SWINE HEALTH INFORMATION CENTER
INTERIM RESEARCH GRANT REPORT FORMAT

Project Title: Development of multiplex real-time PCR- and antibody reagents for the detection of swine acute diarrhea syndrome coronavirus (SADS-CoV) (SHIC 18-146)

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Institution: Cornell University

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I. Stated Objectives from original proposal

The overall goal of the proposed project is to develop rapid diagnostic tools that will allow the timely detection of SADS-CoV nucleic acid and/or antigens in clinical samples. The objectives of the proposed study are:

Objective 1: To develop and evaluate a real-time multiplex PCR for SADs-CoV, PEDV and PDCoV.

Objective 2: To develop antibody reagents for SADs-CoV.

II. Progress toward meeting objectives

Progress has been made in both objectives.

Objective 1: To develop and evaluate a real-time multiplex PCR for SADs-CoV, PEDV and PDCoV. A SADS-CoV-specific Real-time PCR assay was developed and is under validation/evaluation. The assay efficiency was evaluated against in vitro transcribed RNA. Additionally, the newly developed SADS-specific assay was incorporated into the current Tetracore EZ-PED/TGE/PDCoV, in which the TGE assay was removed and replaced by the SADS-CoV-specific assay.

Objective 2. To develop antibody reagents for SADs-CoV. SADS-CoV proteins, including S1/S2 and NP were expressed in E. Coli, purified and used to immunize rabbits or mice for polyclonal and monoclonal antibody production.

III. Status of project in regards to stated timeline

The project is on track and good progress has been made in both objectives. Preliminary qPCR validation was completed and polyclonal antibodies specific for the SADS-CoV proteins are available (see results below). We currently have mice immunized with S1/S2 and should be able to start fusion process for generation of mAbs soon.

Although the project is currently on track with a PCR and antibody reagents already available for SADS-CoV, we would like to request a 12-month extension of this project. As discussed with Dr. Sundberg I will be moving to Cornell University and given the re-organization that this move will require, we will likely need more time to complete the full validation of the assays and reagents proposed on the project.

IV. Modifications of project from original proposal

Nothing to report.

V. Preliminary results

Real-time PCR development. The objective of this study was to develop a wet real-time RT-PCR assay for the specific detection of SADS. Currently there are only a few SADS sequences available on Genbank (Figure 1). These sequences were downloaded and imported into the Sequencher 5.4.5 Software. The software was used to assemble the sequences into a contig. The sequences were visually inspected for conserved region. For Corona Viruses mainly the Nucleocapsid (N) and RNA-dependent RNA polymerase (RdRp) gene regions seem to be highly conserved. For the new SADS rtRT-PCR assay the N region was chosen.

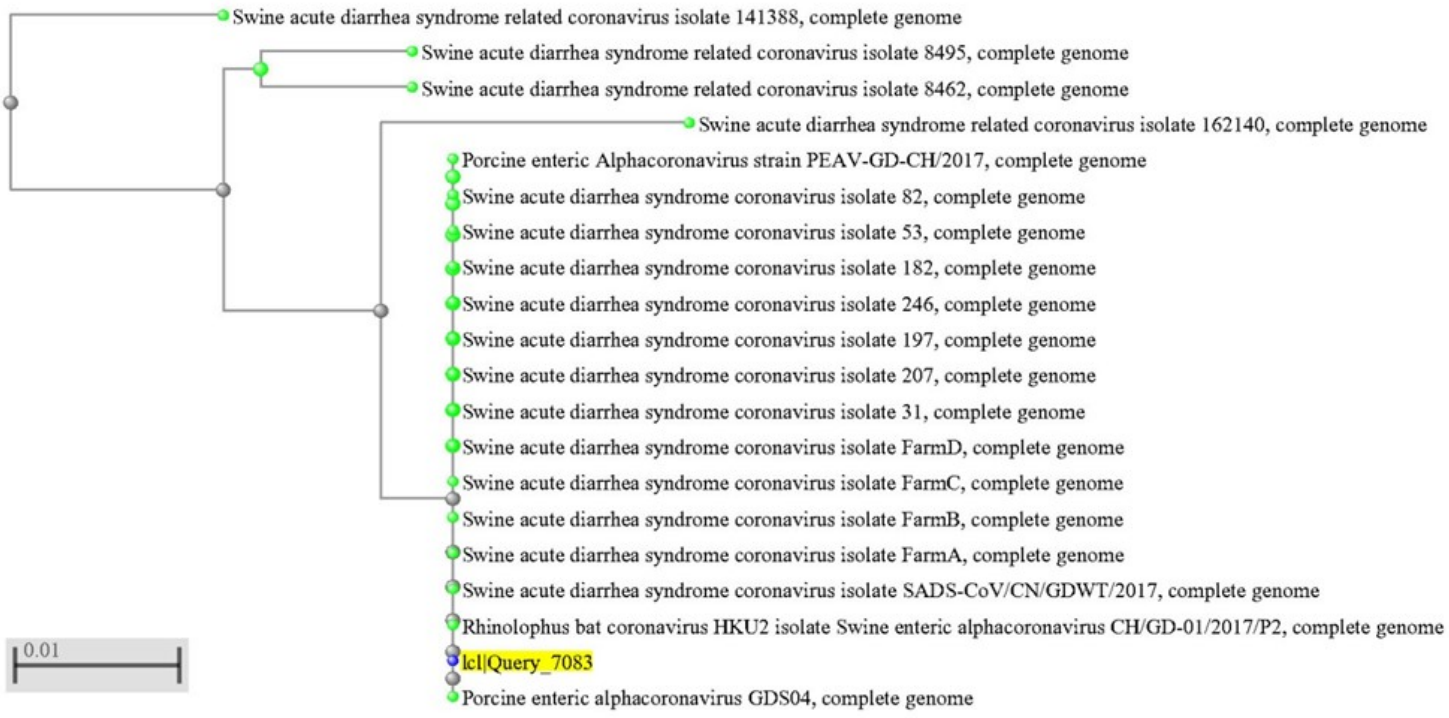


Figure 1. available SADS sequences

An In Vitro Transcript was designed which included the target sequences for the SADS assay. The process to create the IVT began with designing a syntethic oligo. The oligo includes a T7 promotor which is used by the MEGAscript® T7 Kit to transcribe the In Vitro Transcript (IVT).

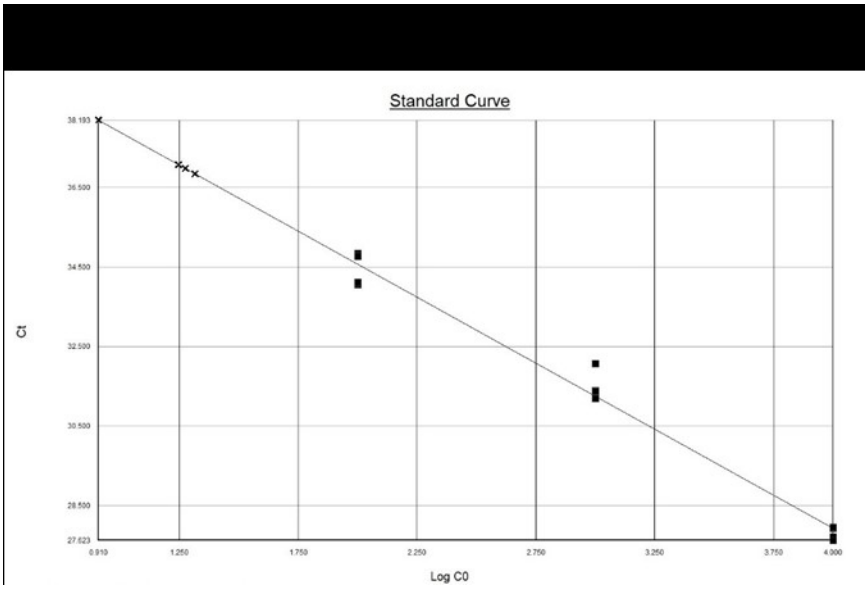


Figure 2. SADS standard curve

The slope of -3.321280 and R2 value of 0.984144 demonstrate that the chosen design of the rtRT-PCR SADS assay is efficient.

The SADS RT-PCR assay was incorporated into the EZ-PED/TGE/PDCoV MPX 1.1 RT–PCR assay. The SADS RT-PCR assay replaced the TGE RT-PCR assay in the multi-plex. A comparison was completed between EZ-PED/TGE/PDCoV MPX 1.1 vs EZ-PED/SADS/PDCoV MPZ. Swine fecal and oral fluid samples were randomly selected and extracted for testing with both assays side-by-side. As shown below results between the two assays was very similar, indicating that the newly developed SADS-CoV is compatible and does not interfere with the PED and PDCoV assays (Table 1 and 2)

Table 1 – Comparison between different versions of EZ-enteric assay with or without the SADS-CoV specific assay. Results for PEDV detection.

EZ-PED/TGE/PDCoV MPX 1.1		**EZ-PED/SADS/PDCoV MPX**
PED (FAM)		
PC	27.40	27.01

PED 1	29.17	29.29
PED 2	33.94	33.23
PED 3	30.03	30.00
PED 4	26.04	26.34
PED 5	32.71	32.74
PED 6	31.01	29.08
PED 7	28.33	28.66
PED 8	0.00	0.00
PED 9	27.37	27.30
PED 10	30.11	30.24

Table 2 – Comparison between different versions of EZ-enteric assay with or without the SADS-CoV specific assay. Results for PDCoV detection.

EZ-PED/TGE/PDCoV MPX 1.1		**EZ-PED/SADS/PDCoV MPX**
PDCOV (CY5)		
PC	30.03	29.23
PDCoV 1	35.86	35.30
PDCoV 2	36.46	36.13
PDCoV 3	28.52	28.20
PDCoV 4	31.07	30.46
PDCoV 5	32.34	31.49
PDCoV 6	24.04	23.78
PDCoV 7	30.23	29.84

PDCoV 8	35.34	33.97
PDCoV 9	0.00	0.00
PDCoV 10	31.17	30.72

Screening of Chinese SADS-CoV samples using the newly developed SADS-CoV real-time PCR. The new multi-plex real-time PCR (**EZ-PED/SADS/PDCoV**) was evaluated in China using samples with known enteric and SADS coronavirus status (**Table 3**). This portion of the project was carried out in the laboratory of Dr. Shao Lun Zhai at the Guangdong Academy of Agricultural Sciences. All the samples tested with the new multiplex PCR had been previously tested with an in-house PCR developed at Dr. Zhai's laboratory. As shown in Table 3, overall results from Dr Zhai's PCR and the newly developed assay here are consistent, with the newly developed assay showing increased capability of detecting a few additional positive samples for all three pathogens (PEDV, PoDV and SADS-CoV) when compared to the in-house PCR. An interesting observation of these data is the fact that most SADS-CoV positive samples were also positive for PEDV or PDCoV. Some samples were also positive for all three pathogens (**Table 3**).

Table 3 – Screening of Chinese clinical samples.

Sample ID	Orignal results				Tetracore results		
	PEDV	PoRV	PDcoV	SADS-CoV	PEDV	PDcoV	SADS-CoV
275	+	-	-	+	+	-	+
276	+	-	-	+	+	-	+
277	+	-	-	+	+	-	+
282	+	-	-	+	+	-	+
286	-	-	-	+	-	-	+
287	+	-	-	+	+	-	+
290	-	-	-	+	+	-	+
293	+	-	-	+	+	-	+
298	+	-	-	+	+	-	+
299	-	-	-	+	+	-	+
302	+	-	-	+	+	-	+
303	+	-	-	+	+	-	+
307	-	-	-	+	+	+	+
312	+	-	-	+	+	-	+
314	+	-	-	+	+	+	+
317	+	-	-	+	+	+	+
318	+	-	-	+	+	+	+
320	-	-	-	+	-	+	+
325	+	-	+	+	+	+	+
328	+	+	-	+	+	+	+
329	+	-	-	+	+	+	+
332	-	-	-	+	+	+	+
330-2	+	-	-	+	+	-	-
338	+	-	+	+	+	+	+
341	-	-	-	+	+	-	+
344	+	-	-	+	+	-	+
345	-	-	-	+	+	-	+
346	+	-	-	+	+	-	+
348	-	-	-	+	-	-	+
352	-	-	-	+	-	-	+
355	+	-	-	+	+	+	-
356	+	-	-	+	+	-	+
357	+	-	-	+	+	+	+
360	-	-	-	+	-	-	+
361	-	-	-	+	+	+	+
364	+	-	-	+	+	-	-
368	-	-	-	+	-	-	+
371	+	+	-	+	+	-	+
372	-	-	-	+	+	+	+
374	+	-	+	+	+	+	+
377	+	-	-	+	+	+	+
382	+	-	-	+	+	-	+
384	-	+	-	+	+	-	+
391	+	-	+	+	+	+	+
392	+	-	+	+	+	+	+
399	+	-	-	+	+	-	+
408	+	-	-	+	+	-	+
411	+	-	+	+	+	+	+
413	-	-	+	+	+	+	+
414	+	-	+	+	+	+	+
291					+	-	-
270					+	-	-
280	+	-	-	-	+	-	-
285	+	-	-	-	+	+	-
288	+	-	-	-	+	-	-
289	+	-	-	-	+	-	+
292	+	-	-	-	+	-	-
294	+	-	-	-	+	-	-
300	+	-	-	-	+	-	-
306	+	-	-	-	+	-	-
308	+	-	-	-	+	-	-
311	+	-	-	-	+	-	-
313	+	-	-	-	+	-	+
315	+	-	-	-	+	-	-
321	+	-	-	-	+	-	-
322	+	-	-	-	+	-	-
323	+	-	-	-	+	-	-
324	+	-	-	-	+	-	-
334	+	+	-	-	+	-	-
335	+	+	-	-	+	-	-
330-1	+	-	-	-	+	-	-
339	+	-	-	-	+	-	-
347	+	-	+	-	+	+	-
354	+	-	-	-	+	-	-
365	+	+	-	-	+	-	-
366	+	-	-	-	+	-	-
367	+	-	-	-	+	-	-
369	+	-	-	-	+	-	-
373	+	-	-	-	+	-	-
376	+	-	-	-	+	-	+
378	+	-	-	-	+	-	-
379	+	-	-	-	+	-	-
381	+	-	-	-	+	-	-
383	+	-	-	-	+	-	-
389-1	+	-	-	-	+	-	-
388	+	-	-	-	+	+	-
389-2	+	-	-	-	+	-	-
393	+	+	-	-	+	+	-
394	+	-	-	-	+	-	+
395	+	-	-	-	+	-	-
400	+	-	-	-	+	-	-
405	+	-	+	-	+	+	+
407	+	+	-	-	+	+	-
410	+	-	-	-	+	+	+

Development of SADS-CoV-specific antibodies. Recombinant SADS-CoV S1/S2 and N proteins were expressed in *E. Coli* and purified using nickel-NTA agarose beads. As shown in Figure 3 both proteins were successfully expressed and purified.

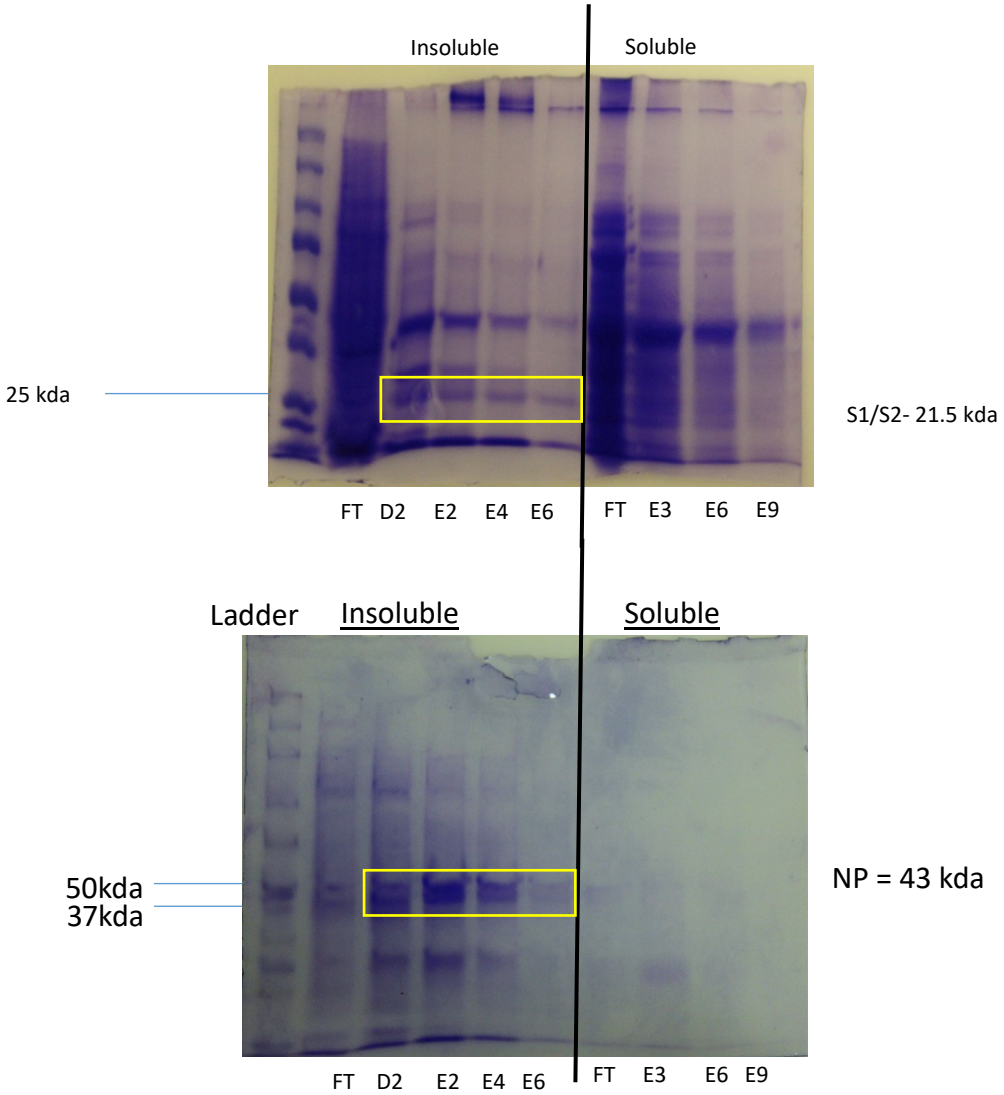


Figure 3. SDS-Page gel stained with comassie blue showing expression of SADS-CoV S1/S2 and N proteins (yellow boxes).

These recombinant proteins were used to immunize rabbits to produce SADS-CoV specific polyclonal antibodies. Animals were immunized and boosted 2 times at 2 week-intervals with the purified recombinant proteins. 3-4 weeks after the second booster each rabbit was exsanguinated and serum containing SADS-specific antibodies was harvested by centrifugation. The specificity of the rabbit polyclonal antibody was tested against the purified SADS-CoV proteins. As shown in Figure 3, the serum from the rabbit immunized with SADS-CoV S1/S2 reacted with the 25kDa protein corresponding to the truncated SADS-CoV S1/S2 protein at all dilutions tested (1:100 to 1:1000).

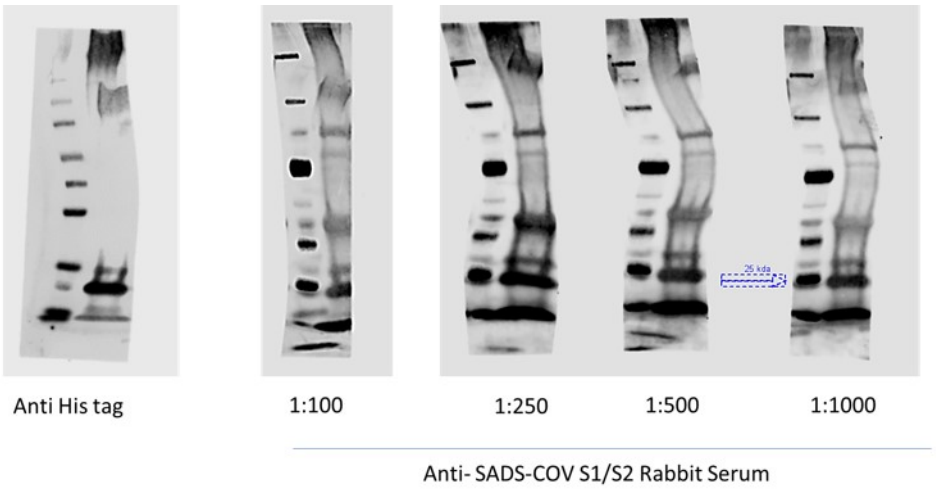


Figure 3 – Western blot confirming the reactivity of rabbit polyclonal serum against SADS-CoV S1/S2 truncated protein. Anti-His tag antibody was used as a positive control as the S1/S2 protein contains a Histidine tag. Blue arrow indicates the correct size of SADS-CoV S1/S2 protein (25 kDa).

Similarly, polyclonal serum harvested from the rabbit immunized with SADS-CoV N protein also recognized the E. coli expressed recombinant protein in Western blot assays (Figure 4).

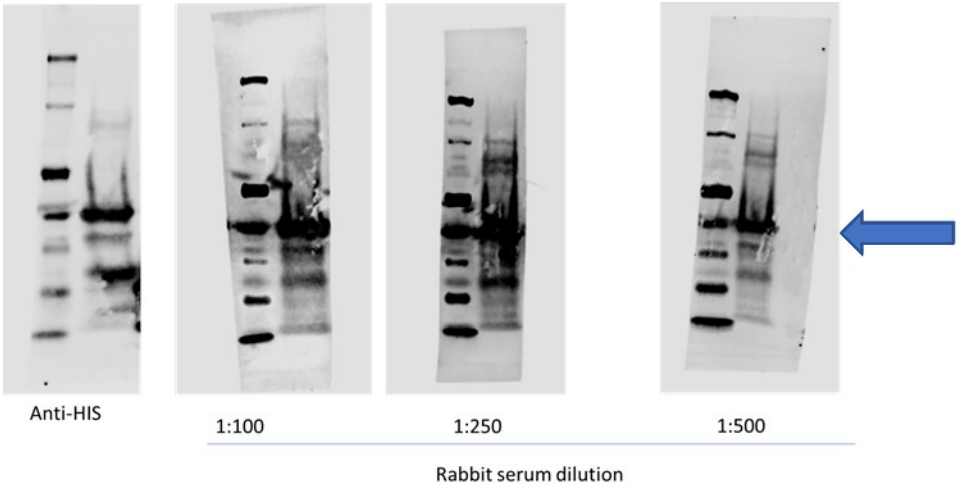
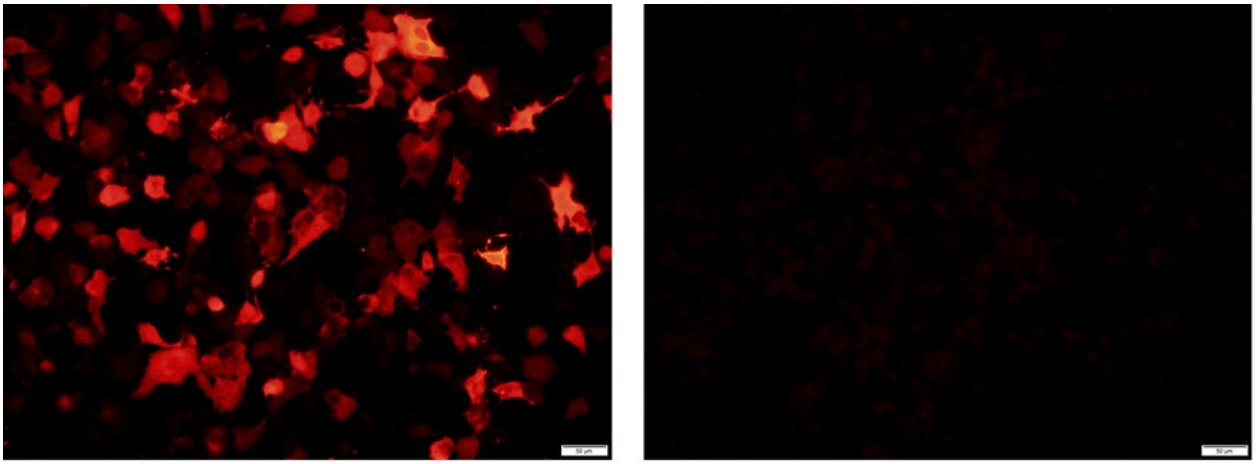


Figure 4 – Western blot confirming the reactivity of rabbit polyclonal serum against SADS-CoV N protein. Anti-His tag antibody was used as a positive control as the N protein contains a Histidine tag. Blue arrow indicates the correct size of SADS-CoV N protein (37 kDa).

The specificity of the SADS-NP polyclonal serum was also evaluated in mammalian cells expressing the recombinant N protein. As shown in Figure 5, bright fluorescent cells expressing the SADS-CoV N protein were detected in cells transfected with an expression plasmid encoding the viral protein.

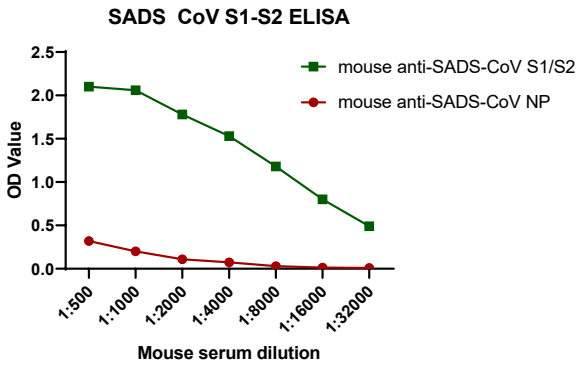


Anti-SADS-COV-NP Rabbit serum 1:250

Figure 5 – Detection of SADS-CoV expressed in H1299 cells with a rabbit polyclonal antibody (Left panel). Non-transfected cells (right panel) were used as negative controls.

Development of monoclonal antibody against SADS-CoV NP. Mice were immunized and the fusion was done. The hybridomas did not survive the selection process. The immunization with SADS-CoV NP is being repeated and animal experiments will start soon.

Development of monoclonal antibody against SADS CoV S1-S2. Mice were immunized with the purified recombinant SADS-CoV to develop SADS-CoV specific mAbs. In order to screen for positive hybridomas secreting SADS-CoV-specific antibodies, we developed an in-house ELISA. For this S1-S2 expressed in E coli was used to coat elisa plates. Different dilutions of mouse anti-SADS COV S1-S2 serum obtained from the mouse immunized from SADS CoV S1-S2 protein was used as the primary antibody. We also used mouse anti-SADS CoV NP obtained from the mouse immunized with SADS CoV NP protein as the negative control. The graph below shows that we were able to develop highly sensitive S1-S2 specific ELISA which was later used for screening hybridomas. It has a high OD value even at a dilution of 1:32000 and does not cross react with NP positive mouse serum.



For mAb production, three mice were immunized with recombinant SADS CoV S1-S2 protein and they were boosted two times. After the third immunization, mouse splenic cells were fused with NS-1 cells lines to obtain hybridoma cells. The hybridoma cells were screened for the presence of S1-S2 specific monoclonal antibody by using ELISA described above. We obtained a total of fifteen S1-S2 specific monoclonal antibody secreting hybridomas. The clone names and their signal strength on ELISA is listed on the table below. We also tested those mAbs for their isotype. Most of them belong to the IgM subtype as shown in the table below.

Clone number	Signal strength via ELISA	Hybridoma antibody subtype
22-231	++++	IgG 2a
120-54	+	IgM
154-1	++++	IgM
154-2	++++	IgM
154-99	++++	IgM
154-168	++++	IgM
154-172	++++	IgM
154-173	++++	IgM
154-180	++++	IgM
194-5	+++	IgM
194-49	+++	IgM
194-184	++	IgM
194-192	+++	IgM
209-156	+++	Not tested
209-160	+++	Not tested

In summary, we have made substantial progress on our project and currently have prototype a good multiplex real-time PCR assay as well as SADS-CoV specific polyclonal and monoclonal antibodies capable of detecting SADS-CoV proteins. These reagents and assays still need to be fully validated. Additionally, monoclonal antibodies are also under development using the recombinant proteins expressed in E. coli. Availability of these reagents will allow proactive interventions by the swine industry should SADS-CoV ever enter the US.