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ORIGINAL ARTICLE

Validating an Autoclave Cycle for Sterilization of Select Agent-Infected Murine Carcasses

J. Patrick Condreay, Kristina Riebe, Rose Asrican, and Scott Alderman*

Abstract

Introduction: Infectious disease research conducted in the Duke Regional Biocontainment Laboratory, a biosafety level-3 facility, often includes work with mice. Infected carcasses must be properly decontaminated at the completion of each study and prior to removal from the facility. Working with Select Agents requires validated methods for complete inactivation of the agent before carcasses can be removed from registered spaces.

Methods: Biological indicators were implanted into the abdomen of unfrozen mouse carcasses, then autoclaved using different cycle parameters and packaging strategies to determine the best approach to ensure complete sterilization.

Results: Both 20-min and 30-min autoclave cycles at 122°C, with three pre-vacuum pulses, failed to consistently sterilize unfrozen mouse carcasses. A 45-min autoclave cycle with the same parameters did result in the successful sterilization of carcasses when packaged at two or four carcasses in an 8″ × 12″ autoclave bag.

Conclusions: This study supports the utilization of an autoclave cycle (122°C sterilization temperature, 45-min sterilization time, three pre-vacuum pulses) to sterilize unfrozen mouse carcasses, up to four per bag.

Keywords: animal biosafety, biocontainment, decontamination, sterilization, BSL-3

Introduction

The Regional Biocontainment Laboratory (RBL) at Duke University was built with funding from the National Institute of Allergy and Infectious Disease to support basic and translational research to develop drugs, diagnostics, and vaccines for emerging and reemerging infections and biodefense. The facility, which includes ∼6,525 square feet of biosafety level-3 (BSL-3) space, received its certificate of occupancy in December of 2006 and final BSL-3 verification in October of 2007. Current research activities include work with multiple Risk Group 3 pathogens, some of which are subject to regulation under the Federal Select Agent Program.

Murine challenge models are routinely utilized for pathogen, vaccine, and therapeutic studies and make up a significant component of the RBL's research portfolio. Final disposal of infectious animal carcasses is through either incineration or tissue digestion by alkaline hydrolysis; however, this requires transport of the material to another facility outside of the RBL containment barrier. To accomplish this the carcasses are autoclaved to render them safe for handling and transfer. However, for experiments involving Select Agents, it is necessary to ensure that the infectious agent is fully inactivated before removing the material from a registered space.

Steam sterilization in an autoclave is a commonly accepted, effective technology for inactivation of infectious agents.[1] For the purposes of Select Agent inactivation efficacy, the exact conditions used must be validated with viability data generated by the institution.[2] Sterilization is generally expressed as the probability of viable organisms surviving a process, and a commonly accepted measure is a probability of less than one surviving

Regional Biocontainment Laboratory, Duke University, Durham, North Carolina, USA.

*Address correspondence to: *Scott Alderman, Duke University, Regional Biocontainment Laboratory, 909 South LaSalle Street, Durham, NC 27710, USA,* Email: scott.alderman@duke.edu

organism in a population of 10^6 organisms. For validation of sterilization processes biological indicators (BIs) containing 10^6 units of an organism that is particularly resistant to the sterilization method are employed. In the case of steam sterilization, the BIs utilized are generally glass vials of 10^6 *Geobacillus stearothemophilus* spores in a growth medium, often with a chemical indicator to detect germination and growth of the spores after the autoclave cycle has been run.[1] An advantage of the vials is the ability to embed them directly into the matrix that one is testing for sterilization and then retrieve them for incubation to determine if the spores have been killed.

Previous studies[3,4] have documented that the composition of animal carcasses often makes it more challenging to achieve proper decontamination while using standard autoclave cycle parameters. In this study we describe a series of experiments with the aim of determining the necessary procedures and cycle parameters to sterilize mouse carcasses used in the RBL. To remove one potential variable in the process, the decision was made to limit our experiments to sterilization of carcasses that were at room temperature; animals were not refrigerated or frozen prior to autoclave treatment. The design of each experiment was based on observations and knowledge gained from the previous runs to devise a validated process that ensures inactivation of Select Agent-infected carcasses before removal from registered spaces.

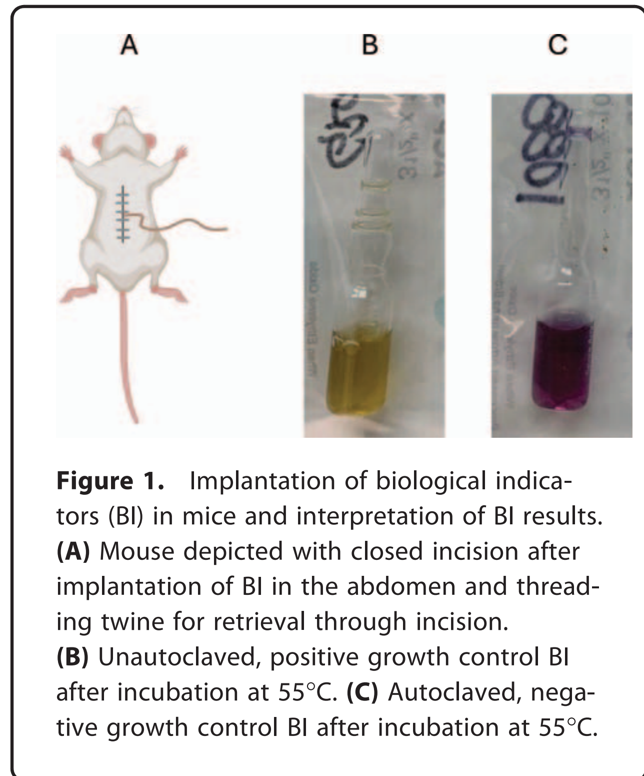

Figure 1. Implantation of biological indicators (BI) in mice and interpretation of BI results. **(A)** Mouse depicted with closed incision after implantation of BI in the abdomen and threading twine for retrieval through incision. **(B)** Unautoclaved, positive growth control BI after incubation at 55°C. **(C)** Autoclaved, negative growth control BI after incubation at 55°C.

Methods

Mouse Carcasses

Naïve BALB/c and C57BL/6 mouse carcasses were obtained after use and euthanasia for purposes directly related to their respective Institutional Animal Care and Use Committee (IACUC) protocols, and therefore no IACUC approval was required for this post-mortem study. Carcasses were ∼20–30 grams each.

Autoclaves and Biological Indicators

Two steam sterilizing autoclaves (model PSS5-B-MSDD, Primus Sterilizer, Omaha, NE) were used for this study. Each unit is currently housed in the RBL's BSL-3 animal suite. The settings for each autoclave cycle used for this study included the following: three pre-vacuum pulses (–5 in. Hg, –10 in. Hg, and –25 in. Hg), a sterilization temperature of 122°C, and two post-vacuum cycles (–25 in. Hg each). The sterilization time was the only cycle parameter that was modified between experiments.

CROSSTEX 1 mL Spore Ampule Biological Indicators (CROSSTEX, Rush, NY), *Geobacillus stearothermophilus*, 10^6 spores/vial, were used for the study. Each BI was labeled, tied to Butcher's twine, and then implanted through a 1-inch incision in the lower abdomen of the carcass. The incision was then closed using surgical staples, ensuring the twine was threaded through the incision site before closing (Figure 1A). Following each autoclave cycle, the twine was used to retrieve the BIs. Vials were placed in individual, pre-labeled envelopes, then placed in an incubator at 55°C and monitored daily for 7 days. The presence of positive growth was identified by turbidity and a color transition from purple to yellow. Conversely, the absence of turbidity and the retention of a purple color signified negative growth, indicating a successful cycle. Positive (Figure 1B) and negative (Figure 1C) growth controls were labeled, incubated, and interpreted for each operational run.

Packaging and Placement of Carcasses

Animal carcasses were placed within biohazard bags designed for autoclaving, with a standard configuration of either one, two, or four carcasses per bag. We ensured good steam contact with the carcasses by loosely closing the autoclave bag and by including the pre-vacuum pulses mentioned above in each cycle. To ensure a uniform, loosely closed bag, we placed a 15 mL conical tube in the top of the bag opening, gathered the neck of

the bag around the tube, and then taped the bag around the tube (Figure 2A). The tube was then removed, leaving a defined-sized opening in the bag.

Subsequently, bags were positioned within a polypropylene autoclavable tray before being placed into the sterilization chamber. The size of each autoclave's chamber allowed the placement of two trays per run, one oriented toward the front of the unit and the other toward the back (Figure 2B). In specific experiments, deliberate efforts were made to arrange carcasses laid out flat in a single layer within the bag or bunched together in a stack to determine whether orientation within the bag contributes to the effectiveness of the autoclave cycle.

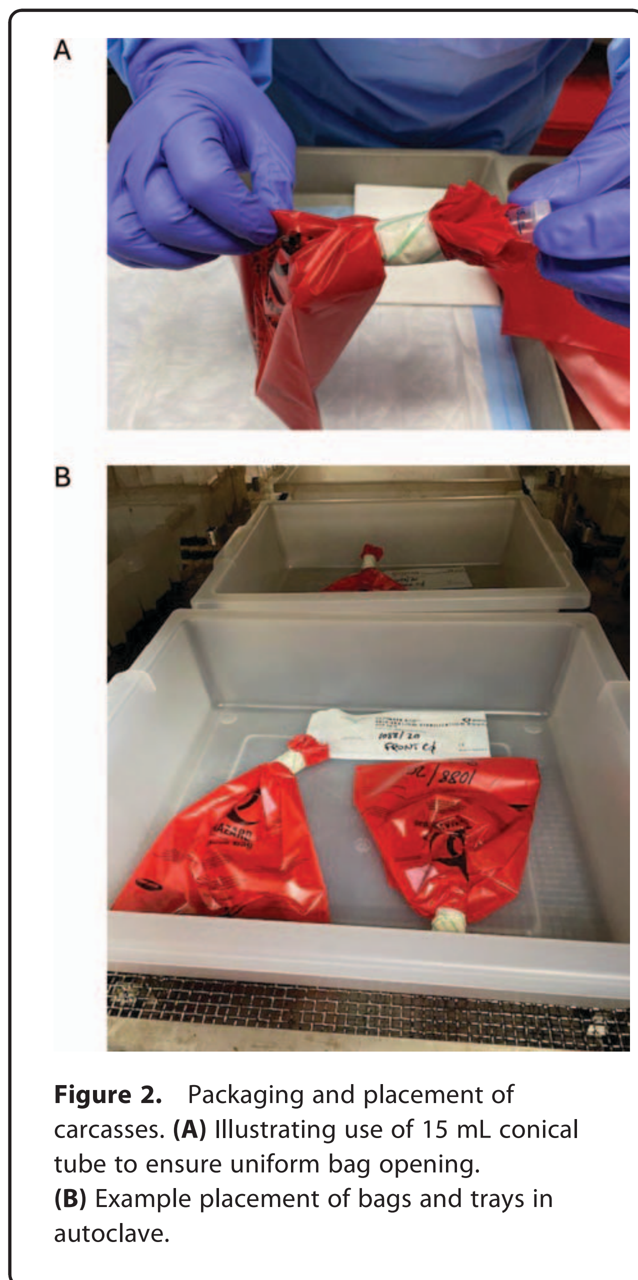

Figure 2. Packaging and placement of carcasses. **(A)** Illustrating use of 15 mL conical tube to ensure uniform bag opening. **(B)** Example placement of bags and trays in autoclave.

Experimental Parameters

Table 1 is a summary of the experiments that were performed, demonstrating the different parameters that were explored in this study. Each experiment was performed in both autoclaves present in the animal suites of the RBL. Furthermore, each autoclave was loaded with two identical trays (front and back of chamber).

Results

Time Range-Finding

The outcomes of Experiments 1–4 were sequentially utilized to progressively establish suitable sterilization time and carcass packaging parameters (see Table 1). Representative BI vials are from Experiment 1 are shown in Figure 3. Control vials demonstrate that a sterilization time as short as 20 min is sufficient to fully inactivate $1x10^6$ *Geobacillus stearothermophilus* spores (compare autoclaved vials [Figure 3B] with the unautoclaved control [Figure 3A]). The option of employing a 20-min cycle for sterilizing mouse carcasses was dismissed from further consideration, as BIs from two carcasses showed growth upon incubation (Figure 3C). However, both 30-min and 60-min autoclave cycles resulted in inactivated BIs in all animals; thus, a 30-min cycle was selected for further exploration.

Testing with 30-min Sterilization Time

Experiment 2 was designed to determine whether the number of carcasses per bag could be expanded to four in a 30-min cycle. Two carcasses that were included in a bag of four were unsuccessfully sterilized, which led us to consider whether the carcasses had stacked upon one another and inhibited adequate steam contact during the cycle. Therefore, in experiment 3, carcasses were deliberately laid out flat to prevent stacking within the bag. All 24 mouse carcasses in this experiment were successfully sterilized using the 30-min cycle. Experiment 4 aimed to investigate the impact of stacking or bunching carcasses within a bag on the sterilization process; results are shown in Table 2. Only 19% (3/16) of bunched carcasses were sterilized successfully, and surprisingly, only 54% (13/24) of single-layered carcasses were sterilized. These findings led us to determine that a 30-min cycle at 122°C would not be adequate to guarantee inactivation of infectious agent in the carcasses.

Testing with 45-min Sterilization Time

Experiments 5 and 6 repeated the packaging and layering of carcasses used in the previous experiment, but the sterilization time was extended to 45 min. The combined results of these experiments are shown in Table 3. The 45-min cycle time resulted in successful sterilization of

Table 1. Summary of tested parameters

Experiment	Sterilization time (min)	Total number of carcasses			Deliberate layering of carcasses
		1/bag	2/bag	4/bag	
1	20	2	8		None
	30	2	8		None
	60	2	8		None
2	30		8	16	None
3	30		8	16	Single layer
4	30		8	16	Single layer
	30			16	Bunched
5	45		4	8	Single layer
	45			8	Bunched
6	45		12	24	Single layer
	45			24	Bunched

all carcasses over four independent autoclave runs, regardless of whether the carcasses were layered on top of each other or not.

Discussion

Proper management of biohazardous waste, including mouse carcasses used in infectious disease research, is a key component of a biosafety program at any high-containment (i.e., BSL-3) research facility. The method (i.e., autoclaving) for decontamination of waste should be validated and compliant with institutional, local, state, and federal requirements.[5] Given that animal research involving select agents is often performed in the RBL, it was essential to establish a packaging process and autoclave cycle that would ensure complete sterilization of infected murine carcasses prior to terminal disposal.[6] Based on our study, our internal standard operating procedure for autoclaving mouse carcasses requires the following autoclave cycle parameters: 122°C sterilization temperature, three pre-vacuum pulses (−5 in. Hg, −10 in. Hg, and −25 in. Hg), and 45-min sterilization time. In addition, no more than four unfrozen mouse carcasses are placed in a single bag, and each bag is loosely closed by a defined procedure to allow steam penetration.

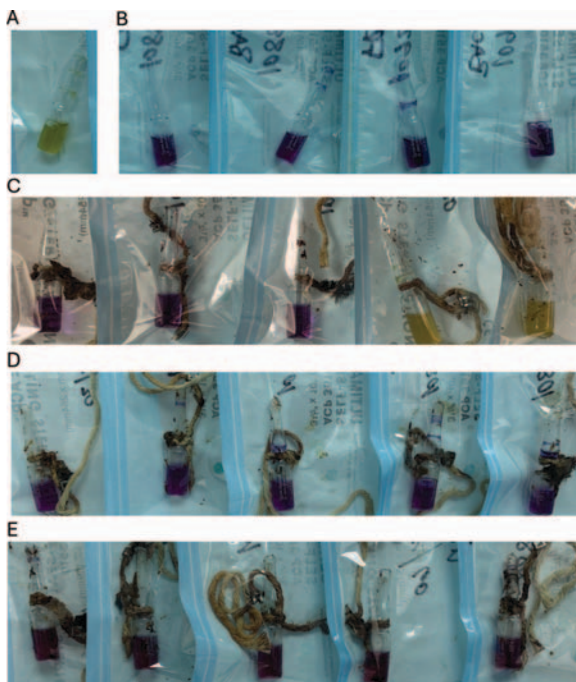

Figure 3. BIs from sterilization time range finding experiment. **(A)** Unautoclaved control BI vial. **(B)** Control vials from 20-min sterilization time cycles. **(C–E)** Vials from individual mice sterilized for 20 **(C)**, 30 **(D)**, and 60 **(E)** minutes. All vials were photographed after incubation at 55°C for 7 days.

In planning these experiments, we considered several factors that could affect the efficacy of sterilization of the carcasses and sought to either control these factors or explore their effect on the results. The temperature of the carcasses was of concern. Baldauf et al.[7] used temperature probes embedded into frozen mouse carcasses and found that significant time (36–42 min) was required in the autoclave before the carcasses reached sterilization temperature. Given our findings that 45 min of sterilization time is required for reliable sterilization of carcasses, this would require a cycle time of at least 90 min if we started with frozen material. Lindinger et al.[8] also noted that frozen carcasses were problematic, requiring extended cycle

Table 2. Results from experiment 4 (30-min sterilization time)

Number of carcasses per bag	Layering	Number of sterilized animals/total number
2	Single layer	5/8 (63%)
4	Single layer	8/16 (50%)
4	Bunched	3/16 (19%)

Table 3. Results with 45-minute sterilization time[a]

Number of carcasses per bag	*Layering*	*Number of sterilized carcasses/total number*
2	Single layer	16/16 (100%)
4	Single layer	32/32 (100%)
4	Bunched	32/32 (100%)

[a]Total number of carcasses from four independent autoclave runs.

times, and thus chose to use thawed carcasses that were at 2–8°C. To reduce the possibility of variability in our experiments, we chose to use carcasses at room temperature that had not been refrigerated or frozen to establish our standardized procedure. Requiring carcasses to reach room temperature before autoclaving introduces additional planning and coordination for laboratory staff. Infected carcasses may need to be processed immediately after daily animal work concludes, while those stored in refrigerators or freezers must be thawed and equilibrated to room temperature prior to loading. These added steps can increase workload and disrupt normal workflows; however, they are essential to ensure complete inactivation when using the autoclave parameters described in this study.

Another aspect related to the efficacy of sterilization is the packaging of carcasses to ensure steam contact with the carcasses.[7,9] Our standard waste autoclave cycles all contain the three pre-vacuum pulses used in this study; however, steam penetration into bagged waste needs to be assured. For this reason, we felt that it was important to establish the simple procedure for bag closure outlined in our study to facilitate steam entry.

Adherence to validated inactivation protocols is crucial to instill confidence in their reproducibility. To achieve adherence to safety protocols, it is important to solicit input from laboratory workers to ensure that the protocols are relevant and practical for them to implement.[10] We first focused on a 30-min sterilization time for our protocol since this matched our standard protocol for decontamination of carcasses infected with non-Select Agent organisms. When this time proved to not be reproducible as we increased the number of carcasses, we considered increasing the sterilization time to 60 min since that had been effective from our range-finding experiment (Figure 3). Input from workers caused us to focus on 45 min instead. This is the sterilization time used in our normal lab waste cycle and would be more practical for their workflow and help ensure that mistakes are not made. Inclusion of bags of layered carcasses in our experiments was done because we considered that this might be a common error that could lead to failure to sterilize material, thus, we sought to mitigate that risk.

An additional consideration is the type of tray used to contain carcasses during autoclaving. In our study, carcasses were placed in polypropylene trays, which are relatively poor conductors of heat but cool rapidly after the cycle, reducing the risk of burns to personnel. Stainless steel trays, by contrast, conduct heat much more efficiently, which can result in shorter heat-up times and more uniform temperature dispersal. While these differences in thermal conductivity could theoretically influence the exposure times required for complete inactivation, the use of polypropylene trays in our work represents a conservative condition. Accordingly, we believe the autoclave parameters validated in this study are broadly applicable across animal facilities, including those that use stainless steel trays.

Conclusions

Animal carcasses differ in composition from other types of solid waste, so specific procedures should be developed and verified if the intent is to fully sterilize the material. This study supports the utilization of an autoclave cycle (122°C sterilization temperature, 45-min sterilization time, three pre-vacuum pulses) to sterilize unfrozen mouse carcasses.

Authors' Note

All authors contributed equally to this work.

Statement of Human and Animal Rights

No live animal or human subjects were involved in this study.

Authors' Disclosure Statement

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