Title: Development of novel reovirus-based mucosal vaccine vectors for PEDV antigen production – NPB #15-146

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Industry Summary

The goals of this project were to identify methods for the high-level expression of the PEDV S protein receptor-binding domain (RBD) from within the genome of the clinically benign virus, Mammalian orthoreovirus (MRV). The eventual goal is to utilize MRV as a replication competent vaccine vector for the mucosal delivery of PEDV antigens to swine. Targeting the mucosa has multiple immunologic and practical advantages including the induction of a more effective immune response and ease and cheaper cost of delivery via oral administration. The work performed in this funding period represents initial benchmarks necessary for achieving these goals, namely identifying the PEDV RBD, as well as a method to drive expression of the PEDV RBD from within the MRV genome.

The MRV genome is very compact, and each gene segment encodes for a protein that is absolutely required for virus replication and cannot be deleted. In our efforts to design MRV for use as a vector, we have previously identified a mechanism to insert foreign genetic sequences within the MRV genome without substantially disrupting the replication and packaging of the virus. The next step in readying the virus for use as a vaccine vector was the subject of this proposal. In order to deliver antigens to cells via MRV, they must not only be encoded within the genome, they must also be expressed as peptides or proteins that can be recognized by the immune system. We tested two methods appropriated from other viruses, the introduction of a sequence, called an internal ribosome entry site (IRES), that can recruit the cellular translation machinery to the internal portion of a gene, and introduction of a sequence that directs the cellular machinery to start translating a protein directly following the termination of an upstream protein to drive dual production of MRV proteins and foreign genetic material (an HIV gp41 peptide and the small fluorescent protein, UnaG). We found that while both of these approaches were capable of driving the production of two proteins from a single mRNA when expressed alone in cells, they were unable to do so when expressed from the virus genome. It is unclear at this time why we were unsuccessful at achieving these goals, and we continue to troubleshoot the assay. Meanwhile, we also were working to identify the PEDV S RBD. In this portion of the project, we were able to identify a small portion of the S protein that when blocked by antibodies, neutralizes virus infection. Future work will include incorporating this region into the MRV genome to confirm expression and detection of the antigen and testing other strategies for dual protein expression from the MRV genome. Contact Cathy Miller (clm@iastate.edu) for additional details.
Keywords

Mammalian orthoreovirus, (MRV) Porcine epidemic diarrheal virus (PEDV), mucosal vaccine, vaccine vector, S protein receptor binding domain (RBD)

Scientific Abstract

Porcine epidemic diarrheal virus (PEDV) is a member of the family Coronaviridae that causes severe enteropathogenic disease in infected pigs resulting in 80-100% mortality in unweaned piglets. Since breaking in 2013, PEDV has resulted in the death of over 8 million piglets and has had a devastating impact on farms throughout Iowa and the United States. Two parenterally delivered vaccines have been conditionally licensed for PEDV in the US. However, immunologic protection for vulnerable piglet populations against this family of viruses has been historically difficult to achieve by parenteral vaccination alone. Based on data from other members of the family Coronaviridae (e.g., transmissible gastroenteritis virus, TGEV), we hypothesize that to achieve the high levels of immunoglobulin in the milk of sows required for piglet protection (lactogenic immunity), a vaccination strategy that includes mucosal vaccines will be necessary. Mammalian orthoreovirus (MRV) is a clinically benign virus that naturally and specifically targets and infects mucosal associated lymphoid tissue, resulting in a high level expression of proteins encoded within the MRV genome in the precise location necessary for induction of mucosal immunity. This suggests MRV is an ideal candidate vector for delivery of PEDV antigens to the mucosa to stimulate lactogenic immunity. We have recently discovered a mechanism that allows manipulation of the MRV genome such that foreign genetic sequences can be packaged into recombinant virus without disrupting viral replication. We have additionally identified a putative neutralizing PEDV antigen comprising the predicted PEDV spike (S) protein receptor binding domain (RBD). In this study, we have begun to test multiple strategies to drive high level expression of the PEDV S RBD antigen from recombinant MRV.

Introduction

A number of basic science benchmarks must be met to develop MRV as a mucosal PEDV vaccine vector. These include, 1) Development of a method to incorporate foreign genetic sequences into the MRV genome while maintaining replication competence, 2) Demonstration of packaging of foreign genetic sequences into recombinant MRV (rMRV) 3) Identification of a minimal antigenic region of the PEDV S protein for incorporation into rMRV vectors, 4) Discovery of a mechanism for expression of the PEDV S antigenic region from replicating or non-replicating rMRV, and 5) Animal studies to examine the efficacy of MRV-based vaccines in protection of pigs from PEDV. The Miller and Yoon labs have already completed three of these benchmarks:

1) A method was developed by the Miller lab to separate overlapping MRV protein open reading frames (ORFs) from packaging sequences (PACs) to create sites within the MRV genome where foreign genetic sequences can be placed without disrupting ORF or PAC sequences required for virus replication (Fig. 1).

![Figure 1](image-url)
2) Multiple non-MRV sequences, including the Hepatitis C internal ribosome entry site (HCV IRES, 300 nts), and a gene encoding the UnaG protein (417 nts) have been cloned into the MRV genome and rescued by the Miller lab, demonstrating that foreign sequences can be packaged into rMRV (Fig. 1).

3) Utilizing polyclonal serum isolated from PEDV infected pigs and a series of plasmids expressing PEDV S gene fragments, an antigenic fragment of S, which is predicted by bioinformatics to include the receptor binding domain (RBD) of the protein, has been identified by the Yoon lab (Fig. 2).

Objectives
The objectives for this funding were to identify the most efficient mechanism for high-level protein expression of the PEDV antigenic S RBD fragment from recombinant mammalian orthoreovirus (rMRV) in mammalian cells, and rescue rMRV expressing PEDV S Receptor Binding Domain (RBD).

Materials and Methods
Traditional molecular cloning methods were used to create plasmids containing MRV genes that expressed foreign genetic sequences (UnaG or an HIV gp41 peptide as surrogates for the PEDV S RBD). These techniques consisted of ordering synthetic DNA encoding either UnaG or Flag-tagged HIV gp41 peptide flanked on either end with restriction sites. The acceptor plasmids and the synthetic DNAs were then cut with the restriction enzymes and the resultant digested DNAs were purified and ligated to create the plasmids. These plasmids were then tested for expression in mammalian cells [Baby Hamster Kidney (BHK) cells that constitutively express the T7 polymerase]. BHK-T7 cells were transfected with the described constructs and at 24 hours post-transfection, cells were fixed with paraformaldehyde and stained with antibodies against MRV proteins and the foreign genetic sequence-encoded protein, followed by staining with fluorescent secondary antibodies. The stained cells were then imaged via fluorescent microscopy. To create viruses containing the foreign genetic material, 4 plasmids containing the other 9 MRV genes, plus the plasmids containing the mutant gene were transfected into BHK-T7 cells. After 5 days incubation, cells were harvested and subjected to three freeze/thaw cycles. The lysates were then utilized in a plaque assay on murine L929 cells. Briefly, L929 cells were plated and after 24 hours, lysates were overlaid on plates and incubated for 60 minutes. Cells were then rinsed and a 1:1 mixture of 2X media and agar were overlaid onto the infected cells. Plates were observed for virus plaques, and if plaques were present, they were picked and amplified by two passages on L929 cells. Purified mutant virus was subjected to RT-PCR to amplify the mutant gene which was then sequenced for accuracy. Mutant virus was additionally used to infect L929 cells, and at 24 hours post-infection, cells were subjected to immunofluorescence microscopy as described above.

Results
Three of the four objectives towards designing a method for expression of the PEDV S RBD from MRV genes have been investigated. We showed that we could introduce and rescue recombinant MRV (rMRV) virus containing the hepatitis C virus (HCV) IRES driving expression of the fluorescent UnaG gene (as a surrogate for PEDV S) from the MRV M3 gene segment. We have been unable, despite repeated attempts, to demonstrate expression of UnaG in cells infected with this recombinant virus. This suggests that there is a problem with our approach. We created HCV IRES-driven UnaG clones with several other MRV gene segments, and were surprisingly unable to demonstrate dual expression of virus protein and UnaG in transfected cells. Taken together with our inability to visualize UnaG expression from the M3/HCV/UnaG rMRV virus, this suggests to us that the dual expression we were documenting in cells transfected with the M3-HCV IRES-UnaG gene likely resulted not from HCV IRES-initiated translation, but instead from expression of UnaG from a termination-reinitiation sequence downstream of the predicted PAC sequence. To maintain expression of the full-length MRV ORF, a wobble sequence comprising the region that overlaps the putative PAC was inserted in frame with the reinitiation AUG.
cryptic promoter within the M3 plasmid construct. This further suggests that the HCV IRES may not be a viable option for IRES expression in our system. We plan to revisit this approach in future work as necessary utilizing other well-defined animal IRES sequences to drive foreign gene expression from the MRV genome. As we were testing this virus, we began construction of clones for the termination/reinitiation approach described above (Fig. 3). We utilized the MRV S1 and L3 genes, which encode MRV proteins σ1 and λ1 respectively. We inserted a Flag-tagged HIV gp41 protein fragment juxtaposed on either side with the virus gene packaging sequence, and the defined stop/start sequence from the Influenza B virus M gene. Upon recovery and amplification of these plasmids, we transfected cells with the plasmids and used immunofluorescence to measure dual protein expression (Fig. 4). We measured robust and specific expression of both the viral proteins and the foreign protein in transfected cells. Unlike what we observed using the HCV IRES approach, we only recorded foreign gene expression in cells also expressing viral protein, suggesting that this is true dual expression from one gene segment, and not from an unidentified cryptic promoter.

In the prior progress report, we had not achieved rescue of any of these viruses by reverse genetics, however at this time, after much trouble shooting of the experiments, we have been able to rescue viruses that putatively contain the modified S1 gene. These viruses were found to be replication defective and have been somewhat difficult to propagate. Moreover, initial testing for dual expression of virus and inserted proteins have not been promising. Final confirmation that the rescued virus contains the inserted sequences is currently underway. We have additionally continued rescue attempts of viruses containing the modified L1 gene. Importantly, because little is known about the architecture of the genome segments within MRV virus capsids, it is likely we will need to insert the foreign genetic material into multiple gene segments to identify an acceptable fit. We are therefore continuing to create clones with the stop/start approach in other MRV genes. Finally, using monoclonal antibodies created from our purified predicted PEDV S RBD, we have made substantial progress confirming that this region of the S protein is in fact involved in virus entry. From multiple monoclonal antibodies, several have been identified that are neutralizing, likely as a result of binding the putative S RBD and preventing virus entry. We are currently in the process of sequencing the S RBD binding sites of these monoclonal antibodies, and have also begun the process of designing the approach we will use for cloning these sequences into our existing stop/start plasmids, as well as other MRV genome segments. Upon completion of this cloning, we will confirm dual expression of the virus protein and S RBD, and move forward with virus rescue.

Discussion

Although our ultimate goal of creating a recombinant replication competent MRV vector for expression of the PEDV S RBD was not met in this funding period, we were able to successfully drive the expression of two proteins from a single MRV gene in transfected cells. This is encouraging as it suggests our goals are possible to achieve and that we simply need to identify the correct approach combined with the correct MRV gene segment for the creation of an MRV PEDV S vaccine vector. Based on our findings, we believe the best approach will be utilizing the Influenza B gene stop/start sequence, as our
IRES approach data suggests there is a possibility of activation of cryptic promoters that can drive the expression of the foreign gene in transfected cells (where the plasmid is introduced to the cellular polymerase in the nucleus) but not in infected cells (MRV will not produce mRNAs in the nucleus). In the stop/start approach, the foreign genetic material is present in frame with the amino-terminus of the existing MRV protein, making it less likely that it harbors a cryptic promoter and more likely that the foreign and viral proteins will only be made by the directed stop/start mechanism. In fact, our data was very strong that this approach can be successful, however, we need to overcome difficulties with rescuing recombinant viruses with the foreign gene inserted. This work will continue in the future. We were also very successful in identifying what is likely the PEDV S RBD, as well as reagents that can be used to detect the peptide in cells. As we continue with this work, we will utilize this RBD fragment instead of surrogate peptides. We expect to be able to create a recombinant MRV virus that strongly expresses the PEDV S RBD in the near future. We predict that this virus will be able to replicate and express the PEDV S antigen in the swine mucosa to generate a strong mucosal immune response that includes neutralizing antibodies against PEDV, and that this increased response, as well as the practical and cost advantages of oral delivery will lead to a superior vaccination approach for enteric viruses of swine in the future.

This work was presented in the following abstract over the funding period: