Cloning, expression, and characterization of potential immunogenic recombinant porcine deltacoronavirus (PDCoV) membrane protein from a synthetic gene.

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Abstract

Porcine Deltacoronavirus (PDCoV) is an emergent swine coronavirus that infects epithelia cells from the small intestine, and inducing watery diarrhea, vomiting and dehydration. Clinical signs are more aggressive in piglets causing high mortality rates (>40%) representing serious economic losses. Despite the importance of PDCoV as an emerging coronavirus, little is known about the currently prevalence in México. We select from GenBank a group of 138 sequences and obtained a consensus PDCoV membrane protein (M-PDCoV) sequence of 216 a.a. A Maximum Likelihood phylogenetic tree was constructed and evaluate the relationship between the 138 sequences. Also, a protein tertiary structure analysis was performed to analyze and compare the topological differences. The phylogeny and the tertiary structure analysis showed that M-PDCoV is highly conserved and therefore suitable to use as an antigen in a diagnostic system. Hence, an expression system performed in *E. coli* BL21 (DE3) using the vector pET-SUMO with a His-tag was prepared, resulting in a synthetic M gene of 654 pb to produce a recombinant M-PDCoV protein (*r*M-PDCoV). Western blot test confirmed the *r*M-PDCoV immune detection in 8 of 17 sera samples. The *r*M-PDCoV was able to successfully stimulate immune response in immunized mice to produce antibodies after day 7 of immunization ($P < 0.001$). Our results show that *r*M-PDCoV is suitable to use in diagnostic systems like an ELISA.

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Summary

Porcine Deltacoronavirus (PDCoV) is an emergent swine coronavirus that infects epithelia cells from the small intestine, and inducing watery diarrhea, vomiting and dehydration. Clinical signs are more aggressive in piglets causing high mortality rates (>40%) representing serious economic losses. Despite the importance

of PDCoV as an emerging coronavirus, little is known about the currently prevalence in México. We select from GenBank a group of 138 sequences and obtained a consensus PDCoV membrane protein (M-PDCoV) sequence of 216 a.a. A Maximum Likelihood phylogenetic tree was constructed and evaluate the relationship between the 138 sequences. Also, a protein tertiary structure analysis was performed to analyze and compare the topological differences. The phylogeny and the tertiary structure analysis showed that M-PDCoV is highly conserved and therefore suitable to use as an antigen in a diagnostic system. Hence, an expression system performed in *E. coli* BL21 (DE3) using the vector pET-SUMO with a His-tag was prepared, resulting in a synthetic M gene of 654 pb to produce a recombinant M-PDCoV protein (*r* M-PDCoV). Western blot test confirmed the *r* M-PDCoV immune detection in 8 of 17 sera samples. The *r* M-PDCoV was able to successfully stimulate immune response in immunized mice to produce antibodies after day 7 of immunization ($P < 0.001$). Our results show that *r* M-PDCoV is suitable to use in diagnostic systems like an ELISA.

Keywords

PDCoV, Recombinant proteins, M protein, Synthetic gene, Porcine deltacoronavirus, Diagnostic system, ELISA.

Introduction

Coronaviruses (CoV) are enveloped viruses that have been identified in humans and in several animals (Cui et al., 2019). Currently, six different swine CoV (SCoV) have been described: Transmissible Gastroenteritis Virus (TGEV), Porcine respiratory coronavirus (PRCV), Porcine epidemic diarrhea virus (PEDV), Swine Acute Diarrhea Syndrome Coronavirus (SADS-CoV), Porcine Hemagglutinating Encephalomyelitis Virus (PHEV) and the emergent Porcine Deltacoronavirus (PDCoV) (Zhang, 2016). PDCoV has a positive single-stranded RNA genome with a length of 25 kb approximately. The genome organization is in the following order: 5' untranslated region (UTR), open reading frame 1a/1b (ORF1a/1b), spike (S), envelope (E), membrane (M), nonstructural protein 6 (NS6), nucleocapsid (N), nonstructural protein 7 (NS7), and 3' UTR (He et al., 2020; Zhang, 2016).

After the first isolation (HKU15) of PDCoV in China in 2012 (Woo et al., 2012), several reports of detection has been informed both in Asia as in America (Ajayi et al., 2018; Le et al., 2018; Lee et al., 2016; Li et al., 2014; Lorsirigool et al., 2017; Lorsirigool et al., 2016; Perez-Rivera et al., 2019). The pathogenesis of the virus preferentially occurs within the enterocytes of the small intestine, where the virus interacts with its antigens and the cells their receptors facilitating the invasion and proliferation into them (Jung et al., 2016). Damage to the epithelia is traduced in watery diarrhea, dehydration and vomit signs that easily affect piglets with mortality rates of $> 40\%$. (Feng et al., 2020; Niederwerder & Hesse, 2018; Wang et al., 2019). PDCoV frequently co-infect with PEDV, TGEV and, Rotavirus C, increasing the intestinal cell damage and inducing a severe infection (Hu et al., 2015; Song et al., 2015). In this scenario, early and suitable diagnosis is of vital importance.

The M protein is required for the virus assembly process is the most abundant component of the virus envelope which is highly conserved (de Haan & Rottier, 2005; Narayanan et al., 2003; Stadler et al., 2003; Wang et al., 2020; Weiss & Leibowitz, 2011). Also, the M protein promote the production of antibodies with virus-neutralizing activity (Weiss & Leibowitz, 2011). Furthermore, cross-reactivity of PDCoV with antibodies to either PEDV or TGEV M protein has not been previously observed (Chen et al., 2015; Jung et al., 2016; Ma et al., 2015). Therefore, the M protein suits as candidate for induction of specific antibodies and diagnosis of PDCoV detection.

Despite that PDCoV as an emerging coronavirus, little is known about the currently prevalence within the pig population in Mexico. The present study deals with the construction and expression in *E. coli* of recombinant proteins from a synthetic gene obtained after *in silico* analysis on 138 sequences of the M protein of PDCoV (*r* M-PDCoV) previously informed at the GenBank.

Materials and Methods

In Silico Analysis of M-PDCoV

A total of 138 PDCoV M protein sequence available in GenBank were obtained. The 138 sequences were from China, Laos, Japan, Vietnam, Thailand, and USA dated from 2012 to 2019 (Table 1). To determine phylogenetic relationships among the 138 sequences, a maximum likelihood phylogenetic tree using RaxML v8.0 was made (Stamatakis, 2014). The sequences were aligned using Clustal Omega online server (Sievers et al., 2011). RaxML was running using a HIVb amino acid substitution model (Nickle et al., 2007) calculated by ProtTest software (Abascal et al., 2005). 1,000 bootstraps were evaluated to assess support values. Also, from the 138 PDCoV M protein sequences available in GenBank we obtained a consensus sequence (217 a.a.) using Jalview software (Clamp et al., 2004). The potential antigenicity sites were predicted whit Jameson-Wolf method using Protean, DNAStar v17.2 (Plasterer, 2000). The hydrophobic and hydrophilic sites were predicted with Kyte and Doolittle and Emini et al. methods using Protean, DNAStar v17.2 (Emini et al., 1985; Kyte & Doolittle, 1982). Additionally, predicted tertiary structure for the consensus sequence, HKU15 (GenBank: JQ065042) and CHzmd2019 (GenBank: MN781985) were determined using I-TASSER (Roy et al., 2010). Also, we include the M protein predicted tertiary structure of the PEDV CV777 (GenBank: AAK38659) and TGEV Miller M6 (GenBank: ABG89300) for comparison. The predicted tertiary structures were selected according to a suitable C-Score value (range of -5 to 2) where higher value signifies a model with a high confidence (Yang et al., 2015; Zhang, 2008). The selected tertiary structures were visualized using PyMOL Molecular Graphics System, Version 2.0 Schrodinger, LLC software. To assess the topological similarities, we determinate the template modeling score (TM-Score). TM-Score ranged from 0 to1, where 1 indicates a perfect match between two structures (Zhang & Skolnick, 2004) (Table 2). Finally, the amino acid identity was determined using the Sequence Manipulation Suite (SMS) on line (Stothard, 2000).

Molecular cloning of M-PDCoV in pETSUMO vector.

Since PDCoV is an emergent disease, it was not possible to collect a PDCoV sample from a pig farm. Therefore, a synthetic gene (654 bp) from the consensus amino acid sequence was obtained from a commercial supplier and was verified with the following primer sequences: Fw 5'-GACGCAGAAGAGTGGCAAATTATT-3' and Rv 5'-GCGCTACTACATATACTTATACAGGCG-3. The synthetic gene was cloned in pETSUMO vector and positive recombinant plasmids were transformed into *E. coli* BL21 (DE3). To perform a competent cell, MgCl2 and CaCl2 protocol was used. Transformants were selected in LB agar plates supplemented with kanamycin, 50 μg/ml. The positive colonies were propagated and used to purify the plasmid DNA, using Wizard® Plus SV Miniprepr kit (Promega). The clones were confirmed by PCR using the primers sequences mentioned above. Also, a PCR using the above forward primer sequence a the reverse T7 sequence primer 5'-TAGTTATTGCTCAGCGGTGG-3' was made to confirm the right direction of the inserted synthetic gene with the vector expression promoter.

Expression of the recombinant M-PDCoV in *E. coli* BL21 (DE3)

A transformant colony was inoculated into 3 ml of LB supplemented with kanamycin (50 μg/ml) medium at 37 °C, 250 rpm, 12 h as a pre-culture. Then, 100 ml of LB medium was inoculated starting 0.1 OD_{600} nm. The medium was incubated at 37 °C, 250 rpm until the culture reached the 0.5 OD_{600} nm. Then, the expression of recombinant M-PDCoV (*r* M-PDCoV) protein was induced by addition of Isopropyl β-D-1-thiogalactopyranoside, (IPTG) (Merck, Germany) at a final concentration of 1.5 mM followed by incubation over night at 37°C.

r M-PDCoV purification.

The induced medium was centrifuged (12,000 g for 10 min), the pellets were resuspended 400 ml of 0.1 M Tris-HCl pH 8.0 buffer. The cells were disrupted mechanically with a cell disrupter (GAULIN) at 550 kg/cm^2, 15 min. The insoluble phase was separated by centrifugation at 15,000 g, 20 min, 4 °C. Then, the inclusion bodies from the insoluble phase, were solubilized into union buffer (7% N-Lauroylsarcosine sodium salt, Tris-HCl 50 mM pH 8) with agitation 250 rpm, 12 h, 25°C. After the inclusion bodies solubilization, the recombinant proteins were purified using Ni-NTA agarose column with His-tag affinity (Amersham), 1.5 ml of resin packed into a vertical column. The column was equilibrated with 5 CV of binding buffer (Tris-HCl 50 mM pH 8). Then, 2 CV of solubilized inclusion bodies (2 mg/ml) were loaded. Other proteins will be

eliminated with 5 CV of wash buffer (Tris-HCl 50 mM pH 8, 30 mM imidazole) and the recombinant proteins were recovered using 4 CV of elution buffer (Tris-HCl 50 mM pH 8, 500 mM imidazole).

SDS-PAGE, Western blot analysis

The purified recombinant protein was separated by 12% SDS-PAGE and confirmed by Western blot. The recombinants proteins were transferred in to polyvinylidene fluoride membrane (PVDF) and blocked with 5% non-fat milk in PBS-Tween buffer (20 mM Tris-HCl, pH 8, 0.15 M NaCl, 0.05% Tween 20) at 4°C for 16 h with moderate agitation. Membrane was washed with PBS-Tween buffer and incubated with primary antibodies. To identify the *r* M-PDCoV an anti-his conjugated by horseradish peroxidase (HRP, Invitrogen) diluted 1:5000 was used and anti-pig IgG+HRP as secondary antibody diluted 1:5000. To visualize the protein bands in western blot, 10 ml development solution (PBS, 12 mg of 3,3'-Diaminobenzidine Tetrahydrochloride, 300 µl 3.4% H_2O_2) was used. Finally, the protein concentration will determinate according to Bradford (Bradford, 1976) using bovine albumin as a standard.

Antigenicity assay of *r* M-PDCoV in swine serum samples

Western blot assay to determinate the recognition by immunodetection of*r* M-PDCoV with 17 swine sera from "El Bajio", Mexico, pig farm. The sera came from pigs affected by lethargy, watery diarrhea, vomiting and dehydration. The Sera were randomly named:

102, 105, 130, 136, and P1-P13 and were diluted 1:1000, the methodology used was the previously mentioned. Negative control serum was from a sow keeping in laboratory

BALBc mice Immunization

The evaluation of antibodies anti-*r* M-PDCoV production was made using three experimental groups. Each group was performed by eight BALBc mice 28 days old. The immunization of Group-1 consisted in 5 µg/mouse of*r* M-PDCOV protein diluted in PBS buffer, 1X pH 7.4. Group-2, 5 µg/mouse of *r* M-PDCOV protein and 5 µg/mouse of using immunostimulating complex, Iscom® (ISCONOVA AB, MATRIX Q, Uppsala, Sweden) as an adjuvant. Group-3, 200 µL de PBS buffer as a negative control. The final volume dose was 200 µL, and two doses were applied to each mouse subcutaneously at day one and at day 14, respectively. The blood sample were collected through the caudal vein at days 7, 14, 21, and 28. The IgG antibodies production were evaluated by indirect ELISA (iELISA). The mice were euthanized using a CO_2 chamber. The IgG antibodies production were evaluated by indirect ELISA. The animals were handled at the house facility at National Microbiology Research Centre (CENID-SAI), *Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias* (INIFAP), (NOM-062-ZOO-1999; SAGARPA).

iELISA for antibody detection

An indirect enzyme-linked immunoassay (iELISA) was performed to determine the presence on IgG in mice sera. The 96 wells plates (microwell plate Batch 011606) were coated with 50 ng of *r* M-PDCoV in 0.05 mol/l carbonates buffer pH 9.6 (Sigma-Aldrich, USA) at 4°C and blocked with 5% skimmed milk at 37°C for 1 hr. After washing four times with PBS buffer, pH 7.4, Tween 0.1%, 100 µl of serum and negative control (Group-3), diluted 1:150, was added to the wells in triplicate and incubated at 37°C for 2 hr. The plates were washed four times and incubated with 100 µl of HRP-conjugated rabbit anti-mouse IgG diluted (Merck KGaA, Darmstadt, Germany) 1:7000 in PBS pH 7.4, Tween 0.1%, with 5% skimmed milk at 37°C for 2 hr. After adding 100 µl of a 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution an incubating at room temperature for 10 min, the reaction was stopped by adding 100 µl of 2N H_2SO_4, and the absorbance at 450 nm was measured. The result was graphed using SigmaPlot software.

Results

Phylogenetic analysis

The phylogenetic analysis confirms the highly conserve of PDCoV M protein. From the 139 sequences (Table 1), we found 111 sequences with 100% of similarity. The 28 rest of the sequences show similarities

> 94% and also, different branch length (Fig 1a). These 28 different sequences were mainly from Asia (China, Japan, Thailand, and Vietnam) and one sequence from USA, the USA/Minnesota292/2014. The sequence CHzm2019 from China, showed the longest branch in the tree with ten different amino acid (Fig 1b). Therefore, a protein M consensus sequence was used due to the high similarity between the sequences

r M-PDCoV protein structure

We made a comparison to evaluate if the protein M consensus sequence has a significant difference at a structural level, (Fig 2). We compare the protein structure from USA/Minnesota292/2014, CHzmd2019, and HKU15 (the first PDCoV isolated reported) and the consensus sequence protein M structure obtained (C-Score values up to -4.6). Also, we include the protein M structure from two different SCoV: PED CV777 isolate and TGE Miller M6 isolate (Fig 2a and 2b). According to TM-Score, the protein structure for CHzmd2019, HKU15 and the consensus sequence protein M has a TM-Score value > 0.8, (Table 2). Also, we found nine potential antigenic sites along the sequence and correlate with hydrophilic sites, indicating that the antigenic sites are in the surface of the protein structure, available to be recognized by the immune system (Fig 2c and 2d).

Expression analysis of *r* M-PDCoV in *E. coli*

A synthetic gene (654 bp) based on the consensus sequence using in this study, was acquired from a commercial supplier. The integrity and weight were evaluated (fig. 3a). The expression was conducted in the *E. coli* BL21 (DE3), using the vector pET-SUMO with a His-tag. After the purification with Ni-NTA agarose His-tag affinity column, the identity of the *r* M-PDCoV was confirmed by Western blotting with a specific antibody *anti* -His. The band size was observed at an estimated size of 37.7 kDa (fig.3b). Also, the *r* M-PDCoV was observed in the insoluble phase (fig.3b), suggesting that *r* M-PDCoV are found as inclusion bodies. After solubilization of inclusion bodies and purification, recombinant proteins were verified from the elutions using SDS-PAGE and western blot. We found the purified *r* M-PDCoV in elution 3 to 5 and the molecular weight was according to expected, ˜37.7 kDa (fig. 3c).

Immunodetection of *r* M-PDCoV in *E. coli*.

The immune reactivity of the *r* M-PDCoV protein with specific*anti* -PDcoV-IgG antibodies in 17 serum samples from pigs infected by PDCoV from "El Bajio" Mexico, was confirmed by Western blot analysis. The Western blot detected a band of approximately 37.7 kDa consistent with the molecular weight of the M protein. The results show eight positive sera: 130, P-1, P-3, P-4 and P-10-P13 (Fig. 4). No bands, of the corresponding molecular size were detected with serum from control non- infected pigs. Negative control serum was from a sow keeping in laboratory.

Antibody response of BALBc/mice to immunization with *r* M-PDCoV protein.

The capability of *r* M-PDCoV to stimulate the immune system to produce antibodies was determined using three mice groups. The antibodies production was evaluated by iELISA (Fig. 5). The antibody response increased after the first dose given from day 7 lasting to the end of the experiment (day 28) being significantly higher ($P < 0.0005$) compared to the PBS control group. Also, the higher antibody production was observed in Group-2: *r* M-PDCOV with immunostimulating complex (Iscom®) being significantly higher ($P < 0.0005$) compared to the PBS control group.

Discussion

Swine coronavirus (SCoV) are cause effect important diseases in pigs. PDCoV is the most resent SCoV discovered during 2012 in China. Later, it spread around the world causing serious economic losses. It has been suggested that PDCoV spread from the United States to Mexico due to the commercial swine exchange and the geographical proximity of these two countries (He et al., 2020; Perez-Rivera et al., 2019). Identification and diagnosis in early stages are necessary to prevent and control the diseases. Therefore, the goal in this study was to develop a recombinant PDCoV protein M based on a consensus sequence from 138 available sequences from China, Laos, Vietnam, Thailand, and USA (Table 1).

We found a highly conserve M protein among the 138 sequences in the phylogenetic analysis (Fig 1). The similarity among the sequences was >94%. The high frequency of M in the virus envelope (molar ratio S:N:M of 1:8:16 and 1:6:15) (de Haan & Rottier, 2005) and its role in the assembly process like a "building block (Wang et al., 2020), are probably the cause of the highly conserve property of M protein.

Moreover, at a structural level (Fig 2a), the consensus sequences folds in three hydrophobic domains alternating with short hydrophilic regions in the N-terminal half of the protein (Fig 2b). This tertiary structure was observed in all sequences compared but not in the TGE Miller M6 sequence. TGE M proteins has a cleavage N-terminal, but it is still not clear the function of this (Kapke et al., 1988). However, this incision could give an alternative configuration like the observed in figure 2a. Similarly, the topological tertiary structure comparison between the PDCoV sequences and the consensus sequence shows a significant TM-Score of 0.8 (Table 2). Overall, the tertiary structure observed for consensus M protein correlates with the reported in the literature (Neuman et al., 2011).

It has been observed that PDCoV is rare among enteric SCoV infections (Perez-Rivera et al., 2019), due to PDCoV is an emergent disease. During the present study, it was not possible to obtain and isolate a viral sample. Therefore, we decided to construct a synthetic gene. The integrity and weight of the synthetic gene was evaluated (Fig. 3a). We successfully expressed and purified a recombinant M protein (r M-PDCoV) from *E. coli* . The r M-PDCoV was evaluated by SDSPAGE and identified by western blotting with ˜37.7 kDa expected molecular weight. Using the r M-PDCoV, we identify eight positive sera by western blot among 17 from "El Bajio" pig farms. Thus, the r M-PDCoV developed in this study, was able to detect antibodies against PDCoV. It has been mentioned that co-infection with PDCoV and PED (19.6%) are likely to occurred (Song et al., 2015). However, the amino acid identity (Stothard, 2000) of r M-PDCoV and the CV777 PEDV M protein (GenBank: AAK38659) is 23.5%, for TGE Miller M6 (GenBank: ABG89300) is 18.89%. This percentage of identity was consistent with the observed by Thachil and cols. These amino acid identity values suggest that the PDCoV consensus protein M used in this study is unlikely to cross-react with PED or TGE (Thachil et al., 2015). Also, this result is in accordance with reported that antibodies cross-reactivity of PDCoV with PED has not been observed (Jung et al., 2016; Ma et al., 2015).

On the other hand, according to the TM-Score value > 0.8 (table 2) the r M-PDCoV protein structure is similar to other reported PDCoV M protein sequences. It has been reported that that structures with a score higher than 0.5 assume roughly the same fold (Zhang & Skolnick, 2004, 2005). Therefore, it is presumed that a vaccine based on r M-PDCoV protein may stimulate the immunity against the disease. To validate the above, three groups of eight mice each was used to determine the r M-PDCoV capability to produce antibodies. Two doses were applied to each mouse subcutaneously at day one and at day 14. The sera were collected through the caudal vein at days 7, 14, 21, and 28. The antibody response induced in BALB/c mice by the r M-PDCoV protein was significantly higher ($P < 0.001$) using immunostimulating complex (Iscom®) (Fig 5). These results are in agreement with previously reported (Sun et al., 2009), who described that vaccines enhanced with Iscom® have been shown to be highly immunogenic, inducing both antibody and cellular immune responses (Sanders et al., 2005) .

Additionally, in figure 5, the line corresponding to r M-PDCoV show an increased antibodies ($P < 0.01$) production along the weeks (Fig 5) In this context, we found nine antigenic sites along the consensus amino acid sequence and from these, five were in the N-terminal region (Fig 2c). Also, the nine antigenic sites lie in hydrophilic regions ready to be recognized by the immune system. (Fig 2d). Furthermore, it has been reported that viral structural proteins, like M protein, possess much higher immunogenicity for T cell responses than the nonstructural proteins (Li et al., 2008). For instance, it has been observed that the M protein N-terminal region play a role as a dominant immunogen for cellular immune response (Liu et al., 2010). Similarly, in alpha, beta, and gamma-coronaviruses, the N-terminal region has an interferogenic activity to produce monoclonal antibodies (Baudoux et al., 1998; Laude et al., 1992).

These results indicates that the r M-PDCoV developed in this study has significant potential to be used as antigen in vaccine or in immune-detection systems. In this scenario, diagnostic system based on recombinant proteins has been frequently developed. Specifically, there has been an interest to develop a recombinant

protein (Balamurugan et al., 2010). For example, several immune assays based on recombinant proteins (S, M, N) from PEDV have been developed using *E. coli* as an expression system (Cao et al., 2013; Pan et al., 2015; Ren et al., 2011; Shenyang et al., 2007). Also, a recombinant TGEV N protein was developed with high sensitivity and specificity (Liu et al., 2001). Additionally, a recombinant S, M, and N protein have been developed to antibodies detection for PDCoV (Luo et al., 2017; Su et al., 2016).

In Mexico, despite PDCoV in an emergent coronavirus, there is no a developed recombinant systems to prevent and control de diseases. Also, in Mexico there is not enough information about the current prevalence of PDCoV. Moreover, recent findings about PDCoV shows an evolutionary change and adaptation leading to human infections by coronaviruses outside of the previously recognized human-associated coronavirus groups (Lednicky et al., 2021), indicating the important risk that PDCoV represents.

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Ethical approval

All procedures were in accordance with Mexican legislation (NOM-062-ZOO-1999-SAGARPA), based on the Guide for The Care and Use of Laboratory Animals, NRC. The experiment was previously approved under a permit from the LACUC (Institutional Animal Care and Use Committee), CENID-MA, INIFAP.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Data availability statement

The data that supports the findings of this study are available in the table 1 of this article

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Figure legends

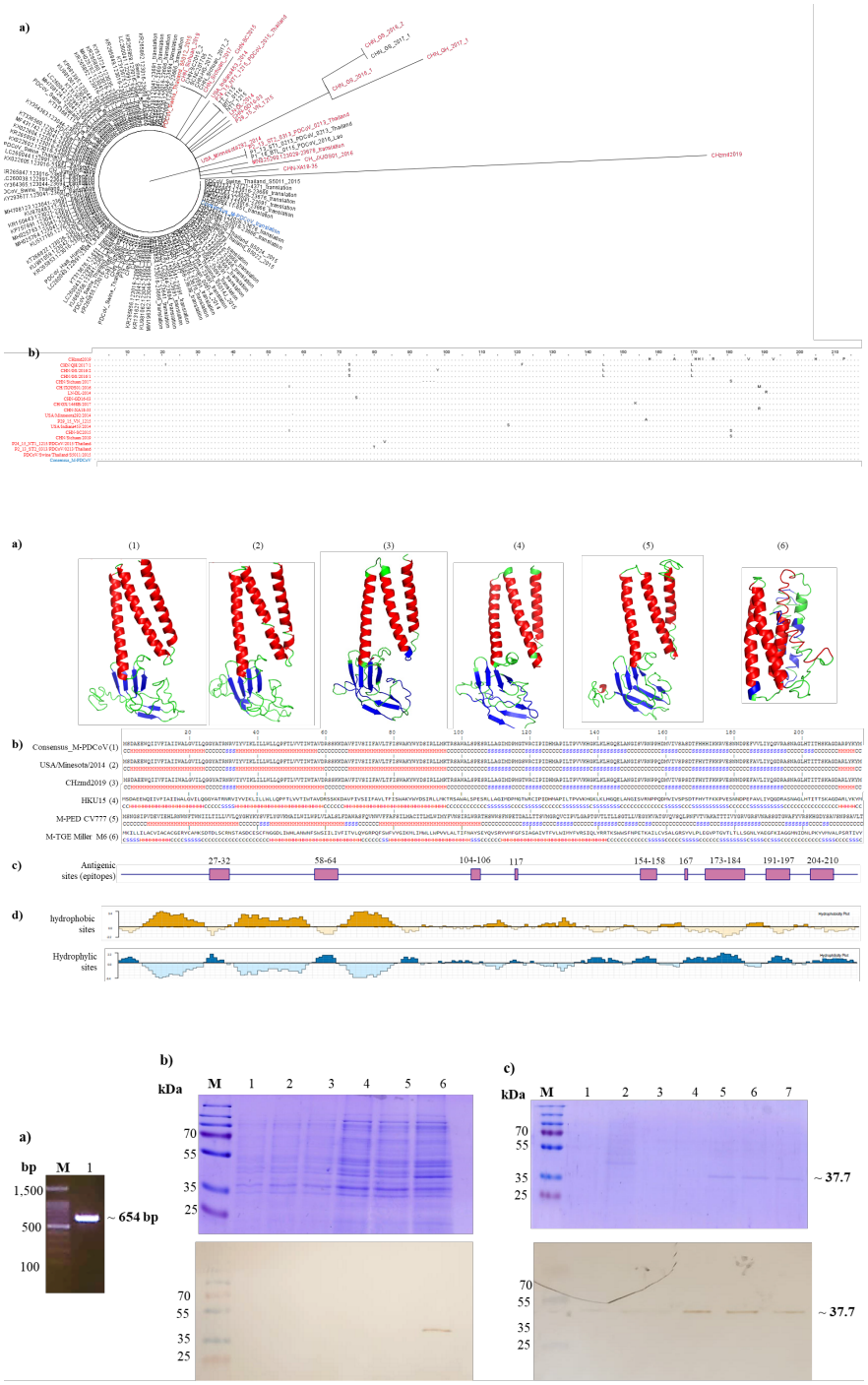
Figure 1. (a) Maximum likelihood phylogenetic tree displaying the 138 PDCoV protein M sequences available in Gen Bank. In blue, the consensus sequences used in this study. In red, 18 sequences showed differences in branch length. (b) Comparison of 18 sequences showed differences at amino acid level.

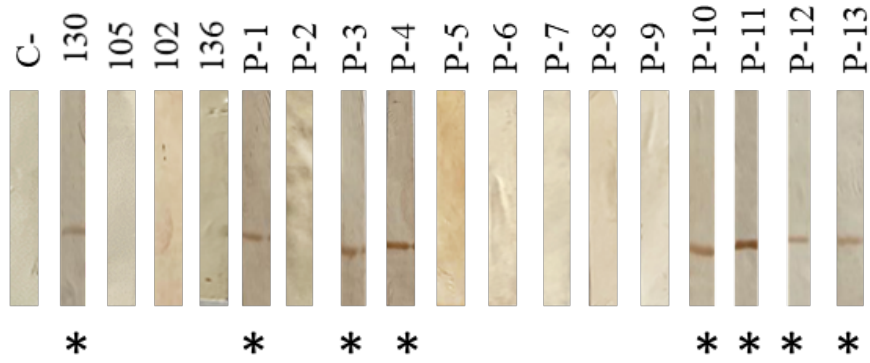
Figure 2 . Comparison of five models predicted by I-TASSER, antigenic sites and surface properties. **(a)** Predicted models for Consensus sequence, Chzmd2019, HKU15, M-PED CV777 and M-TGE Miller M6. **(b)** Amino acid sequence for each model indicated the predicted secondary structure, red: helix, blue: strands, black: coil.**(c)** Nine Antigenic sites predicted and their positions along the consensus sequence. **(d)** Consensus sequence surface properties indicating the hydrophobic and hydrophilic sites.

Figure 3 . **(a)** Integrity and weight verification of PDCoV-M 654 bp synthetic gene **(b)** Western blot and SDS-PAGE to verified *r* M-PDCoV in the expression essay. Lines 1 to 3 are an aliquot of the culture media before cell disruption. Line 4 is an aliquot after cell disruption. Line 5 and 6 are an aliquot after centrifuging the cultures, soluble and insoluble phase, respectively. The *r* M-PDCoV was found in insoluble phase as inclusion bodies.**(c)** Confirmation of *r* M-PDCoV after purification with Ni-NTA agarose column with His-tag affinity. The purified recombinant protein was observed in elutions 5 to 6.

Figure 4 . Western blot to determinate the capability of*r* M-PDCoV to recognized antibodies in 17 pig blood sera from "El Bajio" pig farm. Eight positives sera were determined and are indicate in the image. Negative control serum was from a sow keeping in laboratory

Figure 5. *anti* -PDCoV IgG distribution in sera samples obtained from three groups of eight mice each. Black dots line indicates PBS control group. White dots line indicates *r* M-PDCoV protein group. Triangle line indicates rM-PDCoV + Iscom as an adjuvant to enhance the immune response. Dots and triangle indicated the days of collection sera: 7, 14, 21, and 28 Bars indicated the standard error. The statistical significance P < 0.01 (**) and P < 0.001 (***).





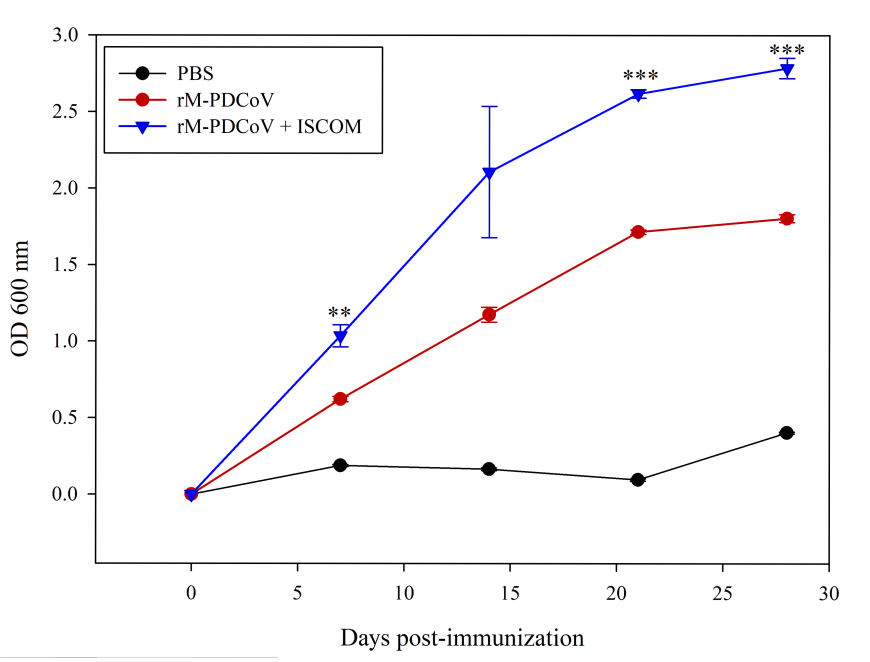


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Table 2.docx available at https://authorea.com/users/459168/articles/555527-cloning-expression-and-characterization-of-potential-immunogenic-recombinant-porcine-deltacoronavirus-pdcov-membrane-protein-from-a-synthetic-gene