Biological Systems – Project 4 | Year 3 Annual Report

Development of a Rapid and Inexpensive Diagnostic Kit for Foot-and-Mouth Disease

DHS Priority Areas Addressed	☐ Prevention ☒ Detection ☒ Response ☒ Recovery ☐ Education/Risk Communication
Proposal Section Addressed	Sections 5.2.1 and 5.3.1
Investigators	UCD: Tilahun Yilma

Objectives	**Deliverables**	Progress Toward Deliverables	Percent Complete
Provide a kit for rapid pen-side diagnosis of FMD	Confirmed sequence of Foot-and-Mouth Disease Virus P3D antigen	The plasmid FMD #502 contains a fragment of 1456 bp that matches the sequence of FMDV type A12 protein 3D. We transformed E. coli cells with the FMD #502 plasmid, and verified fragment sizes of the DNA preparations by restriction enzyme analysis.	100%
	Foot-and-Mouth Disease Virus P3D expressed in baculovirus vector	The FMDV P3D segment was subcloned into the baculovirus transfer vector pMelBac-B to produce clone pCRP3DpMelBacB. Sequence results of pCRP3DpMelBacB matches 100% with the sequence of the original plasmid FMD #502 containing the FMD-P3D. Co-transfection of the pCRP3DpMelBacB transfer vector and the Bac-N-Blue baculovirus expression system was successfully accomplished Recombinant candidates were first tested to confirm recombination using alternate proteins different to FMD-P3D as controls in a western blot system. Confirmed recombinant virus was amplified in Sf9 cell culture. The FMDV-3D protein was extracted from culture, purified using Ni-NTA affinity columns and partially characterized by western blot using guinea pig anti-FMDV sera.	100%
	Demonstrate baculovirus-expressed P3D protein antigen is functional in rapid strip test	The recombinant FMD-3D antigen has been partially standardized for an indirect ELISA with normal sera from mouse, cattle, sheep, goat and pig. Full standardization of the ELISA system will be completed using reference positive and negative serum samples from target species at the Plum Island Animal Disease Center. Development of monoclonal antibodies to FMDV-3D protein is in progress. These MAb's will be used to capture P3D antigen from serum samples in a chromatographic strip test. The P3D gene of FMDV was subcloned into the vector	

		pVax1. The resulting pVax1P3D amplified and used as a DNA vaccine to immunize (prime) groups of BALB/c and C57BL/6 mice. Purified baculovirus recombinant 3D protein was used to boost primed mice. Splenocytes were collected and fused with SP2/0 parental myeloma cells to produce hybridomas. The first round of hybridomas was screened for antibodies to the P3D by ELISA, and three positive candidates were identified. The results of the western blot confirmed the presence of antibodies that recognize the recombinant 3D protein and produced no reaction against wild type parental virus proteins or unrelated proteins expressing a HIS tag used as controls. Positive hybridomas are currently being cloned by limiting dilution. Amplification and partial characterization will be performed at the ILMB for all positive hybridomas obtained. After stabilization of cell line and confirmation for its ability to recognize the FMD-3D protein by ELISA and western blot, monoclonal antibodies will be produced and purified for further testing and characterization at the Plum Island Animal Disease Center. Monoclonal antibodies will be tested to confirm that they recognize all 7 types of FMD at the Plum Island Animal Disease Center. The best candidates will be used as binding and capture antibodies in the rapid strip test.	65%
	Estimate sensitivity & specificity of P3D-based rapid strip test	Validation of the antigen detection test kit will be conducted at the Plum Island Animal Disease Center	0%
	Limited field study of the pen-side strip test for the detection of antibodies against Foot-and-Mouth Disease Virus with repository of bovine sera	Not applicable	--

Interpretive Summary

FMD-P3D antigen detection kit

Foot-and-Mouth Disease is one of the most economically devastating diseases of livestock. Rapid identification of the cause of an outbreak of a vesicular disease in cattle, swine and sheep is an essential tool for controlling outbreaks of this disease. We are currently constructing a rapid diagnostic kit that will detect antigen to the virus that causes foot- and- mouth disease. This kit is based on technology similar to that used in commercially available pregnancy testing kits and will be about the size of a matchbox. The kit will be very simple to use and no special equipment or trained laboratory technicians are required to perform the test. A drop of blood or vesicular fluid from an animal in a suspected outbreak is all that is needed to detect foot-and-mouth disease viral antigen in just a few minutes using this technology. Current tests require sending samples to diagnostic laboratories and results are typically available in days or weeks. Thus, this kit will greatly increase the speed of detection of this virus in the event of an outbreak or for routine epidemiological surveys.

To construct the kit we will use a battery of monoclonal antibodies against a highly conserved viral protein (the viral RNA polymerase or P3D protein) bound to a chromatographic strip. Since this protein is nearly identical in all strains of the foot-and-mouth disease virus it will enable the detection of all serotypes. Thus, only one kit needs to be constructed. To screen for monoclonal antibodies to this protein, it was expressed at high levels in an insect virus (baculovirus), a technique that allows large quantities of a viral protein to be obtained and purified inexpensively and safely. The baculovirus-expressed P3D protein was purified and used to coat ELISA plates as a rapid screening test for monoclonal antibodies recognizing this protein.

Monoclonal antibodies to FMDV-P3D will be developed as capture antigen for the rapid test kit. The antigen-capture monoclonal antibodies will be bound to a chromatographic matrix strip and the reagents for the kit will be optimized. Once the kit is optimized, it will be produced in quantity and validated by using it to test large numbers of blood samples from animals positive or negative to foot-and-mouth disease virus.

We have extensive experience using the baculovirus system for the expression of proteins for use in diagnostic kits; we have developed diagnostic kits for three other diseases of livestock using this technique.

Results and Interpretations

The Foot-and-Mouth Disease virus 3D protein contained in the plasmid FMD #502 was successfully cloned into the baculovirus transfer vector pMelBac-B to produce the plasmid pCRP3DpMelBacB. Sequencing analysis showed that the sequences of the original plasmid (FMD #502) and the plasmid pCRP3DpMelBacB match 100% (Fig 1)

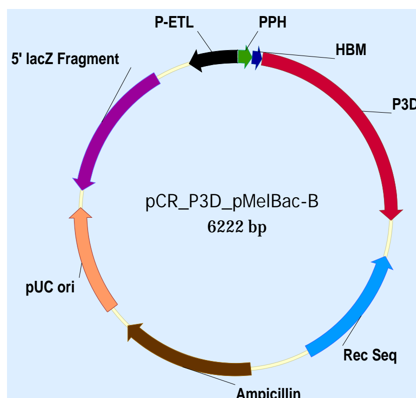
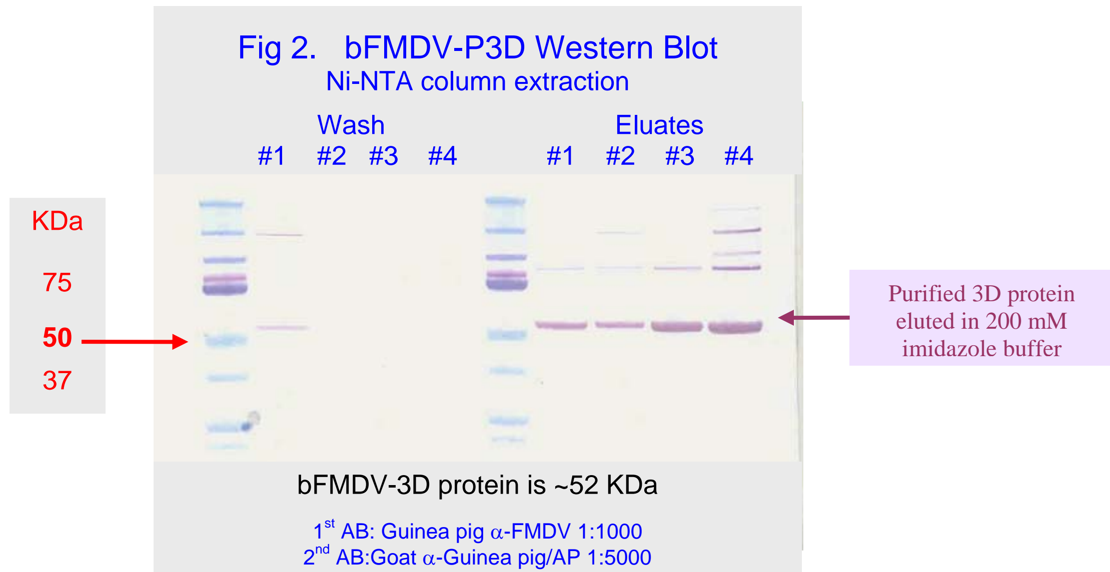


Fig 1. Baculovirus transfer vector pCRP3DpMelBacB

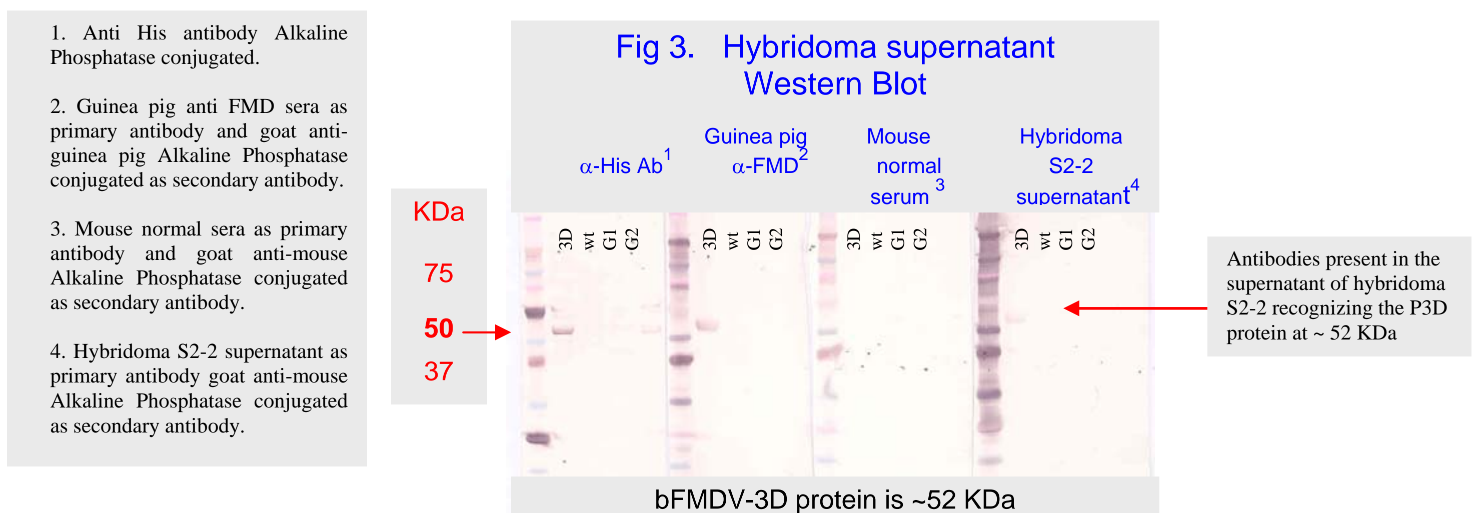
The transfer vector was for co-transfected with the Bac-N-Blue baculovirus expression using Sf9 insect cells. Co-transfection of the pCRP3DpMelBacB transfer vector and the Bac-N-Blue baculovirus expression system was successfully accomplished and recombinant candidates have been tested for confirmation of recombination by PCR and western blot using other baculovirus-expressed viral proteins as controls.

The confirmed recombinant virus was amplified in Sf9 insect cell culture. The FMDV-3D protein was extracted from infected cells, purified using Ni-NTA affinity columns and characterized by western blot using guinea pig anti-FMDV sera (Fig 2). The recombinant FMD-3D antigen has been preliminarily standardized for use in an indirect ELISA with normal sera from mouse, cattle, sheep, goat and pig. Full standardization of the ELISA system will be completed using reference positive and negative serum samples from target species (cattle, sheep, goat and pig) at the Plum Island Animal Disease Center.



Development of monoclonal antibodies to FMDV-3D protein is in progress. These MAb's will be used to capture P3D antigen from blood or vesicular fluid samples in a chromatographic strip test.

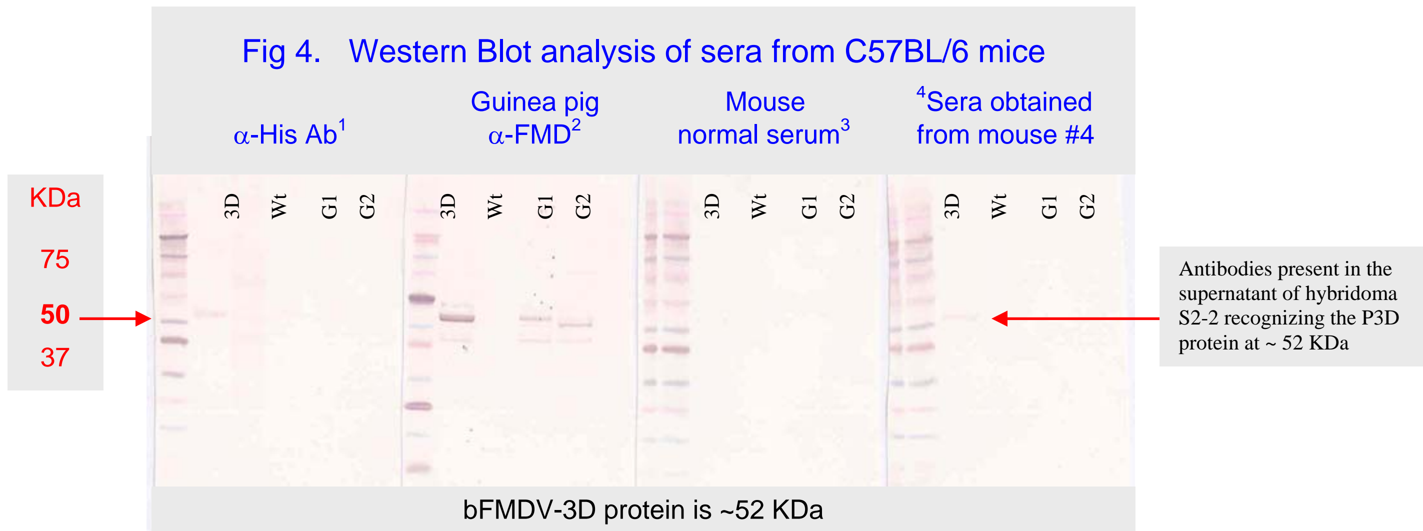
The P3D gene of FMDV was subcloned into the vector pVAX1. The resulting plasmid pVAX1P3D was amplified and used as a DNA vaccine to immunize (prime) groups of twelve BALB/c and twelve C57BL/6 mice. DNA vaccine was administered three times at 30 days intervals. Two weeks after the last DNA vaccination a group of two BALB/c mice were vaccinated with our baculovirus-expressed recombinant FMD-P3D. After three days the spleens were extracted and splenocytes collected and fused with SP2/0 parental myeloma cells to produce hybridomas. Hybridoma supernatants were screened for antibodies to the recombinant 3D protein in an indirect ELISA. Three hybridomas result positive from this –first- fusion (Fig 3).



3D stands for our recombinant baculo FMD-3D protein; wt stands for parental baculovirus wild type virus; G1 and G2 stands for the glycoproteins of Rift Valley Fever virus that express a HIS tag.

After further amplification, the supernatants of the ELISA positive hybridomas were tested by western blot against our baculo 3D protein; a parental baculovirus wild type virus and another two unrelated baculo recombinant proteins expressing a HIS tag. The results of the western blot confirmed the presence of antibodies that recognize the recombinant 3D protein and produce no reaction against wild type parental virus proteins or to the unrelated proteins expressing a HIS tag used as controls.

A second group of two C57BL/6 mice was vaccinated twice, fifteen days apart with the recombinant baculo FMD-P3D. Three days after the second booster the spleens were extracted and splenocytes collected and fussed with SP2/0 parental myeloma cells to produce a second round of hybridomas. Blood was also collected from these two mice and the serum tested by western blot to confirm the presence of antibodies to the recombinant FMDV-3D protein. Both sera were positive to the recombinant 3D protein and negative to the baculovirus wild type parental virus and to the unrelated two proteins expressing a HIS tag used as controls (Fig 4).



3D stands for our recombinant baculo FMD-3D protein; wt stands for parental baculovirus wild type virus; G1 and G2 stands for the glycoproteins of Rift Valley Fever virus that express a HIS tag.

1. Anti His antibody Alkaline Phosphatase conjugated.
2. Guinea pig anti FMD sera as primary antibody and goat anti-guinea pig Alkaline Phosphatase conjugated as secondary antibody.
3. Mouse normal sera as primary antibody and goat anti-mouse Alkaline Phosphatase conjugated as secondary antibody.
4. Blood serum from C57BL/6 mouse #4 as primary antibody goat anti-mouse Alkaline Phosphatase conjugated as secondary antibody.

Positive hybridomas will be cloned by limiting dilution to ensure that the antibodies are monoclonal. Amplification and partial characterization will be then performed to all positive hybridomas obtained. Once the monoclonal antibody is confirmed for its ability to recognize the FMD-3D protein, it will be produced in large quantities and purified for further testing and characterization at the Plum Island Animal Disease Center.

Monoclonal antibodies will be tested to confirm that they recognize all 7 types of FMD at the Plum Island Animal Disease Center. The best candidates will be used as binding and capture antibodies in the rapid strip test. Methodological and statistical validation of the antigen detection test kit will be also performed at the Plum Island Animal Disease Center where samples of different strains of FMDV are available.

Field studies of the rapid test kit will require collaboration with the Plum Island Animal Disease Center or other institution with access to FMDV infected animals under controlled conditions. Field test for FMD negative animals can be performed at any animal production facility in the area.

Renewal Considerations

Technical and methodological complications at the laboratory level have affected the time line and budget initially proposed. It is imperative to adjust and extend both issues in the renewal of the project to successfully complete the expected deliverable

Biological Systems – Project 4B

Development of a Rapid and Inexpensive Diagnostic Kit for Rift Valley Fever

DHS Priority Areas Addressed	☐ Prevention ☒ Detection ☒ Response ☒ Recovery ☐ Education/Risk Communication
Proposal Section Addressed	Section 5.2.1 and 5.3.1
Investigators	UCD: Tilahun Yilma

Objectives	Deliverables	Progress Toward Deliverables	Percent Complete
Provide an ELISA kit for rapid diagnosis of RVF	ELISA kit for the detection of antibody to RVFV Confirmed sequence of N protein of RVFV in transfer vector	The plasmid containing the cDNA of the small (S) segment of the Rift Valley Fever Virus (RVFV) genome was obtained from the Viral Biology Branch, USAMRIID, Fort Detrick, Frederick, MD on December 2004. The plasmid was sequenced and successfully compared with NCBI databases (S segment, accession number NC_002045). The S cDNA segment of RVFV provided by USAMRIID is 1690 bp long and contains a fragment of 735 bp that matches the sequence of the non-structural nucleocapsid (N) gene of RVFV. We designed forward and reverse primers to perform PCR cloning of RVFV N protein using *Pfu* DNA polymerase. The transfer vector was designed with BglII and Xba I restriction enzyme sites into the PCR product. We have purified the PCR product and subcloned the DNA into the baculovirus transfer vector pVL1393. The clone was named pVL1393RVFVN. Insertion of RVFV N protein segment was confirmed by restriction enzyme analysis and by sequence analysis. Sequence results of pVL1393RVFVN matches 100% with the sequence of the original plasmid of the S segment that contains the N protein of RVFV provided by USAMRIID.	100%
	Rift Valley Fever Virus N protein expressed in baculovirus vector	Co-transfection of the pVL1393RVFVN transfer vector and the Bac-N-Blue baculovirus expression system was successfully accomplished. Recombinant candidates have been tested for confirmation of recombination by western blot using mouse and/or rabbit anti-RVFV serums provided by USAMRIID along with alternate proteins different to RVFV as controls. The ILMB was authorized to obtain and use the MP-12 strain of RVFV to produce antibodies for the	100%

		characterization of the N protein antigen. The MP-12 virus strain was amplified in Vero cells, harvested and titrated. Groups of BALB/c and C57/BL mice were immunized with MP-12 strain virus. After a booster, blood was collected by exsanguinations and serum obtained and pooled. Collected sera have been confirmed for antibody reactivity to the recombinant baculovirus RVFV-N protein by western blot analysis.	
	Demonstrate baculovirus-expressed RVFV N protein antigen is functional in ELISA test	The recombinant RVFV-N antigen has been tested for antibody reactivity in an indirect ELISA system using sera from MP-12 vaccinated animals and negative sera from control mice. The indirect ELISA was most sensitive at a dilution of 1:400 of the current stock of RVFV-N protein producing an ELISA pos/neg ratio of 31.5 against pooled mouse sera.	100%
	Estimate sensitivity & specificity of RVFV N protein-based ELISA.	Validation of the RVFV-N ELISA antibody detection test kit with target species serum samples (cattle, sheep and goats) will be conducted in collaboration with the University of Texas Medical Branch or other institutions with access to a larger number of biological samples to statistically validate the test kit RVFV.	0
	Limited field study of the ELISA test for the detection of antibodies against Rift Valley Fever Virus with repository of bovine sera	Field studies of the rapid test kit will be accomplished in collaboration with the University of Texas Medical Branch or other institutions with access to a larger number of biological samples to statistically validate the test kit RVFV. A field test of RVFV negative animals can be performed at any animal production facility in the area.	0

Interpretive Summary

Rift Valley fever virus (RVFV), a member of the Bunyaviridae family, infects most mammalian species, especially small ruminants, with high morbidity and mortality, and is also highly infectious to humans with potentially fatal consequences. This virus is transmitted and maintained by numerous mosquito genera including several native to North America (Aedes, Culex, and Anopheles). Thus, if RVFV is accidentally or deliberately introduced into this country, the disease is highly likely to become endemic in North America. Consequently, this virus has enormous potential as a bioterrorist agent to cause devastating economic losses in the livestock industry as well as illness and death in humans. In the event of an outbreak of RVF in the US, rapid diagnostic techniques would be required to control disease.

We propose using molecular biology techniques to produce an inexpensive and safe source of reagents for an indirect ELISA kit. This test can be run in two to four hours. We have developed three diagnostic kits similar to the one proposed for RVFV that are currently being use to detect antibody to other viral causes of diseases of ruminants: rinderpest virus, peste des petites ruminants virus, and vesicular stomatitis virus. We used the baculovirus expression system to safely produce large quantities of inexpensive reagents for these diagnostic tests that are currently in use in the United States and Africa. We are using the same techniques to express large amounts of a highly immunogenic protein (nucleocapsid) of the RVFV to develop a similar test. We have cloned the gene for this protein into a baculovirus plasmid transfer vector, co-transfected with a baculovirus expression system in Sf9 cells and recombination confirmed. We are in the process of producing anti-RVFV serum in mice for further characterization of the N protein antigen as well as for the standardization of the RVFV-ELISA.

Results and Interpretation

The Rift Valley Fever Virus N protein contained in the plasmid RVF-S was successfully cloned into the baculovirus transfer vector pVL1393 to produce the plasmid pVL1393RVFVN (Fig 3). Sequencing analysis showed that the sequences of the original plasmid (RVFV-S) and the plasmid pVL1393RVFVN match 100% and was co-transfected with the Bac-N-Blue baculovirus expression system using Cellfectin, a cationic liposome-mediated reagent specifically designed for transfection of insect cells.

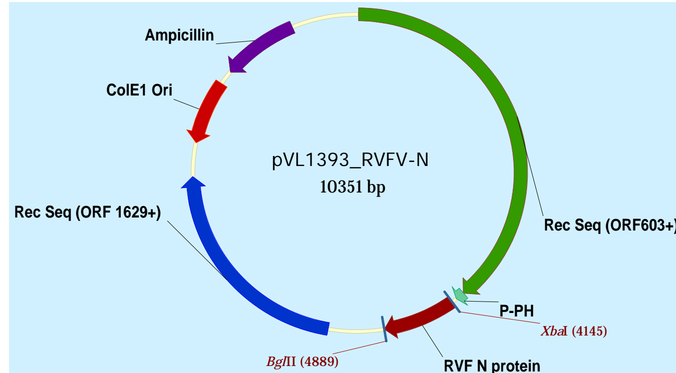
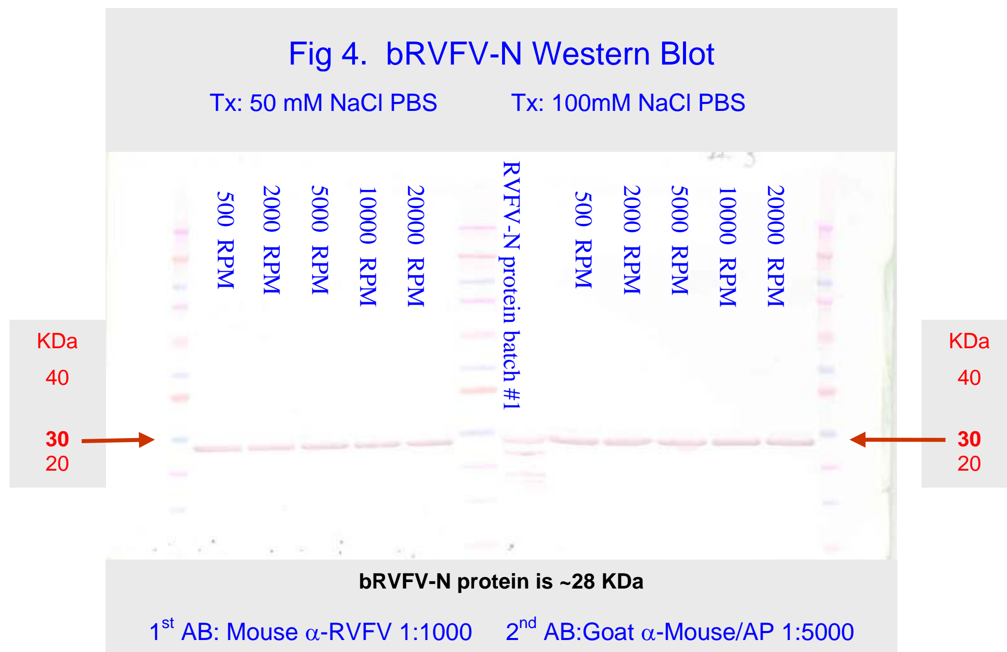


Fig 3. Baculovirus transfer vector pVL1393RVFVN

Simultaneously, a Sf9 cell line was used for co-transfection of the pVL1393RVFVN transfer vector and the Bac-N-Blue baculovirus expression system. Several candidate recombinant baculoviruses were developed in the Sf9 insect cell line. Recombinant virus was confirmed by western blot using a mouse anti-RVFV serum and a rabbit anti-RVFV serum provided by the USAMRIID and alternate proteins different to RVFV.

The recombinant RVFV N protein has been abundantly produced in Sf9 cells and easily extracted using a very simple method that requires PBS buffer and a regular cell homogenizer. The ILMB was authorized to obtain and use the MP-12 strain of RVFV to produce antibodies for the characterization of the N protein antigen (Fig. 4).

The MP-12 virus strain was amplified in Vero cells, harvested and titrated. In order to produce a supply of positive serum against the N protein of RVFV, groups of four BALB/c and four C57BL/6 mice were immunized with MP-12 strain virus. After a booster with MP-12, blood was collected by exsanguinations and serum obtained and pooled. Collected sera was tested for antibody reactivity to the recombinant baculovirus RVFV-N protein by western blot analysis.



The recombinant RVFV-N antigen stock was tested for antibody reactivity in an indirect ELISA system using the MP-12 derived positive and negative sera from control mice (Table 1). The RVFV-N ELISA had the highest ratio of positive to negative sera (31.5) when the antigen was diluted 1:400 and the pooled positive sera was diluted 1:200 (Table 2).

bRVFV-N antigen titration

Antigen	bRVFV-N in carbonate buffer
Positive sera	Mouse α-RVFV-MP-12
Negative sera	Sera from BALB/c mice
Enzyme conj.	Rabbit α-Mouse IgG/HRP 1:10 000
Substrate	TMB + H2O2 in Citrate buffer
Stop	2N H2SO4

Table 1. Standardization of the RVFV-N ELISA

ELISA Pos/Neg ratio

Sera	Ag 1:200	**Ag 1:400**	Ag 1:800
1:100	9.62	7.50	7.00
1:200	18.29	**31.50**	7.67
1:400	14.0	2.50	8.00
1:800	16.83	3.71	3.00

Table 2. Standardization of the RVFV-N ELISA

Validation of the RVFV-N ELISA antibody detection test kit with target species serum samples (cattle, sheep and goats) will be conducted in collaboration with the University of Texas Medical Branch or other institutions with access to a larger number of biological samples to statistically validate the test kit RVFV

Field studies of the rapid test kit will be accomplished in collaboration with the University of Texas Medical Branch or other institutions with access to a larger number of biological samples to statistically validate the test kit RVFV. Field testing of RVFV negative sera can be performed at any animal production facility.

Biological Systems – Project 4C

Development of a Safe and Efficacious Vaccinia virus Recombinant vaccine for Rift Valley Fever

DHS Priority Areas Addressed	☒ Prevention ☐ Detection ☒ Response ☒ Recovery ☐ Education/Risk Communication
Proposal Section Addressed	Sections 5.2.1 and 5.3.1
Investigators	UCD: Tilahun Yilma

Objectives	**Deliverables**	Progress Toward Deliverables	Percent Complete
Construct a recombinant vaccinia virus vaccine for RVF	Verification of deletion of B8R & TK genes in VV by sequence analysis	Complete	100
	Development of RVFV G1 & G2 glycoprotein antigens in VV by western blot analysis	Complete	100
	Confirmation of strong attenuation & enhanced immuno-potentiation of antigenicity of RVFV G1 & G2 expressing VV delete mutant vaccine in vivo in mice	The pathogenicity of the Copenhagen strain of VV has been characterized in SCID and Nude mouse models. Based upon the data obtained we have determined the SCID mouse model to be best fit for measuring attenuation and safety. The CB6F1 mouse model will be used to measure immunogenicity.	80
	Safe and efficacious RVFV vaccine candidate for tested in mice and sheep further field evaluation in Kenya and Senegal	?????	?

Highlight for Research Briefs

The purpose of this project is to develop a highly immunogenic vaccine for the prevention of Rift Valley fever virus (RVFV) infection using recombinant vaccinia virus technology. Our lab has previously employed this technology in the development of successful vaccines against the rabies and rinderpest viruses. This vaccinia virus vector will be highly attenuated by the deletion of two virulence genes, B8R and thymidine kinase (TK), as well as by the expression of human Interferon-gamma (huIFNγ). B8R increases the virulence of vaccinia virus by binding to and inactivating IFNγ, and previous studies have demonstrated the marked reduction of virulence in vaccinia virus negative for TK. The expression of huIFNγ will allow for protection against those who vaccinate in the case of accidental self-inoculation. Previous studies conducted by the International Laboratory of Molecular Biology for Tropical Disease Agents (ILMB) at UC Davis have elucidated the ability of IFNγ expression to attenuate the virus by approximately one million-fold. Efficacy will be achieved by the expression of the highly immunogenic glycoproteins of RVFV, G1 and G2. Neutralizing antibodies to these glycoproteins have previously been demonstrated to protect against infection with RVFV.

The ILMB has created the necessary molecular tools needed for the construction of the recombinant vaccinia virus vectors, including those needed for the insertion of the RVFV G1 and G2 glycoproteins and huIFNγ into the TK gene. These tools have since been used for the creation of the final recombinant vaccinia virus vector. On going work in the laboratory is focused growing stocks of the new RVF vaccine. Once obtained, the stocks will be used in a laboratory mouse model to the test safety and efficacy of the new RVFV vaccine.

Interpretative Summary

The transfer vectors necessary for the construction of the final recombinant virus have been completed. Mainly two plasmids are in use, one for the deletion of B8R, pΔB8R, and a second for the insertion of G1, G2 and huIFNγ into the TK gene, pΔTKG1G2γ. pΔB8R was created from “scratch” using a PCR amplified fragment of the origin of replication and beta-lactamase genes of pUC18. This PCR fragment was combined with a PCR fragment of the 5’ region of the B8R gene amplified from vaccinia virus DNA utilizing complementary restriction sites built into the PCR primers. A 3’ region of the B8R gene was subsequently added to the plasmid creating a partial B8R gene deletion (426 bp, about 52% of the ORF), carefully engineered to prevent expression of a truncated B8R gene without affecting neighboring genes. Finally, a gpt/LacZ cassette was added to the plasmid outside of the B8R gene to allow for selection of recombinant virus by transient dominant selection and creating pΔB8R (6,532 bp). pΔTKG1G2γ will be created by cloning the G1 and G2 proteins of RVFV and huIFNγ into the TK transfer vector designated p2SC11previously used by our laboratory. This plasmid allows for high level expression of the exogenously added genes. A plasmid, pT7-7, containing the M segment of the ZH-501 strain of RVFV has been obtained from Dr. Michael Parker, chief of the Viral Biology Branch of the U.S. Amy Medical Research Institute of Infectious Diseases (USAMRIID) at Fort Detrick, Frederick, MD. The M segment has been completely sequenced and successfully compared to the NCBI database (Accession: NC_002044). The sequence is 100% identical to that found in the database. The G1 and G2 proteins were removed from the plasmid (excluding the NS_M protein) by restriction digestion using NcoI and EcoRI. The resulting fragment was blunted with T4 DNA ligase and cloned into the unique SnaBI site of p2SC11 resulting in the creation of pΔTKG1G2. The huIFNγ gene was obtained from the Invitrogen (Carlsbad, CA, USA) ultimate ORF library. The huIFNγ gene was amplified using primers with flanking XmaI sites and cloned into the unique XmaI sight of pΔTKG1G2 resulting in the second and final transfer vector

pΔTKG1G2γ (11,640 bp). The IFNγ gene was also sequenced and successfully compared to the NCBI database with 100% identity (Accession: AF375790).

The Drillen clone of the VV strain Copenhagen was used to create vCOΔB8R, a recombinant VV deleted for the secreted IFNγ receptor homolog B8R. The development of the recombinant VV was achieved using homologous recombination with pΔB8R and transient dominant selection in Vero cells using the selectable markers gpt and LacZ. vCOΔB8R was confirmed to be free of WT virus after 4 rounds of plaque purification under selection followed by two rounds of limiting dilution. PCR analysis and sequencing of the B8R region confirmed the deletion of the B8R gene. The sequencing data also allowed for the analysis of the neighboring genes B7R and B9R showing no changes or deletions in these genes. The final rVV vaccine for RVFV was completed using the previously constructed vCOΔB8R by homologous recombination with pΔTKG1G2γ and selection in Vero cells using only LacZ as a selectable marker. LacZ has been left in the final recombinant to work as a marker gene for the vaccine virus, allowing it to be distinguished from VVs. Following 7 rounds of plaque purification, vCOΔB8RTK$^-$G1G2γ, was determined to be free of parental virus. Insertion of the huIFNγ and RVFV G1 and G2 genes were confirmed by PCR. Currently the laboratory is working to finalize the characterization of the RVFV vaccine by confirming protein expression through immunoprecipitation and western blot analysis using polyclonal mouse and rabbit sera against RVFV generously provided by Dr. Bob Tesh, UTMB.

<u>Overview and approach</u>

The objective of project 4C is to develop a recombinant vaccinia virus vaccine for protection against Rift Valley Fever virus (RVFV) infection. The recombinant vaccine will be both highly immunogenic and safe. This method has been previously proven by our laboratory with the development of highly efficacious vaccines against the rabies and rinderpest viruses using recombinant vaccinia virus technology. In the development of the strategy for the construction of this vaccine we have built upon our previous knowledge achieved from the creation of the aforementioned vaccines.

Specifically, our approach for construction of this recombinant viral vaccine uses a highly immunogenic strain of vaccinia used for the construction of our previous vaccines, Copenhagen. This strain will also be highly attenuated by the deletion of the virulence genes B8R and thymidine kinase. The B8R gene is a secreted homolog of the IFN-γ receptor that binds and inactivates IFN-γ. Deletion of this gene attenuates the virus by hampering its ability to interfere with the elicited immune response. Loss of the TK gene attenuates vaccinia virus *in vivo* considerably. To further attenuate the recombinant vaccine human interferon-gamma will be expressed from the viral vector. The expression of IFNγ will attenuate the vaccine an additional one million-fold. This attenuation will prevent any serious outcomes due to accidental inoculation of individuals giving the vaccine or caretakers of newly vaccinated animals. However, previous studies in the lab with the WR strain of vaccinia have demonstrated that this high level of attenuation does not result in a reduction of the immune response. The vaccine will be highly immunogenic by expression of the surface glycoproteins of RVFV, G1 and G2. G1 and G2 are expressed from the M segment of the RVFV genome as a single protein and cleaved post-translationally into there mature individual forms. Proper expression of these proteins is dependent on one another, and neutralizing antibodies to these proteins have previously been shown in survivor animals and block re-infection. Therefore we have chosen to express both glycoproteins as a single open reading frame in our recombinant vaccine just as they are produced during natural infection.

Development of recombinant vaccinia virus

Transfer Vectors

The development of the rVV deleted for B8R and TK as well as expressing G1, G2 and huIFNγ has been completed. The vaccine has been characterized and high titer stocks for use in the animal experiments have been grown. Details of the project to date are as follows.

The transfer vectors necessary for the construction of the final recombinant virus have been completed. Mainly two plasmids are in use, one for the deletion of B8R, pΔB8R, and a second for the insertion of G1, G2 and huIFNγ into the TK gene, pΔTKG1G2γ (Figure 4C-1). pΔB8R was created from “scratch” using a PCR amplified fragment of the origin of replication and beta-lactamase genes of pUC18. This PCR fragment was combined with a PCR fragment of the 5’ region of the B8R gene amplified from vaccinia virus DNA utilizing complementary restriction sites built into the PCR primers. A 3’ region of the B8R gene was subsequently added to the plasmid creating a partial B8R gene deletion (426 bp, about 52% fo the ORF), carefully engineered to prevent expression of a truncated B8R gene without affecting neighboring genes. Finally, a gpt/LacZ cassette was added to the plasmid outside of the B8R gene to allow for selection of recombinant virus by transient dominant selection and creating pΔB8R (6,532 bp). pΔTKG1G2γ will be created by cloning the G1 and G2 proteins of RVFV and huIFNγ into the TK transfer vector designated p2SC11previously used by our laboratory. This plasmid allows for high-level expression of the exogenously added genes. A plasmid, pT7-7, containing the M segment of the ZH-501 strain of RVFV has been obtained from Dr. Michael Parker, chief of the Viral Biology Branch of the U.S. Amy Medical Research Institute of Infectious Diseases (USAMRIID) at Fort Detrick, Frederick, MD. The M segment has been completely sequenced and successfully compared to the NCBI database (Accession: NC_002044). The sequence is 100% identical to that found in the database. The G1 and G2 proteins were removed from the plasmid (excluding the NS_M protein) by restriction digestion using NcoI and EcoRI. The resulting fragment was blunted with T4 DNA ligase and cloned into the unique SnaBI site of p2SC11 resulting in the creation of pΔTKG1G2. The huIFNγ gene was obtained from the Invitrogen (Carlsbad, CA, USA) ultimate ORF library. The huIFNγ gene was amplified using primers with flanking XmaI sites and cloned into the unique XmaI sight of pΔTKG1G2 resulting in the second and final transfer vector pΔTKG1G2γ (11,640 bp). The IFNγ gene was also sequenced and successfully compared to the NCBI database with 100% identity (Accession: AF375790).

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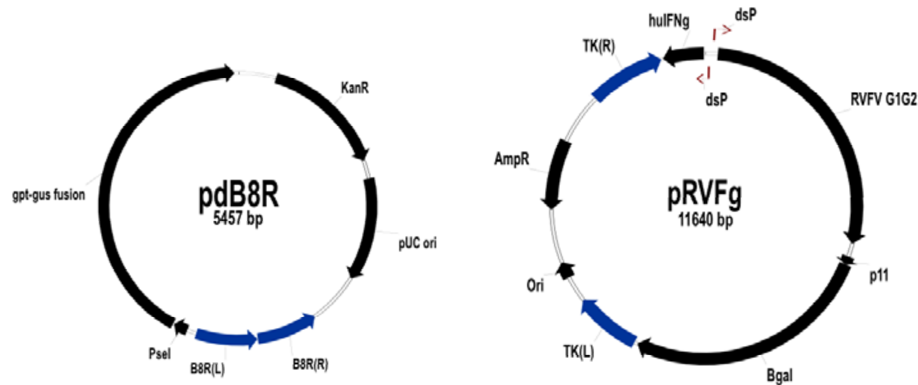
Figure 4C-1

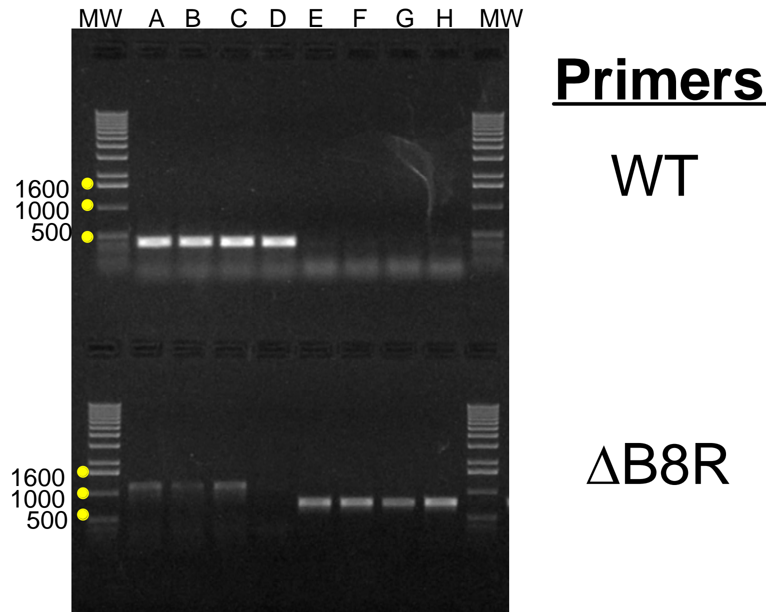


Transfer vectors used in the development of the recombinant VV vaccine for RVF. Panel A, Graphic of the vector used to delete the B8R gene from the Copenhagen strain of VV using transient dominant selection. A gpt/GUS fusion gene was used for selection of B8R deleted clones. Panel B, Graphic of the plasmid used for the insertion of the human IFNγ ORF and the RVFV G1G2 ORF under control of the double strong/synthetic promoter into the TK region of the B8R deleted parental virus. Positive clones were identified and selected using the B-galactosidase gene as a marker. Both panels, blue arrows indicate recombination regions.

Recombinant Vaccinia

Recombinant VV deleted for the B8R gene was created by transient dominant selection in Vero cells grown in serum free medium using the selectable markers gpt and LacZ. This recombinant lacking expression of B8R was purified using three rounds of limiting dilution and used as the parent vector to create the second recombinant with an inactivated TK gene and expressing the exogenous genes G1, G2 and huIFNγ. PCR was used to confirm the deletion of B8R from the Copenhagen strain parental virus (Figure 4C-2).

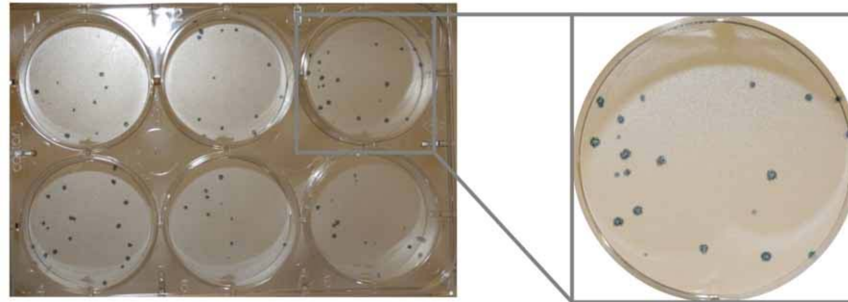
Figure 4C-2



PCR screening was used to identify clones that were deleted for the B8R gene after transient dominant selection and isolation by limiting dilution. Three primer pairs were used to determine the genotype in regards to the status of the B8R gene for each clone isolated. The first primer pair was designed to amplify the polymerase gene from multiple stains of VV only samples positive with this primer pair were considered for further analysis. The second primer pair (WT) was designed to amplify clones that still contained B8R (WT VV) by placing the reverse primer within the targeted deletion of B8R, yielding an amplicon of ~450bp. The third primer pair (ΔB8R) was used to delineate B8R deleted clones from WT VV by amplicon size, B8R yielding an amplicon of ~750bp and WT VV yielding an amplicon of ~1200bp. The figure to the left shows the screening of eight clones after three rounds of transient dominant selection and two rounds of limiting dilution. The upper gel in the figure is of reactions using the WT primer pair, and the lower gel is of reactions with the ΔB8R using the same samples/templates as the upper reactions. Clones negative in the WT reactions and demonstrating an amplicon of ~750bp in the ΔB8R reactions were considered for further use in the insertion of huIFNγ and the RVF glycoproteins into the TK site. Specifically, clone G was used for further vaccine development.

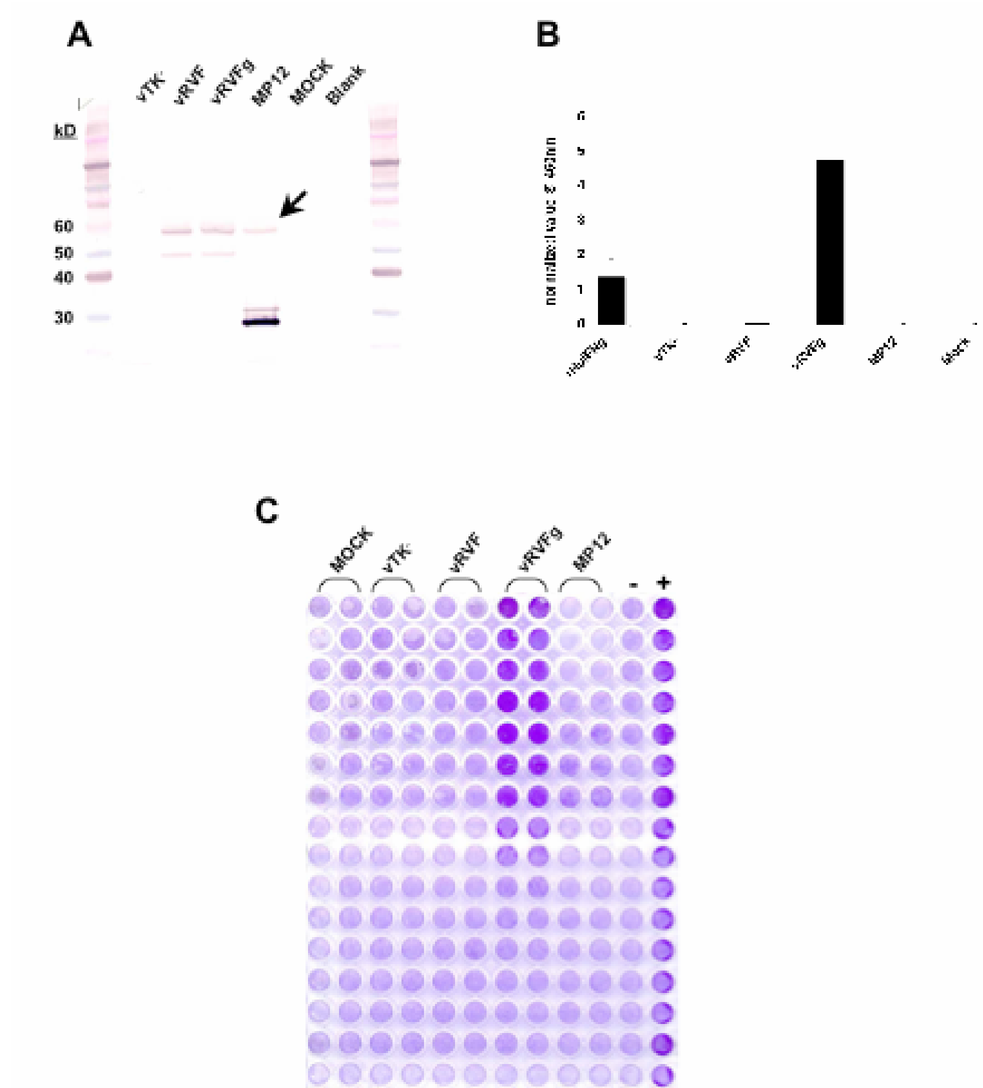
The final recombinant vaccine (vRVFγ) expressing G1, G2 and huIFNγ was also developed in Vero cells grown in serum free medium using the B8R deleted parental virus and the pΔTKG1G2γ transfer vector. Postive clones were selected over eight rounds of plaque purification using the LacZ selectable marker (Figure 4C-3). Incorporation of the beta-galactosidase gene into the final recombinant will serve as a marker gene for the vaccine in the event of an accidental infection with the virus. PCR and restriction fragment length polymorphism has been conducted to confirm the insertion of G1, G2 and huIFNγ, while western blot analysis and an IFNγ bioassay have confirmed high levels of expression of the exogenous genes (Figure 4C-4, data not shown). Currently, this final recombinant vaccine has been purified and grown to high titers for use in animal models to determine the safety, immunogenicity, and efficacy of this new RVF vaccine.

Figure 4C-3



Cloned recombinant VVs were screened for the presence of parental VV contamination using the incorporated marker gene Beta-galactosidase. BSC-1 cell monolayers were infected with VV clones at a known dilution (~20-40 pfu/well in 6-well plates) and histochemically stained 3 days post infection for the presence of the marker gene using X-gal. Blue plaques (see above image) indicate recombinant VV. The absence of colorless plaques confirms the absence of parental VV.

Figure 4C-4



Confirmation of the expression of the heterologous proteins was confirmed using western blot analysis, ELISA, and IFNγ bioassay. BSC-1 cell monolayers were infected at an MOI of 5 with the indicated recombinant VVs. 24 hours post infection cells and culture supernatant were harvested. Panel A, western blot analysis was performed on the cell pellets using mouse anti-RVF polyclonal serum as a primary antibody and goat anti-mouse-AP conjugated secondary antibody (BioRad, CA). The culture supernatants were assayed by ELISA (eBioscience, CA; panel B; results are shown as absorbance at 450nm) and bioassay (panel C) for the presence of huIFNγ. For the bioassay three-fold dilutions of the culture supernatants were used to treat A549 cells for 24 hours and subsequently infected with EMCV. 24 hours post infection viable cells were fixed and stained with crystal violet (note the darker stained cells in the vRVFg and + control rows, samples are shown in duplicate except + and – controls).