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SCALABLE DOWNSTREAM PROCESS FOR PURIFICATION AND INACTIVATION OF MICROCARRIER-PROPAGATED JAPANESE ENCEPHALITIS VIRUS ANTIGEN: PROCESS DEVELOPMENT AND VALIDATION

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ABSTRACT
Japanese encephalitis is a mosquito-borne viral disease of major public-health importance in Asia, and vaccination remains the principal strategy for disease prevention because no specific curative therapy is available for this disease. Inactivated whole-virion vaccines require robust and reproducible manufacturing processes that ensure adequate viral yield, efficient impurity clearance, and reliable viral inactivation for safe product release into the market. The current manuscript describes an integrated process for development, purification, and inactivation of Japanese encephalitis viral antigen propagated in microcarrier-based Vero cell culture and its validation. The upstream platform employed Vero cells expanded on Cytodex 1 microcarriers in a controlled bioreactor environment to support reproducible cell growth and virus production. The viral harvests were concentrated by tangential flow filtration, purified by Celufine Sulfate affinity chromatography, further processed through Sartobind Q membrane filtration for reduction of host-cell DNA, and subsequently inactivated with formaldehyde under defined conditions. The downstream process was assessed across the three independent batches of viral antigen using plaque assay, protein estimation, host-cell protein analysis, host-cell DNA testing, and *in vitro* and *in vivo* inactivation confirmation. The workflow consistently generated purified viral antigen with acceptable impurity reduction and complete loss of detectable infectivity after inactivation. Bioreactor harvests yielded higher virus titers than static cultures, and the downstream processing remained reproducible across three batches prepared using the described workflow. Collectively, these findings support the suitability of the proposed platform for scalable manufacture of purified and inactivated JEV antigen for vaccine applications.

KEYWORDS: Japanese encephalitis virus; Vero cells, Microcarrier culture, Downstream, Vaccines.

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1. INTRODUCTION

Japanese encephalitis (JE) is an inflammatory brain disease that is caused by the Japanese encephalitis virus (JEV) (Erlanger et al., 2009). It is a zoonotic mosquito-borne virus that belongs to the *Flaviviridae* family, the *Flavivirus* genus (Simmonds et al., 2017; Ellis et al., 2000). It is the leading cause of viral encephalitis in Asia, the Western Pacific, and northern Australia (Chugh et al., 2025; Solomon et al., 2003; Burke et al., 1988; Pan et al., 2011). In endemic regions, JE primarily affects children Under 15 age group, but travel-associated cases can occur at any age in the absence of protective immunity (Kuwata et al., 2020). The virus persists within an enzootic cycle, primarily involving Culex mosquitoes and various amplifying vertebrate hosts, such as aquatic birds and domestic pigs (Campbell et al., 2011). Humans are considered terminal hosts, as they typically do not reach sufficiently high levels of JEV in their blood circulation to transmit the virus to mosquitoes during feeding (Weaver et al., 2004).

The effectiveness of vector control strategies is limited due to complex eco-epidemiology of the virus. Severe infection with JEV in humans results in encephalitis (Le Flohic et al., 2013), neurological sequelae, and death, while no specific antiviral treatment is currently available; therefore, immunization continues to be the most effective public-health intervention. The lifecycle of JEV is maintained through an enzootic cycle involving avian reservoir hosts, and due to its ecological persistence across both avian and mammalian populations, the virus remains firmly established in nature, suggesting that global eradication is an improbable goal for the foreseeable future (Kulkarni et al., 2018). Thus, effective antiviral therapy and an ideal vaccine against JEV are a pressing priority. With no specific antiviral treatments available, vaccination is the only effective method to prevent infection and control outbreaks.

Vaccines are available in various forms, including live attenuated, inactivated, and recombinant subunit vaccines, and play a critical role in global public health by preventing infectious diseases. Many viral vaccines are produced using mammalian cell culture systems, either as adherent or suspension cultures. Commonly used adherent cell lines include MRC-5, MDCK, PK-15, CEF, and Vero cells. The manufacturing of many human viral vaccines utilizes the Vero continuous cell line, derived from the kidney of an African green monkey (Yasumura and Kawakita, 1963). The regulatory bodies approve Vero cells for their safety and permissiveness to a broad range of viruses, including poliovirus, rabies virus, and various flaviviruses. Large-scale production involves technologies like roller bottles or microcarriers in stirred-tank bioreactors to provide the necessary surface area for cell growth.

Viruses are complex bioparticles with varying size, shape, composition, and surface structure. The virus surface defines its individual physicochemical characteristics, including the number and distribution of charges, hydrophobic residues, and post-translational modifications (i.e., glycans) of surface proteins. The purification of virus particles based on these unique characteristics and removal of contaminants according to the regulatory guidelines can only be achieved by a combination of different unit operations. Among available vaccine modalities, inactivated whole-virus vaccines retain considerable value because of their established safety profile and compatibility with large-scale immunization programs. However, successful manufacture of such vaccines depends on a production platform that combines efficient upstream propagation with downstream purification strategies capable of removing host-cell-derived impurities while preserving antigenic integrity.

Vero cells are widely used in viral vaccine production because they are well characterized, adaptable to bioreactor operation, and suitable for scalable manufacturing workflows. Traditionally, Vero cells have been cultivated as adherent monolayers on the surface of roller bottles or cell stacks in media supplemented with fetal bovine serum (FBS). However, this method is fraught with significant limitations that are increasingly untenable for modern vaccine production. The reliance on FBS introduces inherent risks of adventitious agent contamination, notable batch-to-batch variability, and high costs, which compromise process consistency and regulatory compliance. Furthermore, the limited surface area of adherent culture systems severely restricts scalability, making it impractical to meet the multi-thousand-liter volumes required for global vaccine campaigns. However, a major challenge in using Vero cells is their anchorage-dependent nature, which complicates upstream processing, is labor-intensive, and limits scalability. Currently, large-scale vaccine production using Vero cells is carried out with microcarriers to increase the available surface area for adhesion; despite achieving remarkable productivity, the process remains limited by surface area.

To address all these issues, the current research has focused on adapting Vero cells using chemically defined, serum-free media. This approach allows for cultivation in bioreactors, which provides a more controlled environment, simplifies process automation, and enables a significantly higher cell density per unit volume, directly translating to increased viral antigen yield and reduced production costs. However, adapting Vero cells to grow in suspension presents challenges, including the formulation of appropriate serum-free media, maintaining high viability, and managing prolonged doubling times. This study aimed to optimize Vero cell growth for flavivirus antigen production by adapting the cells to a serum-free suspension culture medium (SFM) environment using microcarriers in a bioreactor system, and to evaluate the impact on virus productivity. The key optimization parameters include the cell adaptation

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protocol, selecting an SFM that supports high cell densities while minimizing aggregation, and process intensification via perfusion culture. Furthermore, these adapted cells are highly productive for viral antigens, with high-density perfusion culture intensification increasing volumetric productivity. It provides a scalable, safer, and more cost-effective platform for manufacturing flavivirus vaccines, improving global access and pandemic preparedness.

This article describes an overview of individual unit operations currently used for scalable downstream processing for purification and inactivation of Microcarrier-propagated Japanese Encephalitis Virus Antigen. This includes the development and validation of three batches of downstream processes for purification and inactivation of microcarrier-propagated JEV. The study integrates bioreactor-based Vero cell propagation, tangential flow concentration and diafiltration, Celufine Sulfate affinity purification, Sartobind Q filtration, and formaldehyde inactivation in a workflow intended to support scalable vaccine manufacture. Microcarrier-based culture systems are particularly useful for expansion of adherent Vero cells because they provide a large growth surface area within a controlled stirred environment. This configuration facilitates process scale-up and allows improved control of pH, gas transfer, temperature, and nutrient consumption when compared with conventional static systems.

In the present work, the microcarrier-propagated Japanese encephalitis virus was selected as the starting material for the development of an integrated downstream process intended to improve the process consistency and the overall product quality. The purification of Japanese encephalitis virus harvests is the most challenging process because the clarified bulk contains the residual host-cell proteins, host-cell DNA, cell debris, media-derived components, and the process-related contaminants. The study clearly employs Celufine Sulfate affinity chromatography as the primary capture step, taking the advantage of the interaction between sulfated ligands and the Japanese encephalitis viral envelope proteins to selectively recover the desired viral antigen stream. A subsequent Sartobind Q membrane step is incorporated as a polishing operation to reduce the overall residual host-cell DNA, followed by formaldehyde-mediated viral inactivation as a critical safety measure.

2. MATERIALS AND METHODS

2.1 Cell Culture and Virus Preparation

African green monkey kidney cell lines (Vero; CCL-81, ATCC, Manassas, VA, USA) were maintained in serum-free VP-SFM (Gibco, Waltham, MA, USA) with 10 mM HEPES (Gibco) and 10% fetal bovine serum (FBS; Gibco) at 37°C with 5% CO_2. A two-tier banking system, both master cell bank (MCB) and working cell bank (WCB), was established and stored in vapor phase of liquid nitrogen using serum-free media. The SA 14-14-2 strain, a live, attenuated strain of JEV for viral seeding, and the research working virus bank used for this study had a seed stock titer of 8.38×10^6 PFU/mL.

2.2 Serum-free Media Composition

VF-SFM is commercially available, powdered VP-SFM (Gibco) and Cellin-1 (Himedia) were formulated in-house by reconstituting in ambient Water for Injection (WFI) with 4 mM L-glutamine as an energy source for rapid cell growth. The final pH was adjusted to a range of 7.0 to 7.3 using sodium bicarbonate. Neomycin was added at a concentration of 10 mg/L as a growth medium supplement, and the media were sterilized using a 0.2 µm filtration system, followed by sterility testing using Tryptic Soy Broth (TSB) and Sabouraud Dextrose Broth (SDB), resulting in no signs of contamination.

2.3 Preparation of Microcarrier

The microcarriers Cytodex-1, composed of cross-linked dextran polymers with positively charged DEAE groups, were utilized throughout the matrix. These microcarriers were hydrated in sterile phosphate-buffered saline (PBS) for a minimum of three hours, preferably overnight, and subsequently sterilized by autoclaving at 121°C for 30 minutes. The Vero cells were inoculated at a density of 2 to 4×10^5 cells/mL or approximately 15,000±5000 cells/cm² into a 3 L bioreactor containing a 2.2 L working volume of prepared microcarriers. The cultures were maintained at 37±1 °C with an agitation rate of 40 to 100 rpm. Process parameters such as pH (7.2±0.5) and dissolved gas composition were controlled using a tri-gas sparge system to support optimal cell growth, resulting in a cell density of $1\text{-}2\times10^5$ cells/cm² that was typically achieved within 6 days of time frame. To maintain consistent product yield, scheduled medium exchanges were performed, and both physical (pH, temperature, dissolved oxygen, and agitation rate) and biochemical (glucose, lactate, and ammonia) parameters were carefully monitored using a YSI 2950D Biochemical Analyzer.

2.4 Process of Infection and Cell Harvest

Vero cell cultures that were 70-80% confluent was infected with Japanese Encephalitis Virus (JEV) at a multiplicity of infection (MOI) of 0.002 using a well-characterized research seed virus with a titer of 8.38×10^6 PFU/mL. After the process of infection, the Vero cell cultures were incubated at 35±1°C for 24 hours to allow efficient viral adsorption, followed by replacement with fresh culture medium to sustain cell metabolism and viral replication. On the 3rd day post-infection, the infected Vero cell harvests were collected at intervals of 24±4 hours over four consecutive days. Each individual harvest yielded approximately 2.1 to 2.3 L of clarified viral suspension, resulting in a total cumulative harvest volume of 8.5 to 9.0 L per bioreactor batch. All individually collected harvests were sequentially clarified through a 0.22 µm membrane filter to remove cell debris and aggregates, then stored at 2-

8 °C temperature for short-term preservation before purification.

2.5 Tangential Flow Filtration

The clarified viral harvests were subjected to concentration using a 0.5 m² ultrafiltration cassette with a molecular weight cut-off (MWCO) of 100 kilodaltons (KD). Prior to processing, the filtration cassettes were sanitized with 0.5N NaOH, thoroughly rinsed with water for injection (WFI), and subsequently conditioned with 5L of chilled VP-SFM medium to ensure system compatibility and maintain viral integrity. The pooled harvests, stored at 2-8 °C, were processed directly without thawing to preserve viral antigenicity and maintain process reproducibility. The concentration step was carried out until the total volume was reduced to approximately 15-20 x times followed by diafiltration using phosphate-buffered saline (PBS, pH 7.2) to remove residual medium components and exchange the buffer. The diafiltration process was executed using a buffer volume equivalent to five times the retentate volume approx. ~8 to 9L, ensuring effective removal of low-molecular-weight impurities. Upon completion, the viral concentrate was flushed from the system using 1 L of PBS, and the combined eluate was collected to achieve a final working volume of approximately 2.6-2.8 L. The purified concentrate was stored in sterile polycarbonate bottles under controlled conditions (2-8°c) further followed by Celufine Sulfate chromatography purification.

2.6 Celufine Sulfate chromatography purification Method

Celufine Sulfate (CS) is an affinity chromatography matrix designed for the concentration, purification, and dehydrogenation of viruses, viral coats, microbial antigens, and specific proteins. This matrix is based on spherical cellulose beads functionalized with a low concentration of sulphate esters. The low density of sulphate groups gives the gel unique chromatographic selectivity that, in some cases, is similar to immobilized heparin or sulphated dextran. Due to Celufine Sulphate's low exclusion limit of 3 kDa, large molecules adsorb primarily on the packing's exterior, resulting in rapid adsorption and desorption times. Its superior rigidity allows high flow rates and thus rapid processing times at larger scale.

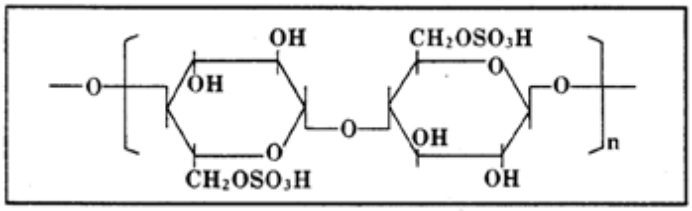

Fig. 1: Partial Specific Chemical Structure of Celufine Sulfate.

The Celufine Sulfate (CS) resin used for the process selectively binds to viral proteins under specified conditions. The material used for purification was 100 kDa, ~20x concentrates from bioreactor harvests. Three consecutive runs were carried out on the same column using the input material from three bioreactor batches. For purification, Column XK 50/30 was manually packed at 15±2 cm^2 bed height with CS resin, corresponding to ~295 mL of column volume with a binding capacity of ~350 mg protein. After packing, the asymmetry was performed, and the values were within specifications at ~0.95; the 100 kDa concentrate of Bioreactor -1, 2, and 3 runs of 1800-2000 mL volume was processed. It was diluted 1:1 to 3600-4000 mL using 1x PB-NaCl pH 7.2±0.2 before loading into the column. The column was operated to have about 10 ±2 min of residence time so that the protein binds to the resin while loading. Once the sample was loaded onto the column, the flow-through was collected separately, and the column was washed with 1x PBS pH 7.2±0.2 until the UV reached baseline (≤2 mAU) further followed by isocratic elution with 1 M NaCl in 1X PBS pH 7.2 ± 0.2.

2.7 Sartobind Q filtration and Virus Inactivation:

The clarified supernatant (CS) elute was diluted to 1:1 with phosphate buffer containing 75 mM NaCl to achieve a target conductivity of 45±10 mS/cm and further processed through a Sartobind Q membrane filter for host cell DNA removal. Prior to processing, the Sartobind Q membrane was preactivated and was sanitized by passing 2 volumes (MV) of 1 M NaOH, followed by a 30-minute incubation. Subsequently, the membrane flushed with 25-30 MV of 1 M NaOH, followed by membrane regeneration with approximately 10 MV of 1 M NaCl through the membrane at a filtration rate of 100 mL/min. Further, the regenerated membrane was equilibrated by washing with 10 MV of 1× PBS until the membrane pH stabilized at 7.2 ± 0.2 and conductivity at 16 ± 2 mS/cm. Once the conditions stabilized, the diluted CS elute was then connected and passed through the Sartobind Q membrane using a peristaltic pump at a flow rate of 10-15 mL/min. Upon completion of filtration, the membrane was washed with a minimum of 4-5 MV of 1× PBS. The filtrate was collected in a polycarbonate bottle containing a magnet and gently stirred for 2-3 minutes post-collection for plaque assay and standard BCA protein analysis.

2.8 Downstream Analytical analysis: The quality of the viral titers, protein content, and purity profile was analyzed using different analytical methods.

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2.8.1 Plaque assay testing

Plaque assay was performed to determine the concentration of infectious viral particles. The serially diluted virus samples were inoculated onto a 70-80% confluent host cell monolayer and overlaid with a semi-solid agar medium to restrict viral spread. Following incubation, the viral replication caused localized cell lysis, resulting in distinct plaques. The plaques were counted, and the viral titer was expressed as plaque-forming units per milliliter (PFU/mL), with each plaque representing a single infectious viral particle.

2.8.2 Protein content Assay

Bio-Rad Protein Assay, based on the Bradford dye-binding principle, was used to determine the concentration of Japanese encephalitis virus (JEV) proteins using IgG as the reference standard. Serial dilutions of IgG standards (1.2-25µg/mL) and JEV protein samples were prepared and mixed with the Bio-Rad dye reagent, followed by incubation at room temperature for 60 minutes to allow dye-protein complex formation. After the process of incubation, the test samples' and standards' absorbance was measured at 595 nm using a spectrophotometer. A standard curve was generated from the IgG standards, and the protein concentration of the JEV samples was calculated by interpolation. It is based on the proportional increase in absorbance resulting from the binding of Coomassie Brilliant Blue dye to proteins.

2.8.3 Host Cell Protein testing using ELISA

The Cygnus Vero Cell 2G HCP ELISA (F975) was used to quantify residual Vero cell host cell proteins (HCPs) based on a two-site sandwich ELISA principle. Microtiter wells pre-coated with affinity-purified anti-Vero antibodies captured HCPs present in the samples, followed by detection with an HRP-conjugated anti-Vero antibody. After washing, tetramethylbenzidine (TMB) substrate was added, and the enzymatic reaction generated a color proportional to the HCP concentration. The reaction was terminated with stop solution, and absorbance was measured at 450 nm with a reference wavelength of 650 nm.

2.8.4 Host Cell DNA testing

The determination of residual host cell DNA (HCD) in biopharmaceutical samples can be accurately conducted using the Invitrogen resDNASEQ Quantitative DNA Kit, which employs TaqMan real-time PCR technology. This sensitive method detects DNA at sub-picogram levels, providing reliable quantification across diverse sample matrices, including in-process samples and final products. Initially, the test control DNA was used for a standard curve by serial dilution to cover a relevant concentration range. In parallel, samples are processed to extract DNA, ensuring removal of impurities that may interfere with PCR. The Reaction mixture containing master mix, assay-specific primers and probes, internal positive controls, and samples/standards is dispensed into a 96-well plate. The plate undergoes thermal cycling using optimized reaction conditions on a compatible real-time PCR instrument to monitor real-time amplification. After amplification, the data analysis evaluates amplification efficiency and curve characteristics, then quantifies sample DNA by comparison to the standard curve. Assay validity is confirmed by internal control performance and replicate consistency. All the results were reported as picograms of residual DNA per millilitre of sample, with accuracy ensured by optimized procedures such as spike recovery and dilution linearity. This approach is well-suited for sensitive monitoring of host cell DNA in vaccine antigen production processes, facilitating regulatory compliance and product safety assurance.

2.8.5 Identity and Antigen content determination

The inactivated Japanese encephalitis virus antigen content was quantified using an indirect enzyme-linked immunosorbent assay (ELISA). High-binding 96-well microplates were coated with optimized conc. Of inactivated antigen prepared in coating buffer and incubated overnight at 2-8°C. After washing, the wells were blocked with a protein-blocking buffer to minimize non-specific binding. The plates were then incubated with a JEV-specific primary antibody, followed by washing and incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody. The tetramethylbenzidine (TMB) substrate was added, and enzymatic reaction was allowed to develop before being terminated with sulfuric acid. The optical density was measured at 450 nm using a microplate reader. This method provides a sensitive, specific, and reproducible approach for quantifying viral antigen during process development and bulk manufacturing, enabling consistent monitoring of antigen recovery and batch-to-batch uniformity.

2.8.6 In Vitro Detection of Japanese Encephalitis Virus in Inactivated Vaccines

The detection of residual live Japanese Encephalitis Virus in inactivated vaccine lots is evaluated using Vero cell monolayers cultivated in tissue culture flasks. Vero cells are seeded and incubated until they achieve a confluent monolayer. The cultures were incubated under controlled temperature and CO_2 conditions and monitored microscopically over a 14-day observation period for evidence of cytopathic effects (CPE)**,** which indicate viral replication. To maintain monolayer integrity, washing and inoculation steps were performed via gentle aspiration and dispensing; following incubation, the inoculum was replaced with an optimized maintenance medium. The monolayers are examined daily for manifestations of CPE, including cellular degeneration, detachment, or plaque formation. The culture medium is replenished at regular intervals to preserve cell viability. In parallel to this, a negative control consisting of uninoculated Vero monolayers is maintained under identical conditions to rule out background cellular artifacts. If the monolayer exhibits no observable CPE throughout the 14-day incubation, the

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culture supernatant is pooled and subjected to definitive *in vivo* testing to confirm complete viral inactivation. Conversely, the manifestation of any CPE constitutes a test failure, establishing the presence of residual live virus. This rigorous dual-testing matrix serves as a critical quality control standard to guarantee vaccine safety prior to lot release.

3. RESULTS

3.1 Harvest Virus yield analysis

3.1.1 Plaque Assay analysis

To evaluate viral productivity following cell scale-up in the bioreactor, Cells were infected with JE SA 14-14-2 virus, and collected harvests were quantified using plaque assay, a standard and widely accepted method for determining infectious viral titers. The test results of 3 independent experimental runs demonstrated higher overall viral yields in the bioreactor compared to traditional Cell Stack (CS-10) static cultures. In bioreactor cultures, the viral titers ranged from 1.83×10^7 pfu/mL to 2.20×10^7 pfu/mL, whereas CS-10 static cultures yielded 1.60×10^7 pfu/mL to 2.00×10^7 pfu/mL. Overall, plaque assay results showed that the bioreactor system demonstrated a consistent ~10-15% viral titer enhancement on average over static control cultures.

Table 1: Comparison of viral titers between bioreactor and static cultures.

Container	H1	H2	H3	H4
Bioreactor (Pfu/mL)	1.83E+07	1.96E+07	2.20E+07	2.05E+07
CS-10(Pfu/mL)	1.60E+07	1.81E+07	2.00E+07	2.04E+07

3.1.2 Protein content Analysis

Quantification of viral antigen protein content represents a critical quality attribute (CQA) in vaccine manufacturing. In this study, Japanese encephalitis (JE) virus antigen levels were quantified using the Bio-Rad protein assay calibrated against an IgG standard. The assay revealed a marked increase in protein content within bioreactor cultures relative to static systems (Figure 2). These findings demonstrate that suspension-adapted Vero cells propagated in bioreactors under serum-free conditions achieve superior viral yields compared to conventional static culture methods. Protein concentrations were determined from a standard curve and expressed as ng/mL. The assay provides a validated quantitation range of 3-200 ng/mL with a detection limit of approximately 1.4 ng/mL, making it suitable for monitoring residual Vero cell proteins during process development, quality control, and final product release. Appropriate controls and sample dilutions were included to ensure accurate and reliable quantification.

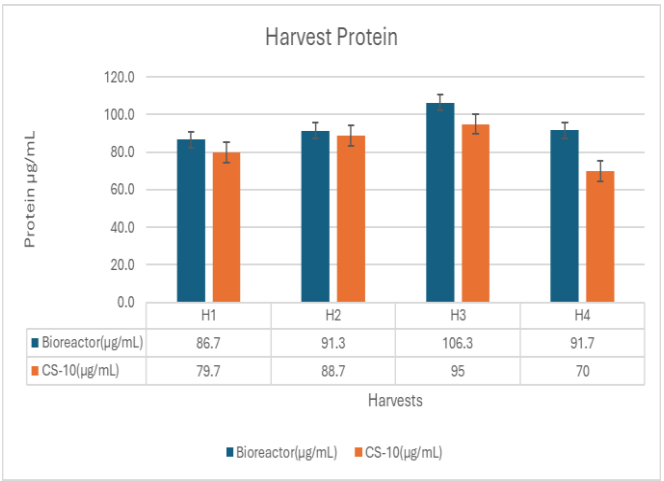

Fig. 2: Virus yields comparison between bioreactor and traditional static cultures.

3.2 Concentration and Celufine Sulfate Purification

The pooled viral harvests from all three individual batches of Japanese encephalitis viral cultures were concentrated using 0.5m2 100 kDa Millipore cassettes for proper concentration of the viral antigen with equipment of cogent M1, followed by diafiltration.

3.2.1 Column Packing Efficiency and Qualification

Prior to preparative sample loading, column bed integrity and packing performance were qualified using a standard conductivity tracer injection test (Figure 3). Following column equilibration, a tracer pulse eluted at a retention volume (V_R) of 454.44 mL with a peak height of 22.35mS/cm. The packed bed exhibited a peak asymmetry factor (A_s) of 1.19, falling within the standard industrial acceptance criteria ($0.8 \leq As \leq 1.8$). Column efficiency analysis yielded a height equivalent to a theoretical plate (HETP) of 0.00758 cm (75.8 um) and 13,186 plates/m. These metrics confirmed a uniform, tightly packed matrix suitable for large-scale preparative processing without risk of channel formation or flow maldistribution.

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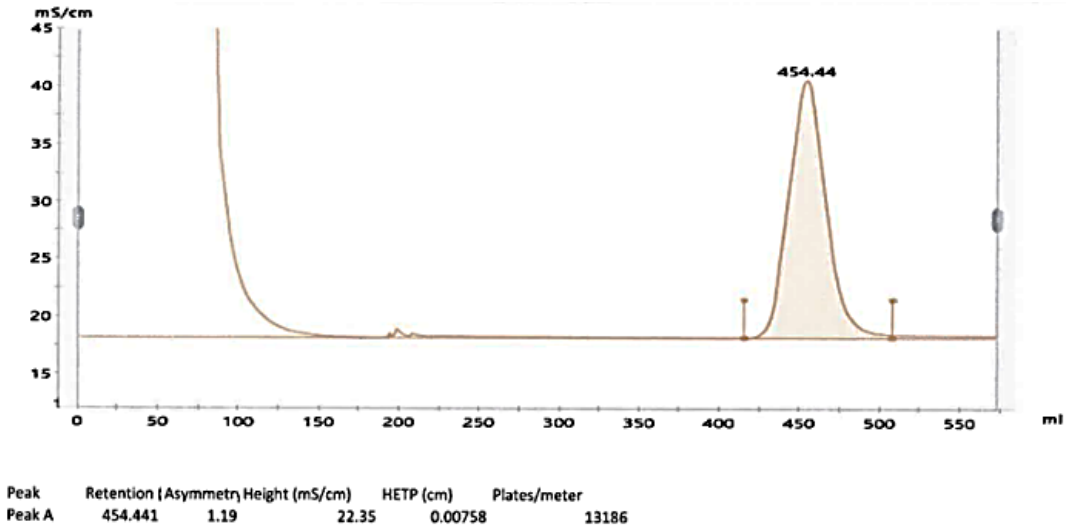

Fig. 3: Column efficiency and packing evaluation chromatogram and Conductivity profile showing the elution of a tracer pulse at 454.44 mL during column testing.

3.2.2 Preparative Protein Purification

Target protein was purified using an automated chromatography protocol. Prior to sample application, the column was equilibrated, establishing a stable conductivity and UV baseline. Unbound host cell contaminants were removed in the flow-through fraction during sample application (5,000 - 18,000 mL) and subsequent post-load wash phases (18,000 - 28,000 mL), during which UV absorbance (A_{280}) returned to baseline. Step elution was induced by buffer change at 28,000 mL, resulting in a sharp, concentrated product elution peak (A_{280} peak maximum ~12.2 AU) collected across a 10 L elution window (28,000 - 38,000 mL). Following product collection, the column was subjected to cleaning-in-place (CIP) and re-equilibration (38,000 - 65,000 mL) to prepare the matrix for subsequent processing cycles.

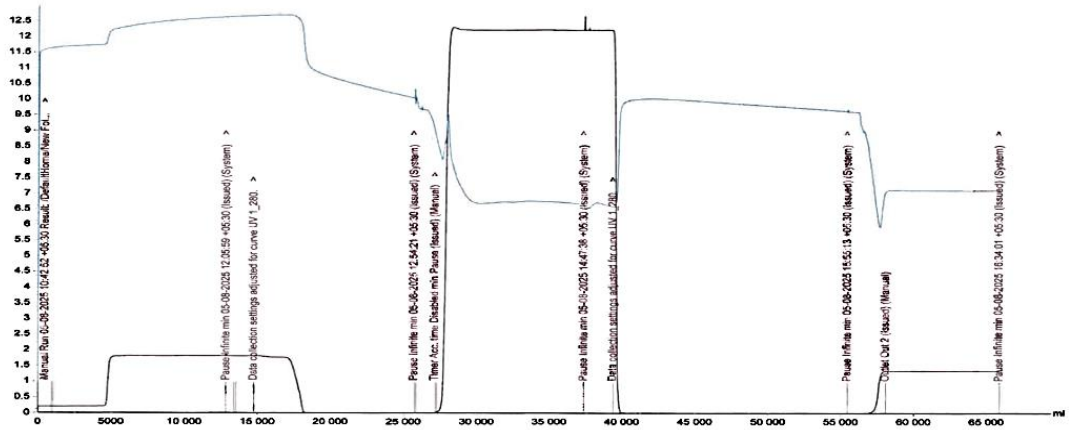

Fig. 4: Preparative scale purification chromatogram. On-line monitoring of UV absorbance ($A_{280,}$ dark curve) and pH/conductivity profile (light blue curve) across loading, wash, elution, and regeneration phases.

3.2.3 Preparative Column Capture

Preparative purification was monitored online via UV absorbance (A_{280}) and conductivity. The viral antigen sample loading across 30,000 mL resulted in a stable flow-through baseline (~150 mAU). Following a post-load wash phase (30,000 - 39,000 mL), step elution was triggered by buffer change, yielding a sharp, concentrated target protein elution peak at ~40,000 mL (A_{280} > 1100 mAU). Peak collection was initiated at 40,500 mL and completed at 44,000 mL.

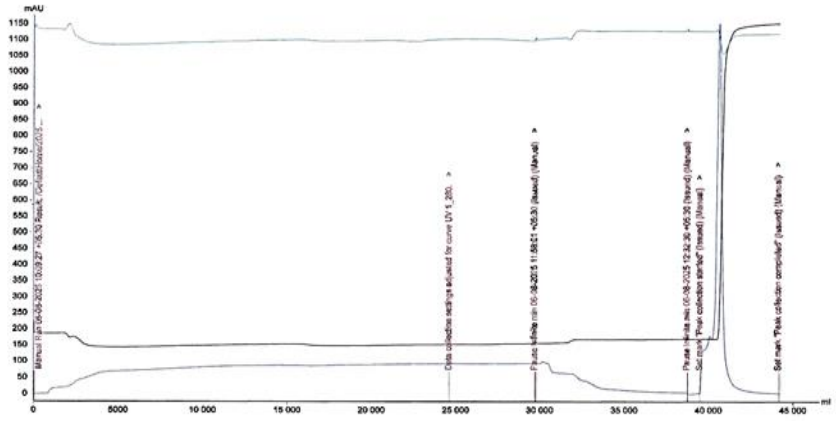

Fig. 5: Preparative purification chromatogram. On-line monitoring of A_{280} and conductivity across loading (0 - 30,000 mL), wash (30,000 - 39,000 mL), and step elution phases (39,000 - 44,000 mL).

3.3 Viral Inactivation Efficiency and Safety Validation

To ensure complete inactivation of Japanese Encephalitis (JE) virus while preserving target antigen integrity, purified harvest from Celufine Sulfate (SQ) affinity chromatography was diluted to 55ug/ml in 1x PBS containing 75mM NaCl. The protein solution was supplemented with sucrose to a final concentration of 8.5 mg/ml (0.015 ml of 60% sucrose per mL) and inactivated using formaldehyde at a final concentration of 0.05% (w/v) (Day 0). On Day 2 post-inactivation, the solution was clarified through a 0.45 um filter to remove insoluble aggregates. Inactivation kinetics and viral clearance were validated across three independent manufacturing batches from both bioreactor and CellStack-10 (CS-10) platforms.

The Plaque Assay for the viral antigen samples collected on Day 2 (post-filtration) and Day 4 post-inactivation yielded 0 PFU/mL across all tested batches, confirming rapid loss of infectivity. The In Vitro Inactivation of Viral antigen samples collected on Day 10 post-inactivation were neutralized with 200 mM sodium metabisulfite to terminate residual formaldehyde activity. Subsequent passage on Vero cell cultures demonstrated complete absence of cytopathic effect (CPE) or live viral replication. The In Vivo Safety Study performed using spent culture fluids from the *in vitro* assay were inoculated into BALB/c mice. No signs of infection, pathology, or morbidity were observed throughout the study period, confirming complete virus inactivation.

4. DISCUSSION

Japanese Encephalitis Virus (JEV) is a neurotropic flavivirus in the family Flaviviridae, and it remains a major cause of viral encephalitis in many countries across Asia, the Western Pacific, and Australia (Chugh et al., 2025; Solomon et al., 2003; Burke et al., 1988). It is a vector-borne flavivirus first reported in 1871 in Japan and first isolated in 1935 from the brain of a fatal JE case (Srivastava et al., 2023). It is one of the major public health problems not only because of infected cases and many deaths but also due to severe neuropsychiatric sequelae that necessitate lifelong support, amounting to a considerable socioeconomic burden (Campbell et al., 2011; Solomon, 2006). Nonetheless, JEV has an enzootic cycle involving mammals and birds, due to which the virus can persist in nature to such an extent that it might be nearly impossible to eradicate the virus in the near future (Kulkarni et al., 2018). Thus, effective antiviral therapy and an ideal vaccine against JEV are of pressing priority.

Vaccination is the most cost-effective therapeutic intervention. Despite challenges, such as a lack of an appropriate drug delivery system or a treatment plan independent of the stage of infection, JEV research has increased rapidly with growing technological advancements, with the primary aim of developing safe and cost-effective therapeutic vaccines for all age groups. The existing studies on JE-VAX are formalin-inactivated virions prepared from JEV-infected mouse brain, which is expensive to manufacture and may carry potential risks such as allergic reactions to brain basic proteins. In addition, there are biosafety issues with propagating infectious JEV in mice. Therefore, the development of second-generation JEV vaccines has been promoted by the WHO (Chambers et al., 1997). Earlier attempts have been initiated to produce high titers of the JEV vaccine strains in Vero cell cultures and to develop improved, inactivated whole-virion JEV vaccines (Srivastava et al., 2001; Sugawara et al., 2002). The Vero cell-derived vaccine is likely to have significant advantages in the absence of mouse brain proteins and in higher antigenicity than brain-derived JE-VAX (Chambers et al., 1997; Kinney and Huang, 2001).

Traditionally, Vero cells have been cultivated as adherent monolayers on the surface of roller bottles or cell stacks in media supplemented with fetal bovine serum (FBS). However, this method is fraught with significant limitations that are increasingly untenable for modern vaccine production. The reliance on FBS introduces inherent risks of adventitious agent contamination, notable batch-to-batch variability, and high costs, which compromise process consistency and regulatory compliance. Furthermore, the limited surface area of

adherent culture systems severely restricts scalability, making it impractical to meet the multi-thousand-liter volumes required for global vaccine campaigns. To address these issues, research has focused on adapting Vero cells to suspension culture and using chemically defined, serum-free media. This approach allows for cultivation in bioreactors, which provides a more controlled environment, simplifies process automation, and enables a significantly higher cell density per unit volume, directly translating to increased viral antigen yield and reduced production costs.

Traditional methods of cultivation in serum-containing media, and tremendous efforts have been made to adapt them to animal-component-free media. Indeed, removing serum reduces the risk of contamination with viruses, mycoplasmas, or prions (Paillet et al., 2009), as well as batch-to-batch variation and purification costs. However, removing serum can reduce cellular growth and density, necessitating medium optimization and cell adaptation. The major concerns of the Vero cell-based vaccine are, however, the safety and cost during vaccine production. A large amount of JEV needs to be propagated in cultures, and then collected and purified before formalin inactivation, procedures requiring high levels of biosafety containment according to Good Manufacturing Practice. This may cause pressure on the cost and may prevent developing countries in the endemic areas from vaccinating large at-risk populations. Therefore, second-generation JEV vaccines available from more efficient, low-cost manufacturing are desirable for mass vaccination. Among the Continuous cell lines currently used in the vaccine industry, such as MDCK, CHO, PER.C6, and Vero cells, have been both the first to be approved by the WHO and the most widely used cell line for viral vaccine production in the last 25 years (Bourigault et al., 2025; Hussain et al., 2016; WHO, 2013). The Vero cells are a continuous adherent cell line isolated from African green monkeys' kidney epithelial cells in 1962 (Yasumura and Kawakita, 1963). In 2014, genome sequencing revealed that the cells originated from a female *Chlorocebus sabaeus* (Osada et al., 2014).

Vero cells were characterized by their spontaneous establishment and aneuploidy, with an average of 58 chromosomes in more than 65% of the cells. They have been used as a substrate for vaccine production since the 1980s. Their widespread use in viral vaccine production stems from their significant advantages in this application. The Vero cell line is susceptible to infection by viruses from various families, including the poliovirus (*Picornaviridae*), the rabies virus (*Rhabdoviridae*), and the influenza virus (*Orthomyxoviridae*) (Velcken et al., 2013). In addition, it facilitates viral replication due to a lack of type 1 interferon production (Hegde, 2015). Since its first US Food and Drug Administration approval in the 1980s (Duigou, 2010), the cell line has received greater attention and is currently deemed non-tumorigenic and safe to use if it stays within the range of 150 passages (Grachev et al., 1998).

Currently, large-scale vaccine production using Vero cells is carried out with microcarriers to increase the available surface area for adhesion. Despite achieving remarkable productivity, the process remains limited by surface area. The current study established a robust, serum-free manufacturing process for the Japanese encephalitis virus vaccine using the SA 14-14-2 strain. By utilizing Vero cells anchored to Cytodex-1 microcarriers and maintained in specialized serum-free media (VP-SFM and Cellin-1), we established a scalable upstream process. This process provides a standardized framework for the industrial-scale production and demonstrates an optimized, scalable upstream process to produce the Japanese encephalitis virus (JEV) vaccine using the live-attenuated SA 14-14-2 strain. By employing Vero cells cultivated on Cytodex-1 microcarriers within a 3L bioreactor and utilizing serum-free media, we established a robust platform for high-titer vaccine manufacturing. Comparative analysis between static (CS-10) and suspension bioreactor cultures revealed that the bioreactor system significantly enhanced cell yields by approximately 30% (2.69×10^5 cells/cm²). Furthermore, viral titers in the bioreactor reached 2.20 × 10^7 PFU/mL, representing a 10–15% increase over static conditions.

The pre-run column qualification demonstrated excellent packing quality, as evidenced by an asymmetry factor of 1.19 and a high theoretical plate count (13.186 plates/m). A peak asymmetry value close to 1.0 indicates minimal axial dispersion and uniform fluid flow across the bed diameter, which is essential for preventing peak broadening and maintaining yield in scale up the chromatography. The downstream preparative runs verified the operational stability of the packed column under high-volume loading conditions (up to 65L). The clear resolution between the unbound flow-through baseline and the step-eluted target fraction confirms robust binding capacity and minimal non-specific retention. Furthermore, the rapid return of conductivity and A_{280} signals to baseline during column regeneration highlights efficient matrix re-equilibration, ensuring consistent performance across repeated purification cycles.

The robust inactivation protocol successfully eliminated JE virus infectivity across both upstream production platforms, the bioreactor and CS-10 systems, without compromising safety margins. The combination of 0.05% (w/v) formaldehyde and sucrose stabilization effectively inactivated viral particles within 2 days (0 pfu/ml), while extended incubation through Day 10 ensured regulatory compliance for viral clearance. Full neutralization using 200 mM Sodium metabisulfite effectively quenched unreacted formaldehyde, preventing cellular toxicity in subsequent cell culture assays. The concordant *in vitro* Vero cell culture and *in vivo* BALB/c mouse model results rigorously confirm total loss of viral virulence, meeting established safety standards for inactivated viral antigen preparations. The

apparent overall yield recovery of 30-50% reflects deliberate removal of process-related impurities rather than target antigen loss. The initial crude concentration steps retain significant quantities of Vero HCP and HCD. The sequential implementation of Celufine Sulfate affinity capture and Sartobind Q membrane filtration produced highly purified antigen free of host cell contamination. This multi-step clearance profile explains the mass balance shift from harvest to final product, establishing a scalable and reproducible downstream purification framework.

These findings provide a standardized framework for industrial-scale, serum-free production of JE vaccines with improved efficiency and quality control. Furthermore, these adapted cells are highly productive for viral antigens, with high-density perfusion culture intensification increasing volumetric productivity by over tenfold. This optimized upstream process provides a scalable, safer, and more cost-effective platform for manufacturing flavivirus vaccines, improving global access and pandemic preparedness. The results indicate that suspension-adapted Vero cells grown in a microcarrier-based bioreactor under serum-free conditions support significantly higher virus and protein yields compared to traditional static cultures. The bioreactor system offers better visualization and control of critical parameters such as pH and dissolved oxygen, which are limited in static containers like Roller bottles or Cell stalks. Although static containers are useful for initial scale-up, they require more intensive manual labor and carry a higher risk of aseptic manipulation failure. The integrated use of YSI biochemical analysis and automated bioreactor controls allows for optimized media conditioning, resulting in a more efficient and predictable production cycle. The transition from static "Cell Stalk" containers to a microcarrier-based bioreactor provides several advantages. Static systems suffer from limited scale-up potential and high labour requirements for aseptic manipulation. Conversely, the bioreactor system utilizes automated control of pH, DO, and temperature, ensuring a more stable environment for Vero cell proliferation. The increased surface-area-to-volume ratio provided by the Cytodex-1 microcarriers allows for high-density growth in a compact footprint. Our data confirm that suspension-adapted Vero cells in serum-free media support superior viral replication, likely due to optimized nutrient availability and efficient metabolite removal through programmed media changes. The highest virus yield was observed using microcarriers with Vero cells in the Bioreactor system compared to cell stalks. Furthermore, to streamline Japanese encephalitis virus cultivation, we employed a serum-free medium devoid of animal-derived components. Accordingly, this study aimed to enhance viral yield by employing an efficient substrate, optimizing conditions for viral replication, and simplifying the bioprocess using a serum-free medium and modern technological approaches.

5. CONCLUSION

This study successfully established a robust and scalable serum-free production process for the JEV SA 14-14-2 vaccine. The transition from static cultures to an optimized 3L bioreactor system utilizing Cytodex-1 microcarriers increased cell density by 30% and viral yields by up to 15%. This methodology provides a robust foundation for high-capacity vaccine manufacturing while maintaining stringent quality and purity standards. With higher cell yields, enhanced viral titers, and better metabolic control, this process provides a robust framework for large-scale vaccine manufacturing. Beyond yield improvements, the integrated bioreactor platform offers superior metabolic process control and improved scalability, which are essential for maintaining stringent quality and purity standards in vaccine production.

This study demonstrates a robust, scalable workflow for downstream purification and complete inactivation of the Japanese Encephalitis virus. Column qualification confirmed optimal packing efficiency of A_s=1.19, 13,186 plates/m. Sequential purification using Celufine Sulfate affinity chromatography and Sartobind Q membrane filtration efficiently removed host cell proteins and host cell DNA, yielding high-purity antigen with a 30–50% overall recovery. Formaldehyde inactivation (0.05% w/v) with 0.45 um aggregate filtration achieved 0 PFU/ml by Day 2. Complete viral safety was verified through 200 mM sodium metabisulfite neutralization and concordant *in vitro* Vero cell and *in vivo* BALB/c mouse studies.

By utilizing serum-free media, the process further minimizes biological variability and reduces contamination risks, making it a safer and more cost-effective solution for the global market. Ultimately, the methodologies presented in this study provide a promising and versatile platform for the large-scale production of flavivirus antigens. This optimized upstream process not only strengthens the infrastructure for current JEV vaccination efforts but also serves as a critical model for rapid-response vaccine manufacturing in the face of emerging global health threats and future pandemics.

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