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Density Gradient Centrifugation of Chikungunya Virus / Cesium Chloride (CsCl) Density Gradient Virus Purification by Ultracentrifugation

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Abstract:- Chikungunya virus was purified with Cesium chloride (CsCl) density gradients by ultracentrifugation from the cell culture supernatant of infected Vero cell. The purification process was done by using combinations of CsCl and PBS (Phosphate buffer saline) and the virus band was found at a density of 1.00 g/ml after centrifugation for 3 hr of ultracentrifugation. The authenticity of the purified Chikungunya virus was confirmed by SDS PAGE. The Purification process has immense application and is useful in making large quantity of purified Chikungunya virus with high titer values for its characterization.

Keywords:- *Chikungunya Virus, Centrifugation, Vero Cells, CsCl, TFF, Antigen.*

I. INTRODUCTION

Density gradient centrifugation stands as a pivotal methodology across molecular biology, virology, and biochemistry owing to its adeptness in segregating particles based on their densities. This technique finds widespread application in the isolation and refinement of diverse biological entities such as viruses, proteins, organelles, and cells, exploiting their distinct density profiles (Araùjo et al., 2008). Notably, its role in purifying viruses from intricate biological matrices holds significance in elucidating viral architecture, replication dynamics, host interactions, and the purification of attenuated viruses pivotal for vaccine development (Nasukawa et al., 2017). Its adaptability and efficacy in sorting and purifying an array of biological particles render it as an indispensable technique in varied scientific domains encompassing environmental science, biotechnology, and medicine, spanning from fundamental research to practical implementations (Spinnrock and Colfen., 2019).

Chikungunya, a viral illness characterized by its transmission from bites of Aedes aegypti and Aedes albopictus species mosquitos, that also serves as vector for other maladies such as dengue fever and zika virus (Monteiro et al., 2019). Chikungunya's onset is marked by a rapid rise in body temperature, accompanied by joint discomfort, muscular pain, headaches, nausea, exhaustion, and a characteristic rash (Kucharz et al., 2012). The consequential joint distress can escalate significantly, leading to severe debilitation, that may last for an extended period, spanning weeks to months.

Chikungunya lacks a targeted antiviral treatment, prompting sole focuson relieving symptoms through rest, hydration, and pain relievers (Rodriguez et al., 2022). Preventive measures include averting mosquito bites through the use of insect repellents, wearing long sleeves and pants, and using mosquito nets to mitigate transmission risks.The application of miniaturized or weakened variants of the virus is one of the tactics commonly used in the creation of a Chikungunya vaccine (Schwameis et al., 2016). Attenuated vaccines are made from live viruses that have been engineered to lower their pathogenicity (ability to cause disease) while still stimulating an immune response.

Attenuated chikungunya virus (ACV) is a weakened or attenuated form of the virus devoid of its pathogenicity. ACVs are useful in the development of vaccines and antiviral treatments.Density gradient centrifugation using cesium chloride (CsCl) serves as significant method for virus purification, considering its ability to create a gradient upon dissolving in water (Nasukawa et al., 2017). The viral particles would be distinguished based on their respective densities in this gradient accordingly. CsCl purification of attenuated chikungunya virus is crucial for multiple reasons. Primarily, it eliminates contaminated protein molecules, nucleic acids, and cellular debris, pivotal for the safety and efficacy of vaccines and antiviral treatments (Wolf and Reichl, 2011). Furthermore, this process enables virus concentration, proving advantageous in specific applications. These purification steps are integral for ensuring the purity and potency of attenuated chikungunya virus preparations, contributing significantly to their clinical utility and research endeavours.

CsCl density gradient centrifugation is a useful method for studying additional viruses in spite of its application in Attenuated chikungunya virus purification. It can be employed to differentiate between distinct genotypes of a virus, to analyse virus organization, and to identify and describe viral proteins. The present research work describes thepurification and characterization of Chikungunya virus (Su et al., 2019). Employing the density gradient ultracentrifugation, pelleting viruses by centrifugation, ultrafiltration, and chromatography. Cesium chloride (CsCl)

density gradient ultracentrifugation has been employed for the purification of chikungunya virus (McNamara et al., 2020).The present communication describes about high concentrated virus purification process for chikungunya virus.

II. MATERIALS AND METHODS

➢ *CHIK Vaccine Live Attenuated*

A small batch of vaccine was made using serum isolates from a Thai patient infected with CHIKV during the 1962 outbreak (CHIK strain 15561) (de Lima Cavalcanti et al., 2022). The vaccine was first passaged in green monkey (GMK) cells and then serially passaged (18 plaque-to-plaque) in MRC-5 cells before CHIK 181/Clone 25 was chosen as the seed strain (Edleman et al., 2000). A lyophilized supernatant from MRC-5 cells infected with an attenuated strain, CHIK 181/Clone 25, makes up the live, attenuated, TSI-GSD-218, Lot 1-85, chikungunya vaccine made at The Salk Institute. It was shown that the vaccination was safe and protected monkeys and mice from challenges by eliciting neutralizing antibodies (Akahata et al.,2010). The United States Army Medical Research and Materiel Command submitted IND 2426, titled "Chikungunya Virus Vaccine Live Attenuated (Strain 181/Clone 25)," to the Food and Drug Administration in July 1986. We got the chikungunya production seed (10 vials, 1.2 ml/vial) from USAMRIID on July 10, 2009, at Passage 32. The strain is CHIK 181/Clone 25. This investigation employed the same strain of ChikV virus. In this investigation, the antigen for the screening assays and the virus-infected cells for immunofluorescence were prepared using the live attenuated strain of the Chikungunya virus.

➢ *Vero Cell Line Propagation:*

A continuous cell line known as Vero was established from kidney epithelial cells obtained from an African green monkey (Ammerman et al., 2008). Many vaccinations, especially those against viruses, rely on these cells for their development and manufacturing (Rodrigues et al., 2015). To keep the Vero cell line viable and growing, it is common practice to subculture or passage the cells throughout propagation (Verma et al., 2020). There were 15 x 106 cells per millilitre of cells cryopreserved in a combination of 50% MEM, 40% FBS, and 10% dimethyl sulfoxide (DMSO). The cells were grown in MEM with 10% FBS (Draper et al., 2001). After reviving the cells, the vial was quickly frozen at 37°C. (Fast thawing). We started by freezing the cells in the vials and then moved them to a 15 ml conical centrifuge tube. The tube already had pre-cooled MEM with 10% FBS in it. We then added pre-cooled growth media drop by drop to gradually dilute the solution. After gently mixing the cell contents, they were centrifuged at 1500 rpm for 6 minutes at 4°C to recover the cells. Placing the cell suspension on a tissue culture flask was the next step after removing the supernatant. The pelleted cells were reconstituted with MEM growth media that included 10% foetal bovine serum. The sterility of the cell-growth medium mix was tested by inoculating it into SCDM (soybean casein digestive medium) and FTM. (a solution of thioglycollate in water"). The confluence, sterility, and health of the revitalized cells were checked daily.

➢ *Preparation of Chikungunya Virus Antigen*

Planting the Vero cell lines into a T150 flask containing MEM containing 10% fetal bovine serum was used. Following 90% cell monolayer confluence, the spent media is removed from the Vero cell lines, and 2 mL of attenuated chikungunya virus (181/25) is added to the flask. The flask is then incubated at 37°C for 30 minutes. After that, 100–150 ml of plain medium is added, and the mixture is incubated for another round (Sudeep et al., 2019). After two or three days, the cytotoxic impact (CPE) became apparent. After cell extraction, the cells were put in tubes and centrifuged at 2000 rpm for six minutes. The soup with the virus was taken out and frozen at -70°C so that it could be used later. The antigen that had been collected was first allowed to cool to room temperature before being treated to increase its clarity. It was then concentrated to 160 ml using a TFF cassette with a 100 KDa filter cartridge. After that, it was subjected to ultracentrifugation at 25,000 rpm for three hours at 4°C. A protein concentration of 0.4 mg/ml was obtained by re-suspending the particle in a 3 ml container. (Bicinconic acid assay for protein estimation). At 4°C, this filtrate was subjected to 3 hours of CsCl Density Gradient Ultra-Centrifugation at 25,000 rpm. Separated parts were taken from three ultra-tubes and put into 15 Eppendorf tubes, one for each. This was done in 1-ml steps, for a total of 45ml (1 x 15 x 3 = 45ml). A 12% resolving gel was used to run all these fractions to get the visible bands. The concentrated antigen still contains BSA, which is a contaminant. Hence, the CsCL density gradient (which increases by 0.5% per milliliter). The virus pellet was re-dissolved in the viral growth medium after centrifugation and kept at -70°C until needed.

➢ *Live Attenuated Chikungunya Virus Antigen Concentration by TFF (Tangential Flow Filtration)*

Traditional applications of the size-based separation technique, tangential flow filtration (TFF), include coarse purification, concentration, and buffer exchange. This research makes use of TFF to selectively remove complexes of attenuated chikungunya antigen (Musumeci et al., 2018). By using a target-protein binding molecule (TPBM) that is an anti-chikungunya antigen polyclonal immunoglobulin G (IgG), this method was shown to restore attenuated chikungunya antigen.

The 300KDa TFF capsule purchased commercially, was used to concentrate a volume of filtered attenuated chikungunya antigen with a pore size of 0.22 µm. The concentrated (attenuated) chikungunya antigen flow that passes through the TFF capsule is collected and filtered through 10 volumes of a buffer comprising 10mM Tris, 150mM NaCl, and one millimolar EDTA, pH 7. The process of diafiltration was repeated ten times, and the resulting concentrate is now in storage.

A Tangential Flow Filtration (TFF) cassette (Merck: 100 kDa) was used to concentrate the attenuated chikungunya virus (CHIKV) (Loewe et al., 2019). The water

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flux (flow rate) was measured while 200mL of 1M NaOH (250C ± 20 C) was passed through the cassette membrane while it was being cleaned in circulation mode with WFI water. After that, 100 ml of 20mM Tris buffer (pH 7.4) was added to the cassette membrane to bring it to pH 7.4. The 400 mL of virus harvest was added to the feed reservoir and linked to the TFF cassette using the Cole Parmer peristaltic pump. The TFF process was started with a flow rate of 10 ml/min, and the retentate and permeate were collected separately before passing them through a 0.22µm pore filter. The feed vessel was drained to establish a consistent permeate flow rate, and the membrane was cleansed with HPLC grade water once the filtering cycle was finished. 2–80C with 1M NaOH was used to store the membrane cassette prior to the subsequent filtering cycle.

The membranes were flushed with 400 mL of 1 M NaOH (40°C, 1 h) using circulation (closed permeate valve) for the TFF tests conducted in concentration mode. Following the measurement of the clean water flow using MilliQ water, the membranes were brought to pH 7.4 by equilibrating them with 100 mL of 20 mM Tris-HCl. After that, 450 mL of MV suspension in either SCM or SFM was added to the feed reservoir. The permeate outlet was closed during the first five minutes, and the feed was circulated. A Sartorius digital balance was used to record the permeate weights, and SciLog pressure sensors (Parker Hannifin, Cleveland, OH, USA) were used to record the retentate, permeate, and feed pressures. The permeate outlet was after that opened. Win Wedge programme was used for the data analysis. For every 50 g of permeate that went through the membrane, about 0.8 mL of sample was obtained from the feed and permeate vessels (Figure 1). The filtering run was terminated when the permeate weight reached 420 g (concentration factor = 15), as reported in Membranes 2019, 9, 160 5 of 22. The feed vessel was drained after each filtering run to maintain a steady permeate flow, and the membrane was flushed with clean water. The membrane was incubated in 1 M NaOH at 40 ◦C for 40-48 hours before the subsequent filtering run. The results of this investigation shed light on a novel approach to TFF-based selective purification of attenuated chikungunya antigen.

- *Ultra-Centrifugation by CsCl*

Dissolving CsCl in PBS at various concentrations (1.0g/ml and 1.5g/ml) was used to produce the CsCl (Strobel et al., 2015). To prepare the CsCl gradients, 10 ml of a solution with a concentration of 1.5 g/ml was added to the tubes first, followed by 10 ml of a solution with a concentration of 1.0 g/ml. Finally, 18 ml of a chikungunya virus stock was slowly added to the tubes. The tubes were placed in an ultracentrifuge and centrifuged at 25,000 rpm while kept at 40°C for three hours. At a density gradient of 1.0 g/ml, the antigen particles (virus) band was detected, and the viral bands were meticulously extracted from the tubes using a micropipette. Following the method previously published for vesicular stomatitis virus (12), the CHIKV virus was separated by ultracentrifugation in density gradients of CsCl. Typically, 0.5 ml of the virus, which had been pelleted in a Spinco #30 rotor at 30,000 rev/min for 1 hour, was spread out across 4.5 ml of a prepared gradient. The virus was then concentrated 10- to 50-fold. In a Spinco model L2-65 ultracentrifuge, the SW-50 rotor was subjected to centrifugation at 36,000 rev/min (141,995 X g). Twenty or forty fractions, each consisting of ten drops, were collected by puncturing the tube's bottom with a piercing device (Buchler Instruments, Inc., Fort Lee, N.J.). This allowed for the fractionation of the gradients. Using an Abbe 3L refractometer to measure the refractive index of each second or third fraction and extrapolating the density using standard curves taken from the International Critical Tables, the density curve for the gradient was generated. Regularly, samples were directly weighed in 100- µl pipettes to ensure the precision of this procedure. Here is how the density gradients used in this research were made. A gradient was produced in 10 ml of 1.5g/ml and 10 ml of 1.0g/ml, with 0.5% increments between each. Dissolving CsCl in PBS was done to prepare the densities. As with gradient 1, 1.5g/ml density CsCl was added to the tube to create the gradient, and for gradient 2, 1.0g/ml density CsCl was added. After 10 milliliters of 1.5 g/ml and 10 milliliters of 1.0 g/ml were layered to produce the gradient, the tubes were filled with viral stock and spun at 25000 rpm at 4°C for three hours.

- *Demonstration of Chikungunya Virus Protein Profile by SDS-PAGE*

To evaluate the purity of the proteins and find their apparent molecular mass, scientists used sodium dodecyl sulphate polyacrylamide gel electrophoresis, often known as SDS PAGE (Nowakowski et al., 2014). After wiping the glass plates with ethanol, they were rinsed with distilled water. Clips were used to secure the silicon rubber tubing between the glass panels to create a watertight seal. To avoid oxygen, which hinders the polymerization of the acrylamide solution, the solution was carefully covered with isobutene after being gently placed between the glass plates. The gel was covered with alcohol once it had set, and then distilled water was used to cleanse the surface. After placing the correct comb on the separating gel, the stacking gel—made of prepared acryl amide solution—was carefully poured on top. Polymerization was allowed to take place for 10 minutes. At the same time, samples were heated for 5 minutes with a 1:4 ratio of sample buffer to samples. The stacking gel is carefully extracted from the wells after polymerization using distilled water, and the gel is then deposited in a gel tank filled with flowing buffer. The gel was run at 12 milliamp power for 5 minutes before use.

Electrophoresis was performed using the correct molecular weight marker after the materials were put into the correct wells using the micropipette. The sample migrated through the stacking gel at 12 milliamps before the power was increased to 15 amps. After 40 minutes, when the tracking dye had settled to the gel's base, the electrophoresis was terminated. The gel was carefully taken from the electrophoresis equipment and washed when the process was finished. For 30 minutes at room temperature on a shaker, the gel was immersed in a tiny box with coomassie blue staining solution. The gel was left to turn colorless in the destainer overnight before the bands could be viewed.

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- *Casting the Resolving Gel:*

To prevent leaking, two oil-free, spotless glass panels were securely fastened between two 0.75 mm thick acrylic spacers. A freshly made resolving gel solution was put between the two plates. The rapid polymerization was made possible by covering it with a layer of water. At room temperature, the gel was given time to polymerize.

- *Casting the Stacking Gel:*

The water that had been poured upon the resolving gel was withdrawn. After the resolving gel was placed on top, a 0.75 mm thick comb was installed, and the stacking gel solution was made just before. This time, the gel was left at room temperature again to polymerize.

- *Loading of the Samples:*

The first well was supplemented with 7 µl of Protein Ladder (Invitrogen, USA), which has a molecular weight ranging from 10 to 220 kDa. To prepare the samples, 10 micrograms of each viral antigen and crude MRC-5 cell lysate were combined with 7.5 µl of sample buffer (3X). The mixture was then heated in a water bath at 100 ° C for five minutes before being added to the wells. At 50 V, electrophoresis was continued until the dye front reached the resolving gel. After that, the voltage was raised to 100 V until the dye front hit the gel's base.

- *Chikungunya Virus Antigen Titration by Plaque Assay:*

After attenuating the CHIKV virus, the infectivity titer was determined using a plaque test, as detailed below (Zhang et al., 2022). Two milliliters of cell culture medium was added to each well of a 6-well plate containing 4 X 10^5 cells per milliliter the day before the plaque test. In MEM with 3% FBS, tenfold successive dilutions of the material (10^{-1} to 10^{-10}), which were then prepared for titration. The Vero cells were adsorbed with the diluted sample for 90 minutes at 37°C in a CO2 incubator. After the first incubation at 37°C for 3 days in a CO2 incubator, 2 ml of 1% methylcellulose was added to the wells. In order to make counting the plaques easier, the plates were dyed with crystal violet dye.

III. RESULTS AND DISCUSSION

➢ *CHIK Vaccine Live Attenuated*

The vaccine was developed using a serum isolate from a patient in Thailand who contracted CHIKV during the 1962 outbreak (CHIK strain 15561). It was first passed through green monkey (GMK) cells and then through 18 plaque-to-plaque passages in MRC-5 cells before the vaccine seed, CHIK 181/Clone 25, was chosen. A lyophilized supernatant from MRC-5 cells infected with an attenuated strain, CHIK 181/Clone 25, makes up the live, attenuated, TSI-GSD-218, Lot 1-85, chikungunya vaccine made at The Salk Institute. It was shown that the vaccination was safe, and it protected monkeys and mice from challenge by eliciting neutralising antibodies.

An investigational new drug application (IND) for a chikungunya virus vaccine (Strain CHIK 181/Clone 25) was filed with the US Food and Drug Administration by the US Army Medical Research and Materiel Command in July 1986. On July 10, 2009, at Passage 32, the chikungunya production seed (Strain CHIK 181/Clone 25) was received from USAMRIID in ten vials, with 1.2 ml of seed per vial. A single strain of ChikV was used for this investigation.This work used a live attenuated strain of Chikungunya virus to prepare the antigen for the screening tests and demonstrate immunofluorescence using virus-infected cells.

➢ *Vero Cell Line Propagation:*

For about 40 years, VERO cells have been widely used in the production of human vaccines; they are now the most well-characterized CCL (Barrett et al., 2009). Despite VERO cells' many natural advantages, serum proteins are necessary for their successful proliferation.

The technique of cultivating Vero cells in culture for use in scientific experimental methods is known as the Vero cell line propagation. The Vero cell line is a continuous cell line type that has its origins in the kidneys of the African green monkey. They are usually cultivated in a specialized medium to ensure the Vero cells' growth and survival. One typical growth media for Vero cells is DMEM, which stands for Dulbecco's Modified Eagle media. In addition to glucose and sodium bicarbonate, it includes a well-rounded blend of vitamins, minerals, amino acids, and other necessary components. Fetal bovine serum (FBS) is a good nutritional and growth factor supplement that may be added to DMEM. Sterile procedures are followed at every step, and the culture medium is created following the manufacturer's instructions. An appropriate density of Vero cells, usually between 10,000 and 100,000 cells per square centimeter, is seeded into a culture vessel (such as a flask or a petri dish). A humidified incubator containing 37°C and 5% CO2 is used to cultivate Vero cells. Constant vigilance is exercised to detect any indications of cell proliferation, including changes in cell number and confluency. When the cells have grown to confluency, they are removed from the culture tube by dissolving the cell-substrate and cell-cell adhesions with a trypsin and EDTA solution. The next step is to move the detached cells to a different culture tube, where they may continue to develop at a reduced density. The culture medium is usually changed every two to three days to keep cells healthy and give them new nutrients. The Vero cells have several potential uses, including the creation of viral vaccines, the expression of proteins, and the assessment of toxicity. Centrifugation separates the cells from the culture medium using trypsin-EDTA; the resulting cell pellet may be used for storage or further processing. Propagation of Vero cell lines is a tried-and-true method with several applications in biomedicine and biotechnology.

➢ *Preparation of Chikungunya Virus Antigen*

The culture supernatant containing the live attenuated Chikungunya virus produced in Vero cells was frozen after each harvest until a substantial amount had been accumulated. After defrosting at room temperature, the viral protein harvest underwent an initial clarity.

The next step was to subject the filtrate to CsCl Density Gradient Ultra-Centrifugation at 25,000 rpm for three hours at 4°C. The separated fractions were transferred to fifteen Eppendorf tubes in one-millilitre increments from three ultra-tubes (1 x 15 x 3= 45ml). These fractions were subjected to a 12% resolving gel run to get the visible bands. The concentrated antigen still contains BSA, which is a contaminant. So, the virus pellet was centrifuged and then resuspended in the same viral growth medium with a CsCL density gradient of 1.5g/ml and 1.0g/ml added in 0.5% increments. It was then kept at -70°C until needed. The SDS-PAGE study of the Chikungunya virus SDS-PAGE was conducted mostly in accordance with the methodology outlined by Laemmli et al., (1971).

- *Live Attenuated Chickengunia Virus Antigen concentratedby TFF (Tangential Flow Filtration):*

One method for the concentration of Chikungunya virus antigens from a sample is tangential flow filtration or TFF. Pressure is applied to a solution while it tangentially flows through a filter to the filter's surface in a technique known as tangential flow filtration (TFF). This paves the way for the isolation of viral antigens and other big particles from other, more minor molecules and contaminants. The following is the technique for isolating Chikungunya viral antigens using TFF.

In order to create a sample that contains the Chikungunya viral antigens, the virus particles infected cells or tissues are collected and processed. The sample is usually cleared using centrifugation or filtering to eliminate cell debris and other impurities. A filter membrane, pressure regulator, and collecting vessel are the components of the specialized filtering system used in the TFF. The size and characteristics of the viral antigens that are being concentrated determine the membrane that is used. The TFF system is linked to a feed reservoir, which is then filled with the sample. Under carefully managed conditions of pressure and flow rate, the sample is pushed through the filter. Filtrate consists of tiny molecules and impurities that pass through the filter, whereas bigger Chikungunya viral antigens are kept on the membrane. The concentration of viral antigens may be increased by repeating the procedure many times. Either changing the direction of flow or using an appropriate elution buffer allows the concentrated Chikungunya viral antigens to be eluted from the filter membrane. The effluent is collected in an independent container and is ready for further processing. Optimizing and validating TFF conditions for each application may be necessary since they will be dependent on the sample's characteristics and the particular viral antigen being concentrated.

A Tangential Flow Filtration (TFF) cassette (Merck: 100 kDa) was used to concentrate the attenuated chikungunya virus (CHIKV). By passing 200 mL of 1M NaOH through the membrane and measuring the water flux (flow rate), the cassette membrane was cleaned in circulation mode with WFI water at 250C ± 20 C. The cassette membrane was then adjusted to pH 7.4 using 100 cc of a 20mM Tris buffer. The virus sample, which amounted to 400 mL, was transferred to the TFF cassette using a peristaltic pump (Cole Parmer). The TFF process was started with a flow rate of 10 ml/min, and the retentate and permeate were collected independently before passing them through a 0.22µm pore filter. The feed vessel was drained to establish a consistent permeate flow rate, and the membrane was cleansed with HPLCgrade water once the filtering cycle was finished. The membrane cassette was kept at 2-80C with 1M NaOH prior to the subsequent filtering operation.

The TFF To prepare the membranes for the TFF studies, they were circulated with 400 mL of 1 M NaOH (40°C, 1 h) while in Concentration Mode, with the permeate valve closed. A 100 mL equilibration solution of 20 mM Tris-HCl (pH 7.4) was added to the membranes after the clean water flow had been measured using MilliQ water. After that, 450 mL of MV suspension in either SCM or SFM was added to the feed reservoir. The permeate exit was closed for the first five minutes, and the feed was cycled. After that, we used SciLog pressure sensors (Parker Hannifin, Cleveland, OH, USA) to record the feed, retentate, and permeate pressures and a Sartorius digital balance to record the permeate weights. The permeate outlet was then opened. The data were analyzed using Win Wedge software. For every 50 g of permeate that went through the membrane, about 0.8 mL of sample was obtained from the feed and permeate vessels (Figure 1). The filtering run was terminated when the permeate weight reached 420 g (concentration factor = 15), as reported in Membranes 2019, 9, 160 5 of 22. The feed vessel was drained after each filtering run to maintain a steady permeate flow, and the membrane was flushed with clean water. To prepare the membrane for the subsequent filtering run, it was incubated in 1 M NaOH at 40 ◦C for 40-48 hours. This research introduces a novel approach to selectively purifying attenuated chikungunya antigen utilizing TFF.

- *Ultra-Centrifugation by CsCl*

Biological samples may be effectively purified using the buoyant density-based approach of CsCl gradient centrifugation. In this method, a CsCl solution is used to create a density gradient in an ultracentrifugation tube, which is then spin at high speeds for several hours. Consequently, the sample is partitioned according to its density, and further analyses or processing may be performed on each partition independently. A typical technique for purifying antigens from the Chikungunya virus is CsCl gradient centrifugation. This method divides the virus particles into parts according to their density by using a gradient made of a dense CsCl solution.

An ultracentrifuge tube is used to transport a CsCl solution produced to the necessary density. A gradient is created by layering the Chikungunya viral antigen, which is concentrated using the Tangential flow filtration (TFF) method on top of the CsCl solution. The tube is then filled with more CsCl solution. After inserting the tube inside an ultracentrifuge, it is spin at very high speeds for several hours. At this point, the Chikungunya viral antigen is being separated by density as it moves along the CsCl gradient. The less dense particles will stay closer to the

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tube's top, while the denser ones will go towards its base. Carefully removing the tube from the ultracentrifuge after centrifugation allows for the fractionation of the contents. The process begins at the base of the tube and works its way up, with each percent being screened for virus particles as it goes. The components that carry the antigens for the Chikungunya virus are combined and concentrated. Chikungunya viral antigens may be effectively purified using the CsCl gradient centrifugation technique. This method may eliminate pollutants and impurities from the sample while separating Chikungunya viral antigens according to their density. It may be necessary to optimize and validate the CsCl gradient centrifugation settings for each application since they will vary depending on the sample characteristics and the particular viral antigens being isolated.

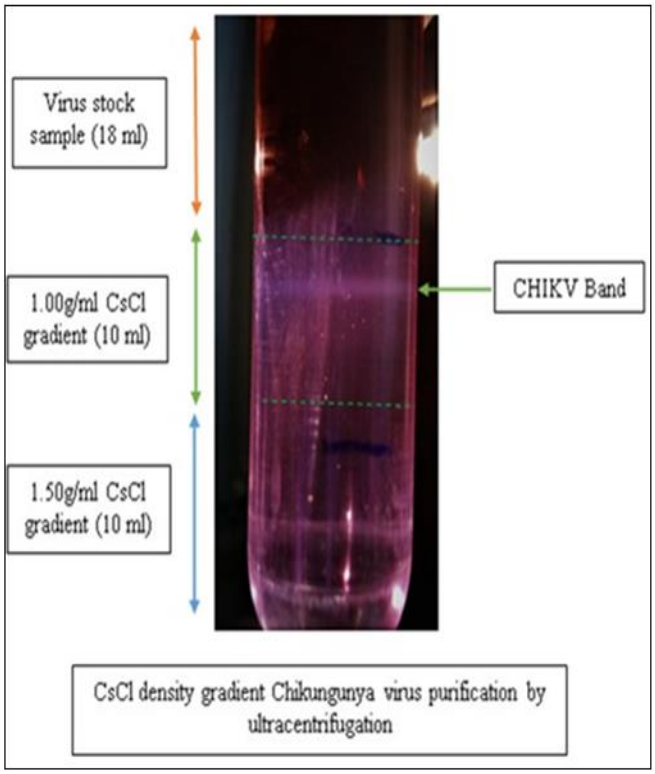

Fig 1 Illustration of the Live Attenuated Chikungunya Virus Antigen (CHICKV-Antigen) band
Separated by CsCl Density Gradient Centrifugation

- *Demonstration of Chikungunya Virus Protein Profile by SDS-PAGE*

One standard method for protein molecular weight separation is sodium dodecyl sulphate polyacrylamide gel electrophoresis or SDS-PAGE. Proteins may be separated and visualized according to their molecular weight using SDS-PAGE. Its analysis of protein samples is widespread in the field. Protein samples, gel characteristics, and staining reagent characteristics all have a role in determining the best SDS-PAGE settings, which may need optimization and validation for individual applications. It uses a molecular sieve made of polyacrylamide gel and an electric field to transport proteins through the gel. The fundamental steps needed to use SDS-PAGE.

- *SDS-PAGE is Employed to Profile the Chikungunya Virus Antigen Proteins*

The concentrated and purified live attenuated chikungunya virus antigen sample was made via TFF and ultra-centrifugation. To separate the proteins accurately according to their molecular weight, it is necessary to denature the proteins and guarantee that all of them have a consistent negative charge. This is accomplished by mixing the purified antigen sample with a buffer that contains SDS and reducing agents. Using a micropipette, the polyacrylamide gel is loaded with this SDS-treated live attenuated chikungunya virus antigen sample. A molecular weight ladder adds proteins with known molecular weights to the gel. The gel is subjected to an electrical field in an electrophoresis equipment. Proteins of varying sizes travel through the gel at different rates; the positively charged electrode attracts the live attenuated chikungunya virus antigen in the sample. Once the electrophoresis is finished, the Chikungunya viral antigen proteins in the gel are dyed with a protein-specific Coomassie blue stain. Imaging methods, such as a UV trans illuminator, allow the protein bands to be seen.

The live attenuated chikungunya virus antigen may be effectively profiled using this SDS-PAGE method. Separating proteins according to their molecular weight makes it possible to learn about the size and quantity of various proteins in a sample. Each application may need optimization and confirmation of the ideal SDS-PAGE settings based on the particular antigen being profiled, gel characteristics, and staining reagents utilized.

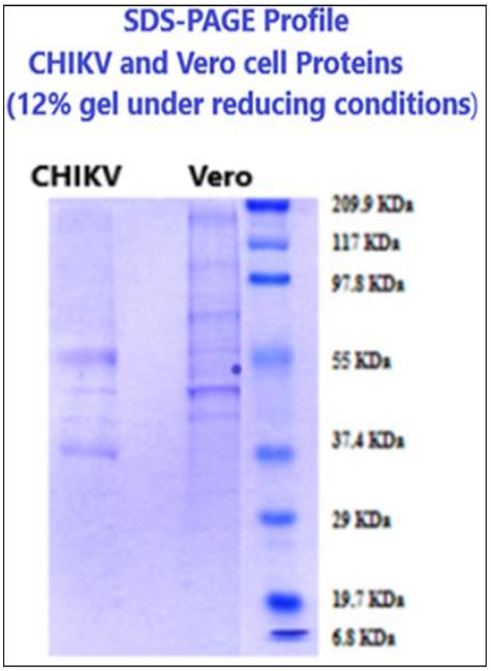

Fig 2 The Polypeptide Profile of the Ultra-Pelleted whole Virus Antigen was Evaluated by SDS-PAGE Under Reducing Conditions. The Structural Proteins Pertaining to Envelope Glycoproteins (~ 55KDa) –E1 and E2, Capsid Protein (~ 37KDa) could be Resolved. The Structural Protein Profile as Resolved by SDS-PAGE is Shown in Figure 2.

➢ *Chikungunya Virus Antigen Titration by Plaque Assay*

A plaque test is one popular way to estimate the quantity of infectious virus particles in a sample. Plaque counting is a technique that uses infected host cells to infect a monolayer and counts the amount of viral plaques, which are visible spots of cell death where the virus has replicated. Establishing a host cell monolayer in a culture plate is the first stage. The particular virus being tested dictates the choice of cell line. Various dilutions are achieved by serially diluting the virus in a diluent, such as culture media or saline. The host cells are cultured in a plate with individual wells for each viral dilution. The cells are given some time to incubate so the virus may infect them. Each well is covered solidly with a semisolid agar medium. While allowing nutrients to diffuse, the agar stops the virus from spreading from infected cells to nearby ones. The virus can reproduce by incubating the culture plate for a specific duration. Following the incubation period, the cover is peeled off, and cells are stained with a dye, such as crystal violet. The viral titer is determined by counting the viral plaques, which are regions of cell death. It may be necessary to optimize and validate the plaque assay settings for each application, as they may differ based on the particular virus being tested and the cell line used.

Using the plaque test, it is usual practice to titrate viruses, such as the Chikungunya virus. Methods for doing a Chikungunya virus plaque assay: Establishing a cell culture dish monolayer of sensitive cells, such as Vero cells, is the first stage. A variety of dilutions of the Chikungunya virus are obtained via serial dilution. Wells in the cell culture plate housing the vulnerable cells are supplemented with each viral dilution. The cells are given some time to incubate so the virus may infect them. To make a solid overlay, an agar medium with a particular concentration of fetal bovine serum is applied to each well. While allowing nutrients to diffuse, the agar stops the virus from spreading from infected cells to nearby ones. The cell culture plate is incubated for a specific duration to facilitate viral replication. Following incubation, the cover is peeled off, and crystal violet dye is applied to the cells. The number of plaques formed by the Chikungunya virus-infected cells in the monolayer is counted to find the viral titer.

The titer results are furnished below for all the three batches.

Table 1 CHIKV Ag Virus Titration by Plaque Assay

Batch No.	Pfu/ml
01/10	2.15 x 107
02/10	2.55 x 107
03/10	2.57 107

IV. CONCLUSION

An overview of the creation and characterization of a live attenuated chikungunya virus (CHIKV) vaccine, TSI-GSD-218, Lot 1-85, which was manufactured at The Salk Institute, is shown in the paper that is being presented. During the epidemic that occurred in 1962, the CHIK 181/Clone 25 strain was originally identified from a patient in Thailand. This strain was used to develop the vaccine. Green monkeys (GMK) and MRC-5 cells were subjected to many passages throughout the procedure, which demonstrated both the safety and effectiveness of the method in safeguarding monkeys and mice. The relevance of the Vero cell line in the production of vaccines is highlighted by the fact that it was essential for the multiplication of the CHIKV virus. The meticulous approach taken to preserve the viability of cell lines is highlighted by the extensive cell culture process, which includes cryopreservation, revival, and testing for sterility and confluence.

Infecting Vero cell monolayers with the attenuated virus was the first stage in creating the CHIKV antigen. Procedures of concentration and purification then followed this. A fresh approach to purification was shown by using tangential flow filtration (TFF) with a 300KDa capsule, followed by diafiltration. This allowed for the selective removal of complexes of attenuated chikungunya antigen. Additional purification of the virus was achieved using CsCl density gradients in ultra-centrifugation, which enabled the separation of separate viral bands. One may better understand the accuracy and technique used in obtaining pure CHIKV by reading the detailed description of the density gradient approach. The SDS-PAGE technique was also used in the research project to assess the molecular mass and purity of the chikungunya viral proteins.

The gel electrophoresis procedure showed an all-encompassing method for analyzing proteins, which included casting the resolving gel, stacking the gel, and loading the samples. The findings are summarised by a plaque test, used to titrate the attenuated CHIKV virus and provide a quantifiable measurement of the virus's infectious potential. The procedure consisted of dilutions, adsorption to Vero cells, and incubation, followed by staining and counting the plaques. In general, the comprehensive technique provided in this research significantly contributes to the creation, multiplication, purification, and characterization of a live attenuated chikungunya virus vaccine. Conventional methods, such as ultra-centrifugation, and cutting-edge technologies, such as TFF, show a complete and efficient strategy for manufacturing vaccines.

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