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EVALUATION OF COMMERCIAL OFF-THE-SHELF SOLUTIONS FOR SUPPORTING VIABILITY RETENTION OF *YERSINIA PESTIS* CELLS

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Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

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There is a pressing need to preserve the viability of biological warfare (BW) threat agents collected in the field. Despite major gains in nucleic acid sequencing technologies, true positive identification and credible threat assessment of BW agents rely on the direct culture (and quantification) of the "live" cells. Solutions aiding the viability sustainment of pathogens contained within clinical samples have been recommended in the past, but many of these have not been examined for their ability to preserve BW cells relevant to the U.S. military (e.g., *Yersinia pestis*). The purpose of this study was to evaluate the effectiveness of 17 different solutions in preserving the viability of non-refrigerated, avirulent *Y. pestis* A1122 cells that were collected from various surfaces. The solutions were evaluated for viability sustainment over a 14 day period at three different temperatures (4, 22, and 40 °C). Polymerase chain reaction analysis was performed on samples that did not show any viable cells at the end of the study to determine if nucleic acids were degraded. The study also evaluated the sampling efficiency and decay rates (at three temperatures) of *Y. pestis* cells that were inoculated on two different surfaces.

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PREFACE

The work described in this report was authorized under the Defense Threat Reduction Agency. The work was started in October 2015 and completed in October 2016.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. The report may not be cited for purposes of advertisement.

This report has been approved for public release.

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EVALUATION OF COMMERCIAL OFF-THE-SHELF SOLUTIONS FOR SUPPORTING VIABILITY RETENTION OF *YERSINIA PESTIS* CELLS

1. INTRODUCTION

Effective biological sampling from environmental surfaces and complex structures is essential to virtually every level of bio-release incident response, including sample collection, investigation, and analysis (Edmonds, 2009; Piepel et al., 2012). In many cases, collected biological samples cannot be analyzed in a timely manner; therefore, they must be preserved or stabilized to minimize cell decay and optimize viability. Typically, this is accomplished through refrigeration or cold storage. Such requirements are often resource intensive and are not available in austere or field conditions. This is a key technical capability gap that needs to be addressed because the ability to culture a sample to determine what microorganisms are present remains critical, despite advances in genomics and proteomics. For example, culturing and growing a biothreat microorganism remains the "gold standard" for identification in a Sample Receipt Facility (Sanderson et al., 2004; USAMRIID, 2014). Identification of the biological warfare (BW) threats using this method ensures that the samples were collected from an active culture and were not the result of residual environmental contamination.

Major threat agents such as *Bacillus anthracis*, *Yersinia pestis*, and *Burkholderia mallei* are endemic in many parts of the world; therefore, a positive polymerase chain reaction (PCR) signal may not confirm a deliberate biological attack. The results from a study that was based on sequencing biological samples collected from the New York City subway system suggested the presence of *Y. pestis* and *B. anthracis*, even though no deliberate contamination was verified (Afshinnekoo et al., 2015). In fact, as of 2015, there have been no reported cases of naturally occurring *Y. pestis* infection within 1000 miles of New York City (Ackelsberg et al., 2015). These examples unequivocally exemplify that for meaningful threat assessment, it is essential to culture the BW agents from field samples within the laboratory. Culturing, growing, and quantifying the number of viable cells are critical inputs for assessing a biothreat and formulating fully informed decisions. These results are expected to provide decision makers with the most accurate information about the presence and the extent of a BW agent release and allow for appropriate operational and consequential responses.

The main objective of this study was to evaluate the potential effectiveness of 17 different solutions for their ability to support and sustain viable cells under non-refrigerated storage for an avirulent strain of *Y. pestis* A1122. The solutions were evaluated for effectiveness over a period of 14 days at three different temperatures (4, 22, and 37 or 40 °C). Real-time PCR analyses were performed on nonviable samples at the end of the 2 week holding period. In additional experiments, the sampling efficiency and decay rates of the *Y. pestis* A1122 strain cells on two different surfaces (stainless steel and painted concrete) were studied.

2. MATERIALS AND METHODS

2.1 Reagents, Mediums, and Biological Preservatives

All chemicals and bacterial mediums were molecular grade and were purchased from either Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA). Butterfield's buffer was prepared according to the U.S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (FDA, 2013). In this study, 17 different potential biological preservative solutions were evaluated. Table 1 lists the selected preservation systems and the associated manufacturers.

Table 1. Preservation Materials

No.	Preservation System	Manufacturer	Catalog Number
1	Butterfield's phosphate buffer	FDA formulation	–
2	All-In-One swab kit	QuickSilver Analytics, Inc. (Belcamp, MD)	10193
3	Biomatrica custom formulation no. 1	Biomatrica, Inc. (San Diego, CA)	–
4	Biomatrica custom formulation no. 2	Biomatrica, Inc.	–
5	BBL CultureSwab	Becton, Dickinson, and Co. (Franklin Lakes, NJ)	220099
6	BD ESwab	Becton, Dickinson, and Co.	220245
7	Buffered peptone water (BPW), 5 g/mL	Sigma-Aldrich Co., LLC	77187
8	BPW, 20 g/mL	Sigma-Aldrich Co., LLC	77187
9	Copan ESwab	Copan Diagnostics, Inc. (Murrieta, CA)	480C
10	Copan swab rinse kit (SRK)	Copan Diagnostics, Inc.	R4160
11	Puritan liquid Amies transport kit	Puritan Medical Products Co. (Guilford, ME)	LA-116
12	Remel Sanicult transport swabs	Thermo Fisher Scientific, Inc.	R723140
13	Skim milk (filtered)	Cloverland Farms Dairy (Baltimore, MD)	–
14	Spent tryptic soy broth (sTSB)	Thermo Fisher Scientific, Inc.	R112731
15	Tryptic soy broth (TSB), diluted 1/50	Thermo Fisher Scientific, Inc.	R112731
16	TSB, diluted 1/100	Thermo Fisher Scientific, Inc.	R112731
17	TSB, diluted 1/1000	Thermo Fisher Scientific, Inc.	R112731

Notes: Shading is used for clarity; –, not applicable.

2.2 BW Agent Simulant Preparation

The avirulent strain of *Y. pestis* A1122 was obtained from the Centers for Disease Control and Prevention (CDC; Fort Collins, CO). Glycerol stocks of strain A1122 were streaked on tryptic soy agar (TSA) plates, and colonies appeared after the plates were incubated at 30 °C for 48 h. A single colony was selected from the plate and individually inoculated into four separate 50 mL, sterile, vented conical tubes that contained 10 mL of sterile brain–heart infusion (BHI) broth. These tubes were then placed in a laboratory shaker at 30 °C and processed at 200 rpm for 48 h. Following this growth step, the cultures were centrifuged, the supernatant was removed, and then the cultures were washed three times with 10 mL of sterile Butterfield's buffer. After the final wash, 50 µL of the washed cultures was added to 950 µL of Butterfield's buffer in 1.5 mL conical tubes. These samples were then centrifuged and resuspended in 1 mL of the experimental preservative solution.

2.3 Viability Testing

The prepared *Y. pestis* cells were stored at 4, 22, or 37–40 °C for up to 14 days in the selected preservatives. At the end of each time point (0, 1, 3, 4, 7, and 14 days), 100 µL of suspension was removed and serially diluted in Butterfield's buffer. These dilutions were then streaked on TSA plates in duplicate. Day 0 samples were taken immediately after cell processing. The plates were placed at 30 °C for 40–48 h, and then the resulting colonies were counted using a QCount colony counter (Advanced Instruments, Inc.; Norwood, MA) to determine the bacterial load. Results are expressed as the Log_{10} of recovered colony-forming units (cfu) or the percentage of viability compared to Day 0 (Rastogi et al., 2010; Hubbard et al., 2011; Betters et al., 2014).

2.4 Real-Time PCR

Real-time PCR was performed on samples that produced no viable cells at the end of the 14 day experimental test period using the 96-well FastBlock format on the Applied Biosystems 7900HT instrument (Thermo Fisher Scientific, Inc.). The *Y. pestis* Target 1, Target 2, and Target 4 FastBlock MasterMix materials (catalog nos. PCR-YPT-1FB-K, PCR-YPT-2FB, and PCR-YPT-4FB-K, respectively) were obtained from the Defense Biological Product Assurance Office (DBPAO; Frederick, MD) and used for PCR analysis. The *Y. pestis* Target 1 100× positive control, *Y. pestis* Target 2 100× positive control, and *Y. pestis* Target 4 100× positive control materials (catalog nos. PCR-YPT-1PCX, PCR-YPT-2PCX, and PCR-YPT-4PCX, respectively) were also obtained from DBPAO and were used as positive controls for each target. PCR runs consisted of 5 µL of nonviable cell suspension. To prepare the samples, 5 µL of sample (negative or positive control or experimental sample) was added to 15 µL of DBPAO MasterMix material and pipetted into the wells of an Applied Biosystems 7900HT Fast Thermal Cycling plate. The samples were run on the PCR instrument in two stages. Stage 1 consisted of 1 cycle of 50 °C for 2 min and 1 cycle of 95 °C for 20 s. Stage 2 consisted of 45 cycles of 95 °C for 1 s and 60 °C for 20 s (Buttner et al., 2004). Targets were considered positive if the cycle threshold (Ct) was <40. For statistical analysis, undetermined samples were given a Ct of 40.

2.5 Decay and Sampling Analysis

In these studies, single colonies of *Y. pestis* cells were selected from a previously streaked TSA plate and individually inoculated into 50 mL conical tubes containing 10 mL of sterile BHI broth. These tubes were then placed in a laboratory shaker at 30 °C at 200 rpm for 48 h. At the end of this incubation, 1 mL of inoculum was spotted on 2 × 2 in. stainless steel (R.G. Collins Glass Co.; Dundalk, MD) and on 2 × 2 in. painted concrete surfaces and allowed to dry for approximately 60–90 min. The paint used for the concrete surfaces was Glidden White Interior 1456 Latex Semi-Gloss (PPG Industries; Pittsburgh, PA). Once the surfaces were dry, they were sampled using Puritan foam-tipped applicators (Puritan Medical Supply; Guilford, ME); additional samples were also taken at a 1 h post-drying time point. These samples were collected according to a surface-sampling method developed for First Responders by the CDC (2012). The swab was initially used in a horizontal overlapping S pattern, followed by a vertical S pattern, and finally in a diagonal S pattern. The swab was placed in a 50 mL conical tube filled with 10 mL of Butterfield's buffer supplemented with 0.01% Tween-80. The tube was then vortexed for 2 min to aid in the removal of the cells from the collection device. Finally, the samples were plated, and the viability was assessed as described in Section 2.3.

3. RESULTS

3.1 Enumeration of *Y. pestis* A1122 Cells

Before the start of the extended storage studies, titers of *Y. pestis* A1122 were evaluated for consistent growth in BHI broth. Three experiments were performed; each consisted of three individual replicates of test strain cells inoculated in 10 mL of TSB. These cells were then grown at 30 °C for 48 h in an upright shaking incubator set at 200 rpm. Following this, the cultures were titered to determine the amount of colony-forming units per milliliter in each (Table 2). Three separate runs were also performed to assess the repeatability of the cell number after washing. The results (Figure 1) show approximately 10^8 cfu in each of the 48 h, 10 mL cultures. Results are expressed as the mean ± standard deviation (SD) of the calculated Log (cfu) from each culture.

Consistent cell numbers were therefore achieved in the samples grown and washed three times with Butterfield solution.

Table 2. *Y. pestis* Cell Density after Inoculation of Colonies in BHI Broth

Sample Replicate #	# of Colonies Inoculated	Dilution	Plate 1	Plate 2	Average	CFU	Log	Average CFU	Average Log	SD
1	1	10-6	55	11	33	330000000	8.52			
2	1	10-6	32	12	22	220000000	8.34			
3	1	10-6	47	41	44	440000000	8.64	3.3E+08	8.52	0.15
4	2	10-7	30	12	21	2100000000	9.32			
5	2	10-6	152	137	144.5	1445000000	9.16			
6	2	10-6	161	97	129	1290000000	9.11	1.61E+09	9.21	0.11
7	3	10-6	136	326	231	2310000000	9.36			
8	3	10-7	22	20	21	2100000000	9.32			
9	3	10-6	208	157	182.5	1825000000	9.26	2.08E+09	9.32	0.05

Note: Colors are used for clarity.

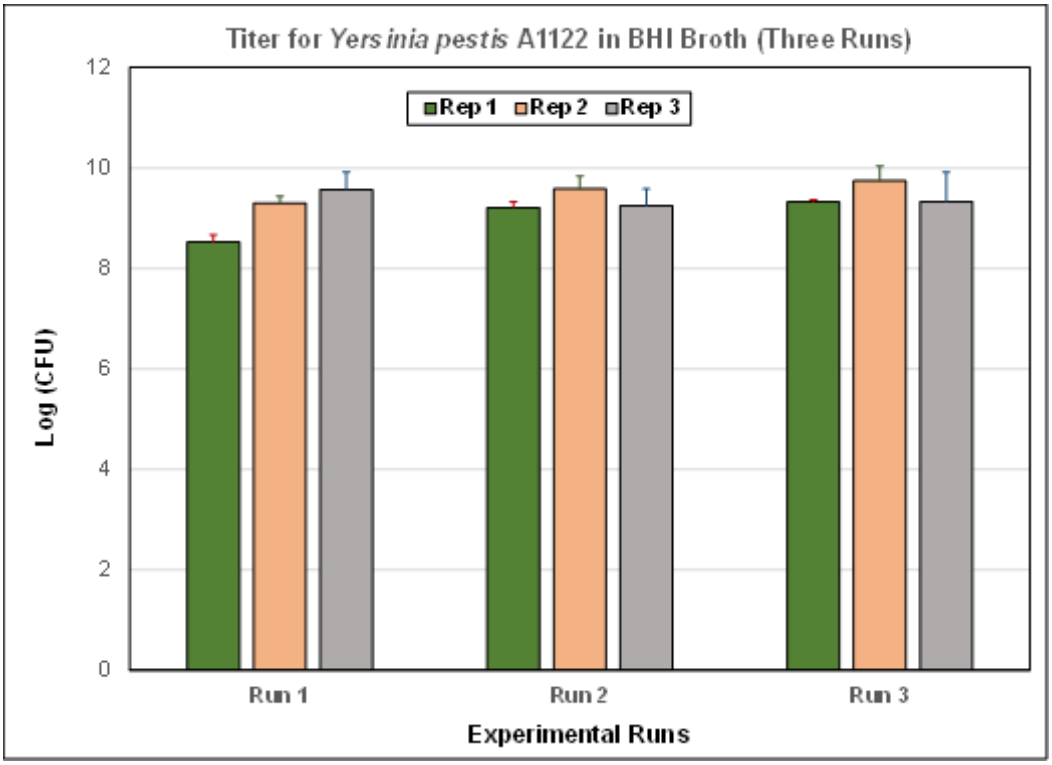
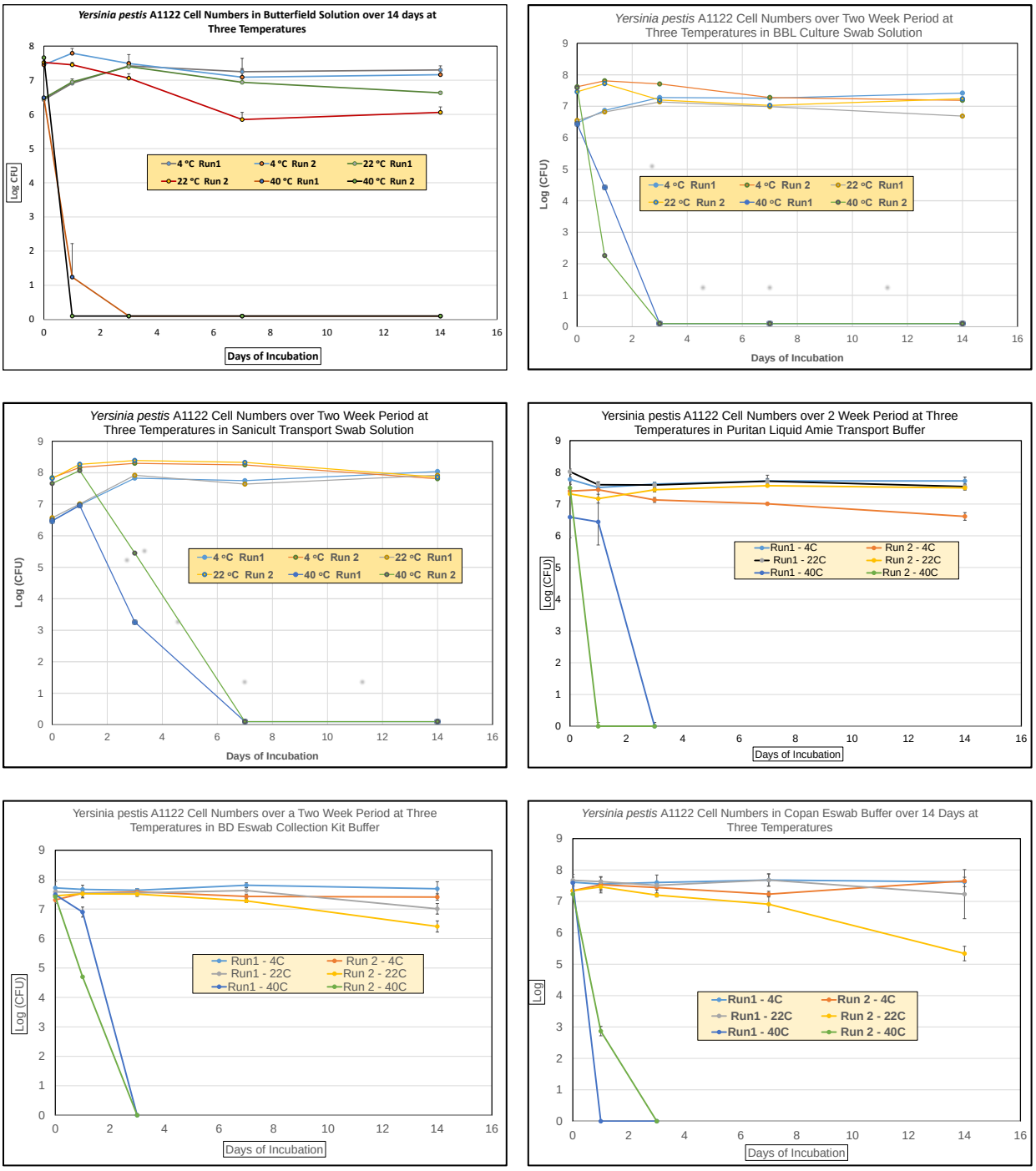


Figure 1. Titer for *Y. pestis* grown in BHI for 48 h.

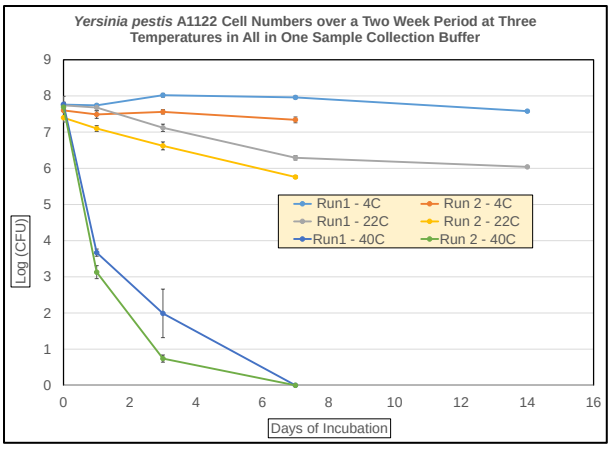
3.2 *Y. pestis* Cell Viability as a Function of Time and Temperature

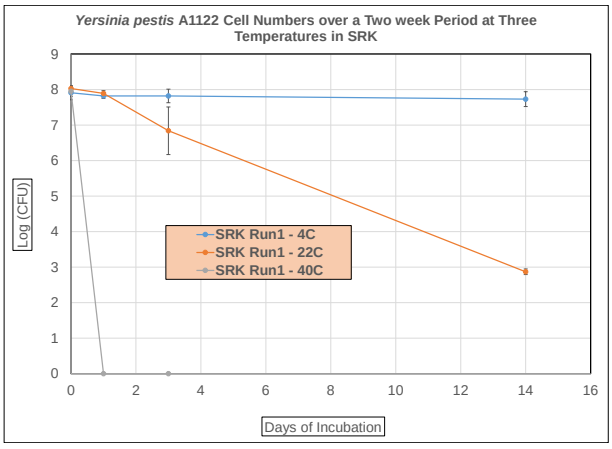
Twelve of the 17 commercial storage solutions (Table 1) were initially evaluated for aiding in the support of viability of *Y. pestis* cells at 4, 22, and 40 °C for up to a period of 14 days. The results summarized in Figure 2 show that none of these 12 solutions sustained viability of *Y. pestis* cells for a period of 2 weeks. All of the tested solutions supported cell viability at 4 and 22 °C (Table 3); however, testing at 40 °C resulted in the complete loss of viability of *Y. pestis* cells (non-permissive environment).

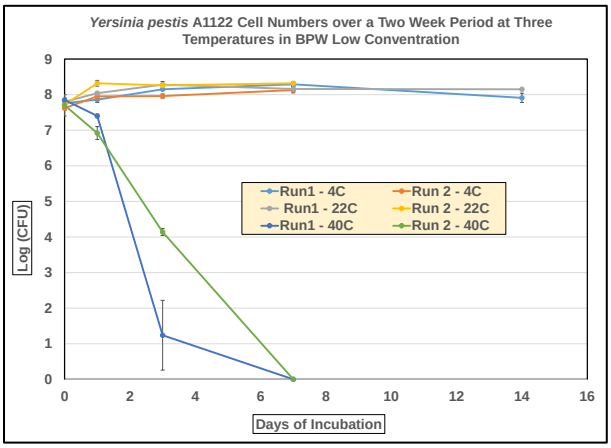


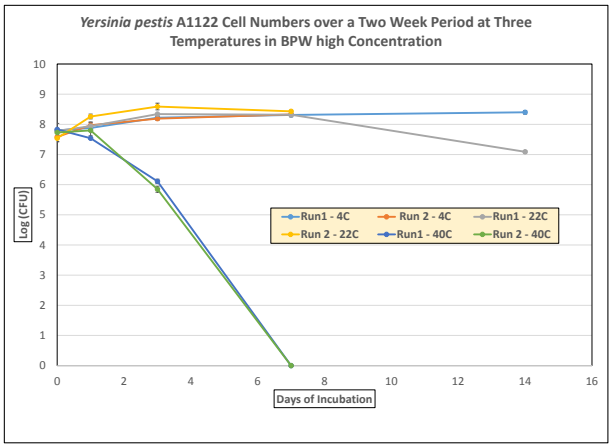
(Figure continued)

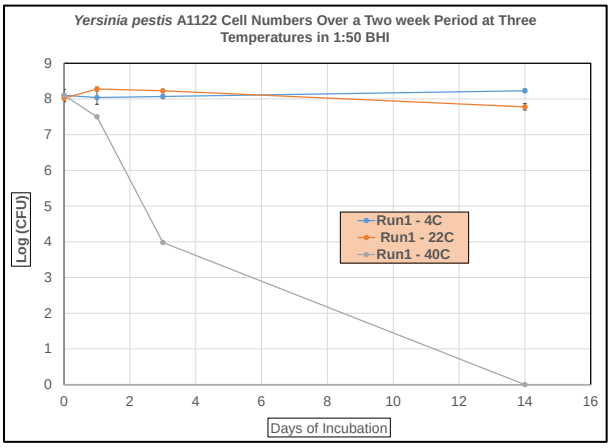
Figure 2. *Y. pestis* cell viability in 12 test solutions over a 2 week period. Results are expressed as the mean ± SD of the reported recovered Log (cfu). Experimental $n \geq 6$ for each data set.











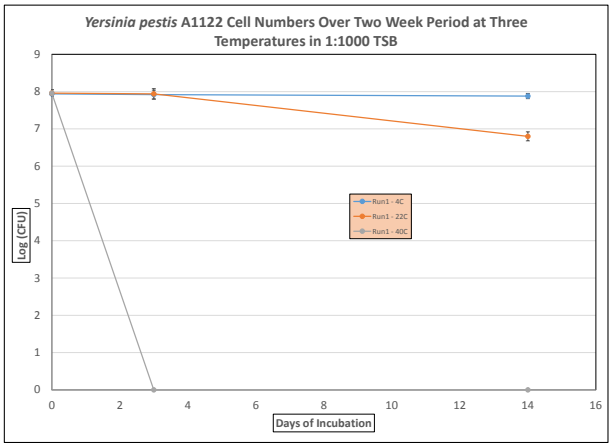


Figure 2. Continued.

Table 3. Summary of Test Solutions for Cell Viability Preservation

Preservative	# Runs	4°C - 7d	22°C - 7d	40°C - 7d	4°C - 14d	22°C - 14d	40°C - 14d	35°C - 7d	35°C - 14d	37°C - 7d	37°C - 14d
All-in-One Swab Kit	2										
BBL Culture Swab	2										
BD Eswab	2										
Biomatrica #1	1										
Biomatrica #2											
BPW (5g/L) - low	2										
BPW (20g/L) - high	2										
Butterfield's Buffer	2										
Copan Eswab	2										
Copan SRK	1	Don't have 7 day data		dead at 3 day							
Puritan Liquid Amies	2										
Sanicult Transport Swab	2										
TSB (1/50)	1										
TSB (1/100)	1										
TSB (1/1000)	1										
BHI 1:50	1										
4% BPW	1										
BHI spent media	1										
1/2 Skim milk	1										
TSB Spent media	1										
1/4 skim milk	1										
YT Full Strength	1										
YT 1/2 strength	1										
BHI 1:10	1										
TSB 1:10	1										

Key
>3-Logs
<3-Logs
No Viability

Blank cells indicate test was not done.
YT is a solution containing yeast extract and tryptone.

3.3 Evaluation of Additional Solutions for Viability Sustainment

Because *Y. pestis* cells were plated on TSA, fresh TSB was also included as a test solution. In addition, the TSB that was recovered after cell culture growth (sTSB) was included in our second round of screening. Four solutions (sTSB; fresh, diluted TSB; skim milk; and YT) were also investigated for their ability to preserve *Y. pestis* cells at 40 °C, but none of the additional solutions were found to sustain cell viability at this temperature (data not shown). Some of these solutions were then screened at 35 and 37 °C because 40 °C may have been too high to preserve the viability of the *Y. pestis* cells (Figures 3–5). In this screening round, skim milk (half- and quarter-strength), yeast extract (full- and half-strength), Biomatrica 1 solution (added because of previous work), and a few of the same solutions from the previous screening (i.e., BPW, BHI, sTSB, and Remel Sanicult) were tested. The results showed that all of the test solutions, with the exception of Biomatrica 1, were observed to sustain *Y. pestis* cell viability at 37 °C.

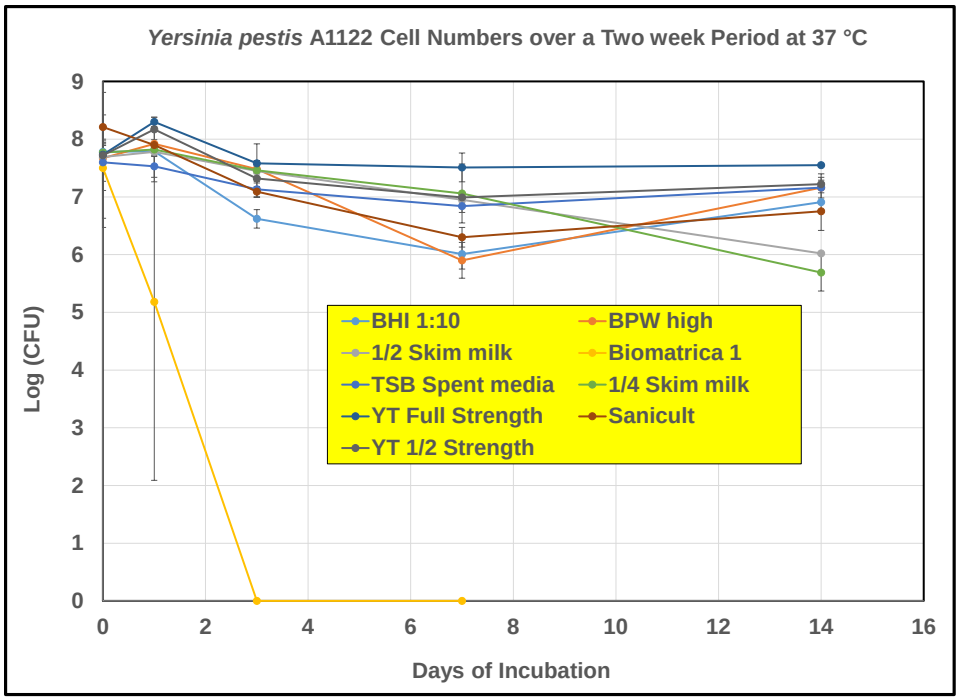


Figure 3. Cell viability sustainment of *Y. pestis* at 37 °C.
Results are expressed as the mean ± SD of the reported recovered Log (cfu).
Experimental $n \geq 6$ for each data set.

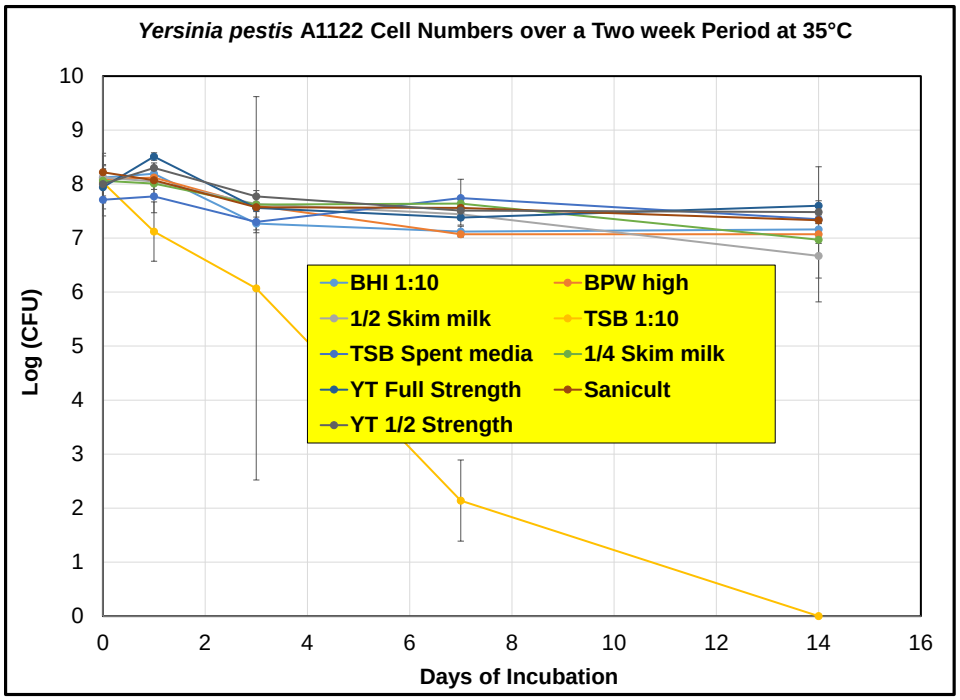


Figure 4. Cell viability sustainment of *Y. pestis* at 35 °C.
Results are expressed as the mean ± SD of the reported recovered Log (cfu).
Experimental $n \geq 6$ for each data set.

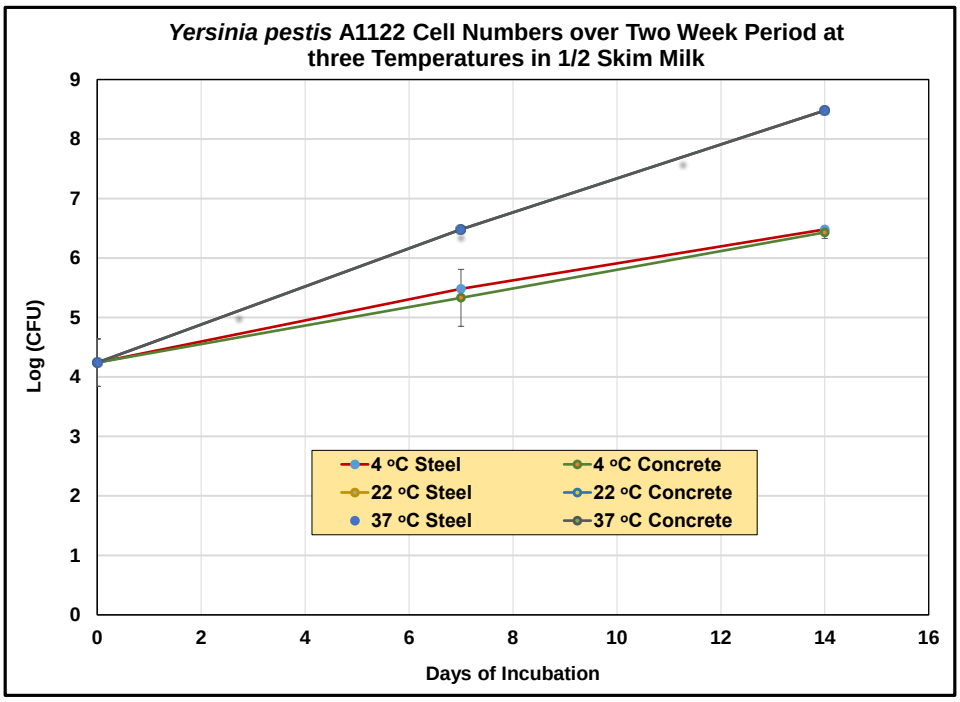


Figure 5. Fate of *Y. pestis* A1122 cells sampled from surfaces and stored in half-strength skim milk. Results are expressed as the mean ± SD of the reported recovered Log (cfu). Experimental $n \geq 6$ for each condition.

3.4 Evaluation of Decay and Sampling Analyses

One of the key tasks of this investigation was to evaluate the ability of down-selected preservatives to sustain the viability of *Y. pestis* cells from two different surfaces: stainless steel (hard, nonporous) and painted concrete. Skim milk and sTSB were evaluated for their ability to sustain cell viability at three holding temperatures of 4, 22, and 37 °C. The results (Figure 6) showed that *Y. pestis* viability was preserved in skim milk at all three temperatures. In fact, whereas a modest increase in cell number was evident at 4 and 22 ºC, a significant increase in cell number was observed at 37 °C. Similar results were observed for sTSB in the support of cell viability and an increase in the number of *Y. pestis* cells at all three temperatures (Figure 7).

In this series of experiments, the effect of interfering Arizona test dust (AZTD) on cell sampling and decay was investigated over 2 weeks at three temperatures. Figures 8 and 9 show that the presence of AZTD made negligible difference in sampling and viability of *Y. pestis* cells over a 2 week period in skim milk and sTSB.

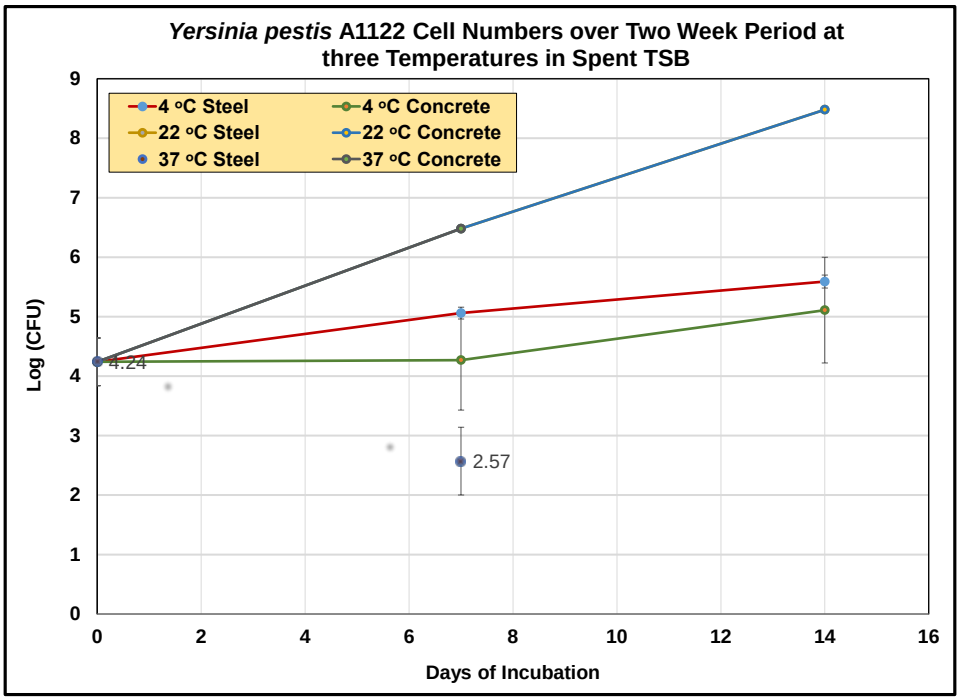


Figure 6. Fate of *Y. pestis* A1122 cells sampled from surfaces and stored in sTSB. Results are expressed as the mean ± SD of the reported recovered Log (cfu). Experimental $n \geq 6$ for each condition.

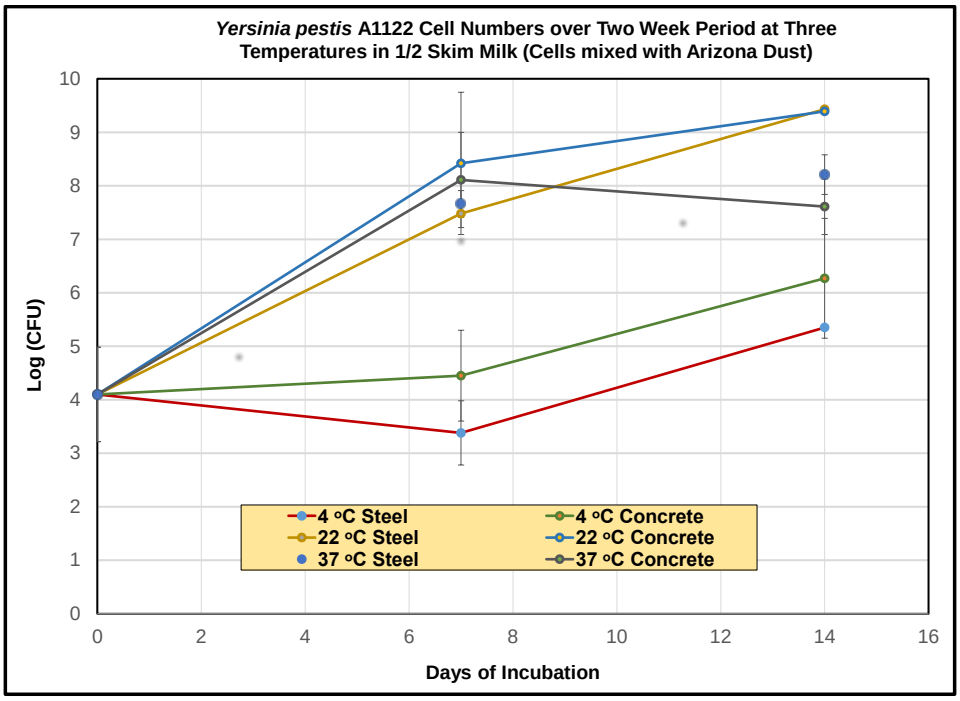


Figure 7. Effect of AZTD on sustained viability of sampled *Y. pestis* in skim milk. Results are expressed as the mean ± SD of the reported recovered Log (cfu). Experimental $n \geq 6$ for each condition.

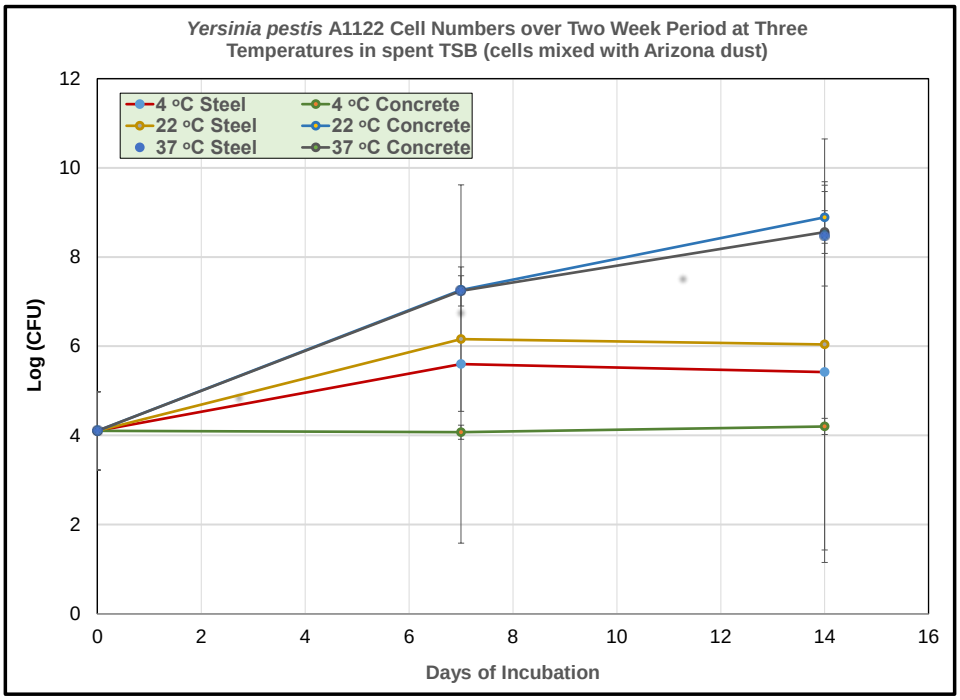
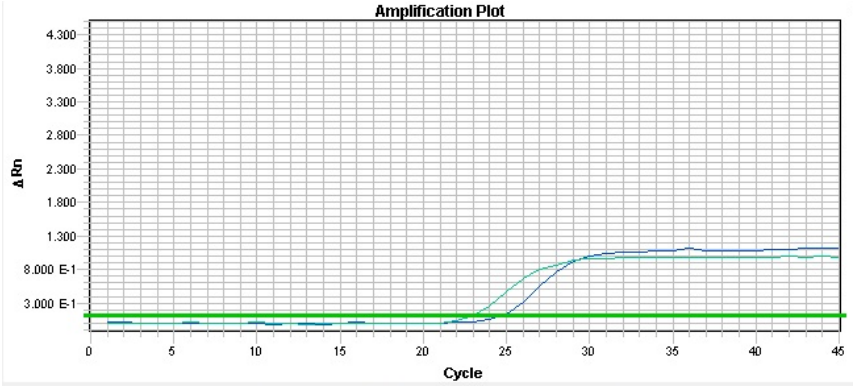


Figure 8. Effect of AZDT on sustained viability of sampled *Y. pestis* in sTSB. Results are expressed as the mean ± SD of the reported recovered Log (cfu). Experimental $n \geq 6$ for each condition.

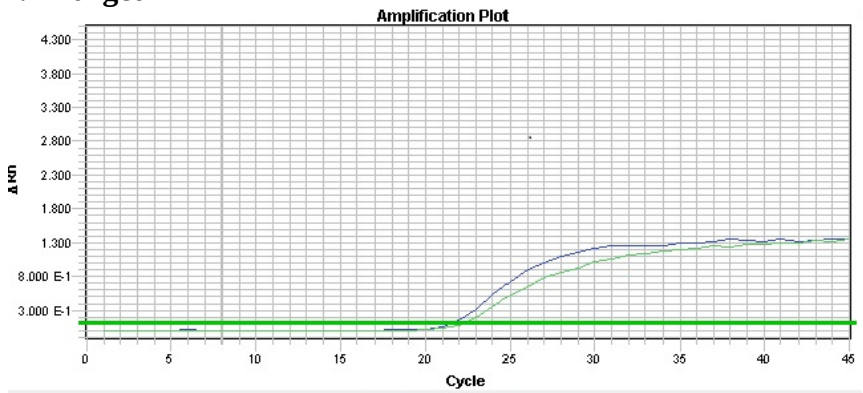
3.5 Genomic DNA Analysis in Nonviable Samples of *Y. pestis*

The recovery and integrity of genomic DNA in nonviable samples of *Y. pestis* was investigated in this series of experiments. Figure 9 shows a typical PCR run after amplification, with three target sites on the genome of *Y. pestis*. A Ct value of around 25–26 demonstrates a high copy number of genomic DNA in the nonviable samples. Clearly, even though the viability was lost, high-quality genomic DNA was recovered in a high copy number.

1. Target 1



2. Target 2



3. Target 4

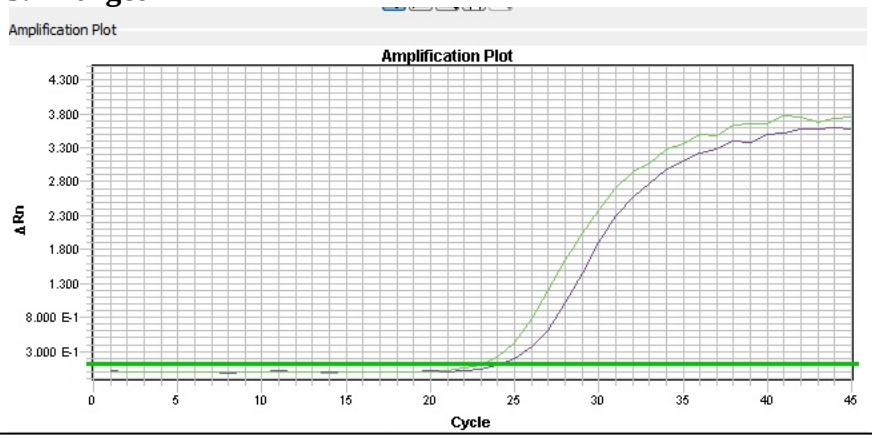


Figure 9. PCR amplification of two nonviable *Y. pestis* samples held at 40 °C.

4. CONCLUSIONS AND DISCUSSION

This study evaluated 17 different solutions (Table 1) that had the potential to preserve the viability of Gram-negative *Y. pestis* A1122 cells for up to 14 days under three different temperature conditions (4, 22, and 40 °C). In addition, spent media, such as BHI and TSB, were included in this initial screen. Whereas none of the solutions preserved the viability of *Y. pestis* cells at 40 ºC, all of the solutions examined were capable of preserving viability at 4 and 22 °C (Figure 2 and Table 3). Because 40 °C proved to be a non-permissive temperature for *Y. pestis*, 37 and 35 °C were tested as alternative high temperatures. In this second series, skim milk (half- and quarter-strength) and sTSB were evaluated. As shown in Figures 3 and 4, with the exception of Biomatrica 1, all other tested solutions (i.e., skim milk, sTSB, BPW, Remel Sanicult, and YT) were all capable of sustaining viability of *Y. pestis* at both 35 and 37 °C.

Y. pestis cells were sampled from two surfaces (steel and concrete); the cells were well preserved in skim milk and sTSB. Similar cell numbers (4 logs out of 7 logs inoculated) were recovered from the two surfaces. In fact, the cell number showed a significant increase at 37 °C (Figures 6 and 7). At the other two temperatures, 4 and 22 °C, a marginal increase in cell number was also evident (Figures 6 and 7). A common environmental interferant, AZTD, did not appear to affect the cell recovery and viability from the same two surfaces, stainless steel and painted concrete (Figures 8 and 9).

The vast majority of nonviable cells in the samples held at 40 °C were found to retain and release intact DNA, as analyzed by PCR amplification of three target sites on *Y. pestis* cells. Based on PCR analysis, all nonviable samples were found to contain intact DNA.

In summary, this study clearly demonstrated that *Y. pestis* A1122 cells lost viability in less than 7 days at 40 °C. Four solutions were identified with the ability to sustain the viability of these cells at 37 °C. It is unclear why a 3 °C temperature difference would have such a marked effect on *Y. pestis* cell viability. With respect to a broader application in environmental sampling, it was interesting that two common solutions sustained the viability of cells sampled from porous and a nonporous surfaces. Interestingly, the same solutions were also found to sustain the viability of Gram-positive *B. anthracis* vegetative cells (Angelini et al., 2017). Future studies should focus on the broad applicability of two common preservatives, skim milk and BPW, with regard to their ability to sustain the viability of other Gram-positive and Gram-negative BW threat agents.

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ACRONYMS AND ABBREVIATIONS

AZTD	Arizona test dust
BHI	brain–heart infusion
BPW	buffered peptone water
BW	biological warfare
CDC	Centers for Disease Control and Prevention
cfu	colony-forming units
Ct	cycle threshold
DBPAO	Defense Biological Product Assurance Office
FDA	U.S. Food and Drug Administration
PCR	polymerase chain reaction
SD	standard deviation
SRK	swab-rinse kit
sTSB	spent tryptic soy broth
TSA	tryptic soy agar
TSB	tryptic soy broth
YT	yeast extract and tryptone

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