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Method development for optimum recovery of *Yersinia pestis* from transport media and swabs

Final Study Report

Office of Research and Development
National Homeland Security Research Center

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Centers for Disease Control and Prevention
National Center for Emerging and Zoonotic Infectious Diseases
Atlanta, Georgia 30329

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Office of Research and Development
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Disclaimer:

The U.S. Environmental Protection Agency (EPA), National Homeland Security Research Center and the Centers for Disease Control and Prevention (CDC), National Center for Emerging and Zoonotic Infectious Diseases, under interagency agreement IA#DW-75-922597-01-0 (CDC IA# CI08-002, CI08-014, CI10-001, and CI10-030), collaborated in the development of the analysis procedure described here.

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List of Acronyms:

µl	microliter
ANOVA	analysis of variance
ATD	Arizona test dust
BB	Butterfield's buffer
BHIB	brain heart infusion broth
C&B	Cary and Blair
CDC	Centers for Disease Control and Prevention
CFU	colony forming units
CHOC	chocolate agar plates
CI	confidence interval
EPA	U.S. Environmental Protection Agency
g	gravity
hms	hemin storage locus
hr	hour
kb	kilo base pairs
LOD	limit of detection
mg	milligram
mL	milliliter
n	number
NB	neutralizing buffer
NHSRC	National Homeland Security Research Center
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.02%Tween® 80
PBSTX	phosphate buffered saline with 0.05 % Triton™ X-100
PCR	polymerase chain reaction
pgm	pigmentation locus
rpm	revolutions per minute
sd	standard deviation
SPSS	Statistical Package for the Social Sciences
TSAII	trypticase soy agar + 5 % Sheep's Blood
TSB+ Isovitalex	trypticase soy agar + Isovitalex™

Executive Summary:

The GAO report investigating the surface sampling methods used during the 2001 mail contamination with *Bacillus anthracis* brought to light certain knowledge gaps that existed regarding environmental sampling [1, 2]. Since then, the Centers for Disease Control and Prevention (CDC) developed and validated sample preparation methods for environmental samples for use in a *B. anthracis* investigation. Should a contamination event occur involving non-spore forming biological select agents, surface sample collection material and transport media specific to those agents will be needed. Under a collaborative interagency agreement between the U.S. Environmental Protection Agency (EPA) and the CDC, CDC executed a research project to evaluate surface sampling materials, transport media, and processing methods for potential bioterrorism agents. EPA's National Homeland Security Research Center (NHSRC) funded the project. Work began with *Francisella tularensis* and *Yersinia pestis*, the causative agents for tularemia and plague, respectively. However, because of continued difficulties obtaining consistent, reproducible *F. tularensis* growth, the study continued with *Y. pestis* only. The study subsequently focused on investigating the recovery of virulent and low virulent strains of *Y. pestis* from four different swab types (polyester, macrofoam, rayon, and cotton) pre-moistened in various buffers and stored in various transport media.

Preliminary work in which six transport media were inoculated with the low virulence strain *Y. pestis* A1122 and held for 7 days at 4°, 25°, and 35°C, revealed that Cary and Blair (C&B) transport medium (modified formulation without calcium chloride [$CaCl_2$] and agar, $C\&B_{mod}$) and the 0.05 % phosphate buffered saline with Triton™ X-100 surfactant (PBSTX) transport medium provided the most stable *Y. pestis* viability. The most favorable sample storage temperature was found to be 4°C, and the remainder of the study was conducted at this temperature.

Phase I of the study involved evaluation of four swab materials; cotton, polyester, rayon, and macrofoam. Each swab type was pre-moistened with either Neutralizing Buffer (NB) or PBSTX, inoculated with 10^4 *Y. pestis* A1122 cells, then the cells were extracted by one of three extraction methods; vortexing, sonicating, or a combination of both vortexing and sonicating. Percent recovery (% recovery) was determined relative to the inoculum. Conditions consisting of macrofoam swabs pre-moistened with NB and vortexed for 3 minutes (% recovery 93.9%, standard deviation [sd] 13.1%), and rayon swabs pre-moistened with NB and sonicated for 3 minutes (% recovery 77.0%, sd 14.4%) were chosen as the two best swab, premoistening medium, and processing method combinations because of the higher % recovery obtained, and these swab conditions were used for the remaining phases of the study.

Phase II of the study investigated the optimum swab conditions over various storage times. Macrofoam and rayon swabs were pre-moistened with NB, inoculated with 10^4 *Y. pestis* A1122 cells, held for 1 hour, and then placed in one of the selected transport media ($C\&B_{mod}$ or PBSTX). The swabs were held at 4°C, with 10 swabs being removed from storage and processed to extract the cells at 0, 12, 18, 24, 48 and 72 hours.

When the rayon swab data from all time points (12 to 72 hours) are combined within each test condition (premoistening agent and transport medium combination), the condition providing the greatest mean % recovery was if pre-moistened with PBSTX and stored in PBSTX as a

transport medium (103.7%, sd 17.2%). When rayon swabs were processed by sonication within 24 or 48 hours, no significant difference was seen between this optimum condition and another condition; pre-moistened with NB and stored in PBSTX. When all macrofoam swab data for (12 to 72 hours) are combined within each test condition (premoistening agent and transport medium combination), the optimum mean % recovery was seen when swabs were pre-moistened with NB and stored in $C\&B_{mod}$ as a transport medium (99.6%, sd 10.9%). When macrofoam swabs were processed within 24 hours (99.2%, sd 11.6%) by vortexing, no significant difference in % recovery was found between this optimum condition and two other conditions (Premoistening agent/transport media: PBSTX/PBSTX and PBSTX/$C\&B_{mod}$).

Phase III of the study involved conducting the same evaluations as in phase II, but with the virulent *Y. pestis* CO 92 strain. The virulent strain behaved somewhat differently than the low virulent strain in that more decline in recovery was seen after 24 hours if macrofoam swabs were pre-moistened with NB, and held in $C\&B_{mod}$. When all phase III macrofoam swab data are combined (12 to 72 hours) within each test condition, the highest mean % recovery was seen when pre-moistened with PBSTX and stored in $C\&B_{mod}$ (101.8%, sd 10.0%). The same conditions provided the optimum % recovery at 24 and 48 hours. When all phase III rayon swab data are combined (12 to 72 hours) within each test condition, the highest mean % recovery was seen if pre-moistened with NB and stored in PBSTX (110.1%, sd 21%). If processed at 24 hours, no significant difference was seen between these optimum conditions and two other conditions; PBSTX/PBSTX and NB/$C\&B_{mod}$.

After some work with *Y. pestis* CO92 was completed, it was found that the C&B liquid medium formulation was altered to exclude $CaCl_2$ because it precipitated out of solution, a problem not seen when the media was prepared as the intended semi-solid formulation. $CaCl_2$ was shown to be a critical ingredient for maintaining the virulence of *Y. pestis* in growth media [3, 4], though no information is available regarding the need for $CaCl_2$ in transport media. Though the modified formulation of the liquid C&B medium did provide good stability, the modified C&B would be difficult to obtain quickly during an investigation because it is not commercially available. We therefore decided that the altered C&B should be replaced with the next-best transport medium, NB, for phase IV of the study.

The recovery of both low virulent and virulent *Y. pestis* strains from swabs in the presence of simulated dust material containing other viable organisms was determined using sixteen combinations of the two optimum swabs, two pre-moistening agents, two transport media and two extraction methods. A mixture of Arizona Test Dust (ATD; Powder Technology Inc., Burnsville, MN) and PBSTX or NB was created and used as the pre-moistening agent before inoculating the swab with 10^4 CFU of *Y. pestis*. Holding and processing was performed as described above.

When ATD was present on macrofoam swabs, the highest mean % recovery of *Y. pestis* A1122 was achieved when the swabs were pre-moistened with PBSTX and held in NB as a transport medium. The % recovery was 106.6%, 106.1%, and 105.1% for the holding times of 24, 48 and 72 hours, respectively. The of *Y. pestis* CO92 was also seen with the same condition

(PBSTX/NB), though the % recovery was somewhat lower than the A1122 strain at 87.7%, 85.4%, or 85.6% for 24, 48, or 72 hours, respectively.

When ATD was present on rayon swabs, the highest mean % recovery of low-virulent *Y. pestis* A1122 was achieved when the swabs were pre-moistened with PBSTX and held in PBSTX as a transport medium: 105.3%, 107.8%, or 107.2% for 24, 48, or 72 hours, respectively. The highest % of the virulent strain CO92 from rayon swabs was slightly lower than A1122, and was seen when the swabs were pre-moistened with NB and held in PBSTX as a transport medium: 82.5%, 84.3%, or 83.7% for 24, 48, or 72 hours, respectively.

Since the optimum pre-moistening agent, transport medium and hold time was not consistent across both strains (*Y. pestis* A1122 and CO92) and selected swabs (rayon and macrofoam), the data were separated according to swab type and strain, and then compared. Within these categories (swab type and strain), data were combined for all storage times from 12 to 72 hours. In a worst case scenario, where sample processing and analyses couldn't occur until 72 hours after sampling, macrofoam swabs pre-moistened with PBSTX and stored in NB as a transport medium performed significantly better than all other conditions, regardless of strain tested (96.0%, $p<0.001$). In the same scenario, rayon swabs had two optimum premoistening medium/transport medium combinations: PBSTX/PBSTX and NB/PBSTX (94.4% and 93.6%, respectively). These two combinations performed equally well, and significantly better than the other two conditions ($p<0.001$).

This research should be considered preliminary, as additional research will be needed to optimize sampling, transport, and extraction protocols for recovering these biothreat agents from surfaces. The results of this research showed that the best recovery of *Y. pestis* from the swabs was obtained using either macrofoam or rayon swabs. Based on the highest % recovery when dust was present (ATD), the optimal conditions for macrofoam swabs were obtained when premoistening with PBSTX and transporting in NB. The optimum recovery of *Y. pestis* A1122 strain and CO92 strain from rayon swabs occurred when the swabs were pre-moistened with either PBSTX or NB, and then placed in tubes containing PBSTX as a transport medium. Regardless of which swab was chosen, the optimum temperature for transport was determined to be 4°C. Additional research will be necessary to apply these findings to a "real world" scenario in which *Y. pestis* is extracted from swabs used to sample from varying surface types and environmental conditions.

1.0 Introduction

After the anthrax attacks in the fall of 2001, the sampling and processing methods used during the investigation were found to be less than optimum. The methods were not well characterized or standardized, therefore post-decontamination sampling data offered little confidence that the buildings were safe to re-occupy [1, 2]. Several ways that the country could better prepare itself in the event of a terrorist attack were identified. One critical need was for validated sampling methods that could be used by all laboratories in the event of a homeland security incident. The U.S. Environmental Protection Agency's *Selected Analytical Methods for Environmental Remediation and Recovery[5]* contains suggested methods for use by laboratories tasked with performing analysis of environmental samples following a homeland security event.

Sample preparation methods need to be optimized for environmental samples containing biological pathogens. Much effort and resources have been allocated to the development of molecular assays and culture techniques; however, the initial sample collection and preparation lags behind in development. Sample preparation involves recovery of the biological agent from the sampling device, and remains the limiting step in the detection techniques, whether those techniques use non-culture methods (e.g., polymerase chain reaction [PCR]), or culture-based methods. Extracting and recovering pathogens and biotoxins from environmental matrices (e.g., air, soil, and water) and sampling devices (swab, wipe, or vacuum device) present challenges, because the matrices and devices are composed of non-target biological and chemical analytes that may inhibit or interfere (compete) with the extraction and detection of the target analyte.

The Centers for Disease Control and Prevention (CDC), part of the Department of Health and Human Services, has extensive knowledge of, and experience with, developing methods for potential bioterrorism agents. The EPA and CDC collaborated to improve and develop methods for sample collection, sample preparation, and sample analysis for biological agents. In this preliminary study, previously developed CDC methods for recovery of *Bacillus anthracis* from non-porous surfaces were investigated for their application to *Yersinia pestis* and *Francisella tularensis* [6, 7] . *Y. pestis* and *F. tularensis* are the causative agents for plague and tularemia, respectively. The primary goal of this project was to determine the best combination of sampling swab, pre-moistening agent, transport media, and extraction method for a high efficiency recovery of *Y. pestis* and *F. tularensis* vegetative cells. The study did not investigate recovery of cells from environmental surfaces. Two strains of *Francisella tularensis* were initially selected as a second pathogen for evaluation during this study, however, it proved to be difficult to obtain consistent growth with either strain, and the organism was subsequently omitted from the study (see section 2.3 and 3.2.2). The limit of detection (LOD) of an analytical method depends on optimized materials, protocols, and conditions that can best maintain the integrity of the sample, and the efficient recovery of the target biological agent from the sampling tools. A secondary goal was to determine the % recovery of these bacteria after they have been held in transport media for

different time intervals (e.g., 24, 48, or 72 hours). In an actual contamination event, it could take from 24 to 72 hours before the sample is processed and analyzed. This research should be considered preliminary, and additional research may be needed to develop sampling, transport, and extraction protocols for recovering these biothreat agents from swabs during an event for use by first responders, EPA's Environmental Response Laboratory Network (ERLN), and the biodefense community as a whole.

2.0 Materials and Methods

2.1 Culture preparations for *Yersinia pestis* and *Francisella tularensis*

2.1.1 *Yersinia pestis*

Preparation of frozen stock: *Y. pestis* Harbin, *Y. pestis* CO92 and *Y. pestis* A1122 were obtained from CDC's Division of Vector Borne Diseases, Ft. Collins, CO, and stored at -70ºC in cryovials with beads (PL170, Prolab Diagnostics, Austin, TX). One bead was removed from a freezer vial and cultured onto trypticase soy agar with 5% sheep blood (TSAII; BD Diagnostic Systems, Sparks, MD) and incubated at 25°C for 48 hours. After 48 hours of incubation, purity of culture was assessed, and then a 0.5 McFarland standard suspension (10^8 colony forming units [CFU] per mL) of the culture was prepared in Butterfield's buffer (BB; Becton Dickinson, Franklin Lakes, NJ). Ten-fold serial dilutions were prepared in BB and spread plated onto TSAII plates in triplicate to determine the concentration of cells. The plates were incubated at 25°C for 48 hours. From the 10^{-4} dilution tube (10^4 CFU/mL), 1 mL was removed and placed in a 250 mL flask containing 99 mL brain heart infusion broth (BHIB; Becton Dickinson, # 237500) and allowed to incubate on a shaker table (100 rpm) at 25°C for 48 hours. After incubation, 1 mL of the culture was placed into a 50 mL glass tube containing 30 ml of BHIB with 10% glycerol. This mixture was vortexed, then dispensed into 1.2 mL volumes in 2 mL Cryovial® tubes (Thermo Scientific, Rochester, NY) and frozen at -80°C.

Preparation of the working suspension: From the frozen stock, 1 mL was added to a 100 mL flask containing 30 mL of BHIB. This was incubated at 25°C, in a shaker-incubator, for 26-30 hours to reach late log phase growth (as determined from the growth curve, refer to section 2.2.1). Cells were harvested by centrifugation at 3000 × *g*, 4°C, for 10 minutes. The supernatant was decanted and the pellet resuspended in 25 mL of sterile phosphate buffered saline (PBS). This wash step was repeated two additional times. After the third wash and re-suspension, a 0.5 McFarland standard suspension was prepared (10^8 CFU/mL), diluted in series, and plated onto TSAII plates to determine the concentration of the cells. Plates were incubated at 25°C for 48 hours. One mL of the 10^{-3} dilution tube (10^5 CFU/mL) was used to inoculate the 9 mL of transport media (resulting in 10^4CFU/mL). One hundred µL of the 10^{-3} dilution tube (10^5 CFU/mL) was used to inoculate the swabs (resulting in 10^4 CFU/swab).

2.1.2 *Francisella tularensis*

Preparation of frozen stock: *F. tularensis* subsp. *holartica* LVS and *F. tularensis* subsp. tularensis Schu S4 were obtained from CDC's Division of Vector Borne Diseases, Ft. Collins, CO, and stored at -70ºC in

cryovials with beads. One bead from a previously frozen vial of *F. tularensis* was removed and cultured on chocolate agar (CHOC; BD Diagnostic Systems,) and incubated at 35°C for 48 hours. After 48 hours, the purity of the culture was ascertained and a 0.5 McFarland standard suspension (~10^8 CFU per mL) of the culture prepared in BB. Ten-fold serial dilutions were prepared in BB and spread plated onto CHOC plates in triplicate to check the titer. The plates were incubated at 35°C for 48 hours. From the 10^{-4} dilution tube (10^4 CFU/mL), 1 mL was removed and placed in a 250 mL flask containing 99 mL trypticase soy broth (TSB) containing 2% Isovitalex™ (Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated on a shaker table (100 rpm) at 35°C for 48 hours. After incubation, 1 mL of the culture was placed in a 50 mL glass tube containing 30 mL of BHIB supplemented with 10% glycerol. This mixture was vortexed and dispensed into 1.2 mL volumes in 2 mL Cryovial tubes and frozen at -80°C.

Preparation of the working suspension: From a frozen stock, 1 mL was added to a 100 mL flask containing 30 mL of TSB+ Isovitalex. This was incubated at 35°C, in a shaker-incubator, until late log phase growth was acquired. After incubation, cells were harvested by centrifuging at 3000 × *g* for 10 minutes at 4°C. The supernatant was then decanted and the pellet resuspended in 25 mL of sterile PBS. These wash steps were repeated two additional times, and the final cell pellet was resuspended in 25 mL of PBS. A 0.5 McFarland standard suspension of the culture was prepared, for a final titer of 10^8 CFU/mL. The titer was checked by performing ten-fold serial dilutions and spread plating onto CHOC. Plates were incubated at 35°C for 48 hours. One mL of the 10^{-3} dilution tube (10^5 CFU/mL) was used to inoculate the 9 mL of transport media (resulting in 10^4 CFU/mL). One hundred µL of the 10^{-3} dilution tube (10^5 CFU/mL) was used to inoculate the swabs (resulting in 10^4 CFU/swab).

2.2 Preliminary Study: Survival of *Y. pestis* in liquid transport media

A preliminary study was conducted to investigate the survival of a low virulence strain (*Y. pestis* A1122, CDC, Ft. Collins, CO) in various transport media. Six liquid transport media were evaluated: (1) Stuart, Toshach, and Patsula medium, (2) Cary and Blair without Calcium chloride ($CaCl_2$) (C&B_{mod}) medium, (3) Amies medium without charcoal, (4) Amies medium with charcoal, (5) phosphate buffered saline with 0.05% Triton X-100 (PBSTX) surfactant, and 6) neutralizing buffer (NB). Transport media 1 – 4 are traditional clinical transport media, meant to be prepared with 0.2 % agar to form a semi-solid mass in a transport tube. Traditionally, the clinical swabs are submerged in the soft media to preserve the cells on the swab. In this application, quantitation is not a goal, but simply preserving any quantity of cell for isolation in the lab in order to identify the organism. For the purposes of this study, however, we required quantitation of cell recovered from the swabs, and found that

significant numbers of cells remained in the semi-solid media upon removing the swab and processing in a separate tube of extraction fluid. We therefore omitted the agar from all formulations to create liquid transport media. In order to evaluate the best transport media, a bacterial suspension of *Y. pestis* A1122 was created in Butterfield buffer (BB; Becton Dickinson, Sparks, MD) from 48 hour plate growth. The suspension was diluted in series to a concentration of 10^5 CFU/mL. Five replicate tubes (9 mL per tube) of each transport medium were inoculated with 1 mL of the 10^5 CFU/mL bacterial suspension. The tubes were held at 4°C, 25°C, or 35°C over a 72-hour holding period, with intermittent sampling over the hold time (see Appendix, Table 1 for test matrix). Tubes were vortexed immediately before samples were taken at 0, 12, 18, 24, 48, and 72 hours. Traditional plate culture methods were used to detect the presence of viable organisms. Samples were plated onto TSAII, incubated at 25°C for 48 hours and colonies counted. The total CFU recovered for the sample was determined and the results were reported as Log_{10} CFU recovered at each time point. Based on the results of the preliminary study (see section 3.1), two transport media were selected for the remainder of the study.

2.3 Growth Curves

For both organisms, 99 mL of broth (BHIB for *Y. pestis* and TSB +Isovitalex for *F. tularensis*) was inoculated with 1 mL from a working stock suspension and allowed to shake at 100 rpm, 25°C (*Y. pestis*) or 35˚C (*F. tularensis*). Two strains of each organism were tested, the low-virulence strains *Y. pestis* A1122 and *F. tularensis* LVS, and the virulent strains *Y. pestis* CO92 (initially *Y. pestis* Harbin until found to be lacking one of its virulence plasmids) and *F. tularensis* Schu S4. The titer of the stock suspension was checked by diluting in series and plating in triplicate on the appropriate media. The flask was allowed to shake at 100 rpm for 24 hours and then sampled periodically to monitor growth. The optical density and the titer were checked at each time point, and a growth curve generated for each isolate. The time required to achieve late logarithmic growth was determined, and all cells used in the study were harvested at that time.

2.4 Phase I: Evaluation of swab extraction methods

Phase 1 was conducted to evaluate the optimum swab materials and extraction methods. Table 2 in the appendix shows the matrix of tests conducted. Four types of swab materials were evaluated: cotton (Baxter Healthcare Corp., Deerfield, IL cat # A5002-5), polyester (Falcon™ # 220690, BD, Franklin Lakes, NJ), macrofoam (Puritan Medical, Guilford, ME, # 25-1607 1PF SC), and rayon (Puritan, # 25-806 1 WR). Two premoistening solutions were evaluated, PBSTX and NB. The swabs were pre-moistened by submerging them in a tube with one of the premoistening liquids for 10 minutes. The swabs were then pressed against the inside wall of the tube as they were removed, to express any excess premoistening liquid. The pre-moistened swabs were directly inoculated with 100 µL of 10^5 CFU/mL working suspension of the test organism, then placed in a 15 mL conical tube to hold for 1 hour at room temperature. After the one hour hold, the swabs were placed into

tubes containing 5 mL of phosphate buffered saline with 0.02% Tween® 80 (PBST) (Sigma-Aldrich, St. Louis, MO). Preliminary work demonstrated that no significant number of cells were lost during the transfer of the swabs from one tube to another after the 1 hour hold. The swabs were then processed by one of four methods: (1) vortexing for 3 minutes (VX-2500 multi-tube vortexer set on the highest speed, VWR, Sewanee, GA, (2) sonicating for 3 minutes (FS 20, a 40-KHz sonic cleaner; Fisher Scientific, Pittsburg, PA), (3) vortexing and sonicating for 30 seconds each – repeated three times for a total of 3 minutes, and (4) no extraction method (submersion in PBST only). After the extraction, the swabs were removed from the transport medium, the excess liquid was expressed from the swab heads by pushing the swabs against the inside wall of the tube. The PBST containing the extracted cells was diluted in series, and each dilution plated in triplicate on TSAII. Positive controls consisted of inoculating 5 mL of PBST (no swab) with the same quantity of cells and processing alongside the swabs. The PBS containing cells was diluted in series, and each dilution plated onto ten plates per dilution. The plates were incubated for 48 hours at 25°C. Colonies were counted and the percent of viable cells recovered was determined, relative to the positive controls, for each test parameter. Each swab material and premoistening solution combination was tested with ten replicate swabs and one positive control.

2.5 Phase II and III: Evaluation of sample storage parameters for both *Y. pestis* strains and sterile swabs

The two best performing transport media and the two best swab materials from the phase I preliminary study were selected for use for the remainder of the study. Various storage conditions, such as swab transport media (top two performers from preliminary studies), temperature (best survival temperature from preliminary studies) and holding time, were evaluated for survival and % recovery of *Y. pestis*. Tables 3 and 4 show the matrices of tests performed in phases II and III. The swabs were pre-moistened with either PBSTX or NB, inoculated with a known concentration of low-virulent *Y. pestis* A1122 (phase II) or virulent *Y. pestis* CO92 (phase III) as described in the methods for phase I, then placed into one of the two best transport media chosen in phase I. The swabs were held at 4°C (found to be optimum temperature in preliminary phase), with 10 swab samples being removed and processed at each of the following time points: 0, 12, 18, 24, 48 and 72 hours. Swabs were processed by the optimum method for each, as determined in phase I.

2.6 Phase IV: Evaluation of sample storage parameters for both *Y. pestis* strains and "dirty" swabs

In order to prepare the "dirty" swabs, a slurry of pre-characterized Arizona test dust (ATD, A-3 Medium, Powder Technology Inc., Burnsville, MN) was prepared to pre-moisten the swabs for experiments which called for "dirty" swabs. One gram of ATD was added to 10 mL of the premoistening agent to achieve a concentration of 100 mg/mL. The slurry was diluted once more, by adding 10 mL to 90 mL of the pre-moistening agent (PBST or NB), creating a final concentration of 10 mg ATD/mL in

the premoistening solution. This slurry was stored at 4°C for up to one week before use. The slurry was re-suspended by vortexing 1 min, then swabs were dipped into the slurry before direct inoculation with the test organism and processed as described in previously mentioned methods in section 2.4 and 2.5, using the transport media selected in phase I. Tables 5 and 6 show the matrices of tests performed in phase IV.

2.7 Statistical Analysis

The preliminary study data are presented as the mean $\log_{10}$ CFU recovered at each temperature, time point, transport medium and organism evaluated. In the preliminary phase, it was necessary to identify the condition(s) that allowed for the least amount of change in cell number (growth or death). To identify the condition(s), the percent of samples with ≤ 0.5 $\log_{10}$ and ≤ 0.3 $\log_{10}$ CFU change in recovered cells, relative to the T_0 (time zero) CFU recovery was calculated for each transport medium, swab type, and temperature. In phase I, the % recovery of cells after each extraction method was calculated relative to the recovery from the positive control. (Positive control was 5 mL of PBS, same inocula and treatment, but with no swab.) In phases II, III, and IV - in order to normalize the % recovery data when comparing storage temperatures, premoistening agents and transport media over several time periods - the % recoveries were calculated relative to the T_0 CFU recovery. The statistical analysis for phases II, III, and IV were SPSS software, version 18 (IBM, Armonk, NY). Each data set was tested for normality. If a normal distribution was found, then ANOVA were performed between various combinations of the two selected swabs, premoistening agents and transport media when grouped 12 to 24 hours, 12 to 48 hours, and 12 to 72 hours ($\alpha \leq 0.05$). Tukey Highest Significant Difference test was performed as a Post Hoc Test. If the data was found to be non-parametric, the Kruskal-Wallis and Mann-Whitney Tests were performed.

3.0 Results and Discussion

3.1 Preliminary Study: Survival of *Y. pestis* in various liquid transport media

3.1.1 Amies medium without Charcoal

The recovery of *Y. pestis* A1122 in Amies without Charcoal medium (Figure 1) was maintained within 0.52 $\log_{10}$ of the T_0 (4.22 $\log_{10}$ CFU/mL) for all time points at 4°C and 25°C. After 48 hours, viable cell count declined when stored at 35°C. No significant difference in recovery was noted between T_0 and cells held at 4°C for 24 hours (p=0.76). Although an increase in cells occurred if the medium were held at 4°C or 25°C for 96 hours, the counts were still within the same order of magnitude as T_0. It appears that 4°C is the better holding temperature for this medium at 24 hours (p<0.001), though no significant difference was seen in recovery between 4° and 25°C at the 48 and 72 hour hold times.

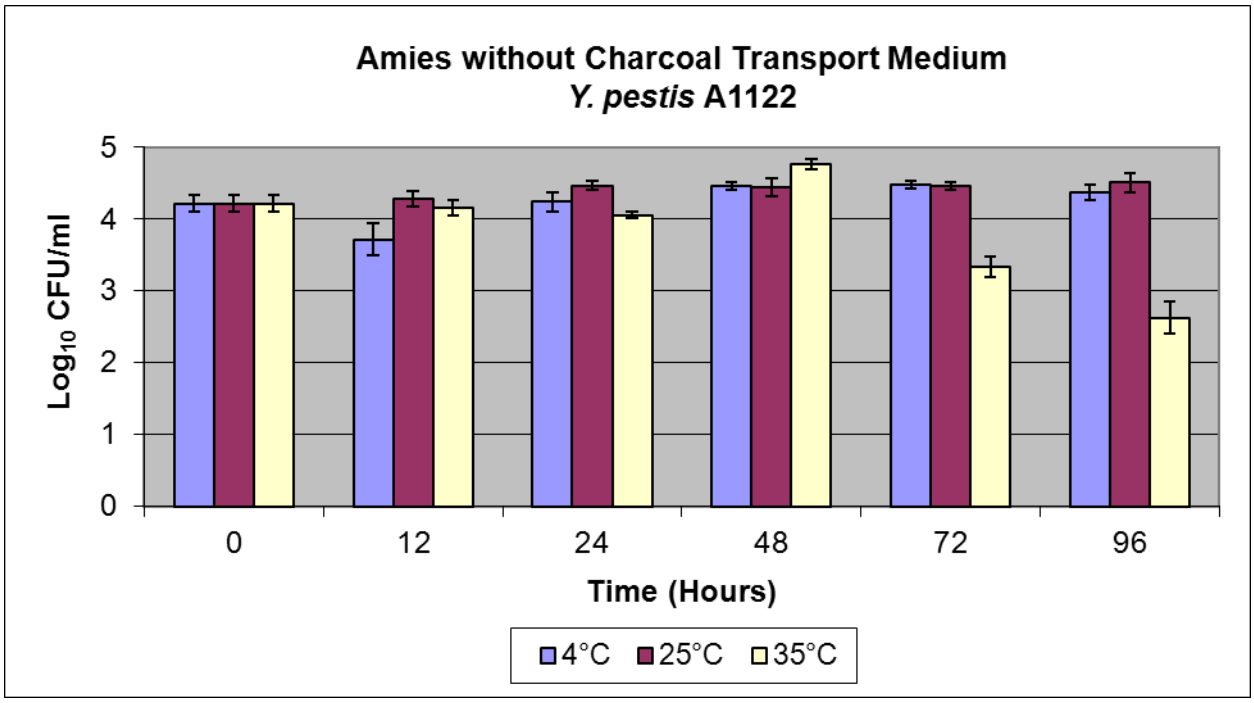

Figure 1: Survival of *Y. pestis* A1122 over storage time in Amies without Charcoal liquid medium. The bars represent the mean $\log_{10}$ CFU/mL recovered over time. The error bars represent the standard deviation (n=5).

3.1.2 Amies medium with Charcoal

The optimal storage temperature for *Y. pestis* A1122 in Amies with Charcoal medium (Figure 2) was 4°C. If held at 4°C or 25°C, recovery at all sample points remained within 0.9 $\log_{10}$ of T_0 (4.6 $\log_{10}$ CFU/mL), though 4°C provided significantly more viable cells than 25°C after storage for 24 and 48 hours ($p<0.05$). When stored at 35°C, a significant decline in viable cells was seen after 24 hours, and by 96 hours, no detectable cells were found, representing greater than 4.3 $\log_{10}$ loss of cells (the limit of detection of the assay was 0.33 $\log_{10}$ CFU/ml).

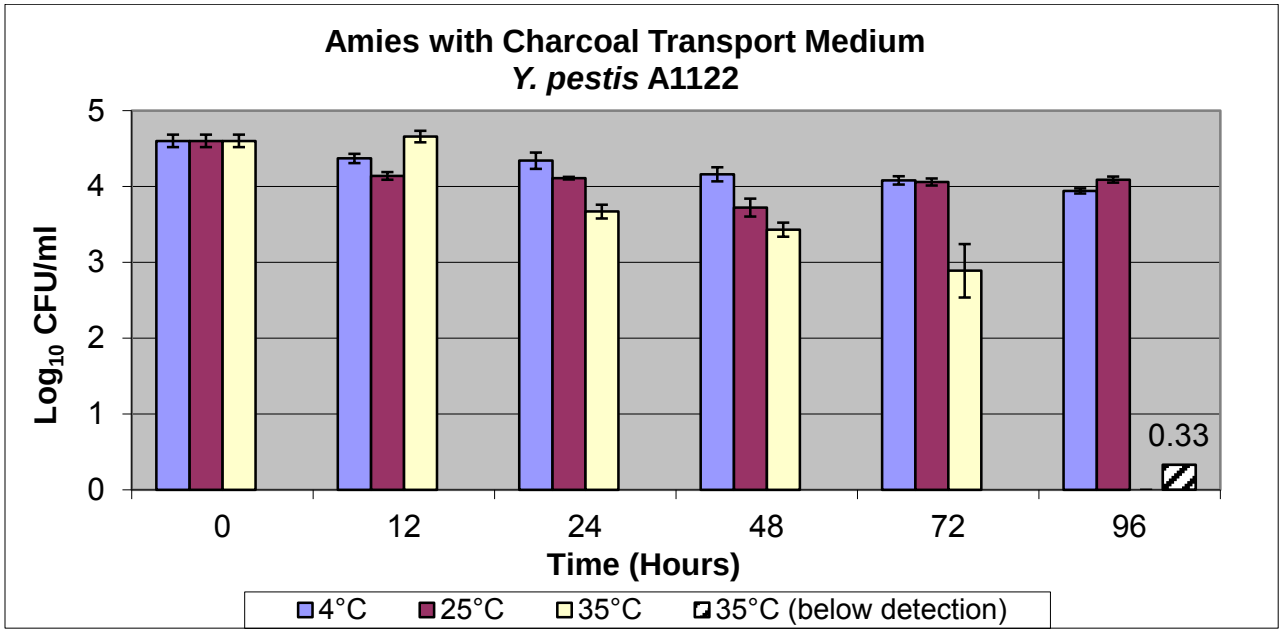

Figure 2 : Survival of *Yersinia pestis* A1122 over storage time in Amies with Charcoal liquid medium. The bars represent the mean $\log_{10}$ CFU/mL recovered over time. The error bars represent the standard deviation (n=5).

3.1.3 Cary and Blair$_{mod}$ medium

There was no more than a 0.35 log_{10} change relative to T_0 (5.16 log_{10} CFU/ml) of cells held at either 4° or 25°C at any time point (Figure 3). The optimal temperature for *Y. pestis* in C&B$_{mod}$ medium was 4°C. When data for all time points are combined, there is no significant difference in the recovery of viable cells between at 4° or 25°C. However, if analyzed at each time period, 4°C provided significantly better recovery at except at 72 hours (when no difference was noted). A decline of viable cells was observed at 35°C with a 2 log_{10} reduction relative to T_0 by 96 hours.

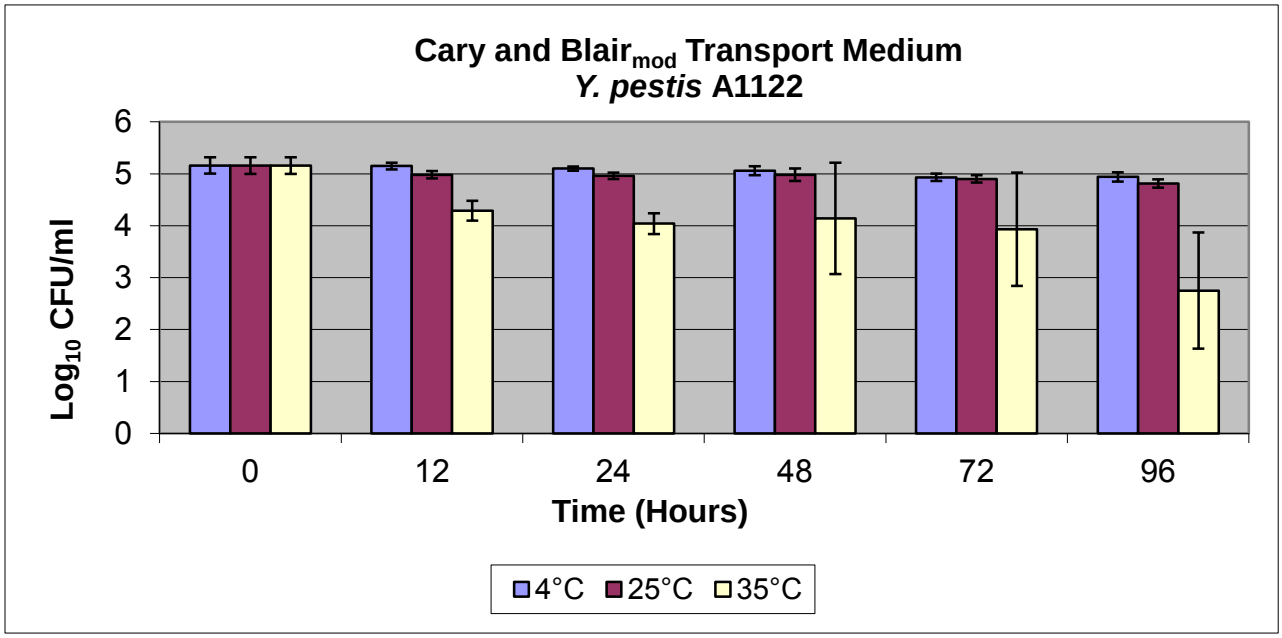

Figure 3: Survival of *Yersinia pestis* A1122 over storage time in Cary and Blair$_{mod}$ liquid medium. The bars represent the mean log_{10} CFU/mL recovered over time. The error bars represent the standard deviation (n=5).

3.1.4 Phosphate buffered saline with 0.05% Triton X-100

When *Y. pestis* A1122 was held in PBSTX medium in at 4°C or 25°C, less than a 0.1 $\log_{10}$ change in recovery was seen relative to T_0 (3.67 $\log_{10}$ CFU/mL) (Figure 4). When data for all time points are combined, 25°C appears to be slightly better in $\log_{10}$ recovery than 4°C, though no significant differences were seen at 24 and 48 hours ($p>0.05$). There was significant loss if held at 35°C at all time periods, and as much as a 3 $\log_{10}$ reduction in recovery by 96 hours.

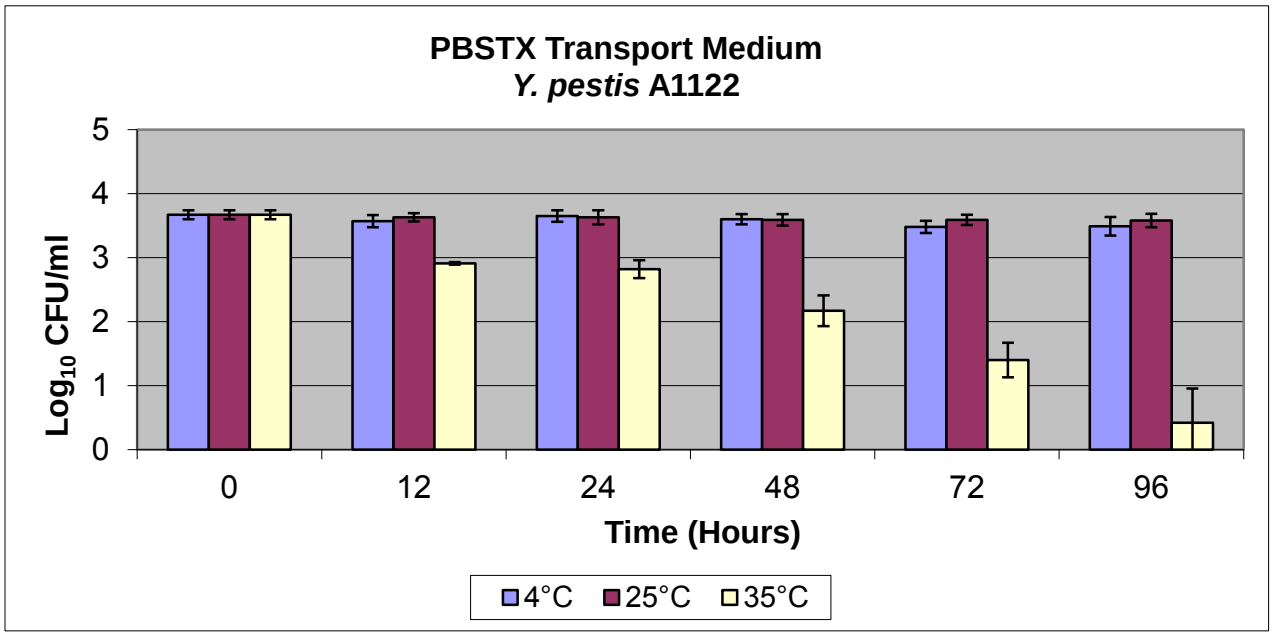

Figure 4: Survival of *Yersinia pestis* A1122 over storage time in PBSTX liquid medium. The bars represent the mean $\log_{10}$ CFU/mL recovered over time. The error bars represent the standard deviation (n=5).

3.1.5 Neutralizing Buffer

Holding cells in NB at 4°C was significantly better than holding at 25°C or 35°C for any period of time ($p<0.05$), with less than a 0.23 $\log_{10}$ decline from the T_0 concentration (4.77 $\log_{10}$ CFU/mL) after 96 hours (Figure 5). When held at 25° and 35°C, a 0.6 $\log_{10}$ and 1.9 $\log_{10}$ decline was seen by 96 hours, respectively.

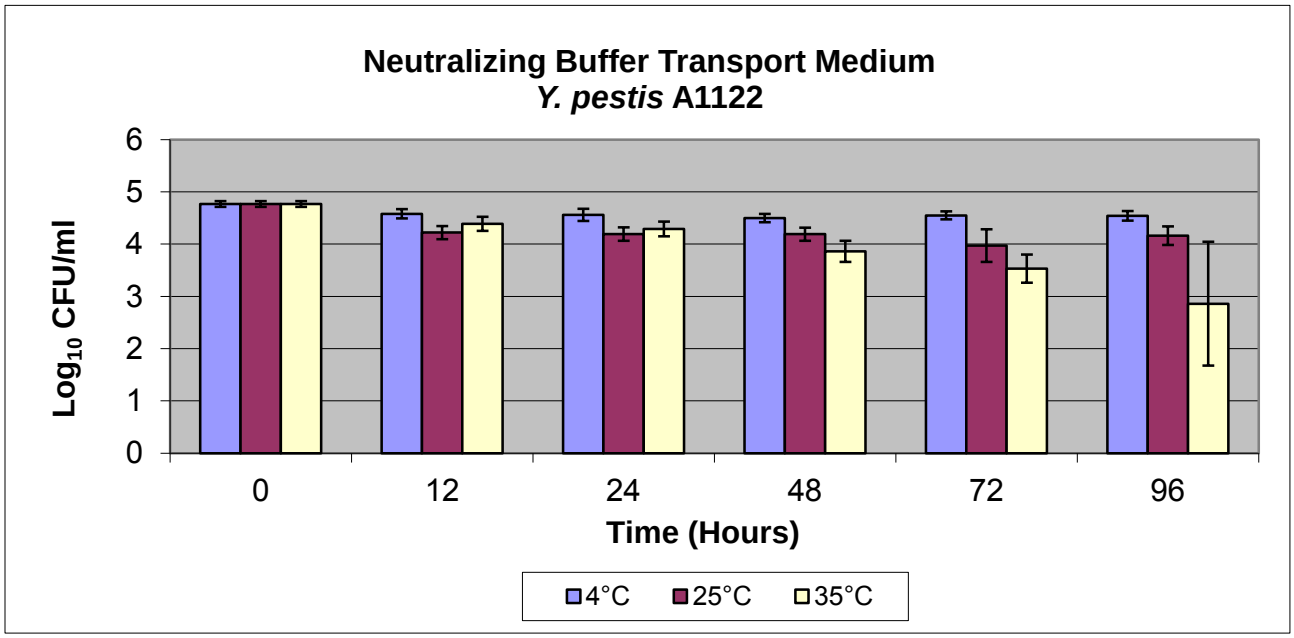

Figure 5: Survival of *Yersinia pestis* A1122 over storage time in NB liquid medium. The bars represent the mean $\log_{10}$ CFU/mL recovered over time. The error bars represent the standard deviation (n=5).

3.1.6 Stuart, Toshach and Patsula medium

When using the Stuart, Toshach and Patsula medium (Figure 6) growth occurred within 12 hours at 25°C or 35°C. When held at 4°C, no significant differences, relative to T_0 (4.64 $\log_{10}$ CFU/mL) were observed in cell recovery ($p>0.05$) across all time points. A greater than 3 $\log_{10}$ increase in cell numbers was observed by 96 hours if held at 35°C, and a 1.8 $\log_{10}$ increase occurred by 96 hours if held at 25°C.

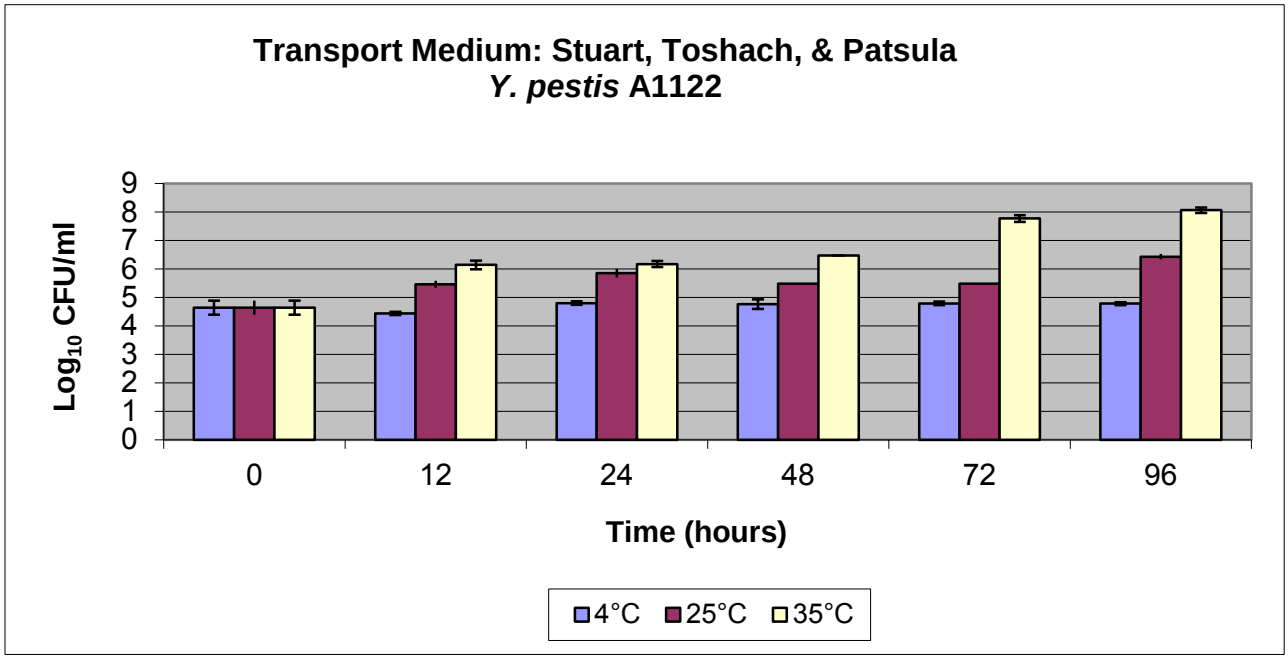

Figure 6: Survival of *Yersinia pestis* A1122 over storage time in Stuart, Toshach and Patsula liquid medium.
The bars represent the mean $\log_{10}$ CFU/mL recovered over time. The error bars represent the standard deviation (n=5).

3.1.7 Selection of transport media for the following study phases

The data for each medium was compiled by temperature from 12 to 72 hours. Though 72 hours is beyond what might be expected for shipment or storage of samples, samples could get lost in transit or be held until supplies or personnel arrived for processing. We must consider how cell viability would be affected beyond optimum storage times. Though there was some variability in inocula between transport media tests, this variability is unlikely to have influenced the results, since the selection criteria was from log transformed data, and the changes compared were determined relative to the recovery at T_0 for each medium. The percent of transport tubes with cells recovered that remained within 0.5 $\log_{10}$ of the recovery at the zero time point was calculated and is presented in Table 1. These data indicate that with three transport media (Amies no charcoal, C&B$_{mod}$, and PBSTX) 100% of samples yielded recoveries within 0.5 $\log_{10}$ of the T_0 recovery. Neutralizing buffer was a close fourth choice, with 100% and 68% of samples yielding recoveries within 0.5 $\log_{10}$ at 4° and 25°C.

Table 1. Percent of *Y. pestis* samples with recoveries of ≤ 0.5 $\log_{10}$ change relative to T_0 for storage times of 12 to 72 hours (n=60) in various transport media

Storage temp	Transport Media					
	Amies with charcoal	Amies no charcoal	Cary & Blair	Neutralizing Buffer	Stuart, Toshach & Patsula	PBSTX
4°C	77	100	100	100	100	100
25°C	55	100	100	68	0	100

The data were then re-examined so that the percent of samples with cell recovery that remained within 0.3 $\log_{10}$ of the recovery at T_0 was calculated. These data are presented in Table 2, and indicate that C&B$_{mod}$ and PBSTX provide the higher percent of swabs with recoveries within 0.3 $\log_{10}$ of the T_0 recovery. PBSTX yielded 92% and 100% of swabs within 0.3 $\log_{10}$ of the T_0 recovery at 4°C and 25°C, respectively, C&B$_{mod}$ yielded 97% and 77% of samples within 0.3 $\log_{10}$ of the T_0 recovery at 4°C and 25°C, respectively. Based on results from the preliminary study, C&B$_{mod}$ and PBSTX were chosen as the two transport media that maintained cell viability well at both temperatures. The optimum storage temperature was found to be 4°C for the transport media chosen. Subsequent evaluations were conducted at this temperature and in these two transport media.

After phase III of the study, the formulation for $C\&B_{mod}$ was found to have been altered by omitting the $CaCl_2$. $C\&B_{mod}$ was replaced with NB because it provided comparable results to $C\&B_{mod}$ at 4° (already chosen as the optimal temperature by the time this altered formulation was discovered) when examining the ≤ 0.3 $\log_{10}$ change data (Table 2), but also because it is immediately commercially available and would be easily obtained without special formulation (as would be needed if the next best choice, Amies without charcoal, was chosen).

Table 2. Percent of *Y. pestis* samples with recoveries of ≤ 0.3 $\log_{10}$ change from T_0 for storage times of 12 to 72 hours (n=60) in various transport media

Storage temp	Transport Media					
	Amies with charcoal	Amies no charcoal	Cary & Blair	Neutralizing Buffer	Stuart, Toshach & Patsula	PBSTX
4°C	67	71	97	77	97	92
25°C	0	85	77	0	0	100

3.2 Growth curves

3.2.1 *Yersinia pestis*

The growth curves for *Y. pestis* A1122 (low virulence), and virulent Harbin and CO92 strains are shown in Figures 7, 8 and 9, respectively. After the growth curve was completed for the *Y. pestis* Harbin strain, testing revealed that this isolate had lost one of the virulence plasmids before we acquired it. Based on these results, CDC and EPA jointly decided to instead use *Y. pestis* CO92 for work calling for a virulent strain. *Y. pestis* CO92 was confirmed as virulent by the Congo red pigment test [8] (indicating the presence of the hemin storage [hms] and pigmentation [pgm] loci) and by confirmation of the presence of the three virulence plasmids (110, 70, and 9.5 kb) as carried out by CDC's Division of Vector Borne Diseases in Ft Collins, CO. Figure 7 shows the growth curve for *Y. pestis* A1122 strain, indicating 60 hours was required to reach the late log phase when starting with 10^4 CFU/mL in BHIB. The *Y. pestis* Harbin strain curve is shown in Figure 8, and late log phase was reached around 42 hours. The *Y. pestis* CO92 strain, seen in Figure 9, reached late log phase by 26 hours.

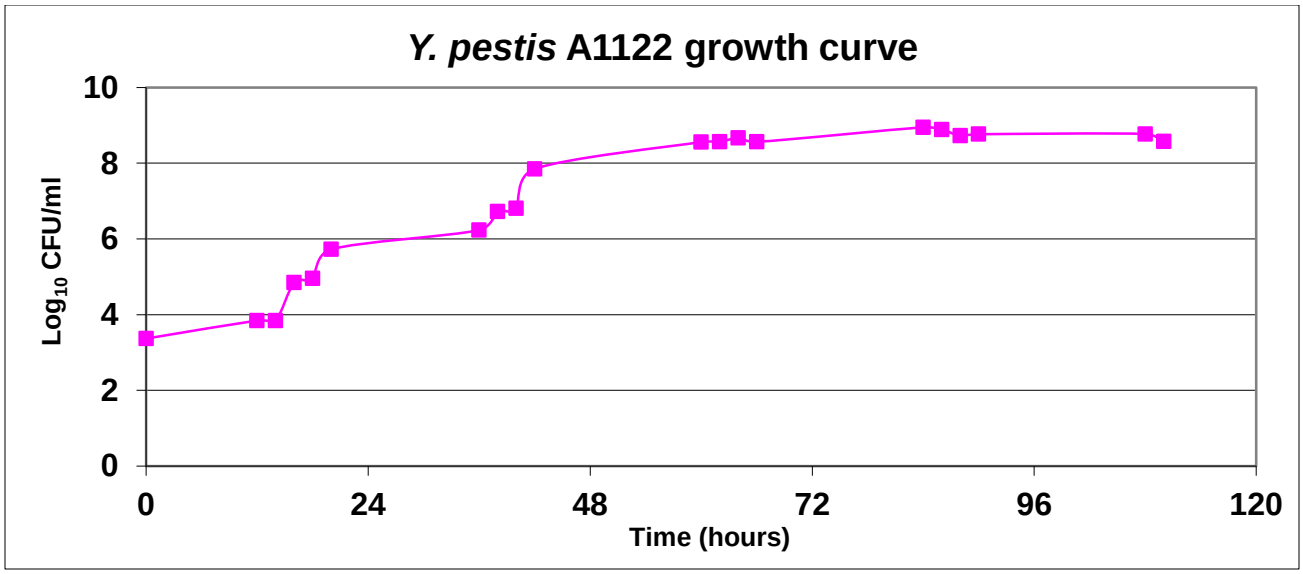

Figure 7: Growth curve for *Yersinia pestis* A1122.

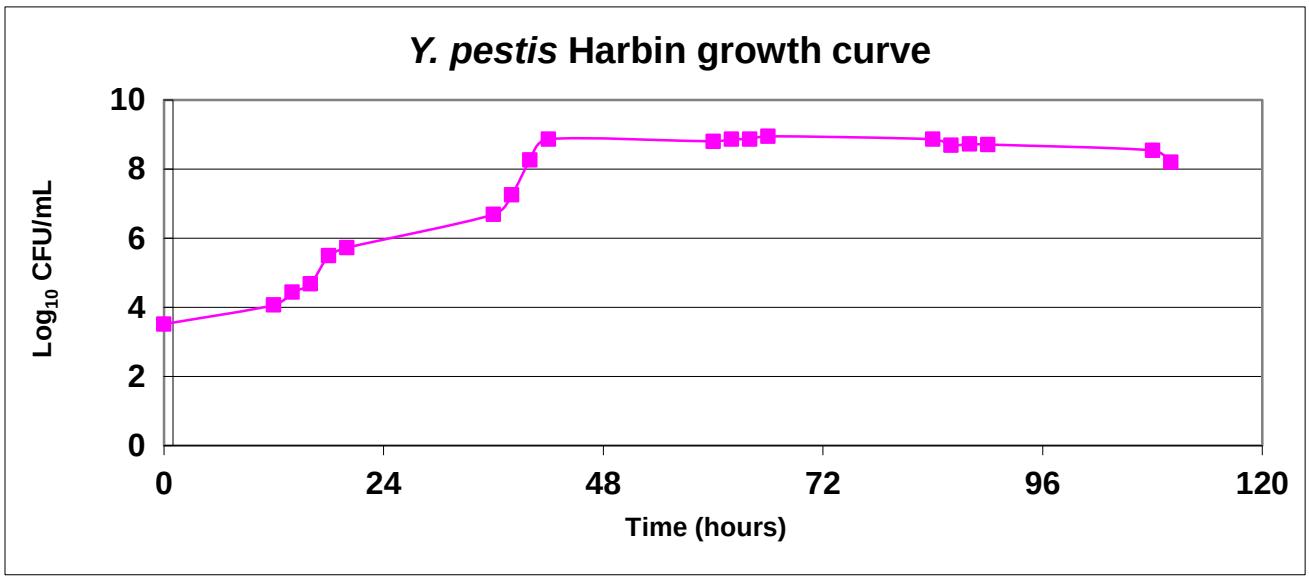

Figure 8: Growth curve for *Yersinia pestis* Harbin.

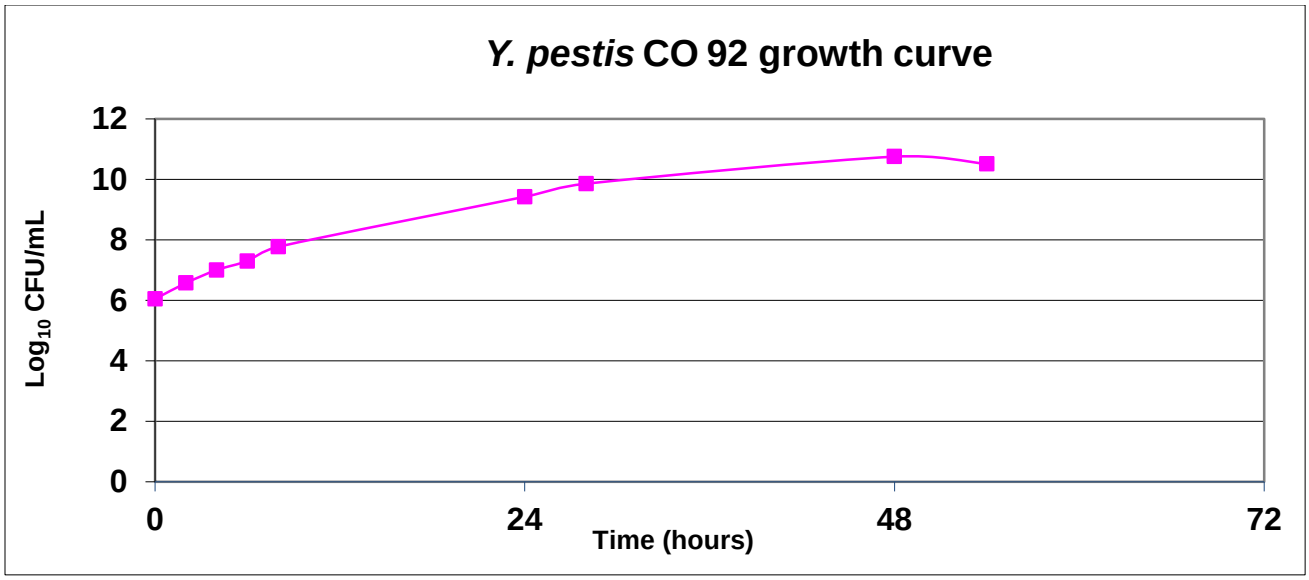

Figure 9: Growth curve for *Yersinia pestis* CO92.

3.2.2 *Francisella tularensis*

The growth curves for *F. tularensis* LVS and Schu S4 strains were found to both require 60 hours to reach late the log phase when starting with 10^4 CFU/mL in trypticase soy broth + 2% Isovitalex (Figures 10 and 11, respectively).

While performing these growth curve studies, problems were encountered with getting consistent growth with the *F. tularensis* strains. *F. tularensis* is known to be a fastidious organism, especially when grown in liquid media[3] with specific nutritional requirements and high inocula required. Though a well characterized medium was used, several times the organisms simply did not grow, or did not grow to the expected titer after extremely long incubation times. These problems set the timeline for the project back significantly. Therefore, after considering time and budget constraints, CDC and EPA jointly decided that all subsequent work would focus only on *Yersinia pestis.*

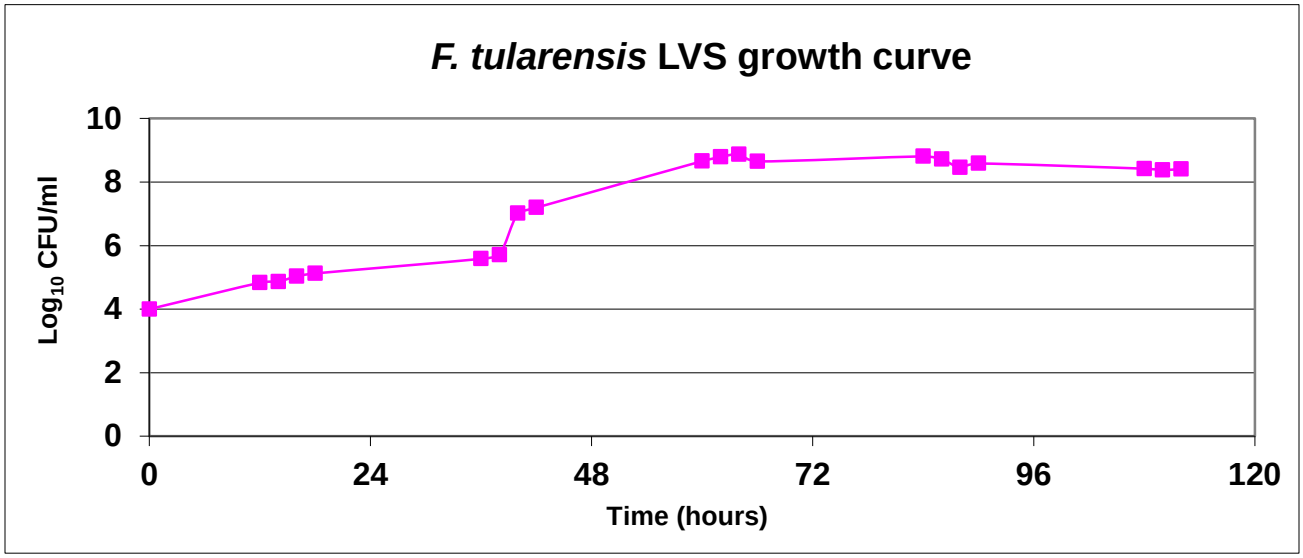

Figure 10: Growth curve for *Francisella tularensis* LVS.

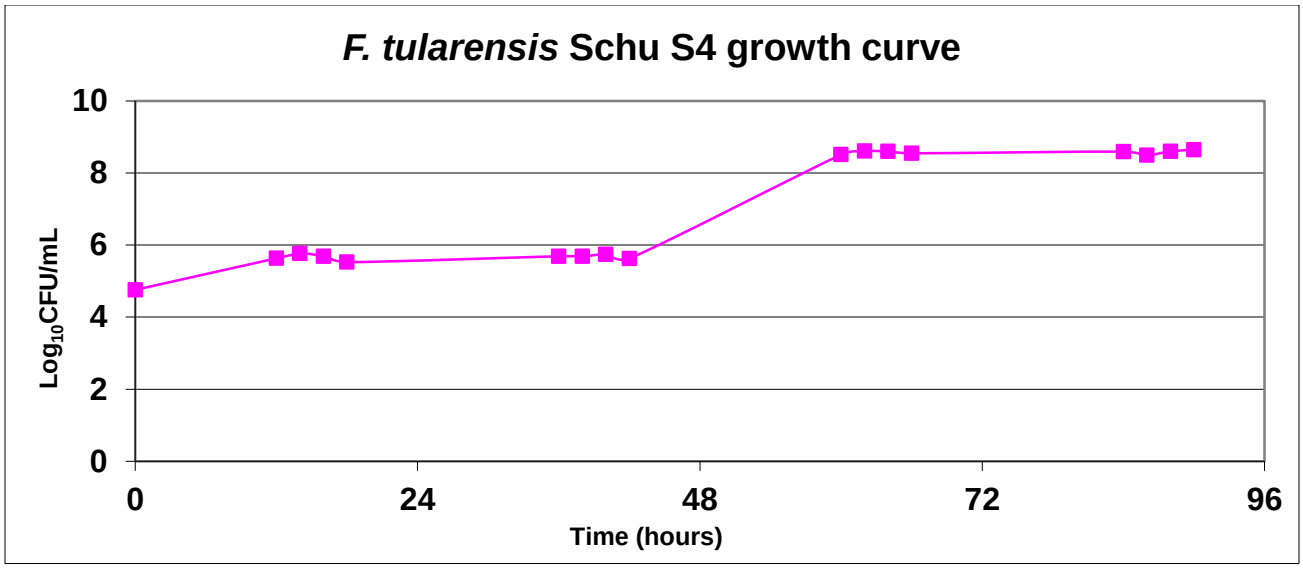

Figure 11: Growth curve for *Francisella tularensis* Schu S4.

3.3 Phase I: Evaluation of swab extraction methods

Table 2 in the appendix shows a matrix of all tests performed in Phase I. Macrofoam swabs pre-moistened with NB and inoculated with *Y. pestis* A1122 that were processed by vortexing only or by sonication and vortexing together yielded the highest % recovery, relative to the positive control (inoculated PBST, no swab) at 93.9% or 93.5%, respectively (Table 3, shaded cells). Since no significant difference was seen between these two processing methods (p=0.94), the method with the fewer steps and simpler equipment (vortexing only) was chosen for subsequent evaluations. Using the no extraction method was the least efficient, with only 68.7% recovered from the swab.

Rayon swabs pre-moistened with NB and inoculated with *Y. pestis* A1122 that were processed using sonication only yielded the highest % recovery at 77.0% (Table 3, shaded cell). Combining sonication and vortexing yielded a % recovery of 73%. The vortex-only method was less efficient for rayon swabs than for macrofoam swabs, at 55.2%. Polyester swabs yielded the lowest % recoveries of all the swab types and were discontinued for future testing. Although the optimal condition for cotton (pre-moistened with PBSTX and vortexed and sonicated) yielded a statistically equivalent % recovery to rayon's optimum (79% and 77%, respectively), cotton swabs were excluded from subsequent evaluations because of the concerns for potential PCR inhibition.

As a result of these findings, subsequent phases of the project focused on the use of macrofoam swabs processed by vortexing (3 minutes) and rayon swabs processed by sonication only (3 minutes).

Table 3. Mean percent recovery for each swab material, pre-moistening liquid, and extraction method (n=10).

Extraction Method* Premoistening agent	**Swab Material**			
	Cotton	**Macrofoam**	**Polyester**	**Rayon**
Vortex Only				
NB	60.9 (8.6)	93.9 (13.1)**	44.6 (7.1)	55.2 (12.2)
PBSTX	69.04 (2.03)	86.53 (2.59)	55.54 (1.86)	68.7 (5.20)
p-value	0.009	0.099	0.000	0.005
Sonicate Only				
NB	53.7 (8.8)	89.0 (12.7)	44.6 (11.7)	77.0 (14.4)
PBSTX	69.43 (2.31)	84.78 (3.89)	50.57 (2.73)	64.6 (5.0)
p-value	0.000	0.330	0.132	0.019
Vortex & Sonicate				
NB	53.6 (11.0)	93.5 (9.4)	53.7 (11.0)	73.0 (15.7)
PBSTX	79.42 (3.08)	86.33 (3.24)	53.65 (3.59)	67.8 (4.6)
p-value	0.000	0.035	0.989	0.328
None				
NB	40.4 (5.0)	68.7 (9.2)	25.1 (5.0)	28.6 (7.8)
PBSTX	48.42 (2.50)	62.45 (3.86)	46.40 (1.43)	29.80 (3.1)
p-value	0.000	0.065	0.000	0.641

*Extractions were performed one hour after swab inoculation. All extractions were performed in PBS+ 0.02% Tween® 80.

** Shading indicates the best extraction methods for the two best swab materials

3.4 Phase II and III: Evaluation of sample storage parameters for both strains and sterile swabs

3.4.1 Phase II: Low-virulent strain, *Yersinia pestis* A1122

The recovery efficiency of low virulent *Y. pestis* (strain A1122) from macrofoam swabs and rayon swabs was evaluated. Tables 3 and 4 in the appendix show the matrices of tests performed in phases II and III. Both swab materials were inoculated directly with 100 µl of a 10^5 CFU/mL suspension (10^4 CFU/swab) and held at room temperature for one hour before placing the swabs in one of the two optimum liquid transport media, either the $C\&B_{mod}$ or the phosphate buffered saline with 0.05% Triton X-100 (PBSTX). The swabs were held in the media at 4°C for the following times: 0, 12, 18, 24, 48, and 72 hours before processing. Two swab preparations (pre-moistened with either PBSTX or NB) and two methods of swab processing (vortexing only for macrofoam swabs and sonication only for rayon swabs) were evaluated. Percent recovery relative to T_0 was determined. We subsequently adopted the convention of denoting each specific condition as pre-moistening medium / transport medium.

3.4.1.1 Macrofoam Swabs

Macrofoam swabs yielded a % recovery range of 46.9-106.2% (Figure 12). When all time points from the 12 to 72 hour storage periods were averaged for each condition (premoistening agent/transport medium), the highest mean % recovery from macrofoam swabs was 99.6%, (sd=10.9, n=49) when pre-moistened with NB and stored at 4°C in C&B$_{mod}$ as the transport medium (denoted as NB/C&B$_{mod}$). When swabs were processed within 24 hours, no significant difference was seen between this optimum condition and two other conditions, PBSTX/PBSTX and PBSTX/C&B$_{mod}$ ($p>0.05$). Swabs pre-moistened with NB and stored in PBSTX (NB/PBSTX) yielded significantly lower % recoveries (Table 4). When swabs were processed within 48 hours, condition NB/C&B$_{mod}$ yielded significantly higher recoveries than two other conditions, PBSTX/C&B$_{mod}$ and NB/PBSTX ($p<0.04$) (Table 5).

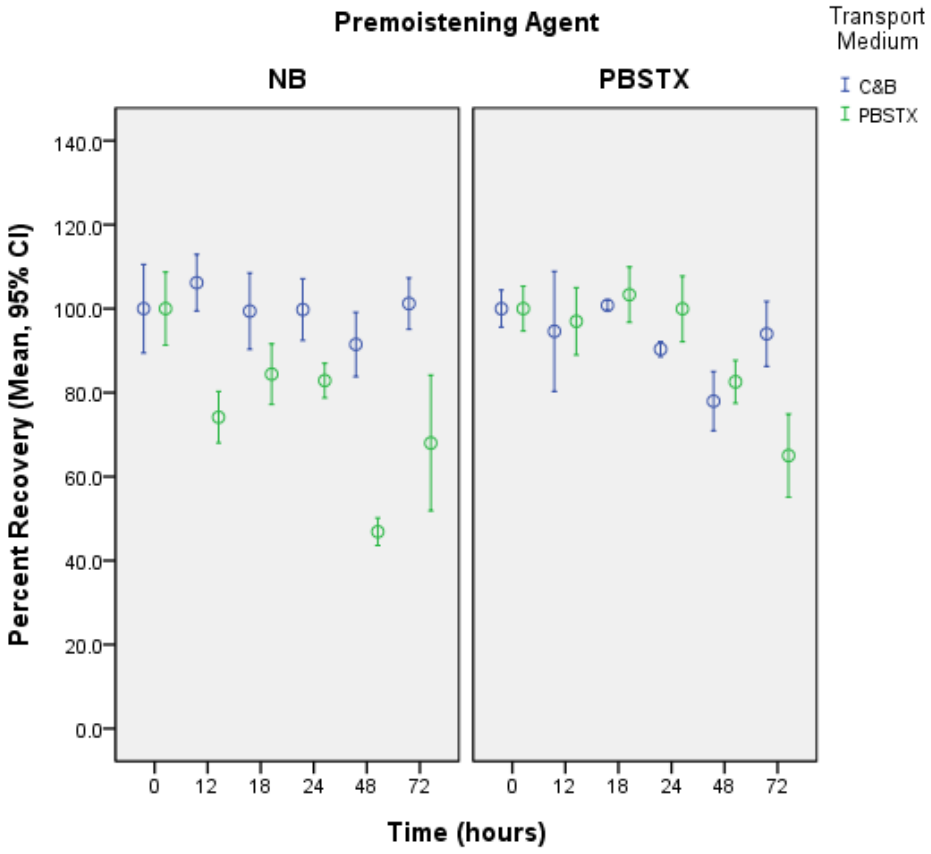

Figure 12: Mean % recovery of *Y. pestis* A1122 (10^4 CFU/ swab) from macrofoam swabs over storage time. Bars represent the 95% confidence interval of the mean % recovery (n=10).

Table 4: Comparison of mean % recovery of *Y. pestis* A1122 from macrofoam swabs stored for 12, 18 and 24 hours, (n=30 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference[b]	Std. Error	p
1	2	4.8433	2.7614	.301
	3	19.6167*	2.7614	.000
	4	-1.7651	2.7851	.921
2	1	-4.8433	2.7614	.301
	3	14.7733*	2.7614	.000
	4	-6.6084	2.7851	.088
3	1	-19.6167*	2.7614	.000
	2	-14.7733*	2.7614	.000
	4	-21.3817*	2.7851	.000
4	1	1.7651	2.7851	.921
	2	6.6084	2.7851	.088
	3	21.3817*	2.7851	.000

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/C&B$_{mod}$, 3=NB/PBSTX, 4=NB/C&B$_{mod}$.

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

Table 5: Comparison of mean % recovery of *Y. pestis* A1122 from macrofoam swabs stored for 12, 18, 24 and 48 (n=40 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	4.7850	3.0859	.410
	3	23.6350*	3.0859	.000
	4	-3.4848	3.1056	.676
2	1	-4.7850	3.0859	.410
	3	18.8500*	3.0859	.000
	4	-8.2698*	3.1056	.042
3	1	-23.6350*	3.0859	.000
	2	-18.8500*	3.0859	.000
	4	-27.1198*	3.1056	.000
4	1	3.4848	3.1056	.676
	2	8.2698*	3.1056	.042
	3	27.1198*	3.1056	.000

[a]Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/C&B$_{mod}$, 3=NB/PBSTX, 4=NB/C&B$_{mod}$.

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

3.4.1.2 Rayon Swabs

Rayon swabs inoculated with *Y. pestis* A1122 yielded a % recovery range of 73.6-118.1% (Figure 13). When all time points within the 12 to 72 hour storage periods were averaged for each condition (premoistening agent/transport medium), the highest mean % recovery was from rayon swabs (103.7%, sd=17.1, n=50) pre-moistened with PBSTX, stored at 4°C in PBSTX as a transport medium. When swabs were processed within 12 to 24 hours or within 12 to 48 hours, no significant difference was seen between this optimum condition (PBSTX/PBSTX) and the NB/PBSTX condition (Tables 6 and 7). The other two conditions (PBSTX/C&B$_{mod}$ or NB/C&B$_{mod}$) yielded significantly lower % recoveries whether processed within 12 to 24 hours ($p<0.03$) or 12 to 48 hours ($p<0.004$).

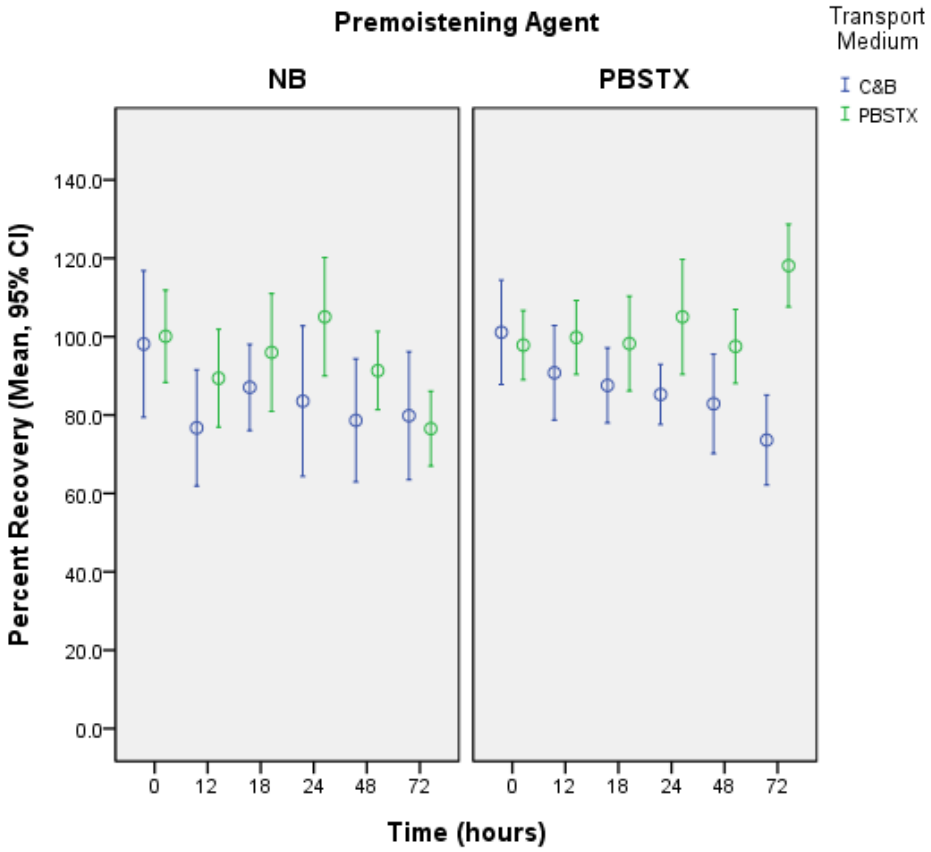

Figure 13: Mean % recovery of *Y. pestis* A1122 (10^4 CFU/ swab) from rayon swabs over storage time. Bars represent the 95% confidence interval of the mean recovery (n=10).

Table 6: Comparison of mean % recovery of *Y. pestis* A1122 from rayon swabs stored for 12, 18 and 24 hours, (n=30 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	13.1567*	4.6354	.027
	3	4.2167	4.6354	.800
	4	18.6130*	4.6752	.001
2	1	-13.1567*	4.6354	.027
	3	-8.9400	4.6354	.222
	4	5.4563	4.6752	.649
3	1	-4.2167	4.6354	.800
	2	8.9400	4.6354	.222
	4	14.3963*	4.6752	.014
4	1	-18.6130*	4.6752	.001
	2	-5.4563	4.6752	.649
	3	-14.3963*	4.6752	.014

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/C&B$_{mod}$, 3=NB/PBSTX, 4=NB/C&B$_{mod}$.

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

Table 7: Comparison of mean % recovery of *Y. pestis* A1122 from rayon swabs stored for 12, 18, 24 and 48 hours (n=40 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	13.5275*	3.9419	.004
	3	4.7075	3.9419	.631
	4	18.7040*	3.9671	.000
2	1	-13.5275*	3.9419	.004
	3	-8.8200	3.9419	.118
	4	5.1765	3.9671	.561
3	1	-4.7075	3.9419	.631
	2	8.8200	3.9419	.118
	4	13.9965*	3.9671	.003
4	1	-18.7040*	3.9671	.000
	2	-5.1765	3.9671	.561
	3	-13.9965*	3.9671	.003

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/C&B$_{mod}$, 3=NB/PBSTX, 4=NB/C&B$_{mod}$.

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

3.4.2 Phase III: Virulent strain, *Yersinia pestis* CO92

The recovery efficiency of virulent *Y. pestis* (strain CO92) from macrofoam swabs and rayon swabs was evaluated. Both swab materials were inoculated directly with 100 µl of a 10^5 CFU/mL suspension and held at room temperature for one hour before placing the swabs in either of the two optimum liquid transport media: C&B$_{mod}$ or PBSTX. The swabs were stored in the medium at 4°C for the following times: 0, 12, 24, 48, and 72 hours before processing. Two swab preparations (pre-moistened with either PBSTX or NB) and two methods of swab processing (vortex only for macrofoam swabs, sonicate only for rayon swabs) were evaluated.

3.4.2.1 Macrofoam Swabs

Macrofoam swabs yielded a % recovery range of 2.3-114.3% for the virulent cells (Figure 14). When all storage periods from 12 to 72 hours were averaged for each condition (premoistening agent/transport medium), the highest mean %% recovery (101.8%, sd=18.0, n=49) was for macrofoam swabs stored at 4°C for the PBSTX/C&B$_{mod}$ condition. Whether processed within 24 hours or 48 hours, the optimum condition

(PBSTX/C&B$_{mod}$) yielded higher % recoveries than all other conditions ($p<0.05$) (Tables 8 & 9).

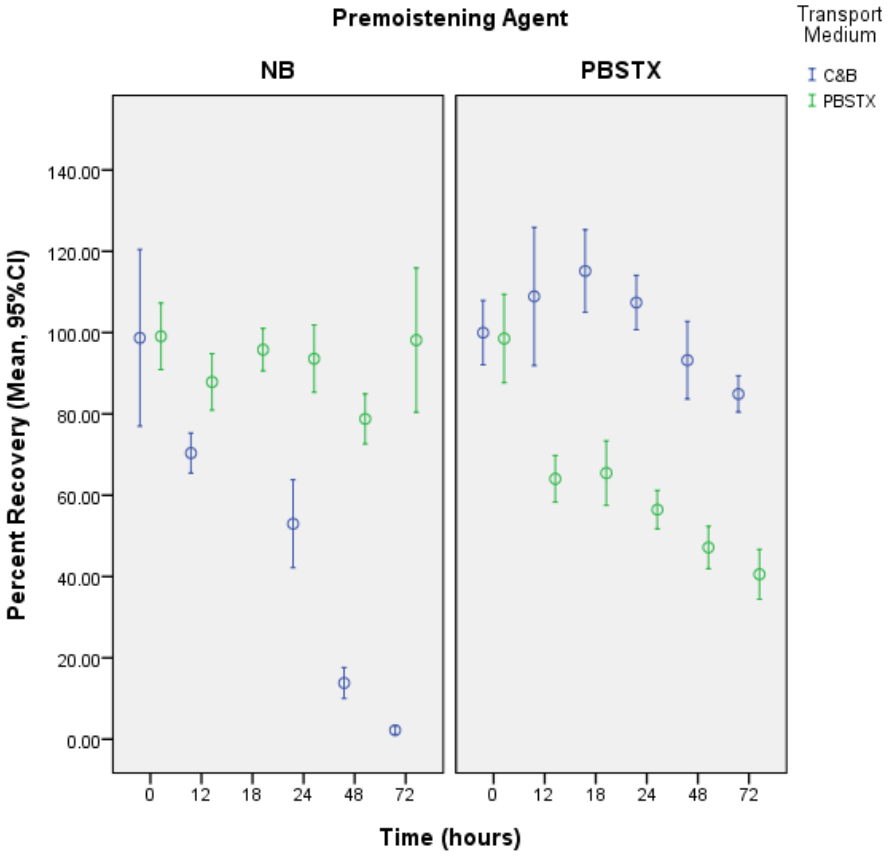

Figure 14: Mean % recovery of *Y. pestis* CO92 (10^4 CFU/ swab) from macrofoam swabs over storage time. Bars represent the 95% confidence interval of the mean recovery (n=10).

Table 8: Comparison of mean % recovery of *Y. pestis* CO92 from macrofoam swabs stored for 12, 18 and 24 hours (n=30 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	-48.4103*	3.3701	.000
	3	-30.2377*	3.3419	.000
	4	.5040	3.7300	.999
2	1	48.4103*	3.3701	.000
	3	18.1726*	3.3419	.000
	4	48.9143*	3.7300	.000
3	1	30.2377*	3.3419	.000
	2	-18.1726*	3.3419	.000
	4	30.7417*	3.7045	.000
4	1	-.5040	3.7300	.999
	2	-48.9143*	3.7300	.000
	3	-30.7417*	3.7045	.000

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/C&B$_{mod}$, 3=NB/PBSTX, 4=NB/C&B$_{mod}$.

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

Table 9: Comparison of mean % recovery of *Y. pestis* CO92 from macrofoam swabs stored for 12, 18, 24 and 48 hours (n = 40 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	-47.8026*	3.8081	.000
	3	-30.6796*	3.7842	.000
	4	12.6046*	4.0837	.013
2	1	47.8026*	3.8081	.000
	3	17.1230*	3.7842	.000
	4	60.4072*	4.0837	.000
3	1	30.6796*	3.7842	.000
	2	-17.1230*	3.7842	.000
	4	43.2842*	4.0615	.000
4	1	-12.6046*	4.0837	.013
	2	-60.4072*	4.0837	.000
	3	-43.2842*	4.0615	.000

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/C&B$_{mod}$, 3=NB/PBSTX, 4=NB/C&B$_{mod}$.

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

3.4.2.2 Rayon Swabs

Rayon swabs yielded a % recovery range of 74.7-126.5% for all storage time points, pre-moistening agents and transport media for the virulent cells (Figure 15). When all time points within the 12 to 72 hour storage period were averaged for each condition (premoistening agent/transport medium), the highest mean recovery (110.1%, sd=21.6, n=40) was from rayon swabs stored at 4°C for the NB/PBSTX condition. When processed after a storage time of 12 or 24 hours, no significant difference was seen between this optimum condition and two others, PBSTX/PBSTX and PBSTX/C&B$_{mod}$ (Table 10). When processed after a storage time of up to 48 hours, no significant difference was seen between this optimum condition (NB/PBSTX) and the PBSTX/C&B$_{mod}$ condition (P>0.06) (Table 11). This optimum condition (NB/PBSTX) yielded significantly higher % recoveries than the PBSTX/PBSTX and NB/C&B$_{mod}$ conditions, when processed after a storage time of up to 48 hours (p=0.01).

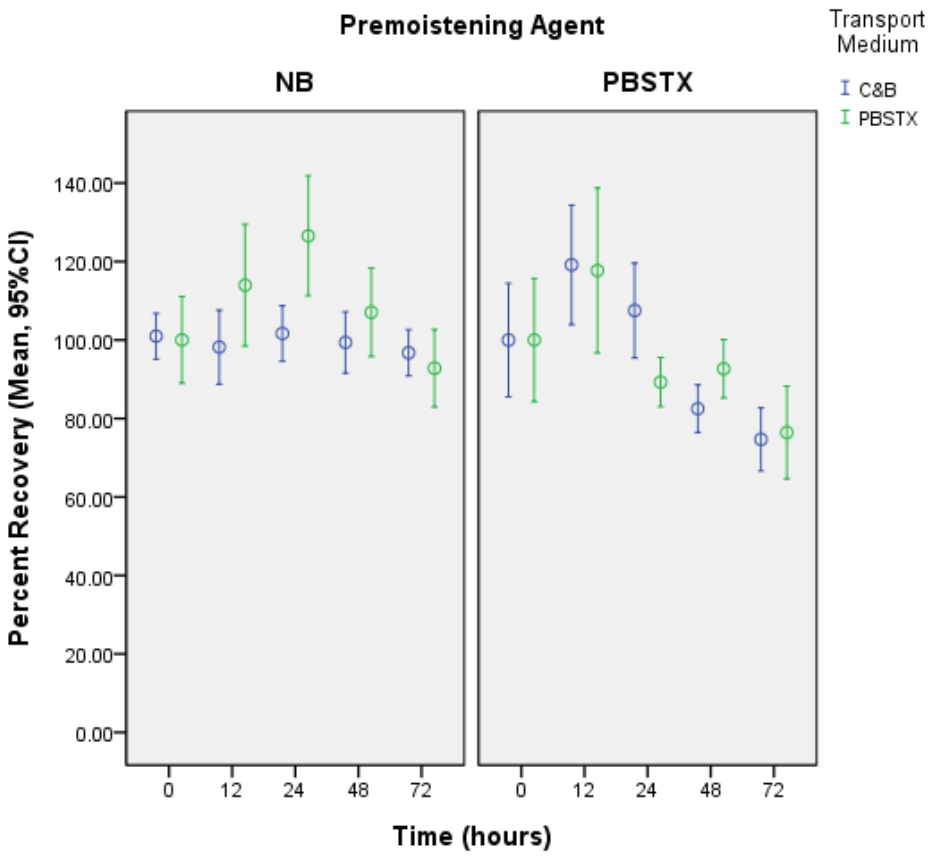

Figure 15: Mean % recovery of *Y. pestis* CO92 (10^4 CFU/ swab) from rayon swabs over storage time. Bars represent the 95% confidence interval of the mean recovery (n=10).

Table 10: Comparison of mean % recovery of *Y. pestis* CO92 from rayon swabs after storage for 12, 18 and 24 hours (n=30 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	-9.8450	6.4382	.425
	3	-16.7750	6.4382	.053
	4	3.5600	6.4382	.945
2	1	9.8450	6.4382	.425
	3	-6.9300	6.4382	.705
	4	13.4050	6.4382	.168
3	1	16.7750	6.4382	.053
	2	6.9300	6.4382	.705
	4	20.3350*	6.4382	.012
4	1	-3.5600	6.4382	.945
	2	-13.4050	6.4382	.168
	3	-20.3350*	6.4382	.012

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/C&B$_{mod}$, 3=NB/PBSTX, 4=NB/C&B$_{mod}$.

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

Table 11: Comparison of mean % recovery of *Y. pestis* CO92 from rayon swabs after storage of 12, 18, 24 and 48 hours (n=40 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	-3.1700	5.0699	.924
	3	-15.9767*	5.0699	.011
	4	.1400	5.0699	1.000
2	1	3.1700	5.0699	.924
	3	-12.8067	5.0699	.061
	4	3.3100	5.0699	.914
3	1	15.9767*	5.0699	.011
	2	12.8067	5.0699	.061
	4	16.1167*	5.0699	.010
4	1	-.1400	5.0699	1.000
	2	-3.3100	5.0699	.914
	3	-16.1167*	5.0699	.010

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/C&B$_{mod}$, 3=NB/PBSTX, 4=NB/C&B$_{mod}$.

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

During this phase of the study, it was discovered that the C&B$_{mod}$ transport media was altered more than simply removing the agar. When the agar was omitted, the calcium chloride was also omitted because it fell out of solution. $CaCl_2$ is an important nutrient for growth media to maintain the virulence of *Y. pestis [3, 4].* Though no data is available indicating $CaCl_2$ is important for transport media, we were not comfortable continuing to use C&B$_{mod}$ with this change in formulation. Because of our uncertainty as to the consequences of omitting the $CaCl_2$, and because the altered formulation is not currently commercially available, the use of C&B$_{mod}$ transport medium was discontinued and replaced with NB when conducting the experiments in phase IV. NB was chosen because it was considered the "next best" performing transport media to C&B (that was immediately commercially available) in the preliminary study, based on 4°C data (Table 2).

3.5 Phase IV: Evaluation of sample storage parameters for both *Y. pestis* strains inoculated on "dirty swabs"

3.5.1 Low-virulent strain, *Yersinia pestis* A1122

The recovery efficiency of *Y. pestis* A1122 from "dirty" macrofoam swabs and rayon swabs was evaluated. Refer to Table 5 in the appendix for a matrix of tests performed. Both swab materials were inoculated directly with a 100 µl of a 10^4 CFU/mL suspension and held at room temperature for one hour before placing the swabs in one of the two optimum liquid transport media, either NB or PBSTX. The swabs were held in the respective medium at 4°C for before the following processing times: 0, 12, 24, 48, and 72 hours. Two swab preparations (pre-moistened with either PBSTX or NB both with a slurry of Arizona Test Dust (ATD) and two methods of swab processing (vortex only for macrofoam swabs, sonicate only for rayon swabs) were evaluated.

3.5.1.1 Macrofoam Swabs

Macrofoam swabs yielded a % recovery range of 65.8-111.6% of the low-virulent cells recovered for all storage time points, pre-moistening agents and transport media (Figure 16). When all time points within the 12 to 72 hour storage period were averaged for each condition (premoistening agent/transport medium), the highest mean % recovery (105.1%, sd=11.7, n=40) was from macrofoam swabs pre-moistened with PBSTX and stored at 4ºC in NB as the transport medium (PBSTX/NB). For storage times of 24 hours or less, the optimum condition (PBSTX/NB) yielded significantly higher % recoveries than PBSTX/PBSTX and NB/NB conditions ($p<0.001$) (Table 12). For storage times of 48 hours or less,

the optimum condition (PBSTX/NB) yielded significantly higher % recoveries than all other conditions ($p<0.025$) (Table 13).

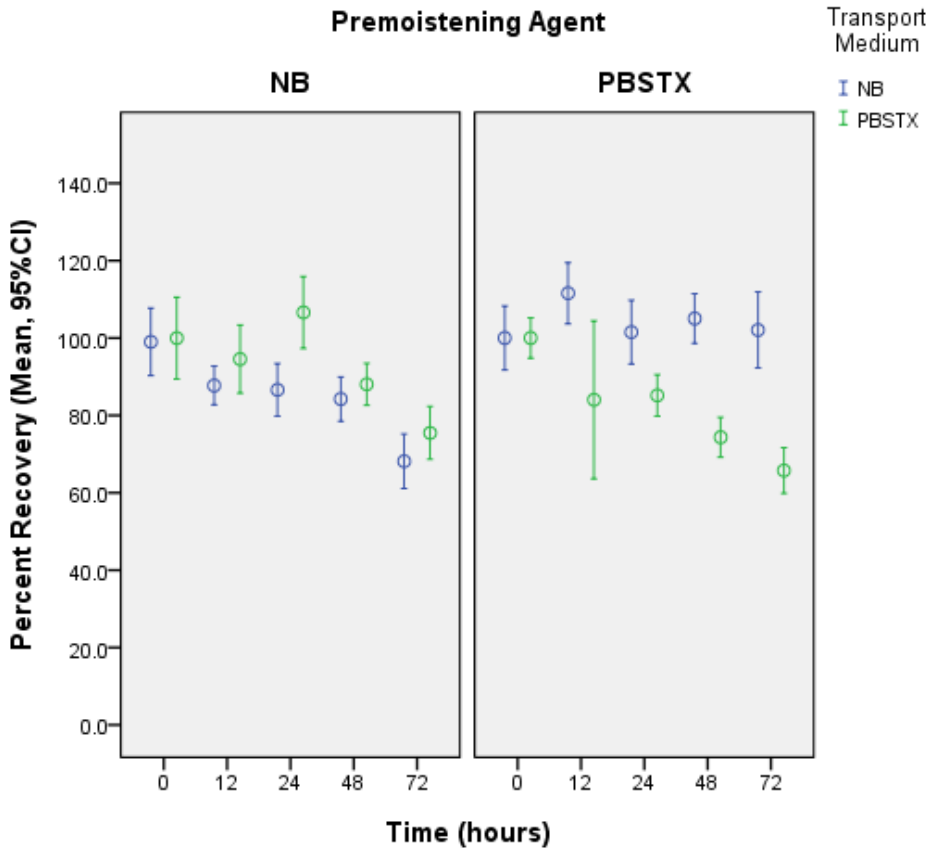

Figure 16: Mean % recovery of *Y. pestis* A1122 (10^4 CFU/ swab) from dirty macrofoam swabs over storage time. Bars represent the 95% confidence interval of the mean recovery (n=10).

Table 12: Comparison of mean % recovery of *Y. pestis* A1122 from dirty macrofoam swabs after storage for 12 and 24 hours (n=20 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	-21.9500*	4.5156	.000
	3	-15.9750*	4.5156	.004
	4	-2.5500	4.5156	.942
2	1	21.9500*	4.5156	.000
	3	5.9750	4.5156	.551
	4	19.4000*	4.5156	.000
3	1	15.9750*	4.5156	.004
	2	-5.9750	4.5156	.551
	4	13.4250*	4.5156	.020
4	1	2.5500	4.5156	.942
	2	-19.4000*	4.5156	.000
	3	-13.4250*	4.5156	.020

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/NB, 3=NB/PBSTX, 4=NB/NB

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

Table 13: ANOVA of *Y. pestis* A1122 mean % recovery from dirty macrofoam swabs after storage for 12, 24 and 48 hours (n=30 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	-24.8633*	3.3572	.000
	3	-15.2133*	3.3572	.000
	4	-4.9967	3.3572	.448
2	1	24.8633*	3.3572	.000
	3	9.6500*	3.3572	.025
	4	19.8667*	3.3572	.000
3	1	15.2133*	3.3572	.000
	2	-9.6500*	3.3572	.025
	4	10.2167*	3.3572	.015
4	1	4.9967	3.3572	.448
	2	-19.8667*	3.3572	.000
	3	-10.2167*	3.3572	.015

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/NB, 3=NB/PBSTX, 4=NB/NB

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

3.5.1.2 Rayon Swabs

Rayon swabs yielded a % recovery range of 79.1-112.8% for the low-virulent cells (Figure 17) from all storage times, pre-moistening agents and transport media. When all time points within the 12 to 72 hour storage period were averaged for each condition (premoistening agent/transport medium),, the highest mean recovery (107.2%, sd=14.3, n=40) from rayon swabs was obtained when pre-moistened with PBSTX and stored at 4°C in PBSTX as the transport medium. This optimum condition (PBSTX/PBSTX) yielded significantly higher % recoveries than all other conditions for storage times of 24 hours ($p<0.02$) or 48 hours ($p<0.001$) (Tables 14 and 15).

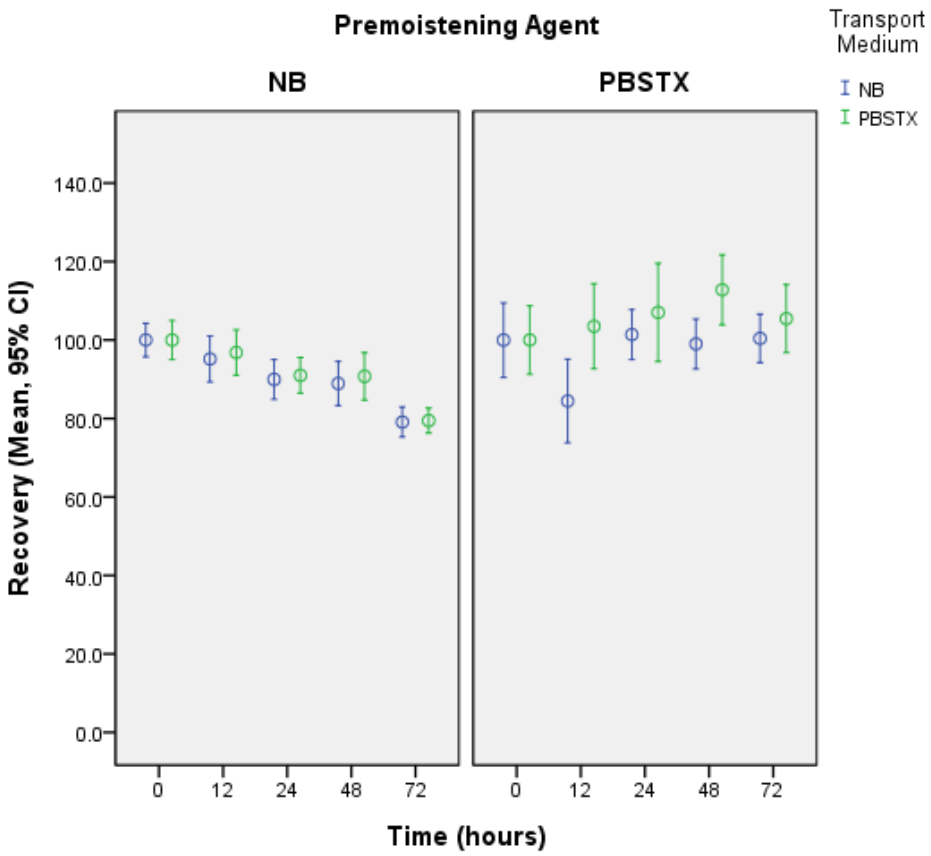

Figure 17: Mean % recovery of *Y. pestis* A1122 (10^4 CFU/ swab) from dirty rayon swabs over storage time. Bars represent the 95% confidence interval of the mean recovery (n=10).

Table 14: Comparison of mean % recovery of *Y. pestis* A1122 from dirty rayon swabs after storage for 12 and 24 hours (n=20 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	12.3450*	3.8618	.011
	3	11.3650*	3.8618	.022
	4	12.7100*	3.8618	.008
2	1	-12.3450*	3.8618	.011
	3	-.9800	3.8618	.994
	4	.3650	3.8618	1.000
3	1	-11.3650*	3.8618	.022
	2	.9800	3.8618	.994
	4	1.3450	3.8618	.985
4	1	-12.7100*	3.8618	.008
	2	-.3650	3.8618	1.000
	3	-1.3450	3.8618	.985

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/C&B$_{mod}$, 3=NB/PBSTX, 4=NB/C&B$_{mod}$.

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

Table 15: Comparison of mean % recovery of *Y. pestis* A1122 from dirty rayon swabs after storage at 12, 24 and 48 hours (n=30 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	12.8333*	2.9779	.000
	3	14.9333*	2.9779	.000
	4	16.4367*	2.9779	.000
2	1	-12.8333*	2.9779	.000
	3	2.1000	2.9779	.895
	4	3.6033	2.9779	.622
3	1	-14.9333*	2.9779	.000
	2	-2.1000	2.9779	.895
	4	1.5033	2.9779	.958
4	1	-16.4367*	2.9779	.000
	2	-3.6033	2.9779	.622
	3	-1.5033	2.9779	.958

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/C&B$_{mod}$, 3=NB/PBSTX, 4=NB/C&B$_{mod}$.

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

3.5.2 Virulent strain, *Yersinia pestis* CO92

The recovery efficiency of *Y. pestis* strain CO92 from "dirty" macrofoam swabs and rayon swabs was evaluated. Refer to Table 6 in the appendix for a matrix of tests performed. Both swab materials were inoculated directly with 100 µl of a 10^4 CFU/mL suspensions and held at room temperature for one hour before placing the swabs in one of the two optimum liquid transport media, either NB or PBSTX. The swabs were held in the respective medium at 4°C for the following times: 0, 12, 24, 48, and 72 hours before processing. Two swab preparations (pre-moistened with either PBSTX or NB both with a slurry of ATD) and two methods of swab processing (vortex only for macrofoam swabs, sonicate only for rayon swabs) were evaluated.

3.5.2.1 Macrofoam Swabs

Macrofoam swabs yielded a % recovery range of 58.0-100% of the virulent cells (Figure 18) from all storage times, pre-moistening agents and transport media. When all time points within the 12 to 72 hour storage periods were averaged for each condition (premoistening

agent/transport medium), three of the conditions yielded similar recoveries. The optimum condition for macrofoam swabs was NB/PBSTX (85.9%, sd=10.6, n=40). No significant difference was seen between this optimum condition (NB/PBSTX) and two other conditions (PBSTX/NB and PBSTX/PBSTX) (Tables 16 and 17). The other condition (NB/NB) yielded a significantly lower % recovery than the optimum condition (NB/PBSTX), whether processed within 24, 48, or 72 hours ($p<0.022$) (Table 16).

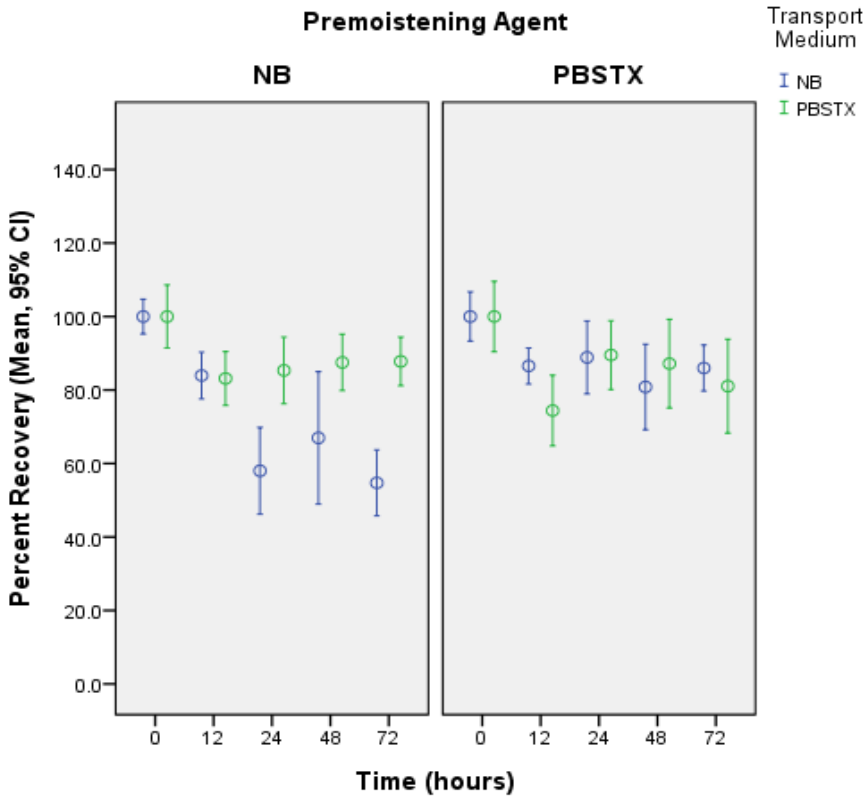

Figure 18: Mean % recovery of *Y. pestis* CO92 (10^4 CFU/ swab) from dirty macrofoam swabs over storage time. Bars represent the 95% confidence interval of the mean recovery (n=10).

Table 16: Comparison of mean % recovery of *Y. pestis* CO92 from dirty macrofoam swabs after storage at 12 and 24 hours (n=20 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	-5.7550	4.5050	.580
	3	-2.2800	4.5050	.957
	4	10.9800	4.5050	.079
2	1	5.7550	4.5050	.580
	3	3.4750	4.5050	.867
	4	16.7350*	4.5050	.002
3	1	2.2800	4.5050	.957
	2	-3.4750	4.5050	.867
	4	13.2600*	4.5050	.022
4	1	-10.9800	4.5050	.079
	2	-16.7350*	4.5050	.002
	3	-13.2600*	4.5050	.022

[a] Experiments (premoistening agent/transport media) were as follows: 1=PBSTX/PBSTX, 2=PBSTX/NB, 3=NB/PBSTX, 4=NB/NB

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

Table 17: Comparison of mean % recovery of *Y. pestis* CO92 from dirty macrofoam swabs after storage for 12, 24 and 48 hours (n=30 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	-1.7167	3.9966	.973
	3	-1.6267	3.9966	.977
	4	14.0600*	3.9966	.003
2	1	1.7167	3.9966	.973
	3	.0900	3.9966	1.000
	4	15.7767*	3.9966	.001
3	1	1.6267	3.9966	.977
	2	-.0900	3.9966	1.000
	4	15.6867*	3.9966	.001
4	1	-14.0600*	3.9966	.003
	2	-15.7767*	3.9966	.001
	3	-15.6867*	3.9966	.001

[a] Experiments (premoistening agent/transport media) were as follows:1=PBSTX/PBSTX, 2=PBSTX/NB, 3=NB/PBSTX, 4=NB/NB

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

3.5.2.2 Rayon Swabs

Rayon swabs yielded a % recovery range of 34.3-100% of the virulent cells (Figure 19) from all storage times, pre-moistening agents and transport media. When all time points within the 12 to 72 hour storage times were averaged for each condition (premoistening agent/transport medium),, the highest mean recovery (83.7%, sd=14.3, n=40) from rayon swabs was obtained at 4°C for the NB/PBSTX condition. This optimum condition (NB/PBSTX) yielded significantly higher % recoveries than PBSTX/NB, for storage times of 24 hours or 48 hours ($p<0.001$). No significant differences were seen between this optimum condition and two others (PBSTX/PBSTX and NB/PBSTX) for storage times up to 24 or up to 48 hours (Tables 18 and 19).

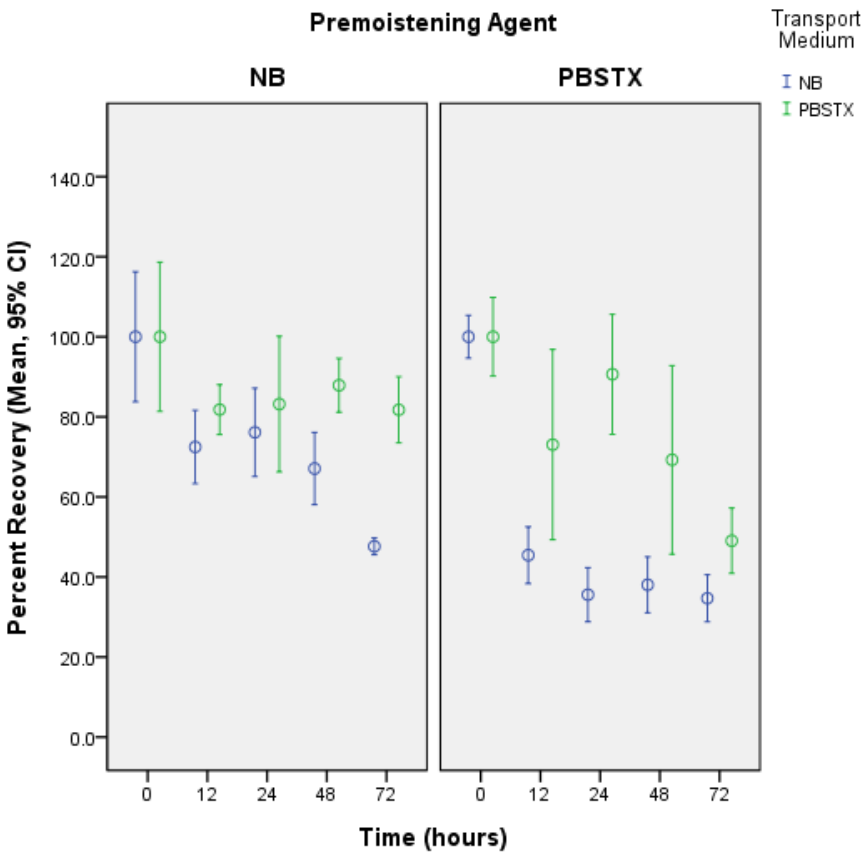

Figure 19: Mean % recovery of *Y. pestis* CO92 (10^4 CFU/ swab) from dirty rayon swabs over storage time. Bars represent the 95% confidence interval of the mean recovery (n=10).

Table 18: Comparison of mean % recovery of *Y. pestis* CO92 from dirty rayon swabs after storage for 12 and 24 hours (n=20 for each experiment) using Tukey HSD analysis.

Reference Experiment [a]	Comparative Experiment	Mean Difference [b]	Std. Error	p
1	2	41.3450*	5.9671	.000
	3	-.6450	5.9671	1.000
	4	7.5500	5.9671	.588
2	1	-41.3450*	5.9671	.000
	3	-41.9900*	5.9671	.000
	4	-33.7950*	5.9671	.000
3	1	.6450	5.9671	1.000
	2	41.9900*	5.9671	.000
	4	8.1950	5.9671	.520
4	1	-7.5500	5.9671	.588
	2	33.7950*	5.9671	.000
	3	-8.1950	5.9671	.520

[a] Experiments (premoistening agent/transport media) were as follows:1=PBSTX/PBSTX, 2=PBSTX/NB, 3=NB/PBSTX, 4=NB/NB

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

Table 19: Comparison of mean % recovery of *Y. pestis* CO92 from dirty rayon swabs after storage for 12, 24 and 48 hours (n=30 for each experiment) using Tukey HSD analysis.

Experiment[‡]	Experiment	Mean Difference	Std. Error	p
1	2	37.9733*	4.8860	.000
	3	-6.6367	4.8860	.528
	4	5.7633	4.8860	.641
2	1	-37.9733*	4.8860	.000
	3	-44.6100*	4.8860	.000
	4	-32.2100*	4.8860	.000
3	1	6.6367	4.8860	.528
	2	44.6100*	4.8860	.000
	4	12.4000	4.8860	.059
4	1	-5.7633	4.8860	.641
	2	32.2100*	4.8860	.000
	3	-12.4000	4.8860	.059

[a] Experiments (premoistening agent/transport media) were as follows:1=PBSTX/PBSTX, 2=PBSTX/NB, 3=NB/PBSTX, 4=NB/NB

[b] mean % recovery of reference experiment minus the mean % recovery of the comparative experiment.

* indicates that the mean difference is significant at the 0.05 level

3.6 Statistical Analysis

When recovery of *Y. pestis* was compared from all conditions tested (clean or dirty, premoistening agent and transport medium), no specific condition was identified as the best for recovery from both macrofoam and rayon swabs (Table 20). Therefore, another approach to analyzing the data was considered. The data for each swab type were combined; that included both strains, both clean and dirty, and data for all time points up to 72 hours (assumes a worst case scenario of a processing time of 72 hours). For these analyses, the data for the substituted transport media (C&B and NB) were treated as one data set (PBSTX/C&B data pooled with PBSTX/NB, and NB/C&B pooled with NB/NB). The combined data for each swab type were not normally distributed, and therefore analyzed using the Kruskal-Wallis and Mann-Whitney tests. The results of these tests show that macrofoam swabs had one condition that resulted in statistically higher recoveries than the others; the PBSTX/C&B$_{mod}$ (data pooled with PBSTX/NB) ($p \leq 0.000$, data not shown). Since the standard C&B formulation was found to be unstable in liquid form and was not commercially available, PBSTX/NB was chosen as the optimum premoistening agent/transport medium for macrofoam swabs. When the data was grouped similarly for rayon swabs (both strains, dirty and clean, all time points), the test results show that the optimum premoistening agent/transport media combinations

were PBSTX/PBSTX ($p \leq 0.000$, data not shown) and NB/PBSTX ($p \leq 0.000$, data not shown).

Table 20: Optimum recovery of low-virulent (A1122) and virulent (CO92) *Y. pestis* from each swab type at the specified condition.

Strain	Swab Type	Clean or Dirty	Pre-moistening Agent	Transport Medium	Percent Recovery (24hrs)†	Percent Recovery (48hrs)‡	Percent Recovery (72hrs)≡
YP A1122	Macro-foam	Clean	NB	$C\&B_{mod}$	101.9%	99.2%	99.6%
YP A1122	Macro-foam	Dirty	PBSTX	NB	106.7%	106.1%	105.1%
YP CO92	Macro-foam	Clean	PBSTX	$C\&B_{mod}$	110.6%	106.1%	101.8%
YP CO92	Macro-foam	Dirty	NB	PBSTX	84.2%	85.3%	85.9%
YP A1122	Rayon	Clean	PBSTX	PBSTX	101.0%	100.1%	103.7%
YP A1122	Rayon	Dirty	PBSTX	PBSTX	105.3%	107.8%	107.2%
YP CO92	Rayon	Clean	NB	PBSTX	120.3%	115.9%	110.1%
YP CO92	Rayon	Dirty	NB	PBSTX	82.5%	84.3%	83.7%

†24 hrs represents the mean percent recovery for 12hr and 24hr combined.
‡48 hrs represents the mean percent recovery for 12hr, 24hr, and 48hr combined.
≡ 72 hrs represents the mean percent recovery for 12hr, 24hr, 48hr, and 72hr combined.

4.0 Conclusions:

Six liquid transport media (no swabs) were evaluated using the low-virulent strain of *Y. pestis* A1122 and holding time of up to 72 hours at 4°, 25°, or 35°C. The most consistent recovery of cells was found when the storage temperature was maintained at 4°C for all transport media. $C\&B_{mod}$ and PBSTX were found to be the best of the six transport media evaluated, and were selected for use in subsequent evaluations. It was later found that the C&B liquid formulation was prepared without calcium chloride, an ingredient that influences the virulence factors of *Y. pestis* in growth media [3, 4] so the next best commercially available transport medium, NB, was substituted after phase III.

Of the four swabs evaluated, rayon and macrofoam were chosen as the best two swabs because they were found to release *Y. pestis* cells significantly better than polyester swabs and they did not have the PCR reagent inhibition concerns associated with cotton swabs. Rayon swab recoveries were higher when processed by sonication for three minutes, and macrofoam swab recoveries were higher when processed by vortexing for three minutes.

The pre-moistening agent and transport medium combinations were analyzed for each strain and swab, dirty or clean, and at each storage time. No single pre-moistening agent/transport media condition stood out as the best for all combinations (both swabs, both isolates, clean and dirty).

The data were separated by swab type and then the results were combined for both strains for both dirty and clean combinations. Considering a worse case storage time of 72 hours, all data from all time points were also combined for these analyses.

The results showed that macrofoam swabs pre-moistened with PBSTX and stored in NB or $C\&B_{mod}$ (PBSTX/NB or PBSTX/$C\&B_{mod}$) provided significantly higher % recovery of *Y. pestis* than any other combinations of premoistening agent and transport media tested. The C&B medium was discontinued after phase III because of the aforementioned formulation problems, so NB was chosen as the optimum transport medium for macrofoam swabs.

When all data for rayon swabs (both strains, dirty and clean, all time points) were combined and analyzed, two combinations of pre-moistening agent/transport medium, PBSTX/PBSTX and NB/PBSTX , stood out as significantly better than the other two. Rayon swab recoveries were higher when clean than when dirty with these two optimum combinations ($p \leq 0.001$). The combination of PBSTX/PBSTX was optimum for the YP A1122 and NB/PBSTX worked best for the YP CO92 strain.

Collection of cells from surfaces was not evaluated in this study. The choice of premoistening agent may affect the collection efficiency of cells from the surface.

It should be noted that in phase III and IV, the swabs were stored and processed in the given transport medium, so the differences in preferred transport medium for each swab may reflect the differences in the need for the surfactant to release the cells during

processing. Each swab has unique electrochemical properties that interact with the cells and influence adherence [9]. This project did not address these properties.

5.0 References

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6.0 Appendix: Study Matrix Tables

1. Preliminary Study: Survival of *Y. pestis* in liquid transport media

Table 1. Preliminary study matrix: Transport media, temperature, holding times evaluated

Transport media	**Temperature (°C)**	**Holding time (h)**
Stuart, Toscach and Patsula	4, 25, 35	0, 12, 18, 24, 48, 72
Cary and Blair	4, 25, 35	0, 12, 18, 24, 48, 72
Amies without charcoal	4, 25, 35	0, 12, 18, 24, 48, 72
Amies with charcoal	4, 25, 35	0, 12, 18, 24, 48, 72
PBS +0.05% Triton X-100	4, 25, 35	0, 12, 18, 24, 48, 72
Neutralizing buffer	4, 25, 35	0, 12, 18, 24, 48, 72

2. Phase I: Evaluation of swab extraction methods

Table 2. Phase I study matrix: Swab material, premoistening agent and extraction methods evaluated. All extractions performed with *Y. pestis* A1122 and all processing performed in PBS + 0.02% Tween 80. N= 10 per extraction method

Swab material	Premoistening agent	Extraction method [a]
Rayon	Triton X -100	V, S, VS, N
Polyester	Triton X -100	V, S, VS, N
Macrofoam	Triton X -100	V, S, VS, N
Cotton	Triton X -100	V, S, VS, N
Rayon	Neutralizing buffer	V, S, VS, N
Polyester	Neutralizing buffer	V, S, VS, N
Macrofoam	Neutralizing buffer	V, S, VS, N
Cotton	Neutralizing buffer	V, S, VS, N

[a] V=vortexing 3 min, S = sonicating 3 min , VS = vortexing and sonicating 30 seconds each, repeated three times for a total of 3 min, N=no extraction method.

3. Phase II: Evaluation of sample storage parameters for *Y. pestis* A1122 on sterile swabs

Table 3. Phase II study matrix; YP strain, swab material, premoistening agent, transport media and holding times evaluated. All swabs were held at 4°C (found to be optimum temperature in preliminary study).

Organism	Swab material[a]	Premoistening agent	Transport media[b]	Holding Time (h)[c]
Phase II YP A1122	macrofoam	Triton X-100	PBSTX	0, 12, 18, 24, 48, 72
	macrofoam	Triton X-100	$C\&B_{mod}$	0, 12, 18, 24, 48, 72
	macrofoam	Neutralizing buffer	PBSTX	0, 12, 18, 24, 48, 72
	macrofoam	Neutralizing buffer	$C\&B_{mod}$	0, 12, 18, 24, 48, 72
	rayon	Triton X-100	PBSTX	0, 12, 18, 24, 48, 72
	rayon	Triton X-100	$C\&B_{mod}$	0, 12, 18, 24, 48, 72
	rayon	Neutralizing buffer	PBSTX	0, 12, 18, 24, 48, 72
	rayon	Neutralizing buffer	$C\&B_{mod}$	0, 12, 18, 24, 48, 72

[a] macrofoam swabs processed by vortexing 3 min, rayon swabs processed by sonicating 3 min (optimum conditions determined for each swab type in phase I).
[b] optimum transport media determined in preliminary study
[c] 10 swabs per holding time

4. Phase III: Evaluation of sample storage parameters for *Y. pestis* CO92 on sterile swabs

Table 4. Phase III study matrix: YP strain, swab material, premoistening agent, transport media and holding times evaluated. All swabs were held at 4°C.

Organism	Swab material[a]	Premoistening agent	Transport media	Holding Time (h)[b]
Phase III YP CO92	macrofoam	Triton X-100	PBSTX	0, 12, 18, 24, 48, 72
	macrofoam	Triton X-100	$C\&B_{mod}$	0, 12, 18, 24, 48, 72
	macrofoam	Neutralizing buffer	PBSTX	0, 12, 18, 24, 48, 72
	macrofoam	Neutralizing buffer	$C\&B_{mod}$	0, 12, 18, 24, 48, 72
	rayon	Triton X-100	PBSTX	0, 12, 24, 48, 72
	rayon	Triton X-100	$C\&B_{mod}$	0, 12, 24, 48, 72
	rayon	Neutralizing buffer	PBSTX	0, 12, 24, 48, 72
	rayon	Neutralizing buffer	$C\&B_{mod}$	0, 12, 24, 48, 72

[a] macrofoam swabs processed by vortexing 3 min, rayon swabs processed by sonicating 3 min (optimum conditions found for each swab type in phase I).
[b] 10 swabs per holding time, the 18 h holding time dropped after macrofoam evaluations complete.

5. Phase IV: Evaluation of sample storage parameters for both *Y. pestis* strains on "dirty" swabs

Table 5. Phase IV study matrix: YP A1122, swab material, premoistening agent, transport media and holding times evaluated. All swabs were held at 4°C. Arizona Test Dust was added to the swabs to simulate background dust and organisms prior to inoculation with YP.

Organism	Swab material	Premoistening agent	Transport media	Holding Time (h)[a]
Phase IV YP A1122	macrofoam	Triton X-100	PBSTX	0, 12, 24, 48, 72
	macrofoam	Triton X-100	NB	0, 12, 24, 48, 72
	macrofoam	Neutralizing buffer	PBSTX	0, 12, 24, 48, 72
	macrofoam	Neutralizing buffer	NB	0, 12, 24, 48, 72
	rayon	Triton X-100	PBSTX	0, 12, 24, 48, 72
	rayon	Triton X-100	NB	0, 12, 24, 48, 72
	rayon	Neutralizing buffer	PBSTX	0, 12, 24, 48, 72
	rayon	Neutralizing buffer	NB	0, 12, 24, 48, 72

[a] 10 swabs per holding time

Table 6. Phase IV study matrix: YP CO92, swab material, premoistening agent, transport media and holding times evaluated. All swabs were held at 4°C. Arizona Test Dust was added to the swabs to simulate background dust and organisms prior to inoculation with YP.

Organism	Swab material	Premoistening agent	Transport media	Holding Time (h)[a]
Phase IV YP CO92	macrofoam	Triton X-100	PBSTX	0, 12, 24, 48, 72
	macrofoam	Triton X-100	NB	0, 12, 24, 48, 72
	macrofoam	Neutralizing buffer	PBSTX	0, 12, 24, 48, 72
	macrofoam	Neutralizing buffer	NB	0, 12,24, 48, 72
	rayon	Triton X-100	PBSTX	0, 12, 24, 48, 72
	rayon	Triton X-100	NB	0, 12, 24, 48, 72
	rayon	Neutralizing buffer	PBSTX	0, 12, 24, 48, 72
	rayon	Neutralizing buffer	NB	0, 12, 24, 48, 72

[a] 10 swabs per holding time

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