SWINE HEALTH INFORMATION CENTER
INTERIM RESEARCH GRANT REPORT FORMAT

I. Project Title and project identification number:

Development of direct detection methods for *in situ* diagnostic of Seneca A virus

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Institution:
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Date Report Submitted:
04/20/2016

II. Stated Objectives from original proposal

The specific aims of this proposal are to develop a set of direct diagnostic tools that allows direct detection of SV-A *in situ*.

A. Development of SVA immunofluorescence assay (IFA) for the detection viral antigen in infected cell culture (*completed*).

B. Development of SVA-IHC for detection of viral antigen in clinical specimens (*currently under development*).

C. Development of SVA *in situ* hybridization (fluorescent and/or chromogenic) for direct visualization of viral nucleic acid in clinical specimens *(completed)***.**

III. Progress toward meeting objectives

PRODUCER UPDATE:

A. Development of SVA immunofluorescence assay (IFA) for the detection viral antigen in infected cell culture **(*Completed*).**

This objective has been completed on time. The main idea was to develop a tool that allows us to confirm the virus in cell cultures. The development of this technique has a tremendous impact for confirmation of virus isolation. Once SVA is isolated from clinical samples direct IFA is the technique of choice to confirm the presence of the virus. Basically, we will be able to stain infected cell having a more objective way to confirm infection through viral staining with specific antibodies (figure 1). As compared to a PCR assay, this allows us to identify presence of LIVE virus.

For this specific objective we developed reagents that were not commercially available and now are not only available for ISU Veterinary Diagnostic Laboratory's diagnostic use, but for researchers and other diagnostic laboratories as well.

B. Development of SVA-IHC for detection of viral antigen in clinical specimens **(*Currently under development*)**.

This objective has been partially achieved. We proposed to develop a technique that allows us to detect SVA in fixed tissues. Since SVA vesicular lesions are non-specific, this technique will allow us detect the virus in lesions and allows us to differentiate SVA from other potential causes of vesicular disease. In order to achieve this goal, we proposed to generate two different types of antibodies (polyclonal and monoclonal). The development of a polyclonal is complete and already evaluated with excellent results (Figure 2). We are able to detect SVA in section of skin with vesicle. Polyclonal antibodies are easier and faster to produce, but they have less specificity than monoclonal antibodies; thus, they may allow for some cross-reaction and can be more difficult to interpret.

In order to provide a more refined diagnostic tool we also proposed to develop a monoclonal antibody. This technique is still under development. The development of this reagent is done in mice and takes approximately 3 months to complete the process, including multiple steps that cannot be accelerated: the mouse must produce sufficient antibodies to test, and as this is an immune response, takes time to build. Unfortunately, our first attempt provided poor quality antibodies therefore we are repeating the production of new candidate monoclonal antibodies to achieve maximal applicability. We are in the process of screening and evaluating the new SVA-Mab.

For this specific objective we developed reagents that were not commercially available and now are not only available for ISU diagnostic laboratory but for researchers and other diagnostic laboratories.

C. Development of SVA *in situ* hybridization (fluorescent and/or chromogenic) for direct visualization of viral nucleic acid in clinical specimens **(Completed).**

This objective has been completed on time. We evaluated a set genetic probes that allows us to confirm the presence of the virus in tissues based on the presence of genetic material. We evaluated two set of fluorescent probes targeting different regions of the virus. One of them (VP1) showed to be adequate to detect SVA genetic material in tissues. (Figure 2, 3). In order to reduce the effort and cost involved in fluorescent detection, we further evaluated this probe for detection of SVA detection material with light microscopy. SVA VP1 probe was shown to be efficient detecting viral genetic material with light microcopy. This technique will allow us to detect SVA efficiently in lesions without the burden of expensive fluorescent scopes. The benefit of this over PCR is that it will allow us to detect the exact location of virus, which will help to understand where and how long the virus persists in tissue.

TECHNICAL UPDATE:

A. Development of SVA immunofluorescence assay (IFA) for the detection viral antigen in infected cell culture **(*Completed*).**

To determine SVA infectivity we developed a whole virus SVA polyclonal antibody (SVA-Pab). ST cells were seeded at a concentration of 2×10^5 cells in 96 well plates at 50-70% confluency and infected with 100 µL of 1 x10^6/mL of contemporary SVA strain or DMEM as negative control. After 72 h, the cells were fixed for 20 minute at 4ºC with 80% acetone and virus infectivity was detected by direct immunofluorescence assay (IFA).

Briefly, after cells were fixed primary antibody concentration was determined, and infected cells were incubated with 150 µl of SVA-Pab at 1:100, 1:500, 1:1000 dilution for 45 minutes and followed by fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG-FITC. The positive fluorescence signal was detected using a fluorescent microscope.

Results: SVA infectivity was confirmed by the presence of sparsely positive fluorescent cells detected by SVA-Pab IFA (Figure 1).

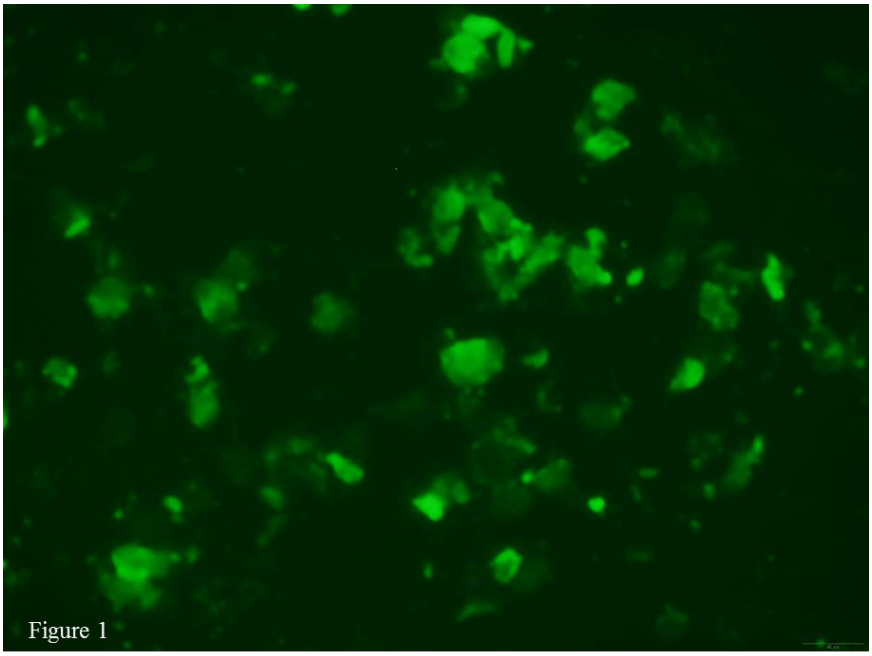


Figure 1

B. Development of SVA-IHC for detection of viral antigen in clinical specimens **(*Currently under development*)**.

We approached this objective by constructing three different sets of antibodies.

SVA Polyclonal antibody (SVA-PAb): Approximately 50 mL of SVA contemporary strain at 10^7 $TCDI_{50}$/mL was purified by ultracentrifugation. Virus pellet was suspended in 800 µl of PBS. Four mice were inoculated with 100µL of virus resuspension, and boosted two weeks after the first inoculation.

Two weeks after a whole virus booster the presence of SVA antibodies was evaluated by a whole VP1 virus ELISA.

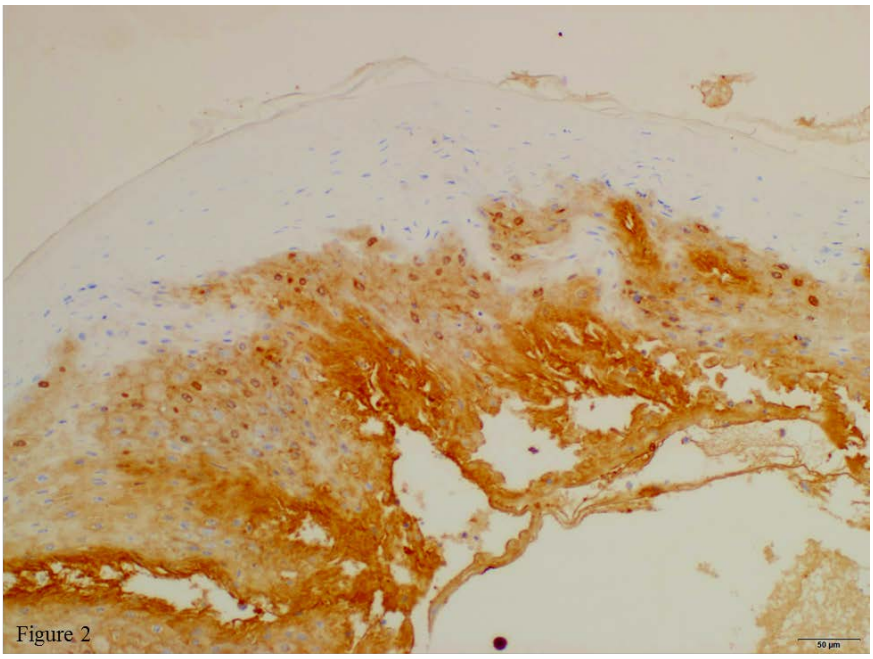
TITER	PRE-BLEEDING	POST-BLEEDING (pAb development)

	SVA WV	SVA whole virus			
	OD_769	OD_769 RR	769_R	769_L	769_N
1:40	0.057	3.351	2.666	3.067	3.074
1:80	0.055	3.231	2.324	2.668	2.805
1:160	0.061	2.761	2.171	2.225	2.379
1:320	0.07	2.451	1.489	1.688	2.064
1:640	0.062	2.162	1.026	1.171	1.564
1:1280	0.047	1.438	0.74	0.747	1.038
1:2560	0.05	1.126	0.389	0.418	0.643
1:5120	0.077	0.597	0.303	0.287	0.391

After detection of circulating SVA antibodies, ascitic fluid was collected from four mice and tested for SVA antigen detection in tissues by immunohistochemistry (IHC) on clinical specimens presented at the ISU VDL. Animals used for IHC tissue evaluation were confirmed SVA positive by PCR in vesicular swab, feces and oral fluids.

Briefly tissue sections were mounted on glass slides and deparaffinized by serial passage through xylene, 100% alcohol, 95% ethanol, and 70% ethanol. In order to determine the antibody concentration, tissue sections were covered with 150 µl of SVA-Pab at 1:100, 1:500, 1:1000 dilution for 45 minutes and followed by a chromogenic anti mouse antibody at concentration of 1:100.

Result: Cells of the dermal basal stratum of the epidermis showed a diffuse intracytoplasmic staining (Figure 2).



SVA Monoclonal antibody (SVA-Mab): An Escherichia coli (E. coli)-codon optimized version of the VP1 (1,359 nt) gene was synthesized *in vitro*. The gene was amplified using the forward primer (5'-CAT CAT CAT CAT CAT CAT ATG TCT ACA GAT AAT GCA GAA ACG-3') and reverse primer (5'-AGA CTG CAG GTC GAC AAG CTT TTA ACC TGA CTG CAT CAG CAT C-3'). PCR product was cloned into pCold II expression plasmid using a NovoRec® PCR One Step Directed Cloning Kit. The construction of pCold II-VP1-SVA was confirmed by sequencing and then transformed into E. coli BL21 (DE3) pLysS. VP1 over-expression was induced by IPTG. VP1 protein (30.9 kDa) was mainly expressed in the

precipitate of cell lysate as an inclusion body. The rVP1 protein (30.9 kDa) was solubilized from inclusion bodies using a denaturing buffer (20mM Tris, 6M Guanidine-HCl, 10mM β-Mercaptoethanol, pH 8.0). Then, the rVP1 protein present in the supernatant of the solubilized inclusion body was refolded in vitro into the native conformation, and concentrated by dialysis against a refolding buffer (50mM Tris, 240mM NaCl, 10mM KCl, 2mM MgCl2, 0.4M Sucrose, 0.5M Arginine, 0.05% Triton X-100, Dithiothreitol, pH 8.2).

Purified protein was used as immunogenic antigen for SVA-VP1 monoclonal antibody production. Four mice were inoculated with 100 µL of purified SVA-VP1 protein mixed with freund's adjuvant, and boosted after two weeks. Two weeks after SVA-VP1 protein booster the presence of SVA-VP1 IgG was evaluated by a SVA-rVP1-ELISA.

TITER	PRE-BLEEDING	POST-BLEEDING (mAb development)		
	SVA rVP1	SVA recombinant VP1		
	OD_770	OD_770 R	OD_770 L	OD_770 N
1:40	0.061	2.655	2.776	2.66
1:80	0.058	2.304	2.848	2.61
1:160	0.05	1.802	2.894	2.204
1:320	0.058	1.228	2.788	1.627
1:640	0.053	0.76	2.675	1.064
1:1280	0.087	0.437	2.775	0.66
1:2560	0.103	0.274	2.197	0.366
1:5120	0.071	0.168	1.629	0.237

Based on pre- and post-bleeding antibody titers mouse 770L was selected for fusion and hybridoma production. The supernatant of 576 clones was evaluated by SVA-rVP1-ELISA After the first screening 26 clones were positive and were expanded for large scale production of SVA-VP1 Mab. During the second screening the 26 selected clones were negative.

Two remaining mice were boosted with 300 µg of SVA-rVP1protein. One week after the second SVA-rVP1-protein booster, the presence of SVA-VP1 antibodies was evaluated by a whole VP1 virus ELISA.

Serum titer	PRE-BLEEDING	1st inoculation		2nd inoculation	
	OD_770	OD_770 R	OD_770 N	OD_770 R	OD_770 N
1:80	0.151	2.703	3.375	4.613	3.928
1:160	0.149	1.893	2.813	4.729	3.593
1:320	0.136	1.206	2.071	4.687	3.006
1:640	0.136	0.784	1.458	4.700	2.108
1:1280	0.125	0.468	0.875	4.568	1.541
1:2560	0.106	0.288	0.532	4.497	0.932
1:5120	0.134	0.218	0.343	4.270	0.569
1:10240	0.152	0.234	0.249	3.710	0.353

Based on first and second inoculation pre- and post- bleeding antibody titers, mouse 770R was selected for fusion and hybridoma production. First clone screening **is schedule for April 26th and second screening for May 10th**. Upon confirmation of SVA antibodies, our plan is

to evaluate the SVA-Mab for antigen detection in tissue.

SVA epitope antibody (SVA-epi Antibody): We have constructed 18 overlapping epitopes comprising the whole VP1 structure with 5 overlapping amino acids. Each epitope is attached to KLH. We are in the process of confirming the most immunogenic epitope based on an epitope array. Upon confirmation with clinical samples, serum induced by a whole virus and monoclonal antibodies generated against VP1, we will develop a panel of specific peptide antibodies for potential IHC or potential blocking ELISA.

C. Development of SVA *in situ* hybridization (fluorescent and/or chromogenic) for direct visualization of viral nucleic acid in clinical specimens **(Completed).**

We developed two sets of fluorescent probes either targeting the SVA 5'UTR or the VP1 region.

To determine the efficiency of SVA fluorescent *in situ* hybridization (FISH), ST cells were seeded at a concentration of 2×10^5 cells in chamber slides and infected at 50-70% confluency with 100 μL of 1 $x10^6$ of contemporary SVA strain or DMEM as negative control. After 72 h, the cells were fixed, and virus infectivity was detected by FISH. Cells were probed for 15 hours. at 42ºC either with 5'UTR or the VP1 fluorescent probes.

Results: Positive cells were sparsely detected by the VP1 fluorescent probe (Figure 3)

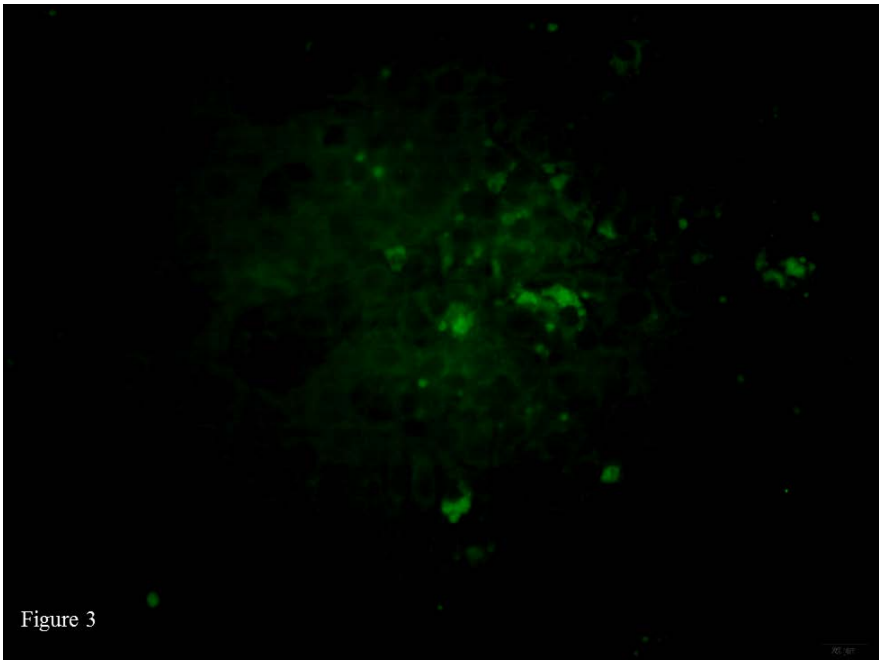


Figure 3

Following FISH SVA confirmation in cell culture we evaluated the probes on clinical specimens presented at the ISU VDL. Animals used for this tissue evaluation were confirmed SVA positive by PCR in vesicular swab, feces, and oral fluids.

Fluorescent molecular probes targeting the VP1 region were applied to sections of formalin-fixed tissue containing characteristic epidermal vesicles. Briefly, tissue sections were mounted on glass slides and deparaffinized by serial passage through xylene, 100% alcohol, 95% ethanol, and 70% ethanol. In order to determine the probe's concentration, sections were

covered with 150 µl of the hybridization solution containing SVA-VP1 probe in different concentration varying from 5 mg to 50 mg. Hybridization was carried out for 6 hours at 42°C.

Results: The epidermis showed diffuse fluorescent cells adjacent to the vesicles (Figure 4). All sections of epidermis were run in parallel with sections of skin not related with SVA cases. The fluorescence obtained by SVA-VP1 probe has a moderate background. However, positive cells presented a dense intracytoplasmic punctate staining similar to the staining pattern observed for positive cells in experimentally infected cells.

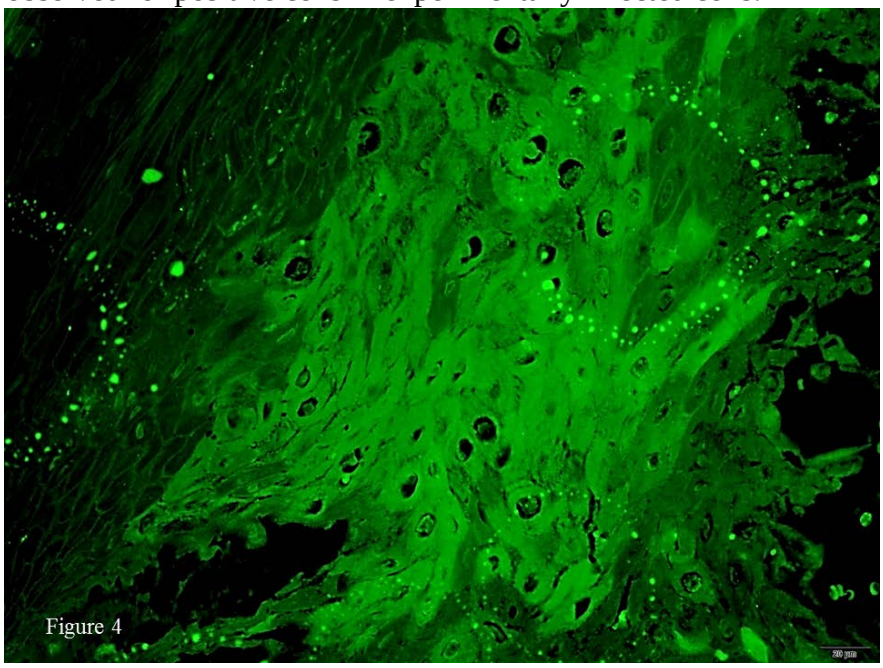


Figure 4

In order to eliminate fluorescent background and facilitate the evaluation of the FISH probes with a regular light microscope we developed a chromogenic detection of the nucleotide probes for direct visualization of viral nucleic acid in clinical specimens.

Skin sections previously tested by FISH were reevaluated for chromogenic probe detection. Tissue sections were probed with a fluorescent VP1 prove, followed by a chromogenic anti-FITC secondary antibody.

Result: Cells of the dermal basal stratum of the epidermis showed a diffuse intracytoplasmic staining (Figure 5)

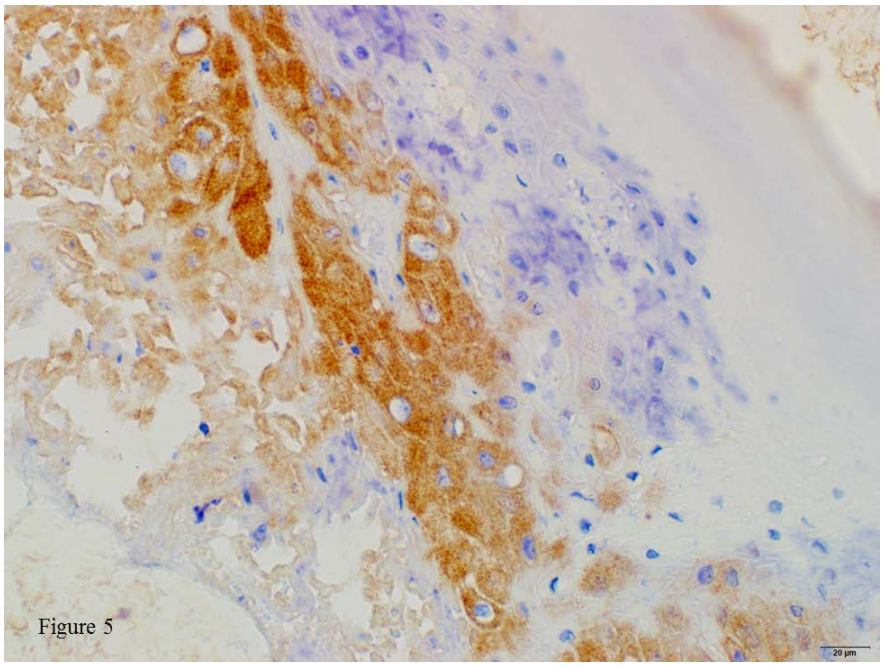


Figure 5

Summary: Objective C "*in situ* hybridization (fluorescent and/or chromogenic) for direct visualization of viral nucleic acid in clinical specimens has been **completed.** The technique has been validated using samples from SVA-PCR-positive clinical cases obtained at ISU and also by evaluation of positive control samples kindly provided by Dr Vannucci from UMN.

IV. Status of project in regards to stated timeline

A. Development of SVA immunofluorescence assay (IFA) for the detection viral antigen in infected cell culture (Completed)

B. Development of SVA-IHC for detection of viral antigen in clinical specimens

- SVA-Pab: (Completed)
- SVA-Mab: (Pending)
- SVA epitope: (Pending)

Due to failure during the first attempt of hybridoma fusion this part of the SVA-IHC development project has been delayed. Therefore, an extension on the proposed original schedule would be necessary.

C. Development of SVA *in situ* hybridization (fluorescent and/or chromogenic) for direct visualization of viral nucleic acid in clinical specimens (Completed)

V. Modifications of project from original proposal

VI. IV. Preliminary results