RESEARCH REPORT
SWINE HEALTH

Title: Optimization of Methods for the Study of Swine Viral Pathogens in Aerosols – **NPB #03-038**

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Abstract: The specific objective of this proposal was to optimize the methods to sample viruses in aerosols, including optimization of medium and comparison of two air samplers (AGI-30 and SCK-biosampler®). The long-term goal of this project is to provide producers the information they need to predict the likelihood of virus transmission between herds under specific atmospheric conditions (temperature, relative humidity, sunlight, wind).

An apparatus was designed and constructed to conduct the air sampler optimization studies consisting of a nebulizer (24-jet Collison nebulizer, BGI Incorporated Waltham MA) capable of aerosolizing 16.5 ml of liquid per hour at 40 psig connected to an 25 liter glass cylinder (aerosol reservoir). The aerosol reservoir was equipped with 6 ports to which impingers (AGI-30's or SKC-biosampler's®) could be connected for air-sampling. The capacity to simultaneously sample the same "cloud" with up to 6 impingers allowed for direct ("head-to-head") comparisons of media treatments, sampling times, and impingers.

Specific accomplishments achieved in this project include: 1) an antifoam with minimal cytopathogenic properties was identified; 2) a effective sorbent for inclusion in the medium was identified; 3) it was determined that ethylene glycol could be included in sampling medium, thereby providing the ability to collect air sample at temperatures below freezing; and 4) infectious virus was detected using both the AGI-30 and the SKC-biosampler® authenticating their use for PRRSV.

Introduction: Aerosol transmission is frequently cited as the means of virus transmission within and between swine herds, but nearly always without evidence or supporting data. The absence of corroborative data may be related to the technical challenges of documenting and describing the complex interactions that occur during airborne transmission. That is, a thorough understanding of how, and under what conditions, an organism is spread by aerosol requires knowledge of several variables:

1. The number of organisms aerosolized by an infected pig per unit of time over the course of the infection.
2. The rate of inactivation of airborne organisms under specific environmental conditions (temperature, light, relative humidity, dust, etc).
3. The number of viable organisms required to infect the next susceptible pig (minimum infectious dose).

When available, such information can be very useful. For example, in March 1981, the French reported outbreaks of FMD (foot-and-mouth disease) on 13 swine farms in Brittany and one in Normandy. Based on the meteorological conditions (relative humidity, windspeed, temperature, cloudy conditions) and equations developed to model the behavior of FMDV, it was correctly predicted that the virus would be carried 300 kilometers to the south coast of England. Forewarned is forearmed, and FMDV infections in cattle in Jersey and on the Isle of Wight were promptly detected and eliminated.

The FMDV example is intended to show the level of information that is required to effectively understand the risk of airborne spread. Unfortunately, FMDV is one of the few agents for which we have relatively good aerobiological data.

EXPERIMENTS AND RESULTS:

Part I. Optimizing aerosol collection medium.

A. Selecting an anti-foam - effects of antifoams on cell viability, virus, or PCR performance.

Antifoams are needed in aerosol collection media to prevent foaming and ensure proper function of the impingers. The possible direct effect of antifoaming compounds on tissue culture or PCR-based assays used to detect viruses has not been described. The objective was to screen antifoaming compounds for any detrimental effect on cell viability or PCR performance.

Effect of anti-foams on cell viability: Six commercially-available antifoams (Antifoams 204 (A26426), A Emulsion (A5758), B Emulsion (A5757), C Emulsion (A8011), O-30 (A8082), and SE-15 (A8582) Sigma, St. Louis, MO) were tested on 3 continuous cell lines using 2 exposure periods. Continuous cell lines tested were African Green monkey kidney (MARC-145), Madin-Darby canine kidney (MDCK), and pig kidney (PK-15). Antifoams were serially 10-fold diluted and 200 µL of each antifoam dilution was added to 3 wells of a 96-well microtitration plate. Two exposure times were tested by incubating cells with antifoam dilutions at 37°C in a 5% CO_2 humidified incubator for either 2 or 24 h. Cells exposed for 2 h were rinsed twice and plates incubated for an additional 22 h. Each treatment was replicated eight times.

The effect of exposure to antifoam on cell viability was determined using a neutral red cell viability assay. The neutral red assay is a method for estimating the percent of viable cells. The principle of the assay is that viable cells retain the dye, whereas damaged cells do not. The results for each well were read using a spectrophotometer. The percent cell population viability for each treatment was calculated as: (mean absorbance of treated wells / mean absorbance of control well) * 100. **Results:** Antifoams were found to decrease cell viability in the 3 continuous cell lines tested following either 2 hr or 24 hr exposure periods. Differences in the degree of cytotoxicity existed between antifoams, but the effect was consistent across cell lines for specific antifoams. Increasing concentrations of antifoams decreased cell viability, but the effect was variable among antifoams. Antifoam C Emulsion was selected for use in future studies due to its non-cytotoxicity at all dilutions tested.

Effect of antifoams on virus (virucidal effects) or PCR performance: The effect of antifoam on virus infectivity was tested on 3 viruses using one dilution (10^{-3}) and exposure period (6 hr). PRRSV, SIV, and PCV-2 were added to media containing antifoam treatments and incubated at 37°C in a 5% CO_2 humidified incubator. $TCID_{50}$'s and Quantitative RT-PCR and $TCID_{50}$'s were performed. **Results:** No significant difference ($P > 0.05$) was found in virus infectivity between control and antifoam treatments for PRRSV and PCV-2. Antifoam 6 was virucidal when compared to control ($P = 0.02$) for SIV; no difference was detected between the remaining antifoams and the control. No antifoam inhibitory effect on PCR-based assays was observed. Based on the neutral red assay and $TCID_{50}$ results, antifoam C Emulsion was selected for use in future studies.

B. Selecting a "protectant"

Protectants are typically used in aerobiology to prevent the inactivation of viruses via the physical disruption caused by impinging (sampling). The objective of this work was to determine which protectant provided the best protection against virus inactivation during the process of impingement.

Three commercially available protectants (mucin, gelatin, and bovine serum albumin) were tested at 0.1, 0.25, 0.50, 0.75 and 1.0% treatment levels. A bank of AGI-30 impingers loaded with 20 ml of collection medium (PBS and HEPES) and protectants were spiked with equivalent amounts of PRRSV and SIV and impinged for 15 minutes at 30° C. Impingers were placed in a waterbath to maintain constant temperature and run concurrently in a class II biological safety cabinet. Temperature and collection media pH were monitored throughout the 30 min trials. After 30 minutes, treatments were reconstituted to the initial volume of 20 ml. Positive (no protectant with impingent) and negative (no protectant without impingement) controls were run for baseline comparisons. Microinfectivity assays ($TCID_{50}$) were conducted to quantify infectious virus in each treatment and quantitative RT-PCR was used to describe total viral load.

Results: The pH of the collection media was 6.8 pre and post impingement. Impinging did not reduce the amount of viral RNA detected by quantitative PCR (see Table 1). Comment: these results are not in agreement with published literature. Therefore, additional trials are being conducted to verify the results.

Table 1. Effect of protectants on virus infectivity during the process of impingement

Level (%)	Run 1						Run 2					
	BSA		Gelatin		Mucin		BSA		Gelatin		Mucin	
	PRRSV[c]	SIV[c]	PRRSV	SIV	PRRSV	SIV	PRRSV	SIV	PRRSV	SIV	PRRSV	SI
0.00[a]	2.90	5.10	2.80	4.30	3.80	5.60	3.90	5.70	3.90	5.70	3.80	6.0
0.00[b]	1.70	4.10	1.90	4.30	2.00	4.50	3.70	5.80	3.50	5.70	3.40	5.7
1.00	2.40	4.90	2.40	4.20	1.20	4.30	3.50	5.30	3.50	5.70	3.10	5.0
0.75	2.10	4.90	2.20	4.60	1.70	4.50	3.30	5.50	3.50	5.70	3.20	5.2
0.50	2.10	4.80	2.10	5.00	1.80	4.70	3.30	5.70	3.40	5.70	3.30	5.1
0.25	1.90	4.70	2.00	5.00	1.90	4.70	3.10	5.30	2.90	5.50	3.40	5.5
0.10	2.20	4.90	2.00	4.90	1.70	4.90	3.20	5.60	3.00	5.60	3.30	5.5

[a]Sample was impinged.
[b]Sample was not impinged.
[c]Values are expressed in logs as is EID50 per 0.2ml.

C. Selecting a "sorbent"

"Sorbents" are materials used to absorb viruses. The use of sorbents (activated carbon, kaolin, etc) has been described for improving the collection/detection of enteric viruses in contaminated water, but their use has not been investigated for air sampling. The advantage of identifying an effective sorbent is that the sensitivity of air sampling could be markedly increased. In this study, we examined two forms of activated carbon (coal-derived and wood-derived) because the distinct microstructure of the two compounds confers different properties of adsorption.

Effect of activated carbon on cell viability: The effect of activated carbon on cell cultures was examined to determine whether tissue culture assays could be used to detect and quantify virus if activated carbon were included in the collection medium. Two types of activated carbon (coal-derived and wood-derived) were tested at five treatment levels (0.2, 1, 2, 10, 20%) for cytotoxic effects on MARC-145 cells. Cells were exposed to treatment levels for 2 hr at 37 C at 5% CO_2 then rinsed with PBS. Cells were observed for cytotoxic effects using a light microscope. **Results:** Cells showed a cytotoxic response to all levels of activated carbon tested. Therefore, alternative diagnostic techniques were implemented to assess the sorbent properties of activated carbon.

Effect of activated carbon on PCR-based assays: The effect of activated carbon on the ability of RT-PCR to detect SIV and PRRSV was tested. Two treatment levels of activated charcoal 1% and 2% (coal-derived and wood-derived) were inoculated with known quantities of SIV and PRRSV and tested by PCR. **Results:** All samples containing virus (PRRSV or SIV) tested positive by RT-PCR and all negative control samples tested negative. Therefore, PCR-based assays could be used to test the sorbency of activated charcoal compounds.

Sorbency of activated carbon: Sampling in the field (in the future) will be done under a variety of environmental conditions (temperature, relative humidity, dust, etc.).

It can be expected that aerosolized compounds, for example endotoxin, will shift the pH of the collection medium. Therefore, we tested the sorbency of activated carbons (coal-derived and wood-derived) at various pH levels. To conduct the study, PRRSV and SIV were added to PBS containing activated carbon treatments and incubated at 37 C and 5% CO_2. The study was conducted at various levels of pH (5.5, 6.0, 6.5, 7.0) to test whether an interaction between pH and adsorption existed. Samples were collected (0, 30, 60, 90, and 120 min), centrifuged and the pellet submitted for quantitative PCR.
Results: Wood-derived activated carbon actively adsorbed both PRRSV and SIV (Table 2). The difference in the rate of sorbency was statistically significant between carbon-derived and wood-derived activated carbon ($P < 0.0001$). The effect was independent of pH and occurred essentially instantaneously. It may be speculated that the large micropore structure of the wood-derived activated carbon interacted more efficiently with the viral particles.

Table 2. Effect of activated carbon type on adsorption of PRRSV and SIV

		PRRSV			SIV		
pH	Time	Control	Carbon	Wood	Control	Carbon	Wood
5.5	0	6.20[a]	0.00	5.20	4.20	0.00	3.30
5.5	30	6.00	1.00	5.40	4.10	0.00	3.70
5.5	60	6.30	3.60	5.50	4.20	0.00	4.00
5.5	90	6.10	1.70	5.50	4.10	0.00	3.90
5.5	120	5.80	1.30	5.70	4.00	0.00	3.90
6.0	0	5.90	0.00	5.30	3.80	0.00	4.00
6.0	30	5.70	3.40	5.60	3.60	1.90	3.80
6.0	60	5.90	1.80	5.60	3.90	0.00	4.10
6.0	90	5.40	1.10	5.80	3.40	0.00	4.00
6.0	120	6.00	1.10	5.60	4.00	0.00	4.10
6.5	0	5.30	0.70	5.10	3.60	0.00	3.30
6.5	30	5.90	0.90	6.30	3.80	0.00	3.70
6.5	60	5.50	0.90	5.40	3.50	0.00	3.70
6.5	90	5.20	1.50	5.30	3.30	0.00	3.80
6.5	120	5.50	2.10	5.60	3.60	0.00	3.90
7.0	0	6.00	2.60	5.40	3.60	1.80	1.80
7.0	30	5.60	2.50	5.60	3.60	0.00	3.90
7.0	60	5.60	2.70	5.50	3.70	1.90	3.90
7.0	90	4.70	2.80	5.40	2.20	1.90	3.70
7.0	120	5.70	0.00	5.60	3.60	0.00	3.80

[a]Values are expressed in logs as is EID50 per 0.2ml.

D. Effects of ethylene glycol on cell lines and virus infectivity.

PRRSV is stable at 4° C, but increasingly liable as ambient temperature increases (Benfield et al. 1992). This suggests that, in temperate climates, the likelihood of transmission via aerosolized virus could be much different in colder vs warmer months

of the year. Therefore, we investigated the possibility of designing a medium that would allow air sampling at temperatures near or below 0° C.

The possible cytotoxic and virucidal effects of ethylene glycol (20%) was first tested. A 20% solution of ethylene glycol in PBS will decrease the medium's freezing point to -8° C. Monolayers of MARC-145 cells on 96 well plates were exposed for 2 hr to a 20% solution of ethylene glycol and PBS at 37°C in a 5% CO_2 humidified incubator. After exposure, the cells were rinsed with PBS, MEM maintenance media was added, and cells were incubated for an additional 22 hr. A neutral red assay was performed and absorbance read using a spectrophotometer to calculate percent cell population viability. **Results:** Ethylene glycol was found to significantly ($P < 0.001$) decrease cell viability of MARC-145 cells when exposed for a 2 hr time period (data not shown). The percent survivability of treated cells compared to control wells was 86.6 percent. However, no significant difference ($P > 0.05$) was detected in virus infectivity between control and ethylene glycol treatment for PRRSV (Table 3), i.e., ethylene glycol was not found to have virucidal effects on PRRSV for exposure periods of up to 2 hrs. Additionally, ethylene glycol did not interfere with quantitative RT-PCR for PRRSV and SIV.

Table 3. Effect of ethylene glycol on virus infectivity and PCR-based assays

Time (min)	0% Ethylene glycol		20% Ethylene glycol	
0	3.5[a]	6.2[b]	4.0	6.5
30	3.5	6.2	4.25	6.6
60	4.0	6.4	4.0	6.6
90	4.0	6.4	4.0	6.3
120	3.75	6.3	3.75	6.6

[a]Values are expressed in tissue culture infectious dose 50's ($TCID_{50}$).
[b]Values are expressed in logs as is EID50 per 0.2ml.

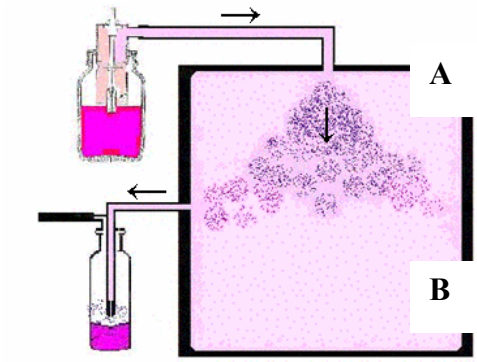
Part II. Ability to generate and capture infectious virus particles

Trincado et al. (2004) described the use of the AGI-30 impinger to sample air for PRRSV. Virus was detected by PCR, but no infectious virus was detected - even using the most sensitive assay, i.e., swine bioassay. Predicting the likelihood of virus transmission between herds is dependent upon our ability to quantify infectious virus, because, by definition, non-infectious virus poses no treat.

Optimization of aerosol collection media demands that we understand the requirements of collecting and quantifying infectious virus particles.

How does impinger sampling time and affect the concentration of virus detected?
To address this question, various levels of PRRSV in PBS were aerosolized into 25 liter glass cylinder (aerosol reservoir)(See diagram). In six replicates, samples were taken at 0, 5, 10, 15, 20, 25, and 30 minutes. For comparison, AGI-30 and SKC-biosampler® impingers were ran in independent replications at each treatment level of PRRSV. Samples were tested by quantitative RT-PCR and microinfectivity assays.

Aerosol Sampling Apparatus:
A: Collison nebulizer, B: Impinger, C: Aerosol Reservoir



C

Results: The Collison nebulizer generated infectious clouds of PRRSV detected by impingers. No significant difference exists in quantity of infectious material in the suspension fluid at the conclusion of each replication. Although, run 3 using the AGI-30 has discrepancies and needs to be repeated. Increased sampling times increased the amount of infectious virus collected. However, PCR detected viral RNA at all time points tested. A sampling time of 30 minutes was optimal (AGI-30 and SKC-biosampler®) for collection of infectious virus using the current model.

Table 4. Results of sampling time and viral load using AGI-30.

Time	Run 1				Run 2				Run 3			
	Nebulizer		Impinger		Nebulizer		Impinger		Nebulizer		Impinger	
	$TCID_{50}$	PCR	$TCID_{50}$	PCR	$TCID_{50}$	PCR	$TCID_{50}$	PCR	$TCID_{50}$	PCR	$TCID_{50}$	PCR
0	4.75[a]	6.70[b]	0.00[a]	0.00[b]	3.75	6.30	0.00	0.00	3.25	6.20	0.00	0.00
5	4.75	6.70	3.00	4.40	4.25	6.20	0.0	4.00	2.50	5.90	0.0	4.10
10	4.75	6.60	3.00	4.60	3.75	6.20	0.0	4.70	2.10	5.90	0.0	4.10
15	4.50	6.50	3.00	4.60	4.00	6.00	2.50	4.60	0.00	5.80	0.0	4.70
20	5.00	6.50	3.12	4.90	3.60	6.10	2.50	4.70	0.00	6.10	0.0	4.50
25	4.50	6.40	3.25	5.00	3.50	6.20	2.60	4.90	0.00	6.20	2.25	5.00
30	4.50	6.30	3.63	5.60	3.50	6.20	2.75	4.70	0.00	6.00	2.25	5.20

[a]Values are expressed as $TCID_{50}$.
[b]Values are expressed in logs as is EID50 per 0.2ml.

Table 5. Results of sampling time and viral load using SKC-biosampler®

Time	Run 1				Run 2				Run 3			
	Nebulizer		Impinger		Nebulizer		Impinger		Nebulizer		Impinger	
	$TCID_{50}$	PCR	$TCID_{50}$	PCR	$TCID_{50}$	PCR	$TCID_{50}$	PCR	$TCID_{50}$	PCR	$TCID_{50}$	PCR
0	4.75[a]	6.50[b]	0.00[a]	0.00[b]	2.75	6.40	0.00	0.00	3.25	5.90	0.00	0.00
5	4.75	6.40	2.50	4.70	2.10	5.80	0.00	4.30	3.00	5.60	2.25	4.20
10	4.75	6.50	2.60	4.70	2.30	5.90	2.20	4.00	2.30	5.70	2.25	4.30
15	4.75	6.50	2.50	5.10	2.10	5.90	2.50	4.30	2.50	5.70	2.25	4.70
20	4.50	6.40	3.00	4.40	2.37	5.70	2.50	4.70	2.37	5.80	2.12	4.70
25	5.00	6.50	2.75	5.60	2.12	5.90	2.20	4.70	2.25	5.50	2.37	4.80
30	4.75	6.70	3.25	5.60	2.37	5.70	2.50	5.20	2.50	5.60	2.37	4.60

[a]Values are expressed as $TCID_{50}$.

[b]Values are expressed in logs as is EID50 per 0.2ml.

Comparison of impinger performance. Two air sampling devices (AGI-30 and SCK-biosamper®) were simultaneously tested to determine the superior impinger performance. The SKC BioSampler® and All Glass Impinger 30 (AGI-30) both sample 12.5 liters of air per minute. An artificial aerosol cloud was generated using a collision nebulizer with varying concentrations of PRRSV in 100 ml of PBS. The cloud was sampled in a carboy equipped with 6 air samplings ports where AGI-30 and SCK-biosamper® were ran for 30 min. No differences were observed in collection of infectious virus or total viral particles between the AGI-30 and SKC-biosampler®.

Table 6. Performance of AGI-30 vs. SKC-biosampler®.

Time	SKC		AGI-30	
	$TCID_{50}$	PCR	$TCID_{50}$	PCR
30	3.75[a]	4.90[b]	3.50	4.90
30	3.25	5.00	3.25	5.00
30	3.50	4.90	3.50	4.70

[a]Values are expressed as $TCID_{50}$.
[b]Values are expressed in logs as is EID50 per 0.2ml.

Lay Interpretation: Understanding the routes of PRRSV transmission and doses required to cause infection within and between herds is critical for control and eradication of PRRSV. Aerosol research is dynamic and complex in nature. Detecting and monitoring bio-aerosols is dependent on many variables including, sampling media, sampling time, impinger type, and pathogen. Describing aerosol behavior requires standardizations of methods for consistent and meaningful results. The current research has attempted to explain these variables and methods in an effort to understand aerosol transmission more clearly. Specifically, we conclude that, among those tested, 1) Antifoam C Emulsion (Sigma A8011), and 2) wood derived activated carbon where superior antifoams and sorbents for use in aerosol collection media. Specific work that remains to be done includes:
1) Further investigation of the efficacy of sorbents using artificially created aerosols.
2) Improved estimates of sampling time for impingers and comparison of optimized media vs. controls.
3) Determination of collection efficiency of the AGI-30 and SKC-biosampler®.
4) Characterizing behavior of viruses in aerosols at different relative humidity's and temperatures.

Proceedings and abstracts:

Hermann J. R., R.B Evans, S.J. Hoff, K. Yoon, and J. Zimmerman. November, 2003. Effect of exposure to commercial antifoams on the viability of three continuous cell lines. Proc 84th Ann Conf of Research Workers in Animal Disease, Absrtact 20.

Hermann J. R., Evans R.B., Hoff S.J., Zimmerman J. J. October, 2003. Effect of exposure to commercial antifoams on the viability of three continuous cell lines. Proc 46th Ann Conf Am Assoc Vet Lab Diagnosticians, pp. 154.

Oral Presentations:

Hermann J. R., K. Yoon, Evans R, Hoff S, Zimmerman J. November 10, 2003. Cell line viability and virus infectivity related to commercial antifoam exposure. 2003 Conference of Research Workers in Animal Diseases. Chicago, IL.

Hermann J. R., Evans RB, Hoff SJ, Zimmerman J. October 12, 2003. Effect of exposure to commercial antifoams on the viability of three continuous cell lines. 46th Annual Conference, American Association of Veterinary Laboratory Diagnosticians. San Diego, California.

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Benfield D.A., Nelson E., Collins J.E., Harris L., Goyal S. M., Robison D., Christianson W. T., Morrison R.B., Gorcyca D., and D. Chladek. 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J Vet Diagn Invest 4:127-133.

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