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Research Article

A multi-epitope-based subunit vaccine candidate against peste des petit generated through reverse vaccinology

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Peste des Petits Ruminant Virus (PPRV) is an acute, highly contagious viral disease of small ruminants, endemic to su-Saharan Africa, Asia and the Arabian Peninsula. The disease is a major constraint to food security, causing significant economic losses to subsistence farmers in affected areas. The fusion and Hemagglutinin proteins are highly immunogenic and produced in large quantities in virus-infected cells. This makes them suitable targets for the host's immune response. This study aimed at developing and evaluating effective multi-epitope vaccine candidate against PPRV developed from two immunogenic proteins Hemagglutinin (H), and Fusion (F), using a bioinformatics approach. In order to construct the subunit vaccine candidates, plasmid pET28a (+) was used as an expression vector, which was later transformed into *E. coli* DH5-α cells, followed by plasmid extraction, PCR amplification, and DNA sequencing. The white recombinant colony was selected, cultured, induced with 50 µm Isopropyl β-D-1-Thiogalactopyranoside (IPTG), and identified using the SDS-PAGE electrophoresis method. The protein were then extracted from the gel and evaluated for their immunogenicity. Results demonstrated that the constructed vaccine candidate was expressed successfully and had the expected molecular weight of 65 kDa *in-vitro* and *in-vivo* testing of the candidate was done to test for immunogenicity and was demonstrated to recognize Peste des Petits Ruminant (PPR) antibodies from vaccinated rabbits and showed a higher antibody titer than the positive control. In conclusion, the immunogenic proteins are potential vaccine candidates for development of a sub-unit vaccine against PPR.

Keywords: Immunogencity, vaccine candidates, peste des petits virus, sero-conversion

INTRODUCTION

Peste des Petits Ruminant's Virus (PPRV) is a highly contagious transboundary viral disease of goats and sheep. The disease is endemic in sub-Saharan Africa and the Arabian Peninsula (Kumar, et al., 2017). The causative agent of this disease, Peste des Petits Ruminant's Virus (PPRV), is an enveloped, single-stranded negative-sense RNA virus belonging to the genus Morbillivirus within the family Paramyxoviridae. It is grouped with Measles Viruses (MV), Canine Distemper Viruses (CDV), and Rinderpest Viruses (RPV) (Vries, et al., 2015). The disease causes significant economic losses, mostly to subsistence farmers who do not have easy access to vaccination. It is considered one of the main constraints to small ruminants' production and is listed as a reportable disease by the Office International des Epizooties (OIE) (European Food Safety Authority (EFSA) 2015). Morbidity and mortality rates vary but can be as high as 100% and 90%, respectively in immunologically compromised populations (Pope, et al., 2013). The vaccines currently in use against PPRV are live attenuated vaccines, which are mired with challenges that need to be addressed. These include the requirement of a cold chain for their preservation and the risk of reversion to virulence. Advancements in the field of bioinformatics and immunology have led to disciplines in vaccine design against diseases *via* computer-aided (*in silico*) epitope predictions. The new concept has been applied successfully in many studies, particularly in the development of vaccines targeting conserved regions within the genome of mutating pathogens (Soria-Guerra, et al., 2015). This has led to the development of specific peptide-based vaccines for most infectious diseases. The viral genome contains six transcriptional units encoding 6 structural proteins

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which include; Nucleocapsid (N) protein, Fusion (F) protein, Hemagglutinin (H) protein, Large (L) protein, Phosphoprotein (P) protein and the Matrix (M) protein (Cai, et al., 2013 and Dhar, et al., 2006). Amongst the structural proteins; the two major surface glycoproteins, the Hemagglutinin (H) and the Fusion (F) proteins, are highly immunogenic and confer protective immunity. Through the use of Bioinformatics, we predicted effective multi-epitope vaccine candidates against Peste des Petits Ruminants' Virus (PPRV) from the two immunogenic proteins Haemagglutinin (H), and fusion using reverse vaccinology as potential vaccine. In this study the potential vaccine candidates were cloned, transformed and analyzed for their immunogenic potential.

MATERIALS AND METHODS

Codon optimization and *in-silico* cloning

A codon optimization approach was used to improve recombinant protein expression. Codon optimization is essential because the genetic code's degeneracy permits most of the amino acids to be encoded by multiple codons.

Java Codon Adaptation Tool (JCat) server (http://www.prodoric.de/JCat) was used in the codon system of *E. coli* (strain K12) to obtain the Codon Adaptation Index (CAI) values and Guanine Cytosine (GC) contents to determine the levels of protein expression.

The best CAI value is 1.0, while >0.8 is regard a good score while the GC content range from 30%-70%. There are unfavorable effects on translation and transcriptional efficiencies beyond this range.

The multi-epitope vaccine's optimized gene sequence was cloned in *E. coli* plasmid vector pET-28a (+), NdeI and HindIII restriction sites were added to the N and C-terminals of the sequence, respectively. Regions of very high (>80%) and/or very low (<30%) GC content were avoided. The adapted codon sequence was then inserted into a pET28a (+) vector (Figure 1).

Finally, the optimized sequence of the final vaccine construct (with restriction sites) was inserted into the plasmid vector pET-28a (+) using the SnapGene software (https://www.snapgene.com/free-trial/, downloaded on) to confirm the expression of the vaccine. The final vaccine codon sequence is represented in yellow while the remaining construct represents the pET28a (+) expression vector. A clone containing 7152 base pairs in length was then formed.

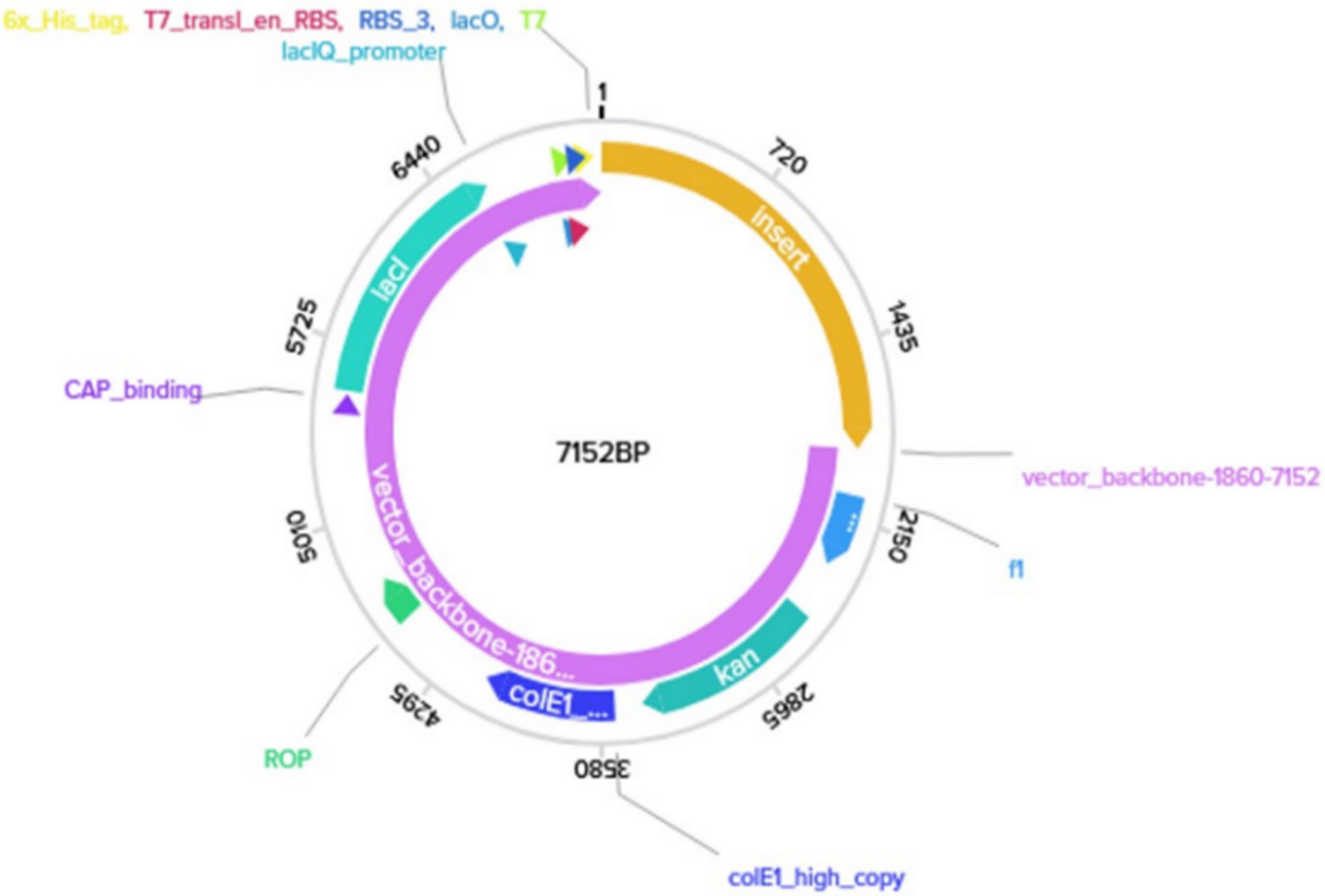

Figure 1. *In-silico* restriction cloning of the final vaccine sequence into the pET28a (+) expression vector. The final vaccine codon sequence is represented in yellow while the remaining construct represents the pET28a (+) expression vector. A clone of 7152 base pairs was then formed.

Proteomics analysis

Competent DH5-Alpha *Escherichia coli* cells (stored in a -80°C freezer) were left to thaw on ice for 20 minutes. This was followed by the addition of 2.5 µl of pET-28a (+) plasmid to 50 µl of the competent cells in a micro-centrifuge tube. The competent cell/DNA mixture was then incubated on ice for 30 minutes and heat shocked at 42°C water bath for 45 seconds and later on ice for 2 minutes.

Heat-shock transformation of chemically competent DH5-Alpha *Escherichia coli* cells: The culture was added to 250 µl of LB media with antibiotics (Kanamycine 50 ug/ml) and incubated at 37°C shaker water bath incubator for 45 minutes at 220 Rotations Per Minute (RPM). Transformed cells were plated (spread plate technique) onto three, initially prepared, 10 cm LB agar plates with 50 µg/ml kanamycin (50 µl of the cells per plate). Non-transformed DH5-Alpha *E.coli* cells were also plated on one LB agar plate (media without kanamycin), acting as a negative control in the experimental set-up.

Protein expression and analysis: Colonies (white in colour) from transformed LB agar plates were picked and inoculated into 5 ml of LB broth containing 50 ug/ml of Kanamycin. The cultures were then incubated overnight at 37°C shaking at 200 rpm. The culture was transferred to 75 ml conical flask of LB media containing (50 µg/ml) Kanamycin and incubated at 37 oC in a shaker water bath for 30 min at 200 rpm until an optical density OD 600 was obtained-determined using a spectrometer at a wave length of 540 nm. The proteins were then induced using IPTG at a concentration of 1 mM. The cultures were grown for an additional 3-4 hours at 37°C with continuous shaking. Thereafter, the cells were harvested by centrifugation for 1 minutes at 12,000 rpm. The supernatant was discarded. The cell pellet was re-suspended in 60 ul of cell lysing buffer containing (50 mM Tris HCL Ph8.0, 50 Mm EDTA Ph8.0; 0.5% Tween 20; 0.5% Triton-X100) 20 ul of 10% SDS and 20 ul of 5X SDS loading dye and lysed by vortexing. The samples were then boiled at 100°C for (5-10 min) with vortexing in between. The samples were then loaded in Sodium Deodycyle Sulphate-Polyacrylamide Gel (SDS-PAGE) and analysed as described by (Sambrook, et al., 1989).

Plasmid and transformed *E. coli* DNA isolation and quantification: Overnight cultures of transformed cells were dispensed in 2 ml eppendorf tubes and span at 14,000 rpm for 3 min at 15°C to harvest cells. The supernatant was completely discarded and the pellet re-suspended in 250 µl P1 buffer which consisted of(25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose) andRNase-A. To the mixture, 250µl P2 buffer containing (1% Sodium Dodecyl Sulfate (SDS) was added and mixed by gently inverting the tubes 6x for 5 min after which 350 µl of neutralizing N3 buffer (4.2 m guanidine hydrochloride, 0.9 m potassium acetate, pH 4.8) was added, contents mixed by inverting the tube 6x until the solution was cloudy. They were later span for 10 min at 14, 000 rpm at 4°C. The content was gently poured off and the eppendorf tubes air dried for 20 mins before adding 100 ul of nuclease free water to re-suspend the DNA. The DNA was quantified using Thermo Scientific Nano-Drop 1000 spectrophotometer and resolved in 1% agarose gel to check for its quality (Figure 2).

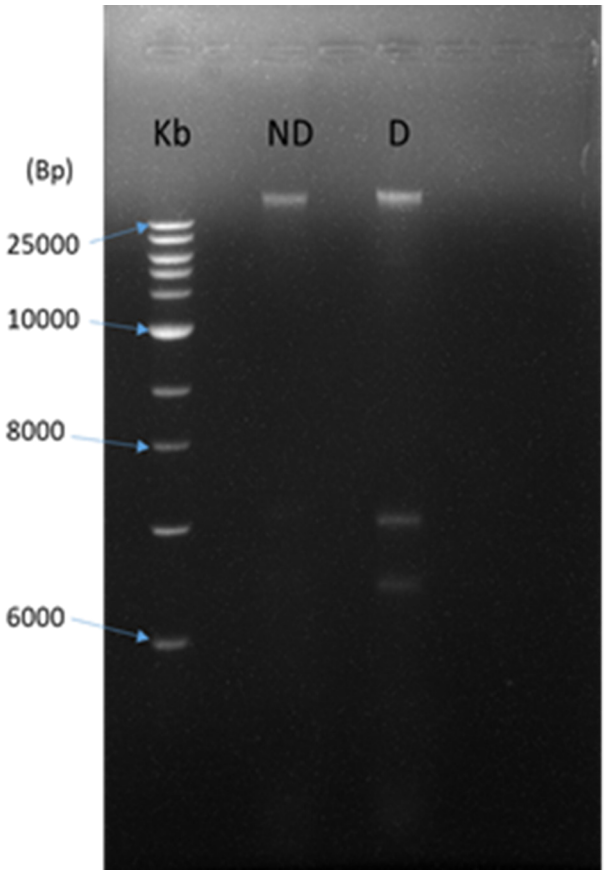

Figure 2. Visualized agarose gel after electrophoresis. DNA ladder 1 kb (1), *E.coli* DNA (2), and plasmid DNA (3) bands are shown, respectively. The above photo was an indication of successful transformation line (D) With an approx band of 72 kd. In lane was a control lane Non-transformed (ND) *E.coli* DH5α that only produced one band this was quite appropriate according to the available literature.DH5α was preferred because it isthe most common laboratory *E.coli* strains utilized for small plasmid propagation withability to achieve high transformation efficiency as shown above.

Protein purification, quantification and expression: The protein purification method was adopted from Qiagen (QIA) expressionist, June 2003 with slight modifications. Sephadex G-200 (Pharmacia, Piscataway, NJ, U.S.A.) in a 40 cm x 1.4 cm diameter column and a 4.5 cm pressure head was eluted with 0.05 m phosphate buffer (pH 7.2 at room temperature) and the flow rate adjusted to 6 ml/h. The extract what volume was added to the column and eluted with the phosphate buffer.Two ml fractions were then collected and stored at -20°C for protein localization, protein concentration curve was used to determine the concentration of purified protein using the Bicinchoninic Acid (BCA) protein assay kit from thermoscientific (Pierce™ BCA Protein Assay Kit). Briefly the expressed cell pellets were removed from -80°C freezer, thawed for 15 min, before adding 8 M urea lysis buffer at a concentration of 10 ml/g wet weight. 0.1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF)-Sigma Aldrich, USA was added to inhibit protease activity. The corning tubes with cells were placed on the rotor and allowed to mix for 60 min at 24 ºC ensuring foaming is avoided. The lysate was span at 10,000 xg for 30 min at 15 ºC to pellet the cellular debris and supernatant saved. An equal volume of 2x SDS page sample buffer was added to 5 ul supernatant and stored at -20°C for further analysis.

Determination of the immune properties of the protein (dot-blot): Ten µl of 100 µg/ml recombinant protein concentration (was blotted on a strip of nitrocellulose membrane. At different concentrations (10^1-10^3) and left to air dry. The membranes were blocked with 5% dry milk in PBS-T (50 mM Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.4) for 1 hour at room temperature. The blocking buffer was discarded before being incubated with primary antibody for 1 hour at room temperature in PBS-T. The membrane was then washed 3 times (10 minutes each) in PBS-T on shaker at rpm speed. Secondary antibody was added at a concentration of 1:1500 and incubated for 1 hour at room temperature in PBS-T. before being washed 3X PBS-T after which 3,3'-Di Amino Benzidinetetrahydrochloride-dihydrate (DAB) substrate (10 µl 30% hydrogen peroxide (H_20_2) was added to 10 ml of 0.05% DAB) and incubated in the dark for 5 minutes. Thereafter, the reaction was stopped by briefly washing the membrane with distilled water and air dried. Further analysis was done using the Western blot technique whereby an aliquot of each eluted protein antigens from collection tubes was used. One volume of each antigen was mixed with two volumes of 3x sample buffer (500 mM Tris/HCl pH 6.8, 4.6% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.004% bromophenol blue) and boiled for 5 min. The protein samples were then loaded onto an SDS-PAGE gel 1.5 mm thick consisting of of 5% stacking and 7.5% linear gradient resolving gel. Electrophoresis was performed at a constant current of 20 mA as described by (Laemmli, et al., 1970). The resolving buffer (pH 8.3) contained 25 mM Tris, 192 mM glycine, and 0.1% SDS. The proteins were later loaded onto the gell and run at a speed of 200 mA overnight.

Western blot was performed as described by (Sambrook, et al., 1989). The separated protein fractions were electrophoretic ally transferred to a nitrocellulose membrane at 350 mA for 1 h. After electrophoresis, the gel was placed onto transfer buffer pre-soaked in 0.22 µm Nitrocellulose membrane (NC) (BA85, Schleicher and Schuell, Dassel, West Germany) laid on two sheets of Whatman 3 MM filter paper which had previously been soaked in transfer buffer (20% methanol, 192 mM glycine, 25 mM Tris-HCl, pH 8.3). Air bubbles were removed carefully and two more Whatman 3 MM filter papers were also presoaked in the transfer buffer placed on top of the gel. The gel nitrocellulose sandwich was also sandwiched between two scotch-brite pads before placing in a transfer-Blot cell apparatus (Hoeffer Scientific Instruments, San Franscisco, Model SE 600) filled with the transfer buffer (25 Mm Tris,192 Mm glycine, pH8.3 with 20% methanol (vol/vol). Electrophoretic transfer of the antigens was carried out for 1.5 hr at a constant voltage of 100V at 4°C. The Nitrocellulose (NC) membrane was removed and briefly air-dried at Room Temperature (RT) (Nkando, et al., 2016).

Immunological validation

Twelve female rabbits Newzealand white (0-3 months of age) were used for animal study. All rabbits had no detectable levels (titers 1:2) of PPRV-neutralizing antibodies. A blind randomization protocol was used. They were all allowed to acclimatize in experimental cages for 14 days and later grouped into three randomized cohorts of 4, the first group (A) was vaccinated with the test vaccine F/H Group (B) was vaccinated obtained vaccine N75/1 from the Kenya Veterinary Vaccine Production Institute (KEVEVAPI), and the last group (C) was vaccinated with placebo-diluent alone sterile Phosphate-Buffered Saline (PBS).

Animal experiments with vaccine candidate: Group A received an intramuscular injection of 0.1 ml F/H subunit vaccine candidate at a predetermined concentration of 100 mg/ml. Group B consisted of four rabbits that received a 0.1ml intramuscular injection of N75/1 vaccine at a concentration of 100 mg/ml this group acted as a positive control group during vaccination. The vaccine candidates were reconstituted with adjuvant Montanide ISA720 at a ratio of 1:1. The Negative control group was vaccinated using a placebo consisting of 1ml diluent. All three groups received booster injections of the original dosage at week three of primary immunization. The rabbits were bleed every week for 35 days and the samples processed for Sera were separated and stored at -20°C until analyzed.

Ethical consideration was obtained from the Animal Institutional Care and Use Committee (AICUC) of Kenya Agricultural and Livestock Research Organization (KALRO) -Biotechnology Research Institute (BRI) for the use of laboratory animals before the onset of the experiment for the use of laboratory animals during this study. KALRO-Biotechnology Research Institute (BRI) permit number C/BIORI/4/325/III/45. All manipulation of the experimental animals satisfied the requirements animal institutional care and use committee.

Screening of serum samples from immunized rabbits using indirect ELISA: The indirect ELISA (iELISA) were done in duplicate and repeated on three different occasions. The negative and positive control sera were placed in duplicates in the first and last row of plates coated with 1 µg/ml of each antigen. No serum sample was placed in the second row and

an OD of this row was subtracted from the OD containing sera. The ELISA plates were coated with 1 µg/ml antigen in coating buffer (100 µl/well) of carbonate: Bicarbonate buffer and incubated at 4°C overnight. The following day, plates were washed 3x in Phosphate-Buffered Saline-Tween (PBS-T). 200 µl blocking buffer (1x PBS-T and 0.5% Horse serum from Gibco Life Technologies™, New Zealand) was added and then tested the serum on dilution plates in each well. For anti-IgG, the initial concentration was obtained at 1/100 and then diluted 4-fold, plates were then incubated at 37°C with gentle shaking for 1 hr, and washed 3x with PBS-T 100 µl of secondary antibody conjugated to Horseradish Peroxidase HRPO at a dilution of 1/10000 in blocking buffer was added in each well and kept for 1 hr with gentle shaking at 37°C. The plates were again washed 3x in PBS-T and 100 µl/well of substrate from Sigma Aldrich, USA added. The plates were placed in the dark for 15 min and read at a wavelength of 405 nm in BioTek Synergy HT, USA plate reader. The results showed that there was indeed seroconversion to the subunit vaccine candidate which was better than the seroconversion observed in N/75 vaccine. Boosting was done on day 14 post-vaccination and hyperimmune sera was collected on the 18th day.

RESULTS

Proteonomic analysis

During analysis to confirm transformation it was observed that transformation was very successful, these wasfollowing the growth of the transformed cells in presence of antibiotic kanamycine. At a concentration of 50 mg/ml This was because the transformed plasmid had kanamycine resistant gene as a marker gene hence its growth in presence of kanamycine was a clear indication of successful transformation as shown in Figures 3a-3c. No growth was observed on the negative control plate that was inoculated with non transformed DH5αcells. (Figure 3d) similar results were obtained when grown on LB both media as observed in Figures 4a-4c.

Confirmation of insert using agarose gell electropherosis

Agar rose gell electrophesosis was performed to confirb the presence of insert within the plasmid the plasmid was isolated and and DNA extracted from it. The DNA was then run on an agaroese gell to show insert. Results indicated that the plasmid containe dthe insert as shown in Figure 2. This was demonstrated by the presence of three bands in line (D) in Figure 2 the bands were Plasmid DNA, *E.coli* DNA and the insert which was approx 72 kda. Single band was observed in lane (ND) which constituted of none transformed *E.coli*. The eresults confirmed that we actually had the right insert for subsequent down stream analysis (Figure 3).

Determination of protein localisation

After the proteins were extracted from the *E.coli* all te alluquates were collected and a protein localisation determined all the 9 alliquates were subjected to a dot blot technique. Amonst them the protein was found to be localised from the 3rd-6th alluqutes as seen in Figure 5. the rest of the alluqutes had no protein of interest hence were discarded the four samples were them concentrated and run on an SDS PAGE to determine moleculer weight of the protein of interest this is as shown in Figure 6. The samples were shown to decrease in intesity as the dillution was increses. Protein ladder. (Natibe Mark™) from fisher scientific was used to determine the moleculer weigh of the protein odf interest which was found to be approx 72 kda. which was the right size of our protein of interest.

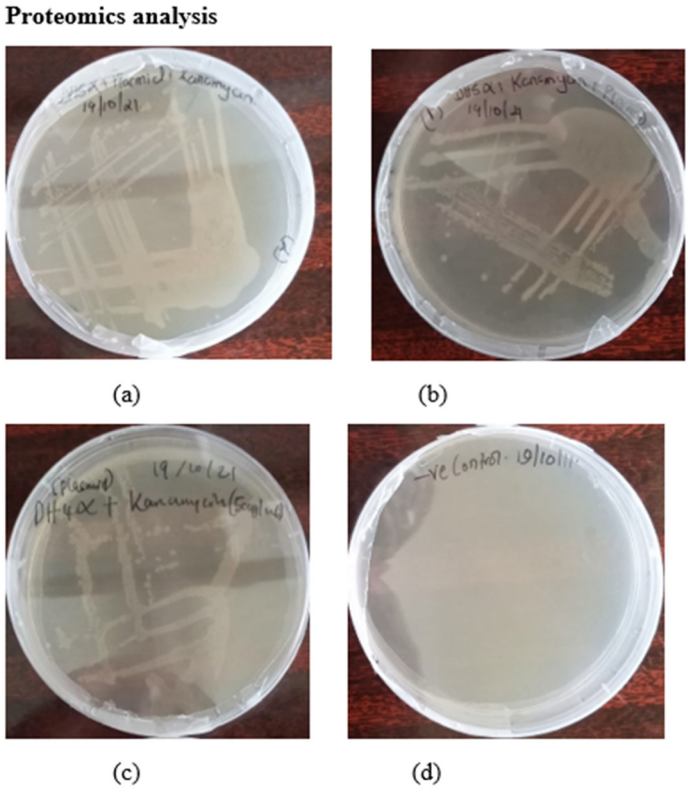

Figure 3. Transformed DH5-α *E.coli* cells cultured on Luria Broth (LB) with agar containing 50 µg/ml kanamycin are shown in a, b, c and d. Non-transformed DH5-α *E.coli* cells cultured on an LB agar with 50 µg/ml kanamycin (negative control) are shown in d. those cultures that were transformed i.e. contained transformed DH5α cells,were able togrow in LB media containing antibiotics (Kanamycine) this was an indication that transformation since the plasmid had kanamycine resistant gene. The cells were selected and grown in broth cultures as shown in Figure 4. Growth was an indication of successful transformation.

Figure 4. (A)Transformed DH5-α *E.coli* cells cultured on LB containing 50 µg/ml kanamycin. (B) Non-transformed DH5-α *E.coli* cells cultured in LB without kanamycin. (C) Non-transformed DH5α *E.coli* cells cultured on LB with 50 µg/ml kanamycin (negative control). cells that were transformed were able to grow in presence of antibiotic. Kanamycine since they possed kanamycine resistant gene marker gene. DNA was extracted and ran on an Agar rose gell to confirm transformation as shown in Figure 2.

Figure 5. Dot blot technique was used to determine to protein localization from the elutes which were from No.1-9 the protein of interest was found in Elute No. 3-6 as shown above. Elutes 7,8 and 9 had no protein of interest hence the elutes were discarded. Those that a signal were concentrated using. Polyethyle Glycol (PEG) and used for SDS analysis Figure 6.

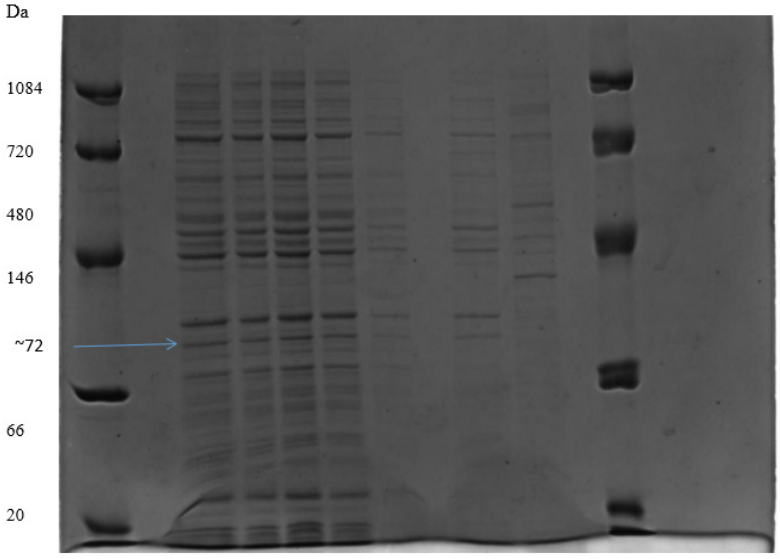

Figure 6. SDS PAGE showing different fractions of the protein bands, our protein of interest was seen at position 72 kDa. Natibe Mark™ unstained protein standardfrom fisher scientific LC075 was used. The purified concentrated protein obtained from the elutes above were loaded onto an SDS page at different concentrations ranging from (10^1-10^7) with bands of higher concentrations becoming more visible and it fades away as the concentration reduces. The molecular weight of the purified protein was determined and it confirmed that we were having the right protein at ~72 kda. The protein was further analyzed for immunogenicity and specificity using a dot blot technique as shown in Figure 7.

In-vitro annalysis of the immunogenicity of the protein F/H

In-vitro immunological assay was performed on the purified protein of interest, during experiment the proeins were loaded onto a nitrocelluler membrane at different concentrations as shown in (Figure 7). The intensity of the blots were shown decreasing with increased dilution. A negative control was also used which included a known New Castle Disease virus (NCD). This was done to be able to test the specificity of the protein of intest. No reaction immunological reaction was observed in the negative control as demonstarted in Figure 7 which was a clear indication of the sensitivity and specificity of the protein of interest. The proteins were further purified prior to *in-vivo* testing of the proteins. Purification was done and purity determined by running an SDS-PAGE. It was observed that purification was successful line (P) as shown in (Figure 8). The none purified protein (NP) was observed to have different bands however the purified protein had only one band that was within the expected range of 67 kda. The gel was further subjected to a western blot technique so as to reconfirm the immunological response of the purified band as shown in Figure 9, the results showed that the expressed recombinant PPRV F/H protein when immunobloted with a strong positive hyper immune serum (Antibodies) there was reaction that indicated sensitivity. Lane 1: 0.5KD pre-stained marker lane 2 and 3: Purified sample (Thermo Scientific 26612) from the results above it was noted that the purified protein was able to recognize the Peste des Petits Ruminant (PPR) virus antibodies when probed with hyper immune sera as shown in lane P hence indicating specificity to PPRV. The purified protein was later quantified the results showed that the protein of interest had very high concentration of 4.4 mg/ml hence for *in vitro* studies the protein has to be diluted further.

In vivo immunological studies on the purified vaccine candidates

The results as shown in Figure 10 demonstrated seroconversion to the subunit vaccine candidate (test) was higher than those observed in the positive control N/75 vaccine (Positive control) the increased rate of seroconversion could be attributed to the specificity of the subunit vaccine candidate that was deamed to offer a much vigourous reaction. Seroconversion was not observed in the negative control group. The results showed similarity in the pattern in antibody production it was observed, an increase in antibody production was observed from day 21 after the boosting with the anigen this was observed in the test group and the positive control group However no reaction was observed in the control group that showed no response it was therefore clearly indicated that the response observed was due to vaccination with the specific antigen.

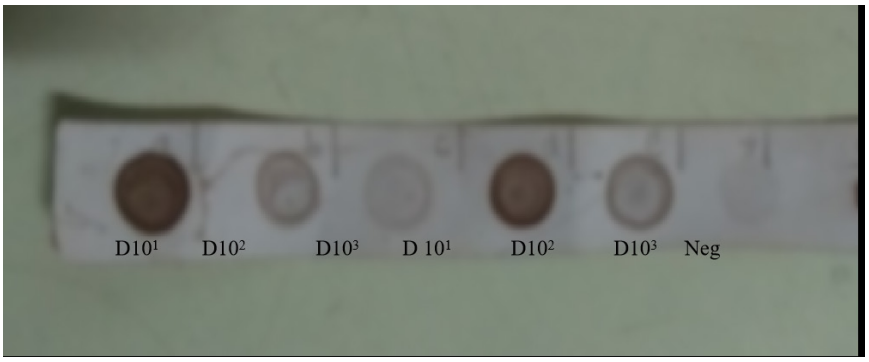

Figure 7. Immunoblotting done to determine the protein concentration samples were diluted tenfold ie 10^1-10^3 this was done in duplicate the proteins were expressed after 2 and 4 hours using Isopropyl β-D-1-Thiogalactopyranoside (IPTG) were blotted on the nitrocellulose membrane at different concentrations (10^{-1} to 10^{-3} aliquots). Neg control was included in the test (Neg) it constituted of *paramyxoviruses* Nucleocapsid (NC) to show specificity of our protein. From the results there was no activity in the negative control this indicated that our protein was specific to PPRV. Highest activity was shown in the neat sample and the activity went on fading with the dilution.

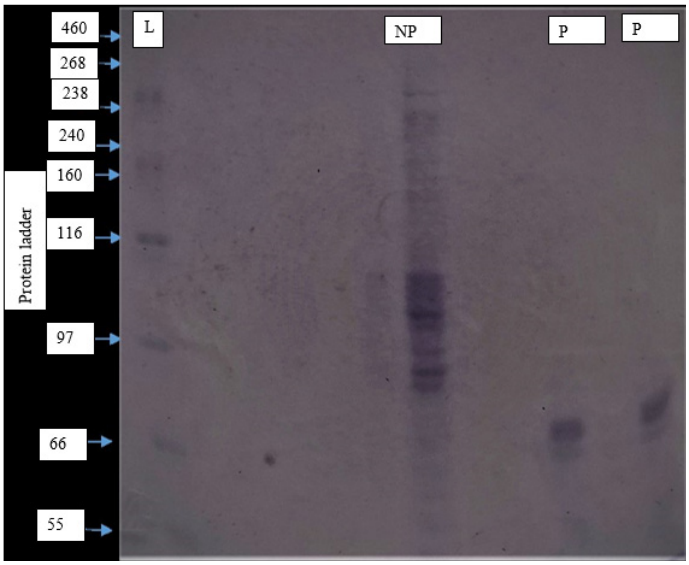

Figure 8. Visualized SDS-PAGE gel. This was done to confirm the purification process and as well as to confirm the molecular weight of the Purified (P) protein of interest The MW was confirmed to be 67 Kd purification process was confirmed since no any other band was observed as compared to the Non Purified protein (NP). (L) protein ladder western blot result. This was done to verify binding ability probing of the samples with secondary antibody after the transfer indicating that there is activity.

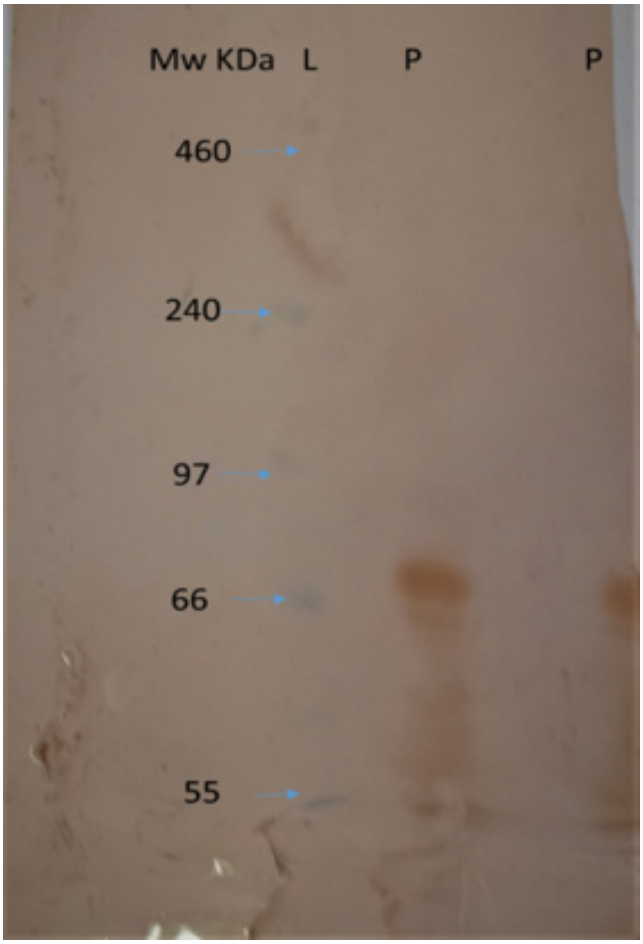

Figure 9. Western blot analysis of the expressed recombinant PPRV F/H protein immunoblotting with standard strong positive hyper immune immune serum; Lane 1: 0.5 Kd pre-stained marker lane 2 and 3: Purified sample (Thermo Scientific 26612) from the results above it was noted that the purified protein was able to recognize the PPR Virus antibodies when probed with hyper immune sera as shown in lane P hence indicating specificity to PPRV. The purified protein vaccine candidates were then quantified as shown in figure 10 below.

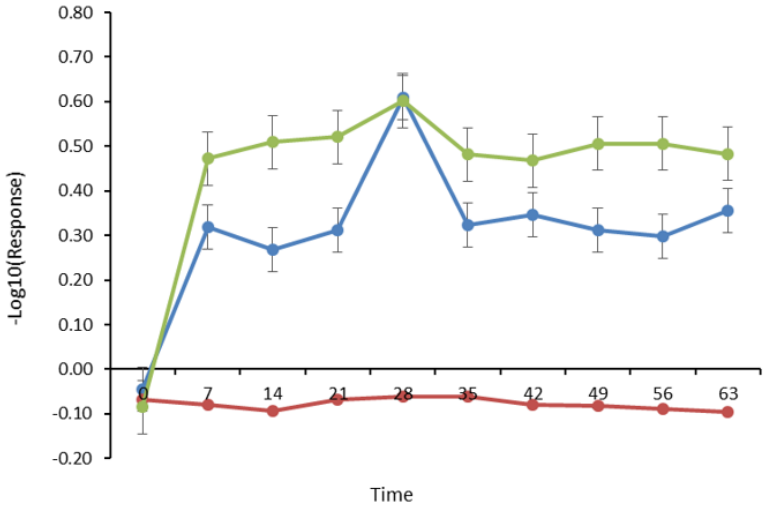

Figure 10. The results showing seroconversion to the subunit vaccine candidate (test) which was better than the Seroconversion observed in N/75 vaccine (Positive control) Seroconversion was not observed in the negative control group. This indicated that the sub unit vaccine candidates could be used as vaccine for the control and management of PPRV in ruminants. **Note:** (—●—): Positive (N/75); (—●—): Negative control; (—●—): Test(F/H).

DISCUSSION

The PPR disease is considered one of the main constraints to small ruminants' production and is listed as a reportable disease by the Office International des Epizooties (OIE) (Berhe, et al., 2003).Morbidity and mortality rates vary but can be as high as 90%-100%, respectively in immunologically naive populations (Pope, et al., 2013).The vaccines currently used against PPRV are live attenuated vaccines, which are mired with challenges that need to be addressed this includes: The requirement of a cold chain for their preservation, and the risk of reversion to virulence. Development of new vaccines starts with the identification of unique components of the microorganism capable of generating a protective immune response. With traditional techniques, this could be a long and arduous process, besides the difficulty of cultivating the microorganism

in the laboratory (Alan, et al., 2010). Advancements in the field of bioinformatics and immunology have led to disciplines in vaccine design against diseases *via* computer-aided (*in silico*) epitope predictions. The new concept has been applied successfully in many studies, particularly in the development of vaccines targeting conserved regions within the genome of mutating pathogens (Gaafar, et al., 2019). This study adopted this methodology and selected proteins with the best values of antigenicity. Antigenicity is the property of the proteins to be recognized by the immune system; hence, it is desirable to find the highest antigenicity value for the selection of the best potential vaccine candidates (Buddle, et al., 2013).

Two external glycoproteins the F and H proteins of the viruses are responsible for inducing protection against the disease in animals. The H and F protein genes of several morbilliviruses have been expressed in various vector systems and used as effective sub-unit vaccines (Diallo, et al., 2007). Based on this concept our initial analysis of the expression efficiency of the cloned protein F and H, the optimized codon sequence had a length of 1860 nucleotides. With the Codon Adaptation Index (CAI) of 0.9682273036751229 and GC content 49.56989247311828, an indication of good expression of the final vaccine clone in the *E.coli* (strain K12). Acording to the optimal percentage range of GC content for good expression in *E.coli* is usually between 30% and 80% and this was quite in line with our results (Redwan, et al., 2006).The 5' end of PPRV F/H gene sequences was amplifed by RT-PCR, cloned into pET28a vector and characterized. In order to express PPRV F/H, BK21 strain of *E. coli* was used for transformation of the characterized recombinant plasmid DNA. After the characterization of the BK21 clones, expression of rPPRF/H was induced using 1 mM IPTG at 37°C. Pre-induction incubation for 3h at 37°C was necessary in-order to achieve mid-log phase growth. On comparison of protein profiles of the recombinant clones with that of the control (vector and BK21), a molecular weight of ~72 kDa expressed protein band was observed in the case of recombinant pET28a+F/H clone, as early as 4 h Post-Induction (PI). The intensity of the bands increased gradually up to 7 hpi. In un-induced and control cultures, such specific bands were not observed even after prolonged incubation. The optimum time of harvest was 5 hpi and not much difference was observed in the rate of expression either at 6 or 7 hpi but thereafter, there was a reduction in expression. This reduction could be attributed to the autolysis of bacterial cells as reported earlier (Yadav, et al., 2009). The proteins resolved in SDS-PAGE and transferred on to a nitro cellulose membrane were detected by using a PPRV specific serum or antibodies. On analysis, ~72 kDa molecular size was observed and confirmed that the expressed recombinant protein was specific to PPRV. The predicted size of the expressed protein from amino acid sequences along with fusion His-tag was ~72 kDa (Dhar, et al., 2009). The calculated size as per a composition along with fused His-tag and as observed by the mobility in SDS-PAGE, was in agreement with the reported size indicating that the ~72 kDa protein was the product from the cloned gene sequences.

Western blot analysis with Monoclonal Antibodies (MAB) against the PPRVNP/polyclonal antibodies or serum indicated that the bands observed in SDS-PAGE are virus-specific. Such His-tag fusion protein has been expressed in the case of PPR (Apsana, et al., 2016). Further, to assess the utility of the expressed protein, they were purified by using Ni-NTA affinity columns to their homogeneity. This method has been successfully used for purification of several other expressed proteins (Catanzariti, et al., 2004). Presence of N-terminal His-tag in the vector as well as the C-terminal His-tag both in cloned gene product and expression vector facilitated easy and efficient Ni-NTA column protein purification. It was found that 300 mM imidazole concentration was optimum for elution of protein. However, various concentrations of imidazole (100-500 mM) have been reported in purifying expressed protein (Catanzariti, et al., 2004). Generally, purification under non-denaturing conditions avoids the re-folding of denatured protein. Moreover, in this study, purification under denaturing conditions followed by re-folding of denatured protein in urea resulted in a good yield of protein in native solubilization form and it was reacted in the immune assays and blot. This was necessary for the retention of confirmation epitopes on the protein and thereby facilitates better reactivity even with MAbs as well as retaining the immunogenicity of the recombinant protein, as reported by (Balamurugan, et al., 2016).During expression the cloned vaccine candidate was able to be cloned in PET28+that has been used widely for recombinant protein production and expression in *Escherichia coli* (Shilling, et al., 2020).The expressed protein was found to be approximately 72 Kd. After the expression and purification of the proteins, their immunogenicity was tested by conducting *in vitro* and *in vivo* assays. Direct ELISA was used methods to evaluate the expressed recombinant protein (Ma, et al., 2011).

The expressed protein were used as coating antigen for the polyclonal antibodies-based assay. The sub-unit vaccine candidates elicited significant seroconversion equal to or more than four-fold rise in antibody titers among the vaccinated rabbits *via* the intramuscular route of inoculation. The high level of seroconversion observed among the vaccines is an indication that the sub-unit vaccine candidates developed were immunogenic and can be used as a vaccine candidate for control of PPR.The response of vaccinated rabbits was consistent with thoseobserved during goat vaccination with homologous PPR vaccine as earlier demonstrated by (Balamurugan, et al., 2014) and chimeric PPR vaccine by (Kumar, et al., 2017). No specific clinical signs or side effects were observed in all the rabbits vaccinated with the developed subunit candidate's vaccine, an indication of the safety of the vaccine. All the vaccinated rabbits exhibited an anamnestic immune response 21 days post-infection. The higher response observed was attributed to the fact that sub-unit vaccination modalities tend to induce particular higher immune effector responses as demonstrated by (Douglas, et al., 2010).

CONCLUSION

We would state that the multi-epitope sub unit vaccine candidate developed could be used as vaccine for the protection of livestock against PPR and hence further *in-vivo* testing should be curried in small ruminants for it to be used as vaccine. However we could say that the vaccine candidates are quite potential as vaccine for the control and management of

PPR in livestock.

CONFLICTS OF INTEREST

The author declares no conflicts of interest.

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