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Original article

Immuno-affinity chromatography for purification of IgG from hyper-immune sera raised against 146S fraction of Foot and Mouth Disease Virus for diagnostic purposes

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Abstract

Immunoaffinity chromatography (IAC) is a fundamental isolation and purification tool which is incorporated in a substantial range of therapeutic and diagnostic applications. This study has reappraised the usefulness of immunoaffinity chromatography for the purification of polyclonal antibodies. Protein A based IAC is a convenient and reliable method for purification of IgG, from hyperimmunesera (HIS) raised in experimental animals such as rabbits, guinea pigs and mice to be utilized in pharmaceutics and diagnostics. The 146S fraction of Foot and Mouth Disease virus (FMDV) TCID50=$10^{5.6}$ was cultured on a baby hamster kidney cell line 21 (BHK-21), concentrated using salt precipitation method using PEG 6000, purified by size exclusion chromatography (SEC) using Sepharose-30 at 254nm absorbance. Purification of 146S FMDV was analyzed using 12% SDS-PAGE which provided two bands of light and heavy chains. The alum-based vaccine, consisting of ≥10µg of 146S FMDV, was applied in 10 male rabbits and 10 male guinea pigs and two animals of each group were taken as a negative control. The titer of serum was calculated using virus neutralization test. A Protein-A kit (Thermo scientific- 44667, 0528.2) was used to purify HIS raised against 146S FMDV and validated using 12% SDS PAGE in reducing condition. The data demonstrate that protein-A affinity chromatography is an efficient tool for the purification of antibodies from hyper-immune sera raised against 146S FMDV and can be used for the production of diagnostic kits e.g. Enzyme linked immuno-sorbent assay (ELISA) and radioimmunoassay.

Keywords: enzyme linked immuno-sorbent assay (ELISA), Foot and Mouth disease virus (FMDV), hyper-immune sera (HIS), immunoglobulin G (IgG), immuno-affinity chromatography (IAC), protein-A, size exclusion chromatography (SEC)

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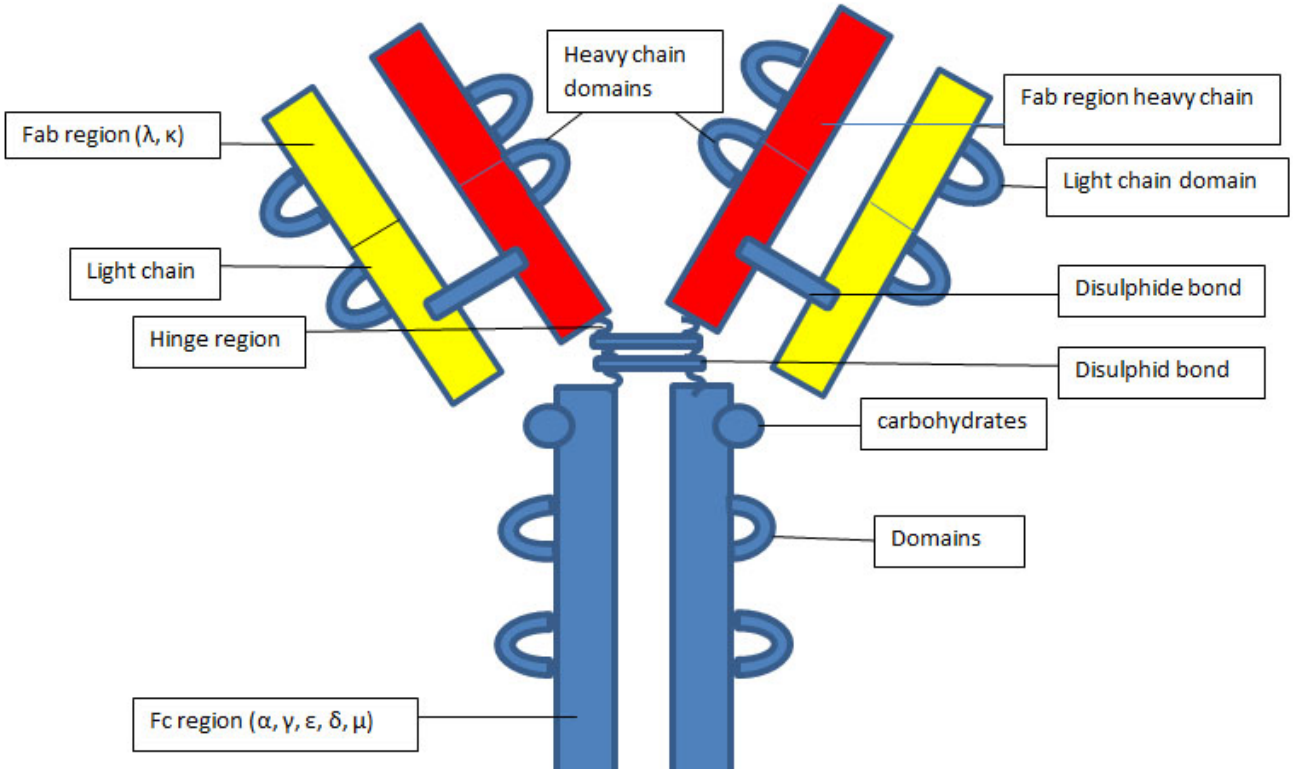

Fig. 1. Classical antibody structure (Immunoglobulin G, IgG)

Introduction

Immunoglobulins are glycoproteins conferring five types divided into IgM, IgG, IgA, IgE, IgD. These Y-shape proteins have a Fab region of 50,000 Da (50 KDa) and a Fc region of 25 KDa (Eivazi et al. 2015). Typically, immunoglobulins have two large heavy chains and two small light chains bound by disulfide bonds. Heavy and light chains can result in the formation of different forms called isotypes (Fig. 1). The heavy chain has five forms such as (Alpha), μ (Mu), γ (Gamma), δ (Delta), ε (Epsilon).

Monoclonal (mAbs) and polyclonal antibodies, which constitute one of the most promising biologics, widely used in diagnostic kits and for treatment of a wide range of diseases, have provoked the development of more effective and affordable downstream purification techniques. The purity of antibodies to be used in various research or therapeutic activities is as crucial as the experiment itself; otherwise impure proteins may hamper the outcome. The market value of these antibodies, which have reached their peak, has been over $70 billion in recent decades (Chames et al. 2009). There are a variety of techniques to purify antibodies, including salt precipitation, column chromatography containing size exclusion, ion exchange, immunoaffinity chromatography (Verdoliva et al. 2002). Among these methods, immunoaffinity chromatography, which uses proteins A, G and L primarily derived from bacteria, is the most promising. Because of its stability, protein A is given precedence over protein G (Rathore and Narnaware 2022)

Protein A is isolated from the cell wall of Staphylococcus aureus. It is a single polypeptide chain protein with a molecular weight of 4.2 KDa and contains a small amount of carbohydrates. Protein A and Protein G have the native ability to bind with the fragment of crystallization part of the antibodies, especially the IgG class. This is heat-resistant and stable at high temperatures, but the column should be kept at 4°C. Protein A contains four binding sites to interact with the Fc portion of antibodies from many species, such as humans, rabbits and guinea pigs (Ayyar et al. 2012). The protein is bound to agarose beads and allowed to settle as a column. This works as the stationary phase for the IgG isolation from the serum. Antibodies from small animals such as rats do not bind to the protein in the column. Overall, Protein A is the best choice for the separation and purification of IgG through Protein A kit (Arora et al. 2017). To enhance binding of IgG with Protein A, 0.1M PBS is used (0.1M sod. phosphate and 0.15M sod. chloride pH 7.2-8.0). Affinity chromatography provides a substantial leap as well as strong potential to purify IgG and other short peptides (Hilbrig and Freitag 2003). A plethora of use was suggested by utilizing the bacterial proteins for the purification

of antibodies specially IgG. The polypeptide chain of Protein A has a molecular weight of 4.2kDa and contains no carbohydrate. Protein A extracted from the bacterial cell wall has four binding sites with high affinity with the Fc part of IgG from several species (Ma and Ramakrishna 2008). Protein A is resistant to heat and temperature variation and can endure denaturing chemicals such as urea. Except for mammals, not all immunoglobulins from other animals can be bound by protein A. Sub classes of IgG such as IgG2 and IgG4 bind tightly with protein A. IgG from small animals, such as immunoglobulins from mice, cannot bind with above mentioned resin. This is an ideal method for purifying the IgG from the serum efficiently (Huse et al. 2002). The basis for purification is the binding of IgG with Protein A or protein G covalently, and then the use of elution buffer to elute the antibodies. For long term storage, elution buffer pH should be neutralized with some buffer (Subramanian 2002). Basagoudanavar et al. (2018) purified the N protein of the PPR virus and analyzed use of the protein in direct and indirect ELISA.

The binding of IgG to protein A binding sites is dependent mainly on the isoelectric point (PI). Below or above PI may cause elution of IgG from protein A (Wu et al. 2011). Since a complete virus is antigenic and broken particles in the vaccine cannot potentially cause immunogenicity in the animals, a complete virion, such as the 146S fraction of FMDV, should be present in the vaccine. Previously, for the creation of the vaccine, only the virus titer was thought to be pivotal. By employing a reference and the widely used sucrose density gradient method, it is possible to determine the 146S fraction of FMDV, but this method is very difficult to automate and optimize. In comparison, a double antibody sandwich ELISA kit may be a more cost-effective, useful, productive approach. It is possible to purify particular antibodies against the 146S FMDV fraction using a variety of column chromatographic methods and various resins (Hosamani et al. 2022). Immuno-affinity chromatography has been used to purify antibodies against FMDV, which can then be used in the double antibody sandwich ELISA kit. This method has been shown to have high sensitivity and specificity in detecting FMDV (Sadeghi et al. 2018), since the IgG antibodies are bound to the resin bed and released with the elution buffer. To elute IgG antibodies, elution buffer is poured to obtain the 146S FMDV raised in the rabbits and guinea pigs. Pre-treatment of the sample is seldom required, depending on the concentration of HIS. The concentration of serum can be increased by salt precipitation using ammonium sulphate (50%) or diluted by adding dilution buffer (Huang et al. 2020).

The current study elaborates on the significance of immune-affinity column chromatography in the purification of various kinds of antibodies, either polyclonal or monoclonal. Purified antibodies raised against 146S FMDV can be used for diagnostic purposes, e.g., in the manufacturing of a double antibody sandwich ELISA kit, radioimmunoassay and western blotting.

Materials and Methods

Source of virus

Epithelial samples of FMDV were collected with the collaboration of the Livestock and Dairy Department (L& DD) from different regions of Punjab-Pakistan. The samples (n=25) were processed, using liquid nitrogen to extract maximum virus from cells by washing with sterile phosphate buffer saline, grinding and centrifugation at 2000rpm/10minutes/25°C, at the Quality Operation Lab (QOL), UVAS, Lahore. FMDV Serotype-O confirmation was done using FMDV antigen detection ELISA kit (serotyping of FMDV O, A, C, ASIA1 by IZSLER, Italy, The Pirbright Institute, UK). An 80-90% confluent monolayer of BHK-21 clone 13 cell line, obtained from SAP, Turkey was given infection for propagation of the virus at 37°C, 5% CO_2, for 36-48 hrs using DMEM and GMEM (Cegrogen®) with 2.5 g Tryptose soya broth (TM MEDIA) and 10 mL (10%) fetal bovine serum (CAPRICORN) in 1 L. After incubation, cytopathic effects (CPE) were observed an under inverted microscope.

Concentration and purification of 146S fraction of FMDV by size exclusion column chromatography (SEC)

The BIO-RAD Econo-Column 15/50 was packed with Sephacryl S-300 (Sigma Aldrich®) resin to purify the 146S fraction of FMDV as a whole virus particle together with 0.011M PBS at pH 7.2 containing 0.15M NaCl (Invitrogen: AM9625) as the elution buffer in SEC. Prior to purification, the virus was concentrated by salt precipitation using Polyethylene glycol (PEG)-6000 (Daejung-Republic of South Korea). The virus was stabilized using 0.1%formaldihide PEG solution (50% PEG solution v/w in Tris buffer) which was added to the virus (8% v/v), stirred for 2 h at room temperature and centrifuged at 3000-3500 rpm for 30 minutes at 4-6°C. An Isco UV/VIS detector, equipped with an Isco Type II Optical unit with 254 nm filter detected the 146S fraction of FMDV and purified 146S fraction from other 12S, 40S and 75S fractions. Blue Dextran dye (2mg/mL) was used as a reference (Hossienizadeh et al. 2021).

Validation of 146S FMDV by SDS-page

For the 12.5% resolving gel preparation, acrylamide and bisacrylamide (bioWORLD) monomer stock solution, resolving gel buffer of pH 8.8, distilled water, 10% SDS solution, 10% APS and TEMED (Thermo Scientific ™) mixed to a specific quantity in a 200 ml beaker (PYREX® – IWAKI GLASS) was prepared to check the presence of purified 146SFMDV. For the staining stock solution of Coomassie brilliant blue G-250 (Sigma-Aldrich) was prepared by mixing the Coomassie brilliant blue G-250 in distilled water. Electrophoresis was carried at 150 volts for 8 hours at room temperature. After 8 hours, the system was stopped when the bromophenol blue dye front was approximately 1cm above the bottom of the resolving gel. Standard marker bands for SDS PAGE Fermentas PAGERULLER SM0661 was used as a scale.

Raising hyper immune sera against 146S fraction of FMDV in rabbits and guinea pigs

The virus was inactivated using 0.1M BEI and 0.04% formalin before vaccine production. For alum-based vaccine preparation, 146S FMDV (3mL) with a maximum antigen load, i.e. 20µg/mL, was homogenized with 3ml of alum gel. The vaccine was injected I/V to rabbits and I/M to guinea pigs, 0.5cc initially and 1cc after two shots. Blood was taken at day zero, 7^{th}, 14^{th}, 21^{st}, 35^{th} and 48^{th} day from rabbits, and day zero, 21^{st} and 48^{th} day from guinea pigs in yellow vacutainers and were kept at 4^{o}C overnight. Centrifugation of serum was then done at 16000 rpm for 5-10 minutes to remove the blood cells and other cellular debris prior to purification of IgG.

Purification of sera by Protein A Immunoaffinity column chromatography

Purification of IgG antibodies against 146S FMDV serotype-O was done using Protein-A kit (Thermoscientific- 44667, 0528.2). The column was first washed with binding buffer. When all the binding buffer was passed through the column, the sample was poured over (3-5mL) using 1mL micropippeter tips. To enhance binding of IgG in HIS with the Protein A column, 0.1M PBS was used (0.1M Sod. phosphate and 0.15M Sod. chloride pH 7.2-8.0). The binding-buffer, at a 1:1 ratio of serum, was used to pour the serum over the column. Elution-buffer (mL) was then added to elute IgG raised against 146S FMDV in rabbits and guinea pigs. The neutralization buffer (300 µl) was added to neutralize the pH of fractions for long term storage. Storage solution, 0.02% Sod. Azide, was used to store the column later.

Confirmation of IgG purification by SDS-PAGE

A sample solution (30µL) with 10 µl pre-stained protein marker (invitrogen™) was loaded into wells. Purified/eluted IgG antibodies from immunoaffinity chromatography were analyzed using 12% resolving gel and 5% stacking gel in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) with 150V for 90 minutes (Rižner 2014).

Concentration of IgG by Nanodrop spectrophotometer and Virus-neutralization test

Concentration of purified IgG was analyzed by Nanodrop-2000C (Thermoscientific) at 280 nm wavelength (Bergmann-Leitner et al. 2008). The serum samples (5 mL) at each blood retrieval day were diluted 5-times by elution buffer and multiple fractions of 1 ml were collected.

Results

Virus propagation, purification and validation by SDS-Page

TCID50 as $10^{5.6}$/mL of virus was selected for propagation over a BHK21 cell line as shown in Fig. 2. CPE observations were rounding of cells, detachment from the cell culture flask, and death of cells, break down of intracellular junctions and freely floating cells. Ultimately, pH and the color of the media in which the cells were grown was changed, which confirmed the propagation of the virus. Serotyping of the isolated and cultured samples of FMDV isolates was done by using the antigen detection and serotyping ELISA. OD was observed using the Thermoscientific Multiskan ELISA reader at 450 nm wavelength. An OD value of >0.1 showed positive results. During purification of the virus by SEC, retention time of peak of virus at 254 nm in Sephacryl-300 based column chromatography was ≥150 minutes providing highly pure virus which was validated by SDS-PAGE as shown in Fig. 3.

Purification and validation of IgG against 146S FMDV

Purification of IgG antibodies was done for both rabbit serum and guinea pig serum by protein A immune affinity column chromatography to remove the impurities and other proteins from the serum. Purity of HIS raised in rabbits and guinea pigs was analyzed using 12% SDS-PAGE. The presence of two distinct bands of heavy chain (H) and light chain (L) shown in Fig. 4. The SDS PAGE analysis showed that purification by immunoaffinity chromatography resulted

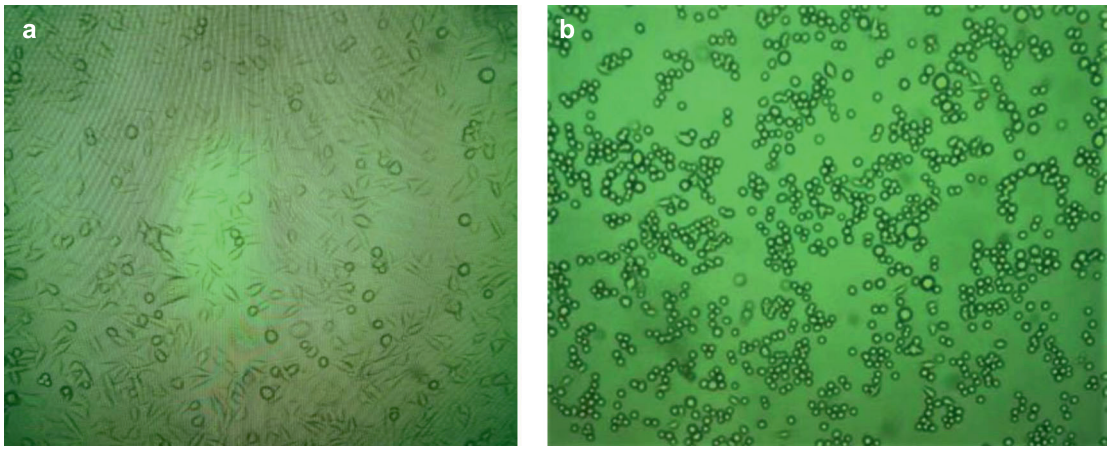

Fig. 2. Baby hamster kidney (BHK21) cell line **(a)** BHK-21 cell line before infection with 146SFMDV. **(b)** Infected cell line showing cytopathic effect (CPE).

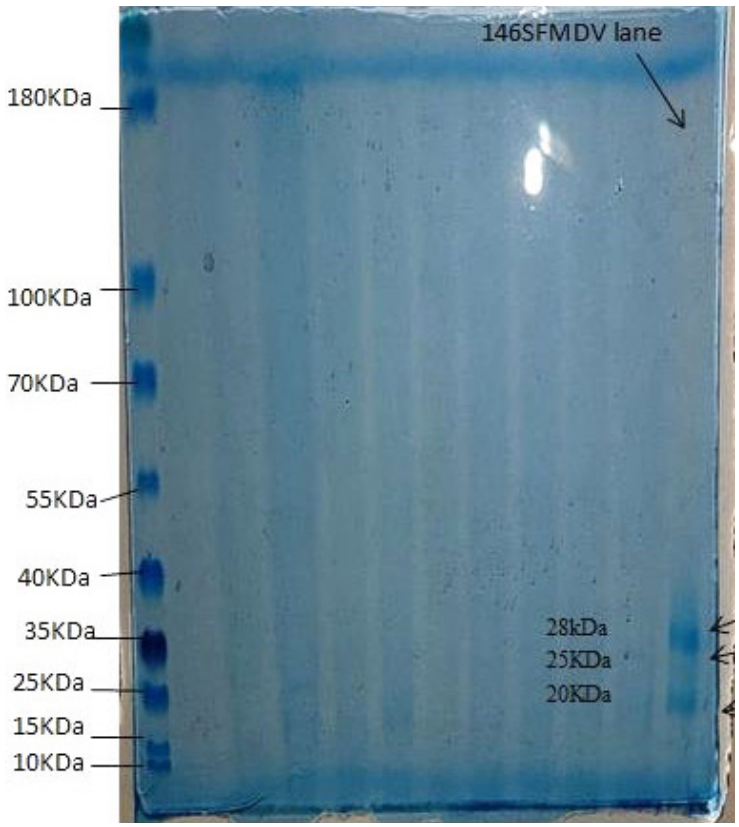

Fig. 3. Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS PAGE) analysis of 146S FMDV purification by size exclusion chromatography (SEC)

in a highly pure product to be used in later experiments with least background noise.

Antibody titer calculation by neutralization test (NT)

Titer of antibodies (rabbit and guinea pig) against 146S FMDV was found using a virus neutralization test as given in Table 1 for rabbit antibody titer and Table 2 for guinea pig antibody titer.

Discussion

Over the years, purification of antibodies by immunoaffinity chromatography has been a stalwart technique utilizing various bacterial origin based proteins e.g. Protein A, G and M required in clinical diagnostics, biological research and industries (Wang et al. 2011). IgG antibody detection and purification tools are not only extremely beneficial in diagnostics but also to assess the performance and excellence of vaccination programs. (Coelho et al. 2012).

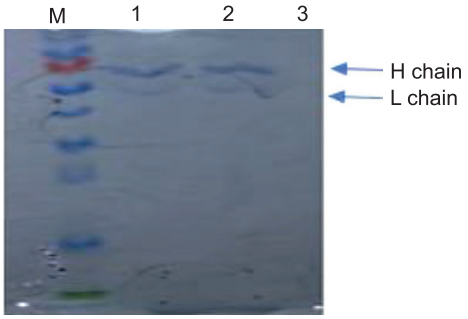

Fig. 4. SDS PAGE analysis of purification of HIS from rabbits and guinea pigs showing heavy chain (H) and light chain (L) of IgG confirmed by 12% SDS PAGE

Table 1. Antibody titer calculation from rabbit sera by neutralization test (NT).

Sr.#	Sampling day	Antibody titer (log_2) in rabbits					Mean titer (log_2 titer)
			2	3	4	5	
1.	1st	00	00	00	00	00	00
2.	14th	4.00	3.00	4.00	4.00	3.00	3.6
3.	21st	5.00	4.00	6.00	4.00	5.00	4.8
4.	35th	6.00	7.00	8.00	6.00	7.00	6.8
5.	48th	9.00	8.00	8.00	8.00	9.00	8.4
Mean titer at 35th and 48th days							7.6

Table 2. Antibody titer calculation from guinea pigs by neutralization test (NT).

Sr.#	Sampling day	Antibody titer (log_2) in guinea pigs					Mean titer (log_2 titer)
		1	2	3	4	5	
	1st	00	00	00	00	00	00
	21st	3.00	3.00	4.00	2.00	3.00	3.00
	48th	6.00	5.00	6.00	7.00	6.00	6.00

In this study, we purified the polyclonal antibodies from the rabbit and guinea pig with a straightforward assessment. These animals produced a significant amount of antibodies, demonstrating the viability of our approach to our needs for research and diagnostics tool production (Roque et al. 2007). Our observation indicate that polyclonal antibodies can also function effectively and satisfactorily to obviate our need for further studies; however, monoclonal antibodies are favored for diagnostic kit production. The size of antibodies, pH, acidity of buffer, immobilization coupling between molecules and elution conditions should all be taken into account before selecting an antibody purification procedure (Yang et al. 2015). De Sousa et al studied various factors for the purification and adsorption kinetics of IgG (de Sousa et al. 2019). Not only protein A, but also proteins G and L are efficient examples of ligands which are derived for natural resources and possess high selectivity for immunoglobulin purification (Abi-Ghanem and Berghman Luc 2015). In this study, purification of both the 146S fraction of FMDV and HIS raised against this whole virus was obtained at more than 95%, which provides a solid basis for their further use. Light and heavy chains of IgG obtained from both sera, analyzed by SDS-PAGE, showed successful purification by IAC. Moreover, the columns are reusable and remarkable amount of sera can be purified with each column. This serological product was suitable for conjugation with enzymes such as HRP, markers and radiolabels. These antibodies can further be utilized in immune electrophoresis, enzyme immunoassays and for immunostaining.

Nevertheless, these antibodies can play a vital role in analyzing the presence of whole virus in vaccine by making an ELISA kit from them, as it has been recognized and verified by research that for an FMDV vaccination programme, complete virion or 146S fraction obtained from ultracentrifugation or size exclusion chromatography must be present in the vaccine and the broken form of the virus cannot produce immunity in animals. The whole virus fraction can be obtained using the reference and standard method of sucrose

density gradient i.e. dependent on extremely skilled personnel and very difficult to optimize and to automate. In comparison, a double antibody sandwich ELISA kit can be a cost effective, time saving and effective method. Specific antibodies against the 146S fraction of FMDV can be purified using various column chromatographic techniques utilizing different resins. Immuno-affinity chromatography has been utilized for this purpose. Although, the most efficient approach to figure out the presence of 146S FMDV in vaccine is to use polyclonal antibodies, few studies have been published so far.

Conclusions

Protein A IA-CC has been established for purification of IgG from hyper-immune sera of rabbits at pH 8.3 of binding buffer and pH ~ 3 for elusion buffer. Highly purified IgG >90% can be obtained and used in further proceedings for various diagnostic purposes.

Acknowledgements

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