protein of interest and helps in the subsequent smooth separation of the VLPs. This partially purified sample running over a linear gradient overnight will allow the VLPs to find their buoyant density. It will tend them to get concentrated at a particular point identical to their density. This step will enable us to get pure and concentrated VLPs required for the structural and functional evaluation. The sandwich ELISA of the fractions with the polyclonal and monoclonal antibody had demonstrated the presence of concentrated VLPs slightly above the middle of the gradient (In fractions 10, 11, 12 and 13 from the bottom) which corresponds to the white band observed. These results are in concordance with the previous findings of Lieberman *et al*., 1976 [13], where they determined a density of 1.308± 0.005 g/ml in cesium chloride for the empty capsid formed during a typical virus infection. The fractions collected beyond this white band level (14,15 and 16 fractions) are also giving higher absorbance in spectroscopic assessment while the OD for corresponding fractions are exhibiting a diminishing trend indicating the presence of non specific lower sized proteins at these regions. Besides, this protocol avoids the pelleting step and averts the demerits of associated structural changes, time consumption for pellet resuspension and protein loss. This protocol allows us to get VLPs in a more concentrated and pure form for laboratory study purposes in a considerably short period. For large-scale production systems like vaccine manufacturing, further studies involving affinity tags or size exclusion chromatography have to be carried out to make this bio-safe vaccine platform a real game-changer.

5. Conclusion

The optimized purification protocol described through this study could effectively remove the host cellular debris and smaller sized proteins co expressed along with the VLPs of FMDV in baculovirus expression system and results in a purified and concentrated lot of VLPs for future structural and functional evaluation

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