Evaluation of Sanitation Procedures in Swine Nursery Facilities Utilizing ATP Bioluminescence

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Summary

Following an *in vivo* animal challenge experiment, 44 pens, which had previously individually housed pigs inoculated with Enterotoxigenic *Escherichia coli* F18 (ETEC), were assigned to one of four sanitation treatment protocols. Pens were assigned to treatment in a randomized complete block design over three separate days of cleaning and disinfection. There were 11 replicates per treatment, and the pen served as the experimental unit. Within each pen, five sampling locations included the feeder lip, exterior of the stainless-steel feeder, center of the metal flooring, flooring in the corner of the pen, and a solid plastic pen divider on the corner wall. Treatments included: 1) hot water (HW, 138.6°F) pressure washing followed by Synergize (1:256), 2) cold water (CW, 73.8°F) pressure washing followed by Synergize, 3) HW pressure washing followed by Virkon S (1:128), 4) pre-treatment of BarnStorm (1:64) then HW followed by Synergize. Synergize, Virkon S, and BarnStorm applications all had ≥ 10 min contact time. Adenosine triphosphate (ATP) bioluminescence sampling was taken before and after sanitation treatment at each sampling location within the pen (UltraSnap surface ATP test and Luminometer, Hygenia, Camarillo, CA). No significant sanitation treatment × sampling location interactions were observed. Pens treated with HW followed by Virkon S showed a reduction ($P < 0.001$) in ATP compared to other sanitation treatments. Differences in residual surface ATP were observed between sampling locations ($P < 0.001$), with the highest residual levels post-sanitation detected on the feeder lip and the lowest on the solid plastic pen divider on the corner wall. These results suggest that pressure washing with HW followed by disinfection with Virkon S was the most effective protocol of those evaluated for reducing surface ATP in swine housing facilities contaminated with ETEC. The feeder lip is the most difficult area to sanitize, highlighting that differences in accessibility and surface type affect cleaning efficacy.

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Introduction

Enterotoxigenic *Escherichia coli* is of high concern to the swine industry as it causes postweaning diarrhea (PWD), a condition that is becoming resistant to current available antimicrobial therapies. Preventing the persistence of ETEC in contaminated environments is critical, as poorly sanitized facilities can lead to repeated infections in successive litters or weanling pigs. Traditionally, cleanliness assessments have relied heavily on visual inspections, which are often subjective.[3] Adenosine triphosphate (ATP) bioluminescence was introduced as a way of determining cleanliness for livestock industries. However, this testing method has a low specificity and cannot distinguish between sources of microbial and non-microbial ATP. The presence of ATP increases the likelihood that infectious agents are present, risking the integrity of biosecurity practices. Previous studies have implemented the use of ATP bioluminescence to measure the cleanliness of livestock trailers,[3] swine farrowing units,[4] as well as other applications. Compared to traditional bacterial culture methods, ATP bioluminescence offers a fast, real-time indicator of cleaning effectiveness, providing insight into ways current sanitation protocols may be failing.

Traditional methods of assessing sanitation efficacy, such as microbiological cultures, viral isolation, or polymerase chain reaction (PCR), are time-consuming, labor-intensive, and costly. Adenosine triphosphate bioluminescence serves as a sensitive, rapid, easy, and inexpensive tool that works by measuring the amount of ATP present on the surface. Adenosine triphosphate is a ubiquitous component of all living cells, including but not limited to bacteria, parasites, fungi, and mammalian cells. Cells are collected and exposed to a liquid-stable reagent in the testing device. When ATP is released from these cells, it reacts with luciferin/luciferase enzymes (present in UltraSnap tests used for sampling), producing light. The amount of light emitted is directly proportional to the ATP concentration and is recorded as relative light units (RLU). Higher RLU readings indicate dirtier surfaces, and lower readings indicate cleaner surfaces. However, the accuracy of ATP bioluminescence can be affected by the type of disinfectant used. Some studies have shown that disinfectants based on quaternary ammonium compounds (QACs), such as Synergize (1:256; Neogen Corporation, Lansing, MI), can artificially increase ATP readings, leading to false positives.[5,6] Other studies have shown hydrogen peroxide-based disinfectants may suppress ATP signals in a process referred to as "quenching," resulting in false negatives.[6,7] The objective of this experiment was to evaluate four different sanitation protocols in a Biosafety Level 2 (BSL-2) facility following a live-animal experiment using Enterotoxigenic F18 *Escherichia coli* to evaluate surface adenosine triphosphate concentration as a marker of sanitation efficacy. The aim was to

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[7] Omidbakhsh, N., F. Ahmadpour, N. Kenny. 2014. How reliable are ATP bioluminescence meters in assessing decontamination of environmental surfaces in healthcare settings? PLoS One. 9(6):e99951. doi: 10.1371/journal.pone.0099951.

identify a superior protocol based on the ability to effectively remove biological contamination following exposure to Enterotoxigenic *Escherichia coli* F18.

Materials and Methods

This study evaluated four disinfection treatment methods to assess their effectiveness in cleaning various surfaces within a pen. The study was conducted at the Kansas State University Swine Enteric Health Research Center following a 14-d Enterotoxigenic *Escherichia coli* (10^9 cfu/mL, 10^8 cfu/mL, or 10^7 cfu/mL) challenge experiment. Forty-four pens were selected, with each disinfection protocol applied to 11 pens. Each pen served as an experimental unit and was allotted using a randomized complete block design into one of four treatment groups, taking into consideration the three dosage treatments from the previous study. The experiment was conducted over three days, with the four treatment groups represented on each of the three days. The pens were treated and sampled as follows: 12 on day 1, 16 on day 2, and 16 on day 3.

The first treatment protocol involved pressure washing with hot water (138.6°F) followed by foam application of Synergize (1:256; Neogen Corporation, Lansing, MI), a disinfectant that contains quaternary ammonium compounds (QACs) and glutaraldehyde. Synergize was diluted to 1:2 using water, then the stock solution was diluted through a foam applicator at 1:128, creating a final dilution of 1:256 applied onto surfaces. It is a representation of the common disinfection protocol used in Kansas State University Swine Enteric Health Research Center and served as the control treatment. The second protocol involved pressure washing with cold water (73.8°F) followed by foam application of Synergize to simulate barn sanitation practices where hot water is not available. The third protocol involved pressure washing with hot water (138.6°F) followed by foam application of Virkon S (1:128; Lanxess Corporation, Pittsburgh, PA), an accelerated hydrogen peroxide potassium peroxymonosulfate-based disinfectant selected for its strong oxidizing and broad-spectrum antimicrobial properties. A 10% Virkon S stock solution was created then diluted through a foam applicator at a 1:10 dilution for a final concentration of 1% applied onto surfaces. The last protocol involved pressure washing with hot water (138.6°F) followed by pre-treatment with BarnStorm (1:64; Preserve International, Zephyr Cove, NV), then a hot water rinse followed by Synergize foam-application. This protocol was built upon the control protocol by adding a pre-treatment step with BarnStorm, an organic acid detergent designed to disrupt biofilms, a known virulence factor of ETEC. This treatment aimed to test the potential improvement on the current protocol's cleaning efficacy with the specific addition of a biofilm-targeting agent. All products were left on surfaces for a minimum contact time of 10 min in coordination with the manufacturer's label recommendation. All pen surfaces were thoroughly rinsed with cold water before post-sanitation sampling occurred.

The four disinfection protocols were evaluated by adenosine triphosphate (ATP) bioluminescence at five different locations within each pen. All sampling locations were approximately 10×10 cm^2. The five sampling locations included the feeder pan lip (both exterior and interior), the exterior side of the feeder, the center of the metal flooring, the corner of the flooring, and the corner wall's solid plastic pen divider (Figure 1).

ATP bioluminescence assay

Adenosine triphosphate bioluminescence sampling occurred before and after sanitation at each of the five sampling locations within each pen (UltraSnap surface ATP test and Hygenia SystemSURE/EnSURE Luminometer, Hygenia, Camarillo, CA). Adenosine triphosphate samples were obtained by removing the swab from the test tube and swabbing an approximately 10 × 10 cm^2 area in horizontal, vertical, and diagonal zigzag directions. The swab was placed back in its original tube. The snap valve at the top was broken by bending the bulb then squeezed two to three times to push the liquid reagent containing luciferin/luciferase into the hollow shaft of the swab and subsequently into the compartment for interaction with the collected sample. The tube was shaken for 10 sec and then placed in the luminometer (Figure 2). Results were produced after approximately 15 sec and recorded in relative light units (RLUs).

Statistical analysis

Data were analyzed as a randomized complete block design for one-way ANOVA using the lmer function from the lme4 package in R (version 3.5.1 (2018-07-02), R Foundation for Statistical Computing, Vienna, Austria) with pen considered as the experimental unit. Sampling location, treatment, and the associated interaction were considered fixed effects in the statistical model, and pen, day, and *E. coli* inoculation dosage were included as random intercepts. For evaluation of the effect of disinfectant residue on the surface ATP concentration, a linear model was fit using the lm function from the lme4 package in R, with pen considered the experimental unit, and disinfectant type a fixed effect in the model. Results were considered significant with $P \leq 0.05$.

Results and Discussion

For surface ATP detection, there were no significant treatment × location interactions for samples collected before sanitation, after sanitation, or reduction in ATP counts (Table 1). This lack of interaction indicates the efficacy of the assigned treatments does not change when applied to different locations in the pen, and all locations sampled are equally affected by the type of treatment used.

As expected, for the main effect of sanitation treatment, there were no differences in surface ATP measurements between treatments before sanitation (Table 2). Following sanitation, the combination of hot water + Virkon S had lower ($P < 0.001$) residual surface ATP compared to the other sanitation treatments, leading to a greater ($P < 0.001$) reduction in surface ATP for hot water + Virkon S compared to all other treatments.

For the main effect of sampling location (Table 3), the exterior of the feeder had a greater ($P < 0.05$) surface ATP measurement before sanitation compared to the center and corner of the flooring and wall in the corner of the pen, with the feeder lip intermediate. Following sanitation, the feeder lip had a greater ($P < 0.05$) amount of residual ATP compared to the outside of the feeder, flooring in the corner of the pen, and wall located in the corner of the pen. The center flooring of the pen had greater ($P < 0.05$) residual ATP than the flooring or wall in the corner of the pen, and the wall in the corner of the pen had the lowest ($P < 0.05$) residual ATP. This resulted in differences in ATP reduction ($P < 0.05$), where the corner wall demonstrated the highest reduction, followed by the exterior feeder and corner floor as intermediate, with the center floor and feeder lip seeing the lowest reduction in surface ATP.

The results of this study indicate that pressure washing with hot water followed by disinfection with Virkon S is the most effective sanitation protocol for reducing surface ATP levels in pig pens contaminated with Enterotoxigenic *Escherichia coli* F18 (ETEC). Alternatively, the results suggest this treatment leaves less bacterial residues, thereby enhancing the reliability of this method when evaluating cleanliness. To test if residue from the disinfectants used to sanitize the surfaces of the pens interferes with the accuracy of the ATP Luminometer, further work is being conducted to detect the total impact of Synergize and Virkon S on the ATP bioluminescence enzymatic reaction.

The study also revealed that the feeder lip is the most challenging area within the pen to clean effectively, whereas the corner wall's solid plastic pen divider was the easiest. Adenosine triphosphate bioluminescence is a quick, easy-to-use method of evaluating cleanliness in pig pens; however, the potential impact of disinfectant residue on the accuracy of ATP readings remains uncertain and warrants further investigation. While ATP bioluminescence has a high sensitivity, it lacks specificity due to its inability to differentiate between microbial and non-microbial sources of ATP. Therefore, it should be utilized as a broad assessment tool of cleanliness in conjunction with other evaluation techniques such as bacterial culture methods.

Table 1. Effect of sanitation protocol and sampling location on detection of surface ATP[1]

Sampling site	Hot water + Synergize	Cold water + Synergize	Hot water + Virkon S	Hot water + BarnStorm + Synergize	SEM	Treatment × location, *P* =
Before sanitation					0.406	0.952
Feeder lip	5.98	6.50	6.17	6.06		
Exterior feeder	6.46	6.66	6.35	6.27		
Center floor	5.97	5.72	5.62	5.75		
Corner floor	5.66	5.89	5.29	5.74		
Corner wall	5.41	5.56	5.78	5.77		
After sanitation[2]					0.300	0.470
Feeder lip	5.28	5.49	4.52	5.18		
Exterior feeder	4.77	4.98	3.90	4.08		
Center floor	5.24	5.28	3.47	4.97		
Corner floor	4.49	4.78	3.05	4.36		
Corner wall	3.44	3.73	2.93	3.36		
Reduction[3]					0.436	0.927
Feeder lip	0.71	1.02	1.65	0.88		
Exterior feeder	1.71	1.68	2.46	2.19		
Center floor	0.75	0.44	2.15	0.78		
Corner floor	1.18	1.11	2.25	1.37		
Corner wall	1.99	1.83	2.86	2.41		

[1] ATP measured in $\log_{10}$ adjusted relative light units (RLU). There were 11 replicates per treatment.
[2] Lower values after sanitation indicate a better job of cleaning and cleaner surfaces. Higher values after sanitation indicate a worse job of cleaning.
[3] Higher reduction values indicate better cleaning.

Table 2. Effect of sanitation protocol treatment on detection of surface ATP[1]

Item	Hot water + Synergize	Cold water + Synergize	Hot water + Virkon S	Hot water + BarnStorm + Synergize	SEM	Treatment, *P* =
Before sanitation	5.90	6.07	5.84	5.92	3.051	0.767
After sanitation[2]	4.64[a]	4.85[a]	3.57[b]	4.39[a]	0.152	< 0.001
Reduction[3]	1.27[b]	1.22[b]	2.28[a]	1.53[b]	0.330	< 0.001

[1] ATP measured in $\log_{10}$ adjusted relative light units (RLU). There were 11 replicates per treatment.
[2] Lower values after sanitation indicate a better job of cleaning and cleaner surfaces. Higher values after sanitation indicate a worse job of cleaning.
[3] Higher reduction values indicate better cleaning.

Table 3. Effect of sampling location on detection of surface ATP[1]

	Sampling location						
Item	**Feeder lip**	**Exterior feeder**	**Center floor**	**Corner floor**	**Corner wall**	**SEM**	**Location, *P* =**
Before sanitation	6.18[ab]	6.44[a]	5.77[b]	5.64[b]	5.63[b]	0.307	< 0.001
After sanitation[2]	5.12[a]	4.43[bc]	4.74[ab]	4.17[c]	3.36[d]	0.162	< 0.001
Reduction[3]	1.06[c]	2.01[ab]	1.03[c]	1.48[bc]	2.27[a]	0.338	< 0.001

[1] ATP measured in $\log_{10}$ adjusted relative light units (RLU). There were 11 replicates per treatment.
[2] Lower values after sanitation indicate a better job of cleaning and cleaner surfaces. Higher values after sanitation indicate a worse job of cleaning.
[3] Higher reduction values indicate better cleaning.

Figure 1. 1a) Sampling of the central outer lip of the pen feeder with the UltraSnap surface ATP Test (Hygenia, Camarillo, CA); 1b) Sampling of exterior side of inverted feeder with the UltraSnap surface ATP Test; 1c) Sampling of center paneling of triangular bar flooring in pen; 1d) Sampling of right back corner paneling of triangular bar flooring in pen; 1e) Sampling of corner wall solid plastic pen divider in between metal bars of pen.

Figure 2. Luminometer (Hygenia, Camarillo, CA) with reading of 52 relative light units (RLU).