

Project Title: Development of New Targets for Antiviral Intervention and New Diagnostics to Detect Foot-and-Mouth Disease Virus Infection

Overall Objectives: Antiviral Agents, Resistance Against Threat Diseases and Rapid and Accurate Detection and Diagnosis of Threat Agents

Objective#1: System to identify targets that can be used to develop antivirals to prevent FMDV or develop animals resistant to FMDV

User Needs and Priorities from Work Plan	Antiviral Agents, Resistance Against Threat Diseases		
Investigators and Institutions	P.W. Mason, S. Watowich, University of Texas Medical Branch, Galveston		
Approach	Deliverables	Progress Toward Deliverables	Percent Complete
1) Produce a system to identify targets that can be used to develop antivirals useful to prevent FMDV infection and/or to identify targets that can be used to develop FMDV-resistant livestock	1A) System for high-efficiency introduction of FMDV replicons into cells	We have succeeded in optimizing electroporation for the introduction of replicons into cell cultures.	100
	1B) Cell libraries that can be used for functional phenotype filtering	We have shown that shRNA against a known host virulence factor can protect cells from pathogen challenge, thus serving as a proof-of-concept for the RNAi functional phenotype filtering approach. In addition, preliminary studies have demonstrated that RNAi molecules that target antiviral genes can transiently protect cells from virus-induced cytotoxic effects, providing proof-of-concept for use of this technology in identifying host-encoded anti-FMDV genes. Finally, we have shown that pathways identified using functional phenotype filtering can be modulated to protect cells from virus (e.g., Sindbis virus) challenge, providing additional proof-of-concept regarding the merits of this approach.	65
	1C) Low pathogenicity replicons	<p>Significant progress has been made in engineering replicons to express multiple antibiotic resistance markers, marker genes and multiple predicted attenuating mutations. However, despite the use of all of these tools to obtain cells lines that can persistently replicate low-pathogenicity replicons in cell culture, our proposed strategy of allowing replicon genomes to accumulate mutations on their own (via the natural lack of fidelity of the viral polymerase) did not prove to be successful.</p> <p>To aggressively attack this problem we undertook a “random” mutagenesis strategy of the cDNAs used to produce the replicons using transposons. This strategy, designed to randomly introduce 15-nucleotide (5-codon) insertions within the FMDV protein-encoding regions of the replicon genomes is currently in the final stages of completion, but to-date, no low-pathogenicity replicons have been obtained.</p> <p>As an alternative method to produce cell lines that can express FMDV replicons, we have undertaken the construction of cells that express viral replicon cDNA in a quiescent form that can be activated via the cre-LoxP system to produce replicons. Although these cells will not have “low-pathogenicity” replicons per se, they will produce the desired antigen-expressing cells needed for Aim 2. We encountered problems with this approach that appeared to be attributed to the cytopathologic properties of uninterrupted FMDV polyprotein sequences present in these cDNA sequences, preventing us from obtaining cells harboring quiescent cDNA molecules.</p> <p>Our most recent approach has used transposon mutagenesis of the 3A and 2C genes to identify sites within the FMDV polyprotein that can be used to insert LoxP sequences for this strategy, overcoming the cytotoxicity cited above.</p>	80

	1D) Replicon-resistant cell lines derived from functional phenotype filtering	We established Mengo virus (a cardiovirus strain) as a surrogate system for FMDV. One-step growth curves and cpe kinetics for this virus were established in MEF and CHO cells. Control challenge experiments against MEF and CHO cells were performed that conclusively showed complete cell death in MEF cells. Functional phenotype filtering experiments were performed using this MEF cell-Mengo virus system and retrovirus insertional mutagenesis; we have not selected Mengo virus-resistant cells following Mengo virus challenge. We are repeating these challenge experiments using cells modified with higher multiplicity of infection retrovirus. In ability to produce cells with low-pathogenicity replicons means that any "hits" from the Mengo system will need to be screened with "live" FMDV at PIADC.	30
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Objective#2: Develop FMDV replicon-based diagnostic assay

User Needs and Priorities from Work Plan	Rapid and Accurate Detection and Diagnosis of FMDV-Infected Livestock		
2) Develop replicon-expressing eukaryotic cells as antigens that can be used to detect the presence of antibodies to FMDV	2A) First-generation antigens produced from replicon-expressing cell lines	To evaluate first-generation antigens, cells transfected with our replicons have been tested for their ability to react with a convalescent serum from an FMDV-infected bovine and monoclonal antibodies (MAbs) obtained from a lab in Italy. These studies showed strong reactivity with the convalescent sera, as well as reactivity with MAbs specific for several different FMDV non-structural proteins.	25
	2B) Cross-reactivity profile of replicon-derived antigens determined with a panel of sera (through PIADC collaborations)	Although originally considered as dependent on Aim 1C and completion of Aim 2A, we have now made a large batch of antigen with our optimized transfection (Aim 1A) protocol using high-pathogenicity replicons. This antigen will be used to test a collection of sera at PIADC. Arrangements are being made to coordinate a trip to PIADC by UTMB staff to test these samples in vitro.	25
	2C) Optimized preparations of antigens	We have compared the utility of highly aggressive, reporter-less replicons and replicons harboring reporter genes (that slow replicon growth) for large-scale production of antigens by high-efficiency electroporation. We have found that antigens produced by both replicons yield similar reactivity to both bovine and mouse monoclonal antibodies specific for FMDV, but we have opted to use reporter-containing replicons due to enhanced yield of antigen.	10

Technology Transition

None to report at this time.

Highlight for Research Briefs:

Recent advances have been made in identifying sites in replicons that can be used for insertion of LoxP sequences in the 2C and 3A genes. These are being used to insert new cre-lox components to produce a cDNA version for the replicons that can be used for help us to obtain the non-cytopathic replicons critical for continuation of this project. Antigens have been prepared from two replicons and are being QCed for transfer to PIADC where they will be tested by UTMB personnel with PIADC-provided sera of known FMDV specificity.

Interpretive Summary

Foot-and-mouth disease (FMD) is a highly contagious disease that affects all economically important US livestock. FMD has not occurred in the US since 1929, but it could be readily introduced, and it would spread rapidly. Introduction of FMD into the US would result in drastic restrictions on animal trade and a loss of faith in the animal agriculture of the country, producing economic hardship and chaos. The US maintains rigid quarantine and import restrictions on animals and animal products to prevent accidental introduction of FMD, but these are unlikely to be effective in deterring an agroterrorist introduction of FMD.

FMD is caused by a positive-strand RNA virus (FMDV). The virus exists as seven serotypes with multiple subtypes, producing antigenic diversity that severely complicates detection and control. Existing control is based on diagnostic testing performed at the USDA/DHS laboratory located on Plum Island, NY, at a considerable distance from the major livestock producers of the US. Chemically inactivated vaccines exist for FMDV. However, due to the cost imposed by needing to vaccinate against multiple serotypes of the virus, it is impracticable for the US to implement prophylactic FMD vaccination. However, the US, Canada, and Mexico maintain a bank of FMD vaccine at Plum Island, that could be released for ring-vaccination to control a US outbreak. Nevertheless, there remain concerns over the utility of this policy, due to the time lag needed for this vaccine to induce protective immunity, and the concern that FMDV-seropositive livestock could confuse eradication.

The goals of this project are to generate new products to protect the US from an agroterrorist attack with FMD. These new products will be developed by the application of new technologies to: #1) identify new targets for development of transgenic, disease resistant animals or therapeutics to thwart the spread of outbreaks of the disease (without generation of FMDV-seropositive animals) and #2) to produce viral components that can be used in diagnostic tests.

To achieve these goals, we have developed two specific objectives.

Our first objective is to produce a system to identify targets that can be used to develop antivirals useful in preventing FMDV infection and/or to identify targets that can be used to develop FMDV-resistant livestock. The system consists of two parts. The first part includes surrogates for live FMDV infection. These surrogates are needed, since live FMDV cannot be used in the US outside of Plum Island. One surrogate being used is a genetically deleted form of the viral genome (a replicon), which cannot infect animals but can autonomously replicate in cell cultures. During the first two years of the project we have made considerable progress in improving our replicon system. Nevertheless, until the end of year two we had not obtained non-cytopathic replicons. *However, in year three we have identified 2C and 3A insertion mutants that display reduced cytopathology we expect that this breakthrough will help us achieve our objectives of non-cytopathic replicons useful for this aim as well as aim #2.* The other surrogate we have chosen is Mengo virus, a relative of FMDV that is infectious for mouse cells, but is not a significant human or animal pathogen. During the first year of the project, we have established the Mengo infection system. The second part of this system is the recently described RNA silencing technology (also known as RNA interference or RNAi), which is capable of silencing individual genes in eukaryotic cells. In our system, RNAi technology will be used to generate a panel of cell lines, in which every expressed gene is individually silenced. Following challenge of this "library" of cells with our surrogate virus (and later with the replicon), the surviving cells will be analyzed to determine if they contain gene products critical for virus (or replicon) activity. In the next step, the identification of the host genes silenced in these cells will help us to define new targets for the development of antiviral therapeutics. Ideally, compounds that interfere with these host gene products will be deliverable in an aerosol format that will aid in response to outbreaks. In addition, the targets discovered may be useful for development of transgenic disease-resistant animals providing new methods of prevention of FMDV. During the first two years of the project, we have obtained and utilized a specific type of RNAi library, consisting of short hairpin RNAs (shRNAs). Libraries of cells expressing these RNAs have been challenged with Mengo virus, and we are now recovering and characterizing Mengo virus-resistant cells.

Our second objective is to develop new diagnostics, capable for use in high-throughput assays to detect FMDV infection. These diagnostic reagents will be obtained from FMDV replicon-bearing cells. To obtain a large and reliable source of these reagents, we are developing an FMDV replicon capable of persisting in cells in culture (*see aim 1 advances*). Our replicons, like FMDV, are highly pathogenic in cells in culture, thus, to create low-pathogenicity replicons that can persist in cells in culture, we have engineered replicons with antibiotic resistance marker genes, and we are introducing these into semi-permissive cells to obtain persistently cell lines. These strategies, which have been applied in our lab to other viruses, should allow us to obtain cells that persistently express FMDV replicons, providing a plentiful and inexpensive source of FMDV proteins that can be used in serological tests to determine if animals have been infected with FMDV. Since replicons share many of the same properties as live FMDV, these viral proteins should be produced in a manner very similar to those produced in FMDV-infected animals, maximizing the utility of these proteins in diagnostic tests. During the first year, we have reached improved replicon transfection efficiencies in multiple cell types and reached an important milestone: demonstrating that the cells containing our cytopathic replicons are a potent source of antigen for ELISA assays that can detect the presence of FMDV-specific antibodies in the sera obtained from an infected bovine.

Results and Interpretations

Aim 1:

1A: System for high-efficiency introduction of FMDV replicons into cells

To achieve this aim, we optimized synthetic replicon RNA transfection in BHK cells. Using high concentrations of RNA, and by altering reporter gene cassette composition, we have obtained transfection efficiencies of between 50 and 80 percent. These are sufficient to test transfected cells as a source for antigen (see Aim 2), and have helped us in completion of subAim 1C.

1B: Cell libraries that can be used for Functional Phenotype Filtering

To achieve this aim we obtained a "library" of plasmids that encode ~3 million distinct shRNA elements corresponding to all expressed mRNA in mouse embryo cells. Random members of this library were isolated and sequenced, and analysis of the sequence data indicated no apparent nucleotide, codon, or gene bias in the constructed library. All but one of the sequenced genes corresponded to recognized mouse genes. The shRNA plasmid library was used to transfect a MEF cell line to produce a cell library where each cell is uniquely modified by specific gene down regulation. This cell library was challenged by Mengo virus infection, and we attempted to isolate Mengo-virus resistant cells. Unfortunately, both the parental MEF cell line and cells transfected with the shRNA plasmid library were completely sensitive to Mengo virus challenge. Plasmids

containing the shRNA library are being packaged into recombinant retrovirus, with each retrovirus encoding a single shRNA gene, to increase shRNA expression levels, enable more efficient delivery of the shRNA into mammalian cells, and allow stable integration of the shRNA gene within the host cell genome. As proof-of-principle, we have demonstrated that shRNA directed against specific host cell genes necessary for microbial pathogenesis can protect cells from microbe-induced cell death. In these experiments, HeLa cells were transiently transfected with plasmids encoding shRNA designed to down-regulate anthrax toxin receptor 2. Modified cells survived subsequent challenge by anthrax lethal toxin, whereas mock-transfected CHO cells or CHO cells transfected with plasmids encoding shRNA for the luciferase gene died ~24-48 hours post exposure to anthrax toxin. In addition, we are generating a new library of shRNA plasmids that encode all genes expressed in the MEF cell line sensitive to infection by Mengo virus. Delivery time on this sub-aim is 1/2 way through year two of the project, so we are near our planned schedule.

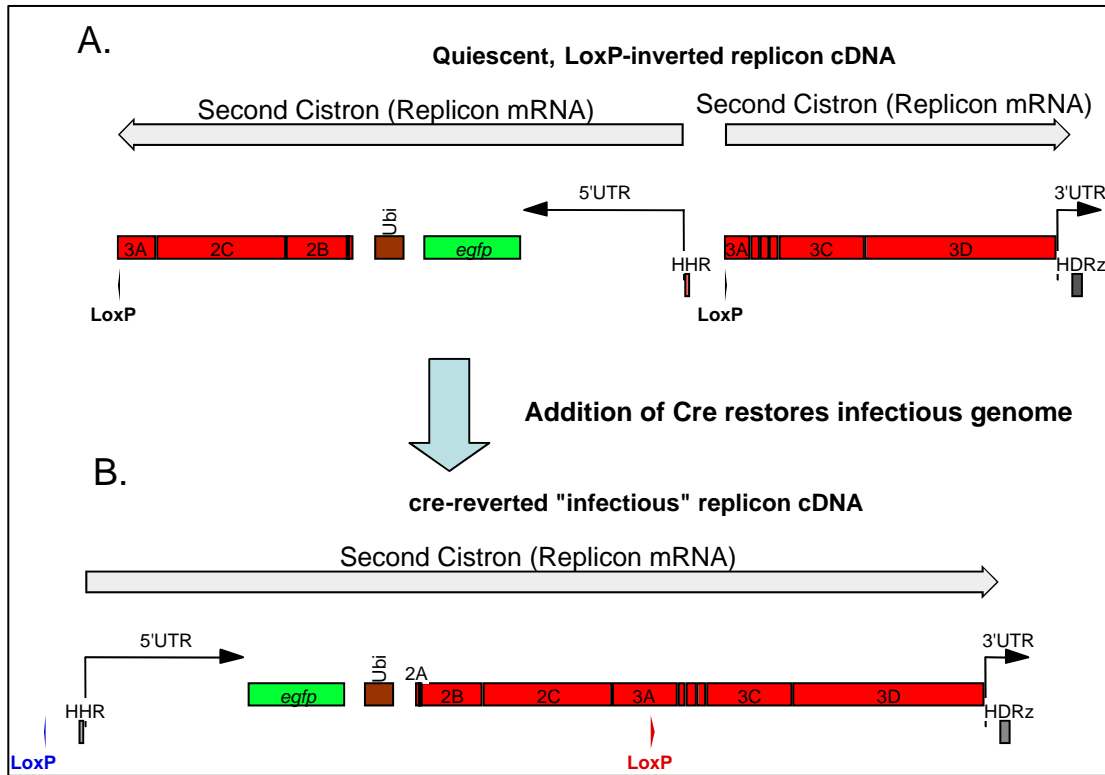


Fig.1: Schematic diagram showing: Panel A. structure of a quiescent replicon cDNA generated by inversion of a portion of the genome at an insertion of LoxP element into a site in the FMDV polyprotein in the gene for protein 3A. Panel B. structure of the cDNA that will be created by Cre-inversion of the inverted section, restoring the replication-competent polyprotein/replicon.

that would be more likely to persist. Although we have demonstrated that this strategy produces genomes that are somewhat defective in replication, we have not succeeded in obtaining clones from these libraries that satisfy our criteria for a low-pathogenicity replicons.

As an alternative strategy to the production of low-pathogenicity replicons, we have started to clone viral replicons in a "quiescent" cDNA format that will be quiescent in mammalian cell cultures, and plan to "launch" the replicons from these cells to produce large-scale cultures of high-pathogenicity replicon-bearing

1C: Low pathogenicity replicons.

This aim, which is critical to the completion of Aim #2 has proven much more difficult to achieve than expected. To obtain the low-pathogenicity replicons, we have focused on selection of cells that persistently replicate the FMDV replicon. Specifically, we have attempted to engineer replicons to encode antibiotic resistance genes, with the hopes of using antibiotic selection to select the "rare" low-pathogenicity replicons that arise in cultures transfected with the replicons, due to the inherent error-prone nature of the viral RNA polymerase. This strategy relies on the ability of antibiotics G418 or puromycin, to kill cells that do not harbor the replicon-encoded resistance genes for neomycin phosphotransferase (NEO), or puromycin acetyl transferase (PAC), respectively. Initial results with the insertion of NEO in the untranslated portion of the FMDV replicon genome produced replicons with unacceptably low transfection efficiencies. To overcome this, these genes were inserted within the polyprotein-encoding region of the FMDV genome, and replicons were obtained that expressed the viral polyprotein and the resistance genes. However, despite the improved transfection efficiency, we were unable to obtain low-pathogenicity replicons that conferred antibiotic-resistance and did not kill their host cells.

To enhance our chances of obtaining cells that harbor replicons, we have replaced the genes in our replicons with genes from strains of FMDV that were attenuated. However, even with

this added "twist", we were not able to identify low-pathogenicity replicon that could evolve from the electroporated RNAs. To further improve our odds, we have now used a random mutagenesis approach to add short (15 nucleotide) segments of nucleic acid to random location in a library of replicon RNAs, and then used these RNAs to transfect the cells. This strategy was used to help to "force" evolution to produce attenuated genomes,

cells. Although this strategy, which relies on the insertion of a LoxP element in the genome, does not produce low-pathogenicity replicons per se, it produces a cell line that can be used as an unlimited source of antigen that is needed for Aim #2. When these cDNAs were assembled, we discovered that they could not be stably maintained in BHK cells. Our analyses to date suggest that this is due to generation of transcripts from fortuitous promoters within the cDNA that produced transcripts encoding toxic FMDV products.

In year three we targeted “specific” insertion of transposons into the 3A and 2C genes of FMDV to identify sites in which we could insert LoxP elements, overcoming the toxicity of these fortuitously transcribed FMDV polyprotein products. By investigating the phenotype of 100s of these insertion mutants we have identified a handful that did not interfere with replicon RNA replication. We are now working to use these to assemble several LoxP genomes with this structure, one of which is shown in Fig.1.

1D: Replicon-resistant cell lines derived from Functional Phenotype Filtering

As discussed in 1B, shRNA libraries corresponding to expressed genes from mouse embryo cells have been obtained. Additional shRNA libraries corresponding to genes expressed in cultured MEF cells are being produced. Cells modified with an shRNA library were challenged with Mengo virus; however no surviving cells were identified. As an alternative approach, we are continuing experiments to select Mengo virus resistant cells from MEF cells modified by insertional mutagenesis with high multiplicity of infection retrovirus.

In related systems, we have challenged cell libraries with cytotoxic pathogens, and produced clonal cells that resist pathogen-mediated cell death. These preliminary studies provide the motivation for selecting clonal cells that resist Mengo and FMD viruses. Delivery time on this sub-aim is at the end of year three of the project, so we should be able to accomplish this sub-aim on schedule.

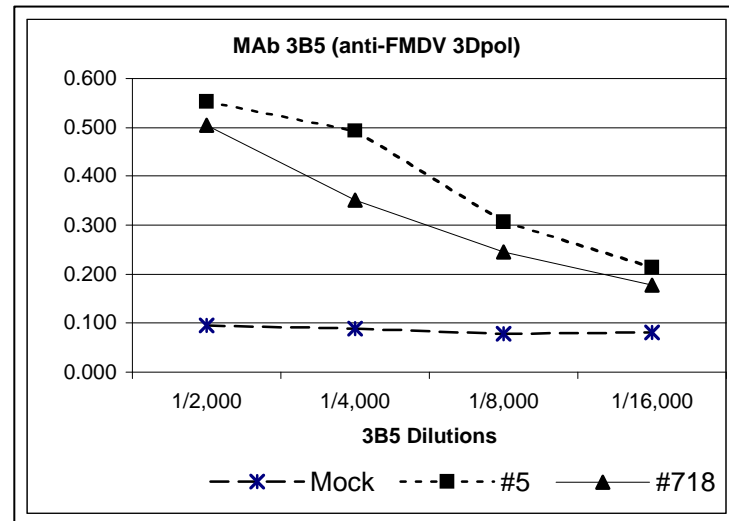


Fig.2. ELISA performed using extracts of BHK cells electroporated with the indicated FMDV replicons (#5 = reporter containing replicon of serotype A; #718 = reporterless replicon of serotype O) or “Mock” electroporated cells. Immunolon II plates were coated with cell extracts at a concentration of 15 ug/mL, and then incubated with the indicated dilutions of MAb shown on the horizontal axis, washed, incubated with a goat-anti-mouse serum, and the bound secondary antibody was detected with a TMB substrate. Vertical axis shows the optical density of the TMB reaction product.

Aim 2:

2A: First generation antigens produced from replicon-expressing cell lines.

The completion of this Aim is dependent on completion of Aims 1A and 1C. Clearly from the standpoint of cost and ease of production the use of cell lines that persistently express FMDV antigen (either in the form of low-pathogenicity replicons or in the form of a quiescent replicon cDNAs) as a source of antigen is an advantage. However, to begin to understand the characteristics of our replicon system, we have used BHK cells transiently replicating our FMDV replicon as an antigen for an enzyme-linked immunosorbent assay (ELISA), a format universally available for veterinary diagnostic work in the US. To this end, ELISAs were conducted with our replicon cell antigen and a gamma-irradiated (for safety reasons) “positive control” serum from a bovine infected with FMDV at PIADC. The results of these tests were quite impressive; they showed that a specific reaction could be obtained with highly diluted sera (1/1000 dilution in the case of this positive-control sera). In addition, the antigen was highly potent; antigen prepared from a single experiment (only 5 million BHK cells) is sufficient for at least 2,000 tests. Using our optimized transfection methods, we have now produced a large batch of antigen suitable for testing with a panel of sera that is being assembled by our DHS colleagues at PIADC, and which should be shipped to UTMB this fall.

2B: Cross-reactivity profile of replicon-derived antigens determined with a panel of sera (through PIADC collaborations).

No progress has been made in this area as of yet, sera should be here this fall, and depending on its makeup (controlled by our DHS colleagues) we plan to start using this sera with the antigen described in aim 2A this fall.

2C: Optimized preparations of antigens.

We have generated optimized antigens for delivery to PIADC with UTMB staff, as soon as arrangements can be made for the visit. Fig.2 shows an ELISA assay prepared with a serotype O antigen made from a reporter-less replicon (#718) and a serotype A antigen made from a replicon containing a reporter gene (replicon #5) that slows its replication, facilitating collection of samples

before cells were lysed by the replicon during preparation. Both preparations reacted well with a monoclonal antibody (MAb) to the FMDV polymerase protein (3D) (Fig.2). Attempts to demonstrate reactivity of this antigen to a gamma-irradiated bovine sera used in earlier studies (see above) revealed a high background reaction of the sera to BHK cells, which we have attributed to deterioration of the antisera upon storage. These replicon antigen samples are now ready for shipment to PIADC for more extensive serological testing once arrangements can be made for sending UTMB personnel to the island for testing.

Issues to be addressed by management:

Most important issue is "status of funding", please see below.

Status of Funding:

Status of funding, and in particular the availability of funds for years 4 and 5 is a major concern. The portion of the project (namely objective #2 - antigen preparation) that will be continued into years 4 and 5 is being accomplished by a single postdoctoral associate (100% support) with a small amount of assistance from a student (20% support) who is currently approved for entry to the facility at PIADC. Continued uncertainty about the budget might require the PI to recommend that the Associate begin the process of identifying a new laboratory to continue his training in, and eliminating support for the student.