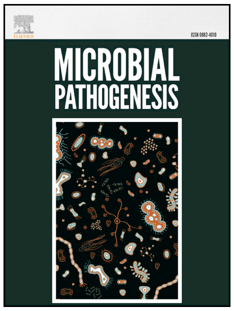

The cloning of the virus envelope glycoprotein F of canine distemper virus expressed in *Pichia pastoris*

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Declaration of competing interest

The authors declare that they have no conflicto of interest.

The cloning of the virus envelope glycoprotein F of canine distemper virus expressed in *Pichia pastoris*.

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Abstract

Canine distemper virus (CDV) is a pathogen which affects members of the Canidae family, causing an acute, often fatal, systemic disease. CDV is an RNA virus of the family *Paramyxoviridae* that contains two envelope glycoproteins: F and HA. In this study, we focused on the envelope glycoprotein F as the main target for neutralizing antibodies produced after infection or vaccination. The complete coding region of the protein (60 kDa) was expressed in the methylotrophic yeast *Pichia pastoris*, obtained in a recombinant form and secreted to the culture medium. Later, to analyze its immunogenicity, the protein was combined with an oily adjuvant and used to inoculate mice. The results provide evidence supporting a potential application of this recombinant protein as a subunit vaccine.

Keywords: canine distemper virus; glycoprotein F; *Pichia pastoris*

1.Introduction

Canine distemper virus (CDV) belongs to the genus *Morbillivirus* of the family *Paramyxoviridae* [1, 2]. Infection by CDV induces clinical signs associated with the respiratory, gastrointestinal and central nervous systems. In addition, CDV induces high morbidity and mortality in dogs and exhibits an ever-increasing host range in wild aquatic and terrestrial carnivores, often with devastating consequences [3, 4, 5]. The genome of CDV is a single-stranded, negative-sense RNA molecule of approximately 15.5 kb in length that encodes eight proteins [6]. The genomic RNA is tightly associated with three viral proteins: the nucleocapsid, RNA polymerase and phosphoprotein. The envelope is constituted by a double lipid layer derived from the host cell, whose inner face contains the matrix protein and whose outer face contains two glycoproteins: hemagglutinin (H) and the fusion protein (F) [7]. The fusion protein F is a type I glycoprotein [8, 9] that is synthesized as a pre-protein of 662 amino acids (pre-F_0). However, some authors have suggested that the initiation of translation of F might occur either in the first initiation codon (AUG1) or a second initiation codon (AUG61) [10, 11]. Both pre-F_0 (1) and pre-F_0 (61) are translocated within the lumen of the endoplasmic reticulum and are cleaved between amino acids 135 and 136 by a signal peptidase (SPase) [11]. This result into two unusually long signal peptides of 75 or 135 amino acids depending on the start codon and a second immature precursor F_0 that trimerizes in the endoplasmic reticulum and is cleaved by furin in the late Golgi apparatus and trans-Golgi network to yield active complexes containing covalently linked F_1 and F_2 subunits [12]. Afterwards, the mature protein is inserted into the plasma membrane of the infected cell [11].

The estimated molecular weight of F_0 is 60 kDa, whereas that of the F_1 subunit is approximately 41 kDa and F2 subunit is approximately 26 kDa [13]. F_1 has an N-terminal fusion peptide (which is inserted into the cell plasma membrane during fusion) [14, 15], a transmembrane C-terminal domain and two hepta repetitions (HRA) and (HRB), which are critical for membrane fusion [16], whereas the F_2 subunit is about 100 amino acids long and is associated with the regulation of fusion [17]. The main role of the fusion protein is to insert itself into the plasma membrane of the host cell, favouring the fusion between the plasma membrane and the envelope of the viral particle [18].

Considering that protection against CDV is based mainly on the immune response against envelope glycoproteins (fusion protein and hemagglutinin), in the present study, we analyzed the ability of the methylotrophic yeast *Pichia pastoris* to express the fusion protein F_0. We also made a preliminary evaluation of its immunogenic properties in mice, to determine whether the recombinant protein could be used as an alternative method to produce a subunit vaccine against canine distemper.

2.Materials and methods

2.1.Strains, plasmids, cells and culture media

The CDV strain Onderstepoort (i.e. the strain used for the CDV vaccine) was used as a standard laboratory strain [13]. Vero cells were grown in Eagles's minimum essential medium -MEM- (Gibco) supplemented with 10 or 2% fetal calf serum –FCS- (Internegocios). *Escherichia coli* strain TOP10, *P. pastoris* strain GS115, and the pPIC9 expression vector from Invitrogen Co. (USA) were used. Yeast extract-peptone-dextrose –YPD- medium (1% yeast extract, 2% peptone, 2% dextrose), minimal glycerol –MGY- medium (1.34% Yeast Nitrogen Base–YNB-, 1% glycerol, 4 x 10^{-5}% biotin), buffered methanol medium–BMM- (100mM potassium phosphate pH 6, 1.34% YNB, 0.5% methanol,4 x 10^{-5}% biotin), Luria Bertani plates containing 100 µg/mL ampicillin (LB-amp), plates with minimal dextrose –MD- (1.34% YNB, 2% dextrose, 4 x 10^{-5}% biotin and 1.5% agar) and plates with minimal methanol–MM- (1.34% YNB, 0.5% methanol, 4 x 10^{-5}% biotin and 1.5% agar) as described in the Invitrogen *Pichia* expression kit (USA) were used as culture media.

2.2.Amplification of the CDV fusion protein gene by RT-PCR

Viral RNA was extracted from the CDC strain grown in Vero cells until infected cells showed 80-90% cytopathic effects. Then, 300 µL of supernatant was mixed with 500 µL of TRIzol (GibcoBRL) and 220 µL of chloroform (Merck) and vortexed vigorously for 10 min. This mixture was then centrifuged at 12,000 rpm and 4 °C for 10 min. The supernatant obtained (600 µL) was transferred onto a new tube containing 750 µL Isopropanol (Anedra) and, after mixing by inversion, centrifuged at 12,000 rpm and 4 °C for 15 min. Subsequently, the supernatant was discarded and the pellet obtained was washed with 750 µL of 70% cold ethanol. Finally, the RNA was resuspended in 15 µL of nuclease-free water (Biodynamics) and quantified by spectrophotometry. Retrotranscription was carried out using a reverse transcriptase MMLV (Promega). The viral RNA (5 µg) and random hexamer primers (1 µg) (Promega) were denatured at 65 °C for 5 min and then cooled to 4 °C. To synthesize the first-strand cDNA, the RNA and primers were mixed with 5 µL reaction buffer (5x), 1 µL of MMLV enzyme (200 U/µL) (Promega), 1 µL of dNTPs (200 µM) and 2 U of RNase inhibitor (Promega) and 3 µL of H_2O. The mixture was incubated at 37 °C for 60 min. PCR was then performed with 5 µL cDNA product, 0.2 µM of each forward primer (PFf) 5'AGGCCTATGCCAGTCTCTTTCTTTGTT 3' and reverse primer (PFr) 5' GCGGCCGCTCAGTGTGATCTCACATA 3' (IDT) containing the *Stu*I and *Not*I (Promega) sites (underlined), respectively, and 1.25 U Taq DNA polymerase (Fermentas) for 29 cycles (95 °C for 1 min, annealing at 59 ºC for 2 min and extension at 72 °C for 2 min). Products were then run in 1.5% agarose with TBE buffer and then stained with ethidium bromide. The full-length PCR products from the F gene were sequenced using the same primers by automatic sequencing (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina).

2.3.Expression in *P. pastoris*

Plasmid pCR2.1TOPO-F_0 was initially generated by inserting a PCR fragment of the F_0 gene into the pCR2.1TOPO vector (Invitrogen). Then, a 1620-bp *Stu*I–*Not*I fragment isolated from pCR2.1TOPO-F_0 was then subcloned into the *SnaB*I-*Not*I-plasmid pPIC9 (Invitrogen). The *Stu*I and *SnaB*I digestion produces blunt ends and after ligation of both products pPIC9 and F_0, ORF F_0 is in reading frame with the export signal to the cell outside. The resulting plasmid was named pPIC9-F_0. The F_0 reading frame was placed under the control of the AOXI (methanol inducible) promoter. In addition, the resulting protein was fused to the α-factor, which directs the secretion of the protein to the culture medium. The ligation mixture of pPIC9-F_0 was transformed into chemically competent *E. coli* bacteria strain TOP10 and then grown in LP-amp. Then pPIC9-F_0 plasmid DNA was purified with a commercial kit (Promega) from the bacterial colonies and digested with the *Eco*RV restriction enzyme (Promega) to verify the presence of the F_0 fragment. To generate homologous recombination between the yeast genome and the transfer vector, pPIC9-F_0 was linearized with the *Bgl*II enzyme (Promega). For this purpose, 5 µg of vector, 3 µL of enzyme and 4 µL of buffer were used in a final volume of 40 µL and incubated at 37 °C for 18 h. The enzyme was then inactivated at 65 °C for 15 min. *P. pastoris* (GS115) cells were transformed with 1 µg of linearized pPIC9-F_0 in Cell-Porator (Gibco) through a 480V pulse, with a capacitance of 10 µF and low resistance (Ωlow). Then, 1 mL of cold sorbitol was added to each cuvette and 600 µL was plated in MD plates. Then, the plates were incubated 30 °C 48 h until the appearance of the colonies. Colonies grown on these plates were spiked onto minimal medium with methanol without histidine plates (MM) and grown for 48 h at 30 °C. After incubation, the presence of the F_0 fragment was confirmed by colony PCR using specific primers to the AOX1 region: 5'AOX1 (5' GAC TGG TTC CAA TTG ACA AGC 3') and 3'AOX1 (5' GCA AAT GGC ATT CTG ACA TCC 3').

2.4.Sequence analysis

To confirm the sequence of the insert, plasmids were sequenced using M13 primers. Sequences were aligned in MEGA program version 4.0 using the ClustalW algorithm and compared with the reference strain Ondersterpoot (Acc. X65509.1, AF305419.1, AF378705.1). The predicted amino acid sequence was compared with reference strain and analysis of N-glycosylation sites was performed (NetNGlyc - www.cbs.dtu.dk/services/NetNGlyc/-). This program predicts asparagines to be N-glycosylated according to the Asn-Xaa-Ser/Thr sequons (where Xaa is not Pro), with a threshold of 0.5.

2.5.Small-scale protein expression

To check the expression of the F_0 recombinant protein, liquid cultures of the GS115-F_0 clones obtained were performed in 100 mL of MGY medium and incubated on an orbital shaker at 120 rpm for 20 h until reaching an OD(600 nm) of 4-6. The yeasts were then collected under sterile conditions and centrifuged at 3000 rpm for 15 min at room temperature. Expression was induced in BMM medium. Cell pellets were resuspended in 1/10 of the initial volume and cultured at 30 °C for 5 days with gentle shaking (120 rpm). Samples of secreted proteins were taken every 24 hours and analyzed by SDS-PAGE and Western blot. The expression remained induced for 108 hours by addition of 100% methanol every 24 h until a final concentration of 0.5% v/v. Culture supernatants were harvested by centrifugation and proteins were precipitated with 60% $(NH_4)_2SO_4$ by centrifugation at 5000 rpm for 60 min. The ammonium sulphate was then removed by extraction with methanol/chloroform (partially purified protein).

2.6.Detection of the recombinant F-protein by SDS PAGE-Western blot

Partially purified protein obtained after 72, 96 and 108 h induction were mixed with 2X Laemmli sample buffer containing 2% (2-Mercaptoethanol), boiled at 99 °C for 2 min, and fractionated on 10% SDS polyacrylamide gels under denaturing conditions. Separated proteins were stained with Coomassie Brilliant Blue R250 (Sigma). For Western blot analyses, the proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes (Sigma) in a Trans-blot Semi Dry Electroforetic Transfer Cell (BioRad) according to the manufacturer’s recommendations. After blocking in PBS containing 0.1% Tween-20 (PBS-T) and 5% of skim milk powder at room temperature for 1 h, the membranes were incubated with a polyclonal mouse antibody anti-CDV glycoproteins produced previously in the Laboratory, in PBS-T–3% skim milk powder at room temperature for 2 h. This serum was obtained after immunization of three conventional six-week-old BALB/c mice with the commercial attenuated canine distemper vaccine -0.05 mL by intramuscular route- (Novibac Puppy DP MSD) with two boosters every 15 days. Subsequently, the membranes were washed in PBS-T at least three times followed by incubation with 1/500 dilution of horseradish peroxidase-conjugated anti-mouse antibody (Sigma Aldrich) in PBS-T–3% skim milk powder at room temperature for 1.5 h. After extensive washing, the membranes were placed in 0.3 mg/mL of DAB solution and H_2O_2 for 15 min until the appearance of the bands.

2.7.Immunization experiment in mice

To evaluate the immunogenicity of the recombinant protein *in vivo*, an immunization scheme was performed in mice. The partially purified protein was run in a SDS-PAGE (in a continuous comb) and cut and eluted from gel at 4 °C overnight in PBS (passive diffusion) (concentrated protein). Subsequently, the protein was quantified by UV spectrophotometry. For this purpose, the protein was diluted in sterile PBS to a final concentration of 10 μg/mL. Specific pathogen-free six-week-old male BALB/c mice were provided by the Laboratory of Experimental Animals (School of Veterinary Sciences, National University of La Plata, Buenos Aires, Argentina) and kept in conventional animal facilities. Animal handling and all experimental procedures were carried out in compliance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Research Council of Argentina. Three groups of three BALB/c mice were immunized three times with purified F_0 (group A), 0.03 mL of 10^5 TCID50/mL (tissue culture infectious dose) attenuated commercial vaccine (group B) or PBS-Specol (group C). Experimental animals were reduced to a minimum, in accordance with laws related to animal welfare. Immunizations were performed intramuscularly in the inguinal plexus with 0.5 µg of protein administered, combined with Specol adjuvant in equal volumes, every 15 days [19]. Only the first immunization was performed with adjuvant. The animals were sacrificed and bled 15 days after the last immunization. To obtain the sera, blood was left to coagulate at room temperature and after coagulation the samples were centrifuged at 1000 rpm for 15 min. The sera obtained were stored at -20 °C until used.

2.8.Immunogenicity assays

In order to analyze the immunogenicity of the F_0 protein, Western blot was performed. Vero cell cultures infected with CDV strain were harvested at 72 h postinfection, lysed directly in loading buffer (crude antigen), run in a SDS-PAGE and then transferred onto nitrocellulose membranes as described above [19]. After transfer, the membranes were cut into strips of approximately 0.5 cm and placed in test tubes (1 cm in diameter; 10 cm long) containing 2 mL of the blocking solution. Sera from each group were mixed in a single pool (A, B, or C) and used in a 1/100 dilution. They were then incubated for 90 min with gentle shaking, always taking care that the membranes were immersed in the blocking solution. Then, three washes were performed and the membranes further incubated with a 1/500 dilution of the conjugated anti-mouse antibody (Sigma). Finally, after three more washes, the strips were revealed using DAB solution as described above.

2.9.Virus neutralization

Virus grown in Vero cells showed 80-90% cytopathic effects was frozen twice at -80° C and centrifuged at 3,000 rpm for 10 min. Infectivity titre of the virus was measured by TCID50/50μL in Vero cells in a 96-wells tissue culture plate. After 72 hs of incubation a titre of 10^3 TCID50/50μL was obtained. Serum samples from each inoculated mouse (A, B and C) were heat inactivated at 56 °C for 30 min. Virus neutralization assays were performed with serial twofold dilutions of sera prepared using MEM in a 96-well flat-bottomed tissue (Nunc, Rochester, NY), mixed with 100 TCID50 in 25 μL and then incubated at 37 °C for 1 h in a 5% CO_2, followed by addition of 100 μL of Vero cell suspension (3×10^5 cells/mL) to each well and incubation for 72 h, as described previously. The VN antibody titre was expressed as the reciprocal of the highest dilution the inhibited cytopathic effect completely [20].

3.Results

3.1.RT-PCR and generation of the recombinant protein in *P. pastoris*

The complete F_0 gene of CDV was successfully amplified by RT-PCR from a commercial vaccine strain. The correct reading frame of the inserted gene was evaluated by sequencing, using the primer AOX1 F and R. Blast (n) analysis of the sequence resulted in 99% homology to the GenBank AF305419.1, AF378705.1 and X65509.1 sequences belonging to the gene of the CDV fusion protein corresponding to the Onderstepoort strain**,** and the predicted sequence of the recombinant F_0 protein is shown in **Figure 1**. The recombinant F_0 protein was expressed and identified and the putative N-linked glycosylation sites are same as reference strain. A PCR fragment bearing the entire coding region (1624 nt; 537 aa) of the F gene was cloned into the expression vector pPIC9, generating a construct expressing the recombinant F_0 gene fused to an export sequence that allowed releasing the protein to the culture medium.

3.2.Small-scale protein expression and detection by SDS-PAGE/Western blot

The expression of the F_0 recombinant protein was induced by addition of 0.5 % of methanol every 24 h to GS115 cells containing the pPIC9-F_0 plasmid. Then, purified supernatants as described above were run in SDS-PAGE. The recombinant protein was detected in low level by Western blot. The estimated molecular weight was about 60 kDa, which corresponded to the molecular weight of the expected F_0 protein **(Figures 2a & 2b).**

3.3.Immunogenicity assay

Purified protein F_0 (partially purified and concentrated) was run in a SDS-PAGE in order to check purity **(Figure 3a).** Immunogenic capacity was checked in BALB/c mice. A single band of approximately 60 kDa, corresponding to the F_0 protein, was detected by Western blot -group A- and in group B more than one band **(Figure 3b)**. The virus neutralization antibody titres in mice sera from groups A and B were relatively low: A1 and A2:1/16; A3 1/8; B1: 1/32; B2: 1/16; B3: 1/32.

4.Discussion

In this study, we were able to clone and express the complete coding region of the CDV F gene. The fusion protein F is constituted by two subunits originated from a protein precursor pre-F_0 [11], which suggests that the peptide is not part of the mature protein. For that reason, we decided to express only the precursor F_0, eliminating the signal peptide located at the N-terminal of the protein.

The expression system used to obtain the F_0 protein, based on the methylotrophic yeast *P. pastoris*, turned out to be relatively simple and efficient for several reasons. One of these reasons was the simplicity to genetically manipulate the system and select the recombinant yeasts by auxotrophy to histidine. The latter allowed cloning the genes simply and rapidly. In addition, the insertion of the genes of interest into the yeast genome allowed performing cultures of high cell density without requiring a constant selection pressure such as an antibiotic [21, 22, 23].

The expression levels obtained for the F_0 protein were low (35 µg/mL); however, the expression system in *P. pastoris* allowed the production of the recombinant polypeptide without the need for as many steps of optimization of expression as those required for other expression systems [24]. Besides, the metabolism of *P. pastoris* is mainly aerobic and unlike *Sacharomyces cereviceae* it can grow in fermenters in large-scale cultures, reaching very high cell densities. In addition, no phenomena of hyperglycosylation of proteins expressed in *P. pastoris* have been observed that could generate an exacerbated immune response preventing their therapeutic use [25]. The recombinant F_0 protein showed a molecular weight of about 60 kDa, a value that corresponded to that estimated by Iwatsuki *et al*. (1998) [13] for the glycosylated form. This suggests that the recombinant F_0 contains some post-translational modification.

To evaluate the immunogenicity of the recombinant F_0 protein, BALB/c mice were used as the *in vivo* model. The protein was combined with an oily adjuvant (Specol) to improve the immune response, because many recombinant proteins are usually poor as immunogens. Some authors have used Specol as an alternative to Freund's Adjuvant, since its administration causes fewer adverse effects [26, 27]. Based on the results obtained by Western blot in **Figure 3b (line 1),** we concluded that the mice inoculated with the F_0 protein produced antibodies that recognized the proteins homonymous to CDV. The use of Specol allowed increasing the immune system response of such mice.

Accessory bands obtained in Western blot **(Figure 3b lane 2**) may be due to the presence of impurities in crude antigen. The non-specificity observed could be due to the fact that the specific antibodies against have reacted with protein products expressed in the infected Vero cells used as crude antigen in Western blot. One possibility is that CDV vaccine is produced in Vero or similar cells.

VN titres greater than 1:100 are considered completely protective in domestic canines obtained with CDV attenuated virus vaccines. Although in this work we have not been able to achieve titles of that magnitude, we must emphasize that the murine model used for the production of specific antibodies does not represent the best model for the study of canine distemper. Since we do not have facilities that can produce SPF canines, only members of the Mustelidae family as a mink or ferret could have been used as replacements, which are very expensive and difficult to keep in captivity. For these reasons we limit ourselves to the use of rodents as experimental animals.

The protection against CDV by vaccination is based primarily on the immune system response against envelope glycoproteins (hemagglutinin and fusion protein) [28]. The immunity to the F antigen has been shown to block the replication of challenge virus and to prevent the emergence of symptoms in animals after virus replication. This suggests that the F antigen may suffice act as an immunogen for protection against canine distemper. Attenuated vaccines have been used to prevent CDV infection worldwide, however there has been an increase in clinical cases [29, 30, 31]. A recent increase in clinical disease has also been detected in Argentina including vaccinated dogs [32]. Despite the above, Pardo *et al*., 1997 [33]**,** have described in their studies that although the levels of antibodies obtained in canines immunized with recombinant vaccines were low, they were sufficient to protect them against the challenge of a pathogenic CDV strain .

The results obtained in our study showed that the CDV F protein could be efficiently expressed in the *P. pastoris* system, indicating the potential of F protein expressed in this system as a subunit vaccine candidate for the control of the disease. The cost of production and secondary consecuences after vaccination could be reduced by using new immunogens. While the best way to achieve lasting and effective immunity is with the use of attenuated virus vaccines, however they can cause encephalitis [34, 35], or reversion to virulent strains, not to mention the low rate of replication of viral strains currently used [36, 37]. Nevertheless, use of a subunit vaccine may overcome these disadvantages.

There is not other studies in family *Paramyxoviridae*, with the exception on Newcastle virus. Kang et al, 2016 [38], produced the recombinant F protein expressed by *P. pastoris* as a subunit vaccine candidate and considered that is an efficient and economic protein expression system, more suitable for expressing viral proteins because of its posttranslational modification. This strategy allows culture time reduction and minimizes the cost of production which is important for industrial applications.

The viral proteins obtained by recombinant technology would allow eliminating the cellular proteins found in the CDV attenuated vaccines currently used obtained by successive passages in cell cultures or embryonated eggs, reducing adverse reactions, and also reducing production costs such as concentration and viral purification. Significant advantages of this system include proper protein folding, posttranslational modifications, and glycosylation of recombinant proteins in the correct sites which is important for protein stability systems [39]. Besides, mammalian expression systems grow slowly and the relevant nutrient requirement is costly and yeasts are widely used for the expression of several proteins in vaccine and pharmaceutical production. Subunit viral vaccines are completely safe and cost□effective and for its production *P. pastoris* is more famous than other expression systems as was summarized in a recent review (Dengue, Chikungunya, Foot and Mouth disease, SARS and Newcastle Disease) [40]. Recombinant CDV proteins might be useful for the development of safer vaccines for future. At this time our working group is expressing other CDV proteins on *P. pastoris* with promisory results that will be the subject of a comparative analysis in the future.

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Figure captions

Figure 1: Alignment of the predicted amino acid sequences for the F_0 protein of the Onderstepoort strains from GenBank. The potential glycosylation sites are enclosed by rectangles

Figure 2a: Small scale F_0 protein expression analysis by SDS-PAGE. Five-fold concentrated by $(NH_4)_2SO_4$ culture supernatants (15 µL) after methanol induction: lane 1: prestained molecular weight marker (Fermentas); lane 2: non-transformed *P. pastoris* (negative control); lane 3: 72 h post-induction; lane 4: 96 h post-induction, lane 5: 108 h post-induction

Figure 2b: Small scale F_0protein expression by Western blot analysis. Five-fold concentrated by $(NH_4)_2SO_4$ culture supernatants (15 µL) after methanol induction: lane 1: non-transformed *P. pastoris* (negative control); lane 2: 72 h post-induction; lane 3: 96 h post-induction, lane 4: 108 h post-induction, lane 5: prestained molecular weight marker (Fermentas). As primary antibody mice sera inoculated with commercial attenuated canine distemper vaccine was used.

Figure 3a: F_0 analysis in SDS-PAGE. Lane 1: partially purified protein; lane 2: concentrated F protein (eluted from gel); lane 3: prestained molecular weight marker (Fermentas).

Figure 3b: Western blot analysis using 2 mL of 1/100 dilutions of polyclonal sera of mice inoculated. Crude antigen obtained from Vero-inoculated CDV was used. Lane 1: serum pool group A (mice immunized three times with concentrated F_0), lane 2: serum pool group B (mice immunized with attenuated commercial CDV vaccine), lane 3: serum pool group C (mice inoculated with PBS-Specol), lane 4: prestained molecular weight marker (Fermentas).

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CLUSTAL O(1.2.4) multiple sequence alignment


RecF            MASLFLCSKAQIHWNNLSTIGIIGTDSVHYKIMTRPSHQYLVIKLMPNVSLIDNCTKAEL 60
AF378705.1      IASLFLCSKAQIHWNNLSTIGIIGTDSVHYKIMTRPSHQYLVIKLMPNVSLIDNCTKAEL 60
AF305419.1      MASLFLCSKAQIHWNNLSTIGIIGTDSVHYKIMTRPSHQYLVIKLMPNVSLIENCTKAEL 60
X65509.1        MASLFLCSKAQIHWNNLSTIGIIGTDSVHYKIMTRPSHQYLVIKLMPNVSLIENCTKAEL 60
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RecF            GEYEKLLNSVLEPINQALTLMTKNVKPLQSLGSGRRQRRFVGVVLAGAALGVATAAQITA 120
AF378705.1      GEYEKLLNSVLEPINQALTLMTKNVKPLQSLGSGRRQRRFAGVVLAGAALGVATAAQITA 120
AF305419.1      GEYEKLLNSVLEPINQALTLMTKNVKPLQSLGSGRRQRRFAGVVLAGVALGVATAAQITA 120
X65509.1        GEYEKLLNSVLEPINQALTLMTKNVKPLQSLGSGRRQRRFAGVVLAGVALGVATAAQITA 120
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RecF            GIALHQSNLNAQAIQSLRTSLEQSNKAIEEIREATQETVIAVQGVQDYVNNELVPAMQHM 180
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AF305419.1      GIALHQSNLNAQAIQSLRTSLEQSNKAIEEIREATQETVIAVQGVQDYVNNELVPAMQHM 180
X65509.1        GIALHQSNLNAQAIQSLRTSLEQSNKAIEEIREATQETVIAVQGVQDYVNNELVPAMQHM 180
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AF305419.1      SCELVGQRLGLRLLRYYTELLSIFGPSLRDPISAEISIQALIYALGGEIHKILEKLGYSG 240
X65509.1        SCELVGQRLGLRLLRYYTELLSIFGPSLRDPISAEISIQALIYALGGEIHKILEKLGYSG 240
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RecF            GDMIAILESRGIKTKITHVDLPGKFIILSISYPTLSEVKGVIVHRLEAVSYNIGSQEWYT 300
AF378705.1      GDMIAILESRGIKTKITHVDLPGKFIILSISYPTLSEVKGVIVHRLEAVSYNIGSQEWYT 300
AF305419.1      SDMIAILESRGIKTKITHVDLPGKFIILSISYPTLSEVKGVIVHRLEAVSYNIGSQEWYT 300
X65509.1        SDMIAILESRGIKTKITHVDLPGKFIILSISYPTLSEVKGVIVHRLEAVSYNIGSQEWYT 300
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RecF            TVPRYIATNGYLISNFDESSCVFVSKSAICSQNSLYPMSPLLQQCIRGDTSSCARTLVSG 360
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AF305419.1      TVPRYIATNGYLISNFDESSCVFVSESAICSQNSLYPMSPLLQQCIRGDTSSCARTLVSG 360
X65509.1        TVPRYIATNGYLISNFDESSCVFVSESAICSQNSLYPMSPLLQQCIRGDTSSCARTLVSG 360
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AF305419.1      TMGNKFILSKGNIVANCASILCKCYSTSTIINQSPDKLLTFIASDTCPLVEIDGATIQVG 420
X65509.1        TMGNKFILSKGNIVANCASILCKCYSTSTIINQSPDKLLTFIASDTCPLVEIDGATIQVG 420
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AF305419.1      GRQYPDMVYEGKVALGPAISLDRLDVGTNLGNALKKLDDAKVLIDSSNQILETVRRSSFN 480
X65509.1        GRQYPDMVYEGKVALGPAISLDRLDVGTNLGNALKKLDDAKVLIDSSNQILETVRRSSFN 480
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RecF            FGSLLSVPILSCTALALLLLIYCCKRRYQQTLEQHTKVDPAFKPDLTGTSKSYVRSH    537
AF378705.1      FGSLLSVPILSCTALALLLLIYCCKRRYQQTLKQHTKVDPAFKPDLTGTSKSYVRSL    537
AF305419.1      FGSLLSVPILSCTALALLLLIYCCKRRYQQTLKQHTKVDPAFKPDLTGTSKSYVRSL    537
X65509.1        FGSLLSVPILSCTALALLLLIYCCKRRYQQTLKQHTKVDPAFKPDLTGTSKSYVRSH    537
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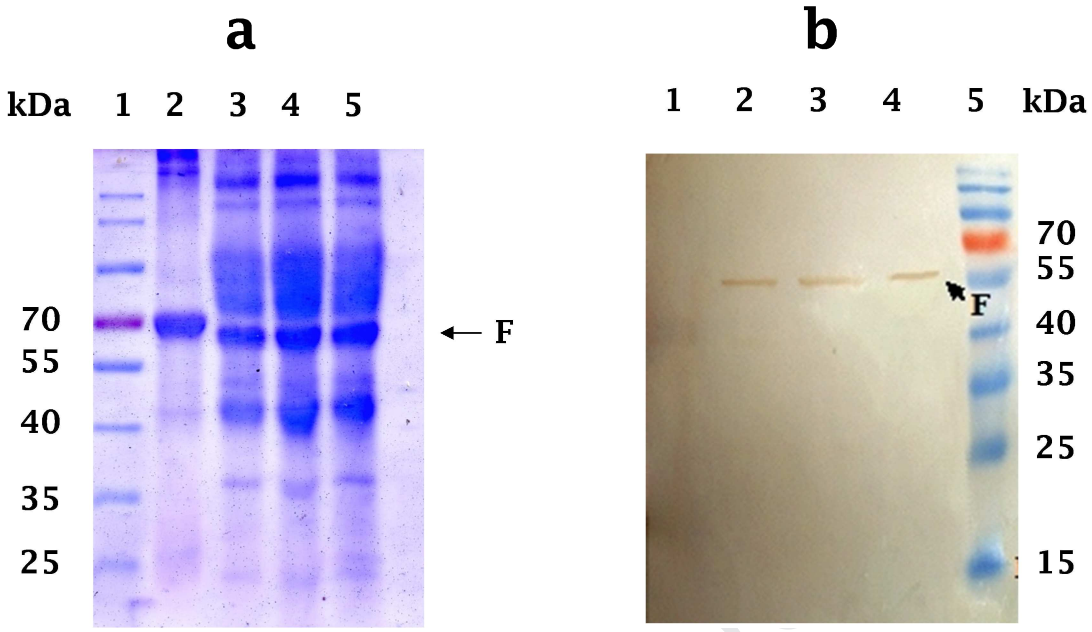
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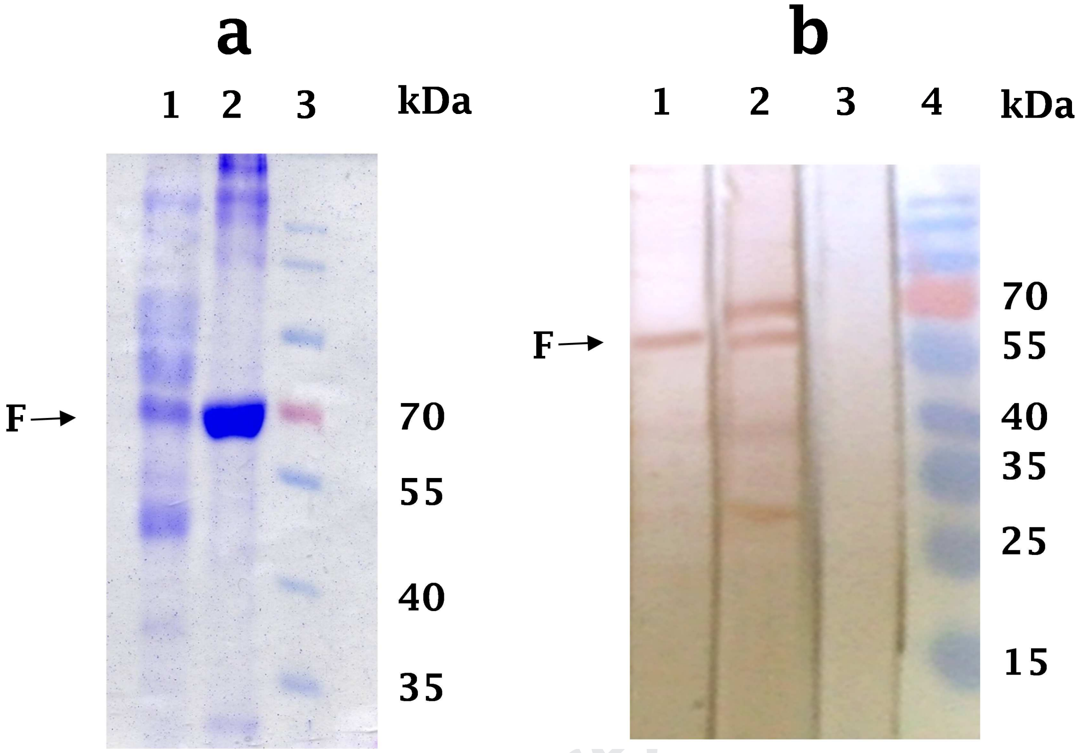
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Highlights

The complete coding region of the F protein of CDV was expressed in *Pichia pastoris*

Mice inoculated with the F_0 protein produced antibodies that recognized CDV protein

The recombinant protein could be used as a subunit vaccine against canine distemper