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Novel Approach for Validating Autoclave Cycles for Biomass in BSL-3/-4

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Abstract

The National Biodefense Analysis and Countermeasures Center (NBACC) conducts research at Animal Biosafety Level 3 (ABSL-3) and ABSL-4. At NBACC, all solid waste in ABSL-3 and ABSL-4 must be sterilized by a validated method before it leaves the containment suites for disposal. Carcass biomass differs across laboratory animal species and its composition differs compared to solid disposable laboratory waste and reusable materials like scrub suits. Successfully sterilizing carcasses requires cycle parameters that differ from those used to sterilize other forms of solid waste and smaller animal species. Surgical placement of the biological indicators (BI) was employed to ensure steam penetrated the carcasses completely. In this article the authors describe a process for validating autoclave cycles using readily available animal carcasses that mimic the biomass of the non-human primate and guinea pig carcasses planned for use in future studies, to accurately develop a cycle that takes into account weight, mass, and muscle depth as well as the moisture content inherent in carcasses. The results demonstrate the extended time duration required for the sterilization phase of the cycle and the effect of increased carcass mass on the ability to validate autoclave cycles.

Keywords

Autoclave Cycle, Validation, Non-human Primates, Biomass, BSL-3, and BSL-4

Introduction

The National Biodefense Analysis and Countermeasures Center (NBACC) conducts research on highly pathogenic organisms to better understand and prepare for current and future biological threats. In some cases the best or only animal model that mimics the effects of infection with a pathogen and the response to treatment seen in humans is the non-human primate (NHP). All studies involving animals undergo a risk assessment analysis by the Health and Safety Office and are then submitted for review by the Institutional Animal Care and Use Committee (IACUC). Any study must be approved by both groups prior to commencement of the work.

Work with highly pathogenic material in animals is conducted in either ABSL-3 or ABSL-4 based on the risk assessment specific to the project. Staff must demonstrate proficiency not only for standard procedures in containment but also for project-specific processes and those related to animal care and use. In ABSL-3, safety enhancements in the form of personal protective equipment with increased protection factors (i.e., use of a powered air purifying respirator in place of an N100 respirator), immunizations, engineering controls (containment cages), and other measures are employed to further reduce risk to staff and the laboratory environment.

One responsibility of the Health and Safety Office involves validating waste decontamination and sterilization

methods. While this article does not involve the use of NHP, it does describe the methods used to validate autoclave cycles used in sterilizing NHP carcasses to ensure no pathogens survive before carcasses are transported offsite for incineration.

National and international guidelines identify the use of an autoclave as a suitable means for sterilization of potentially infectious material, and it is required equipment for work in high-containment (U.S. HHS, 2009; PHAC/CFIA, 2013; WHO, 2014.). Manufacturers may provide recommendations that serve as a starting point for validating autoclave cycles for liquids, utensils, and dry goods (CSS, 2014), but information regarding sterilization cycles for carcasses of NHP or other animal species is not easily found in the literature or in manufacturers' recommendations. Challenges to the steam penetration needed for sterilization of NHP carcasses include not only greater size and weight (weight range is approximately 2.5-8.3 kg (5.5-18.3 lb) depending on age and sex), but also the solid muscle mass in the chest and larger organ bulk as compared with small laboratory species (i.e., mice, rats, guinea pigs) (Cawthon-Lang, 2006). This article describes the validation of autoclave cycles that considers the biomass of the carcasses of one or more NHPs and small laboratory animal species.

For this project, turkeys were chosen to represent NHP carcasses (*Cynomolgus macaques*) as the proportionately heavy muscle mass found in the breast is similar to that of the largest animals anticipated to be used in research studies (5-7 kg (11-15.4 lb). Cornish game hens were chosen as surrogates for guinea pigs based on the similar weight and length of the body; with regard to weight, these could also serve as surrogates for a group of mice or hamsters. Not surprisingly, a comprehensive review of the literature failed to find papers that directly compare data regarding similarities and differences between macaque and turkey, and guinea pig and Cornish hen average body and organ mass. In consultation with the experienced NBACC veterinary staff familiar with animal models, it was decided these surrogates provide a valid substitute that models the difficulty in achieving sterilization for the species planned to be used in these research studies. Turkeys and Cornish hens were autoclaved together as several experimental protocols anticipated using both species in side-by-side comparative studies.

Materials and Methods

Two representative autoclave models were validated: Getinge GEB91422 and Getinge GEB6915 (Getinge, Rochester, NY) (Table 1).

For each cycle, two fresh, unfrozen turkeys (average 4.5-5.4 kg/12-14 lb) and one Cornish hen (0.5-0.9 kg/1-2 lb) were used for each model autoclave being validated. Unfrozen carcasses were used as they are representative of containment operations at NBACC where carcasses are refrigerated (not frozen) after animals are humanely euthanized. The Health and Safety Office, in consultation with the attending veterinarian, decided that should the need arise to freeze carcasses inside containment, they would be thawed to 4ºC ± 2ºC prior to autoclaving. Carcass temperatures were validated to be 4ºC ± 2ºC by inserting a temperature probe into the deepest part of the muscle in the breast. Carcass weights were recorded prior to the placement of the biological indicators (BI).

Two BIs per bird (MagnaAmps, SGM Biotech, Bozeman, MT) were placed in each carcass and sutured in place, one next to the breast bone and one in between the breast bone and the breast meat. The indicators were placed at a depth of approximately 4-6 inches for the turkey and 0.5-1.0 inch for the Cornish hen. A veterinary technician sutured the BIs in place to prevent them from dislodging during the cycle and placed other stitches required to return the bird to its normal anatomic position. The neck, heart, kidneys, and other "giblets" were placed back in the carcasses and the skin of the carcass was sutured closed. One negative control was placed inside the bird to account for possible media discoloration in the BIs. Exposure to extended heat has been proven to "caramelize" the media and affect the pH indicator (Nyberg, 2014). Therefore, the negative control should undergo the same thermal insult as the test indicators to accurately compare the color result. Media that have been caramelized have been proven to still support microbial growth and provide a useful sterilization test. A positive growth control BI was used during the incubation phase for each autoclave validation test.

The temperature of the carcass was verified after placement of the BIs as described above just prior to placing it into the autoclave. If the carcass was not between 4ºC ± 2ºC, it was placed back in the refrigerator until the correct temperature was attained. Each carcass was placed in an autoclave bag, with the top loosely folded and taped, then placed in a second autoclave bag which was also loosely folded and taped. The bag was then placed inside a leak-proof container—in this case a cardboard "hatbox" (5 gallon size, Greif Bros. Corporation, Delaware, OH) per NBACC SOP 144-009-SOP, Disposition of Animal Carcasses. The hatbox was placed within an autoclave bag and each was placed in a separate, open, leak-proof autoclave container (Nalgene, model 6900-0020, Rochester, New

Table 1

Autoclave cycle parameters tested.

	Sterilizing Temperature	Absolute Pressure	Sterilizing Time	Cooling temperature
ABSL-3 Cycle	121ºC	31.5 psi	2.00 Hours	95.0ºC
ABSL-4 Cycle	121ºC	31.5 psi	4.00 Hours	95.0ºC

York). A temperature probe was inserted into a Nalgene container filled with 5.9 kg (13 lb) of water to simulate that carcasses consist mostly of water. The probe in the Nalgene container over-rode the autoclave chamber probe for determining when 121ºC was reliably maintained, thus triggering the start of the ABSL-3 and ABSL-4 "kill" or sterilization portion of the cycle. The sterilization cycle tested at ABSL-3 was 121ºC with 31.5 pounds per square inch (PSI) chamber pressure for 2 hours, and for ABSL-4, 121ºC with 31.5 PSI chamber pressure for 4 hours. Completion of the autoclave cycle was verified by a review of the cycle parameters via equipment printout, and the carcasses were removed as soon as safely possible, allowing time for the autoclave to cool before reaching inside to remove the box containing the carcasses. Test BIs along with a positive-growth control were incubated for 48 hours at 60ºC, per manufacturer's recommendation.

Results

The first validation tests used turkey carcasses that on average weighed 7.5 kg (16.41 lb), which was an approximate 24% increase in weight over the average of 5.7 kg (12.53 lb) carcasses used for subsequent tests. Data in Table 2 demonstrate the failure to sterilize the heavier turkey carcasses tested on April 9, 2011 and April 16, 2011, though the Cornish hen carcasses passed. During the repeat of the ABSL-3 autoclave validation on May 8, 2013 and May 21, 2013, the same autoclave parameters were used with lighter turkey carcasses and all BIs retrieved from the carcasses where killed. Subsequent validation tests of the ABSL-4 autoclaves operating with an increased sterilization time of 4 hours resulted in sterilization of all carcass BIs. All positive control BIs grew (data not shown).

Use of the Nalgene container filled with 5.9 kg (13 lb) of water (to simulate carcass composition) as the trigger for initiating the sterilization cycle resulted in a significantly extended "heat-up" cycle, or time to reach 121ºC as shown in Table 3. This increase in overall cycle time contributes to an operational cost beyond that associated with utility and maintenance costs, and represents a significant amount of time the autoclave cannot be used for other purposes.

Discussion

As part of its health and safety program, NBACC budgeted time and funding to validate autoclave cycles during the facility endurance testing process (Colella, 2013) prior to commencement of work with select agents. This was required from a safety perspective, but it also provided the added benefit of not competing for autoclave resources once the laboratory became active. A project of this scope would be significantly more challenging to complete during normal laboratory operations.

Selection and Placement of BIs

In consultation with veterinary staff, the authors determined that the temperature achieved by placing BIs between the breastbone and breast meat and next to the breastbone would represent a sterilization challenge and would reveal whether steam penetration was adequately achieved. The size of these turkeys allowed both placement of the BIs at a depth of 3-5 inches from the surface and placement between muscle and bone. The 3-5 inches approximate the center of mass for a very large NHP, probably much larger than would be used in an actual study, rep-

Table 2

Results of autoclave validation tests.

Cycle	Model #	Date	Pass/Fail	Sterilization Cycle Time	Cycle #	Turkey #1 Weight (lbs/kg)	Turkey #2 Weight (lbs/kg)	Cornish Hen Weight (lbs/kg)	Total Biomass Tested (lbs/kg)
ABSL-3	GEB 91422	9-Apr-11	Turkey Fail/ Hen Pass	2 hours	1	16.08/ 7.3	16.73/ 7.6	1.99/ 0.9	34.80/ 15.8
ABSL-3	GEB 91422	16-Apr-11	Turkey Fail/ Hen Pass	2 hours	2	16.08/ 7.7	16.73/ 7.6	2.10/ 0.95	34.91/ 15.8
ABSL-4	GEB 91422	18-Jul-11	All Pass	4 hours	1	13.10/ 5.9	13.15/ 6.0	1.58/ 0.7	27.83/ 12.6
ABSL-4	GEB 91422	16-Jul-11	All Pass	4 hours	2	12.44/ 5.6	13.63/ 6.2	1.57/ 0.7	27.64/ 12.5
ABSL-3	GEB 91422	8-May-13	All Pass	2 hours	3	12.46/ 5.7	12.01/ 5.4	1.60/ 0.7	26.07/ 11.8
ABSL-3	GEB 91422	21-May-13	All Pass	2 hours	4	12.66/ 5.7	12.16/ 5.5	1.51/ 0.7	26.33/ 11.9
ABSL-4	GEB 6915	17-Sep-13	All Pass	4 hours	1	12.41/ 5.6	12.41/ 5.6	1.34/ 0.6	26.16/ 11.9
ABSL-4	GEB 6915	18-Sep-13	All Pass	4 hours	2	12.57/ 5.7	11.3/ 5.1	1.41/ 0.6	25.28/ 11.5

Table 3
Comparison of total cycle times for carcasses and laundry in ABSL-3 and ABSL-4.

Cycle Type	Average Time to Reach 121ºC	Sterilization Time 121ºC	Total Cycle Time
ABSL-3 Carcass	5.0 hr	2.0 hr	8 hr 20 min
ABSL-3 Laundry	25 min	1.5 hr	2 hr
ABSL-4 Carcass	6.0 hr	4.0 hr	11 hr 30 min
ABSL-4 Laundry	40 min	4.0 hr	5 hr

resenting a worst-case scenario in challenging the sterilizer. MagnaAmps (SGM Biotech, Bozeman, MT) were chosen as the indicator because a "completely sealed" ampoule system was required for this process. The 3M Attest BI (3M, St. Paul, MN) was originally tested, but animal body fluids and fats leached into the indicator, obscuring any color change. Like the 3M Attest indicators, the MagnaAmp indicators also had over a 10^6 population of *G. stearothermophilus*. A complete kill of these indicators is required to validate sterilization.

Selection of Carcasses and Sterilization Cycles

As no peer-reviewed guidance describing the cycle parameters for autoclaving solid biomass existed, the parameters chosen for each cycle were based on the past experience of the NBACC staff who had worked with macaques, guinea pigs, hamsters, and mice. Throughout testing the objective was to minimize variation across carcass sizes by selectively choosing identical-weight carcasses. This turned out to be more difficult than initially thought, especially throughout different times in the year. During procurement of the turkey carcasses for the first tests, the only turkeys available had a significantly larger biomass than the largest macaque size estimates of 5-7 kg (11-15 lb) per animal. The authors decided to commence with the validation process although this represented stressing the autoclave cycle beyond a worst-case scenario. The first two autoclave tests failed as demonstrated by the growth of the test BIs in the turkeys. This raised the question as to whether the autoclave cycle was too short, the unit itself was defective, or the increased biomass was the cause of the failure. For the next validation, the weight of the available turkeys was lower than during the first validation attempt. The 4-hour sterilization cycle time was successful in killing the BIs, but as two variables had changed, the authors did not know whether the weight difference, sterilization time difference, or a combination of the two played a role in the success of the cycle validation. Unfortunately, before a retest of the ABSL-3 autoclave with a smaller-sized carcass could be undertaken, the facility entered phases of test and balance and commissioning that mandated suspension of the autoclave validations.

When autoclave validation recommenced, the average turkey sizes were still smaller in weight than those originally procured in 2011. The authors achieved repeated success with the smaller biomass using the 2-hour sterilization cycle for ABSL-3 and the 4-hour sterilization cycle for ABSL-4. The conclusion was that the problem in the initial ABSL-3 tests was the biomass autoclaved was too great to achieve sterilization. NBACC currently processes laundry out of the ABSL-3 and ABSL-4 containment suites with 1.5-hour and 4-hour sterilization cycles at 31.5 PSI chamber pressure and 121ºC (controlled by the chamber temperature sensor), respectively. Note that the difference in total cycle times between carcasses and laundry bags is considerable as the time it takes to heat the water in the Nalgene container adds approximately 4.5 hours to the ABSL-3 carcass cycle and 6.0 hours to the ABSL-4 cycle as compared to the laundry bag cycle times to reach temperature. Laundry bags in excess of 11.3 kg (25 lb) per bag have been validated and pose no problem in achieving sterilization, while the results of this study show that using the same cycle parameters for dense animal biomass over 11.3 kg (25 lb) would result in incomplete sterilization.

One must also consider that a load probe in a Nalgene container of water is not identical to an animal carcass with fur and muscle. Doing this study enabled the authors to "trick" the autoclave chamber into extending the heat-up time and then trigger the start of the ABSL-3 and ABSL-4 "kill" or sterilization portion of the cycle when 121ºC was reached. A more accurate way of performing this process would be to temperature map the carcass load with resistance temperature detectors (RTDs) to really visualize heat distribution as well as to inform on a more accurate cycle time required for sterilization. While this study achieved its goal, the authors do not know if this cycle could have been shortened; time and resources did not permit more testing beyond what was done. Temperature mapping would most definitely be useful, but would also require more funding.

This study demonstrates the importance of validating autoclave cycles for biomass and liquid content, as typical laboratory waste, laundry, or reusable tools are less dense than animal muscle and are primarily solid while carcasses are largely comprised of liquid, resulting in a significantly longer time to reach sterilizing temperature.

Future Work

With oversight of the Health and Safety Office, optimizing and defining the cycle time required for carcass sterilization would be advantageous to provide savings in the form of reduced energy consumption, as well as reduced costs for parts and labor. An additional benefit of increased autoclave availability would also be realized as cycle and maintenance down-time decrease. The authors are also considering validating cycles sufficient for increased biomass closer to 13.6 kg (30 lb), to accommodate four lagomorph carcasses as opposed to the current limit of three per cycle. More efficient processing requires less storage space within containment for the carcasses and less autoclave cycles per study.

Acknowledgments

The authors would like to thank the NBACC Comparative Medicine group as well as the NBACC Environmental Operations group for their contributions to this endeavor. *Correspondence should be addressed to Jill Santacroce at jill.santacroce@nbacc.dhs.gov.

Disclosure

This work was funded under Agreement No. HSHQDC-07-C-00020 awarded by the Department of Homeland Security Science and Technology Directorate (DHS/S&T) for the management and operation of the National Biodefense Analysis and Countermeasures Center (NBACC), a federally funded research and development center. The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the U.S. Department of Homeland Security. In no event shall the DHS, NBACC, or Battelle National Biodefense Institute (BNBI) have any responsibility or liability for any use, misuse, inability to use, or reliance upon the information contained herein. The Department of Homeland Security does not endorse any products or commercial services mentioned in this publication.

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