RESEARCH REPORT
SWINE HEALTH

Title: Development of pseudotyped reporter viruses for detection and characterization of neutralizing antibody response to PEDV and Deltacoronavirus – **NPB #14-181**

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Industry Summary
The overall objective of this research was to generate biological reagents for detection and characterization of neutralizing antibody to PEDV and Porcine Deltacoronavirus. (PDCoV). The specific goal was to generate luciferase reporter viruses that express the spike (S) protein of PEDV and PDCoV and to use these reagents for detection and quantitation of virus-neutralizing antibody. We were able to generate PEDV virus-like particles but they were found to be poorly infectious in vitro. A variety of cell-culture conditions were tested to improve yield and infectivity of PEDV reporter virus, but we were not able to reproducibly generate high-titered PEDV pseudovirus stocks need for virus neutralization assays. At present, live infectious virus will be needed for analyses of neutralizing antibody in PEDV-infected or suspect pigs.

Keywords: PEDV, PDCoV, reporter virus, pseudovirus, virus neutralization reagents

Scientific Abstract:
One impediment to current PEDV and PDCoV research is the lack of good cell cultures systems to assess virus replication. The overall objective of this research was to generate biological reagents for detection and characterization of neutralizing antibody to PEDV and Porcine Deltacoronavirus. (PDCoV). The specific goal was to generate luciferase reporter viruses that express the spike (S) protein of PEDV and PDCoV and to use these reagents for detection and quantitation of virus-neutralizing antibody. The PEDV and PDCoV S genes were synthesized, inserted into pcDNA3 expression plasmids, and shown to express high levels of protein in cells. To generate reporter virus, the PEDV and PDCoV S gene expression plasmids were co-transfected with plasmids containing lentivirus core genes and luciferase reporter genes. Virus-like particles were produced but were found to be poorly infectious in Vero cells and MDCK. Expression plasmids containing deletions or mutations in the endoplasmic retention signal were made, and resulted in increased expression of the PEDV S protein at the cell surface; however, this did not restore infectivity of the reporter virus. A variety of cell-culture conditions were tested to improve yield and infectivity of PEDV reporter virus, but we were not able to reproducibly generate high-titered PEDV pseudovirus stocks. PEDV appears to differ from SARS and MERS coronaviruses in that the S protein alone may not be sufficient for production of infectious pseudotyped virus. At present, live infectious virus will be needed for analyses of neutralizing antibody in PEDV-infected or suspect pigs.

Introduction

The overall objective of this research was to generate biological reagents for detection and characterization of neutralizing antibody to PEDV and Porcine Deltacoronavirus. (PDCoV). The S protein of coronaviruses plays an essential role in receptor binding and virus entry, and is the major target of virus neutralizing antibody. Reporter viruses that express the spike (S) protein of PEDV or PDCoV would have the receptor binding and cell entry properties of wild-type, replication competent PEDV or PDCoV. As such, infection of target cells by the reporter virus would be mediated through interactions between the PEDV or PDCoV S protein on the reporter virus surface and the receptors and co-receptors on the surface of susceptible cells. If successful, development of the PEDV and PDCoV envelope pseudotyped reporter viruses would allow rapid, high-throughput, and virus-specific detection of neutralizing antibody. The PEDV and PDCoV pseudoviruses will mimic replication competent viruses with respect to in vitro host range and neutralization by homologous and heterologous sera, and can be used in high-throughput assays to detect, quantify, and characterize the neutralizing antibody response to PEDV and PDCoV. Because these assays do not utilize infectious, replication viruses, they can be conducted using BSL2 laboratory safety conditions.

Objectives:

1. **Generate pseudotyped luciferase reporter viruses expressing the spike protein from PEDV or PDCoV.** The objective of this aim was to develop a lentivirus-based luciferase reporter virus that incorporates the spike protein (S) from either PEDV or PDCoV, such that virus attachment and entry is mediated solely by the coronavirus S protein.

2. **Develop an in vitro assay to quantify infectivity of pseudotyped reporter viruses** In this aim, the infectivity of the pseudotyped reporter viruses was tested in various cell types known or hypothesized to support PEDV and/or PDCoV infectivity. Serial dilutions of virus stock will be inoculated onto cells, and infectivity will be assessed at 72 hr post infection by quantification of luciferase reporter gene activity.

3. **Develop a virus neutralization assay to quantify neutralizing antibody to PEDV or PDCoV.** Using the in vitro assay developed in Aim 2, the objective of this aim was develop a pseudovirus inhibition assay to detect and quantify neutralizing antibody to PEDV or PDCoV.

Materials & Methods:

Overall experimental design

The overall approach for generating PEDV and PDCoV pseudotyped reporter viruses was similar to that used for the pseudovirus incorporating the MERS coronavirus S protein, as well as our previous studies with Influenza A H5N1 and equine infectious anemia virus pseudoviruses. Briefly, the S protein from PEDV was cloned and expressed in the eukaryotic expression plasmid pcDNA3.1. Protein expression was confirmed in transient expression assays using immunofluorescence. To generate pseudovirus, the PEDV-S plasmid were co-transfected with the lentivirus core plasmid and the luciferase reporter plasmid, which expresses the structural and regulatory genes and viral RNA needed for assembly of the pseudovirus. Infectivity of the PEDV pseudotyped virions were assayed by titration in Vero cells.

Molecular cloning and expression of PEDV S genes. PEDV spike gene sequences were obtained from Genbank and were commercially synthesized and then were cloned into the eukaryotic expression plasmid, pcDNA 3.1 using standard molecular biology protocols. To confirm protein expression and evaluate sub-cellular localization, plasmid DNA was transfected into HEK293T cells. After 48 hr, cells were fixed and examined by immunofluorescence using PEDV-specific monoclonal antibody.

Production of PEDV pseudotyped reporter virus. PEDV-S plasmids were co-transfected into HEK293T cells with 5 μg of pEV-53B encoding the lentiviral core proteins of equine infectious anemia virus and 5.5 μg of the luciferase reporter plasmid plgSIN6.1Luc. At various times post-transfection, cells were incubated with trypsin to induce the release of PEDV-pseudovirus from the surface of the producer cells. Supernatants were collected 48 h post-transfection, clarified by centrifugation, and stored at −80°C.

Titration of PEDV-pseudovirus. Reporter virus pseudotyped with PEDV-S were titered on Madin-Darby canine kidney cells (MDCK) and Vero cells. Cells were seeded in 96-well plates at 2 x 10^4 cells/well in Dulbecco's modified Eagle medium supplemented with antibiotics and heat-inactivated fetal bovine serum. The following day, cells were inoculated in triplicate with 10-fold serial dilutions of pseudovirus stock in the presence of 8 μg/mL polybrene. At 48 h post-transduction, cells were lysed and assayed for luciferase activity using the ONE-Glo Luciferase assay system (Promega). Luciferase activity was quantified using a Centro XS3 LB960 illuminometer (Berthold Technologies) and results reported as relative light units (RLU)/mL supernatant.

Results:

Objective 1. Generate pseudotyped luciferase reporter viruses expressing the spike protein from PEDV or PDCoV

We successfully constructed a number of PEDV S protein expression plasmids and demonstrated protein expression in transfected cells. Expression of the wild-type PEDV spike protein (Genbank: AFV59240, CH/HBXQ/10) was confirmed by immunofluorescence using monoclonal antibody to the PEDV spike protein (Figure 1A).

The production of pseudovirus requires encapsidation of the lentivirus core by the PEDV envelope glycoprotein, the S protein. In retroviruses, the viral core proteins and envelope proteins traffic to the plasma membrane and assembly occurs at the plasma membrane. Coronaviruses do not assemble at the plasma membrane, and it is possible that the PEDV spike protein would be retained ER. If so, the S protein would assemble with the lentivirus core and no pseudovirus would be released. To test this, we mutated the ER retention signal in the spike protein and generated a second PEDV pseudovirus, designated PEDV-HBXQ-ERM. To differentiate between cytoplasmic and cell-surface expression, transfected cells were fixed in either methanol:acetone or paraformaldehyde, respectively. Both the wild-type and mutant S protein was expressed in the cytoplasm (Figure 1B, methanol:acetone); however only the spike protein mutated the ER retention signal localized to the cell surface (Figure 1B, paraformaldehyde). That suggests that mutation of the ER retention signal of the spike protein was required to target the spike protein to the cell plasma membrane. Because the ER retention signal is located at the C-terminus of the S gene, deletion of the signal would not be expected to alter the antigenicity of the protein. The wild-type PEDV does not readily grow in cell culture, so we synthesized the S gene from a cell-culture adapted strain, caPEDV and generated expression plasmids containing either the full-length caPEDV-S or the caPEDV-S-ERM mutant.

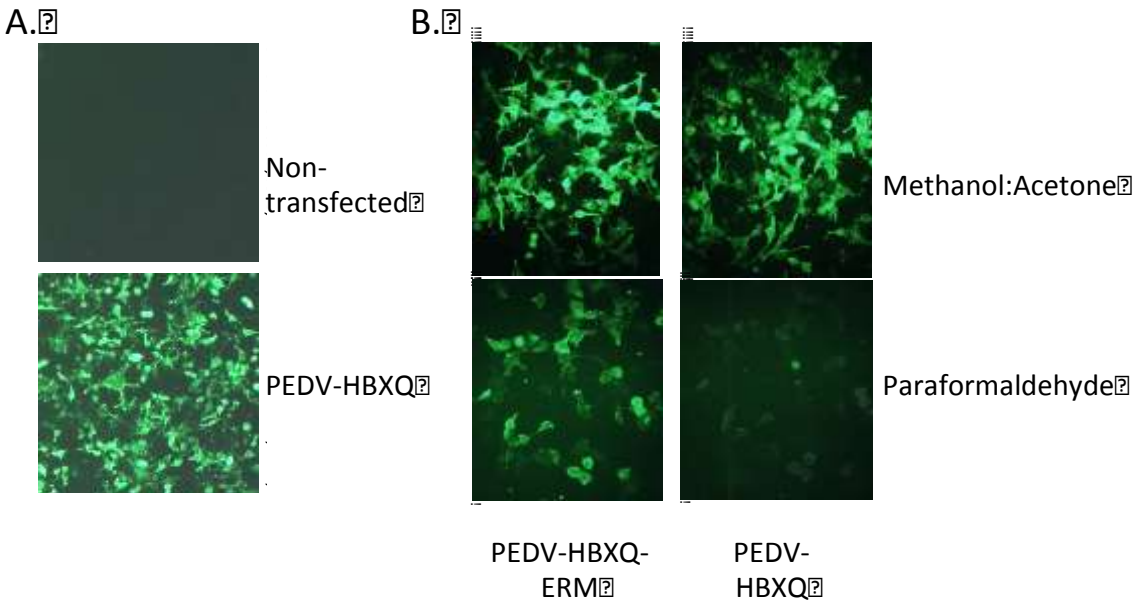

Figure 1. Detection of PEDV S protein by immunofluorescence using monoclonal antibody HV37-11

In summary, we generated four expression plasmids containing the spike protein of PEDV, including the full length ER retention mutants of the S gene from the wild-type HBXQ and the cell-culture adapted strain (caPEDV-S). All constructs were shown to express in transfected HEK293T cells; however, only the ER retention mutants were expressed on the cell surface. In addition, we generated an expression plasmid using the S gene from the HKU15 strain of PDCoV.

Objective 2. Develop an in vitro assay to quantify infectivity of pseudotyped reporter viruses

The goal of this aim was to develop and in vitro method to quantify the infectivity of pseudotyped reporter viruses. To generate stocks of PEDV pseudovirus, HEK293T cells were co-transfected with plasmid DNA containing the various S genes expression plasmids described above along with retroviral core genes and the luciferase expression vector. At 48-72 hours after transfection, supernatants were collected, aliquoted, and assayed for infectivity of pseudotyped reporter viruses, as measure by luciferase expression in target cells. Briefly, pseudovirus stocks were serially diluted, inoculated onto target cells and at 48-72 hr following infection cells were collected, lysed, and assayed for luciferase activity in a luminometer.

Initial studies using the S protein from either the wild-type PEDV or the HKU15 strain of PDCoV isolate showed no luciferase activity, indicating that these pseudoviruses were not infectious. We hypothesized that the lack of infectivity was due to the cell tropism of the S gene and/or incorporation of the S protein into the budding virions. To test this, subsequent studies focused on PEDV, using the S gene from cell-culture adapted strains and constructs with mutations in the ER retention signal, designated ER mutants (ERM). Results from these studies showed a low, but significant level of luciferase activity in Vero cells infected with either of the caPEDV-ERM pseudotyped virus, or with the HBXQ-ERM pseudotyped virus. Thereafter, a number of studies were done to develop high-titered PEDV pseudotyped reporter virus.

It is known that trypsin plays an important role in virus infectivity. Therefore, we examined the effect of adding porcine trypsin during pseudovirus production and/or during titration in Vero cells. We again observed a low but significant level of pseudovirus infectivity with both wild type and cell culture adapted PEDV genotypes that contained mutations in ER retention signal. Additional variables tested included: the amount of DNA used

during the production of pseudovirus stocks; the effect of growth media and media supplements; and the concentration, source, and timing of trypsin treatment.

Based on these findings, we have worked to optimize the conditions that increase pseudovirus titer. Variables tested included the amount of DNA transfected (0.5ug to 3ug), different type of media (DMEM+0.3%BSA, aMEM+0.3%TPB) during pseudovirus production. We have also tested various concentrations of trypsin added during pseudovirus production (1 – 9ug/ml) as well as during infection of Vero cells and duration of trypsin treatment. Addition of trypsin during infection in Vero cells has resulted in more than a 10-fold increase in pseudovirus titer.

The highest titers of PEDV pseudovirus were generated using with 1.5ug of PEDV S gene plasmid, 1.5ug lentivirus core and 1.5ug of reporter plasmid in MEM+0.3%TPB containing 3ug/ml porcine trypsin, and titered on Vero cells in incubated 5hrs prior to transfection in media containing 5ug/ml porcine trypsin. Using these conditions, we generated titers of $3x10^4$ RLU/ml from HBXQ-ERM pseudovirus and $1x10^5$ RLU/ml from pseudovirus with caPEDV-ERM pseudovirus.

Based on these results, we scaled up the culture conditions to generate enough pseudovirus stock to use in virus neutralization assays. Unfortunately, despite numerous attempts and continued modification in experimental protocols, we were not able to generate high-titered PEDV pseudovirus stocks.

Objective 3. Develop a virus neutralization assay to quantify neutralizing antibody to PEDV or PDCoV.

The goal of this aim was to use the in vitro assay developed in Aim 2 to develop a pseudovirus inhibition assay to detect and quantify neutralizing antibody to PEDV or PDCoV. Because we were not able to generated high-titered PEDV-pseudovirus stocks, we were not able to complete this objective.

Discussion:

The S protein of coronaviruses plays an essential role in receptor binding and virus entry, and is the major target of virus neutralizing antibody. The goal of the project was to produce of luciferase reporter viruses expressing PEDV or PDCoV S proteins that would mimic replication competent viruses with respect to in vitro host range and neutralization by homologous and heterologous sera. If successful, these reagents could be be used in high-throughput assays to detect, quantify, and characterize the neutralizing antibody response to PEDV and PDCoV. Because these assays do not utilize infectious, replication viruses, they can be conducted using BSL2 laboratory safety conditions. Unfortunately, we were not able to successfully produce high-titered PEDV or PDCoV pseudovirus.

The reasons for the lack of infectivity of PEDV pseudovirus are not clear. Our analyses indicated a high level of S protein expression in transfected cells, and we determined that deletion of the ER retention signal was required for trafficking of the S protein to cell-surface. The importance of cell-surface expression of the S protein was confirmed in our infectivity assays, as higher titers were observed with pseudovirus stocks generated with ERM plasmids than with full length. HBXQ or caPEDV plasmids.

Conformational rearrangement of viral envelope glycoproteins can play an important role in virus entry. In many cases, the rearrangements are induced by proteolytic cleavage of the glycoprotein. To mimic cleavage events that take place during natural infections, in vitro studies often add trypsin during early and/or late stages in the virus replication cycle. We explored the effects of trypsin treatment both during production of PEDV pseudovirus as well as during infection of target cells. Although we did get some encouraging evidence of infectivity, the results were not consistent or reproducible. It is possible that cleavage alone is not sufficient to induce the proper rearrangement needed for binding and entry, and that other viral and/or cellular co-factors are needed to generate an entry-competent PEDV pseudovirus.

Summary

The goal of the project was to produce of luciferase reporter viruses expressing PEDV or PDCoV S proteins that would mimic replication competent viruses with respect to in vitro host range and neutralization by homologous and heterologous sera. The PEDV and PDCoV S genes were synthesized, inserted into expression plasmids, and shown to express high levels of protein in cells. Virus-like particles were produced but were found to be poorly infectious in Vero cells and MDCK. A variety of cell-culture conditions were tested to improve infectivity, but we were not able to reproducibly generate high-titered PEDV pseudovirus stocks. PEDV appears to differ from SARS and MERS coronaviruses in that the S protein alone may not be sufficient for production of infectious pseudotyped virus. At present, live infectious virus will be needed for analyses of neutralizing antibody in PEDV-infected or suspect pigs.