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Validation of an Autoclave Procedure for Sterilization of Mouse (*Mus musculus*) Carcasses

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The sterilization of potentially infectious animal carcasses is an important biologic safety issue in animal facilities operating as infection or quarantine barriers. However, the literature lacks a validated protocol. Here we describe the validation of an autoclave program suitable for daily use in a small rodent biocontainment unit. We evaluated several procedures for processing mouse carcasses in a standard autoclave. Heat sensors and biologic indicators were implanted inside the peritoneal cavity of dead mice, which were loaded at various densities into IVC cages or metal boxes. Heat sensors revealed broad differences in temperature inside carcasses compared with the autoclave chamber. Achieving the appropriate sterilization temperature was considerably prolonged in carcasses compared with typical laboratory waste material. We show that for 5 cadavers placed well separated inside an IVC, a modified program for mouse cage sterilization using 134 °C for 15 min is suitable. To sterilize approximately 1 kg of carcasses in autoclavable boxes, a period of 6 h is required to reach an effective temperature of 121 °C for 60 min at the center of the waste by using an autoclave program for liquids. In conclusion, we here validated 2 protocols for the sterilization of potentially infectious mouse carcasses, to ensure the application of efficacious procedures.

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Biosafety in Microbiological and Biomedical Laboratories states quite simply: "Decontaminate all potentially infectious materials before disposal using an effective method,"[11], no description of an effective method for the decontamination of animal carcasses is given. The *Guide for the Care and Use of Laboratory Animals* mentions: "Hazardous wastes must be rendered safe by sterilization, containment, or other appropriate means before their removal from the facility. … Infectious animal carcasses can be incinerated onsite or collected by a licensed contractor."[3] Because carcasses from an experimental animal barrier might harbor substantial amounts of infectious agents, effective decontamination is crucial for the protection of personnel and environment. German authorities require the biologic validation of decontamination protocols every 6 mo for ABSL2 and 3 laboratories.[2] The most widely used animal species for biomedical research are mice, and the number of mouse carcasses for decontamination can be quite substantial. However, literature searches for published protocols were not very fruitful; in fact, we found only one detailed description of validation of animal carcass decontamination, in which turkey and Cornish hen carcasses were used to substitute for those of NHP and guinea pigs.[10] We found no further published data regarding validation of the decontamination of mouse carcasses.

The 3 main techniques described for the decontamination of carcasses are incineration, alkaline hydrolysis, and rendering. Incineration is considered one of the biologically safest methods: the carcass is burned to ash in a controlled atmosphere; commercial units with oil or gas burners, automatic timers, and smoke discharge stacks are available.[1] Alkaline hydrolysis digestion uses aqueous solutions of alkali metal hydroxides, such as NaOH or KOH. In this method, the carcass is covered with alkali solution (0.02 kg of a 50% NaOH solution for every 1 kg of carcass weight) and heated to 110 to 120 °C in a stainless steel pressure vessel for 18 h.[1,4] This process destroys all proteins and is suitable for the decontamination of many infectious agents, including prions.[4,6] Rendering describes the controlled crushing of carcass material and its subsequent heating to 115 to 145 °C for 40 to 90 min.[5]

However, each of these 3 methods requires specific equipment not usually present in animal facilities housing small animals or in typical research laboratories. Therefore mouse carcasses are most commonly decontaminated by using an autoclave. International[13] and national[7,9] guidelines recommend sterilization temperatures and times. In Germany, standard programs using a sterilization temperature of 121 °C for 20 min or 134 °C for 12 min are the most common protocols for decontamination.[8] Some autoclaves have specific programs for animal carcasses, but these programs usually are similar to those already mentioned. The use of *Geobacillus stearothermophilus* spores is generally recommended for biologic validation of the autoclaving of biohazardous waste.[12]

Materials and Methods

In the context of commissioning a new animal facility, several sterilization procedures were assessed by using mouse *(Mus musculus*) carcasses in a standard steam autoclave (double-door, 4-m^3 chamber volume, Vakulab PL 181215-2GR, Münchener Medizin Mechanik, Planegg, Germany). Mice from breeding colonies (that is, unusable genotype or age; research-naive) were euthanized by cervical dislocation in accordance with Directive 2010/63/EU and the recommendations of the local animal welfare body. Cages and boxes were placed on a stainless-steel rack (IWT, Tecniplast, Buguggiate, Italy). In the first set of experiments (tests 1 through 3), heat sensors (Valisystem 14046-0404, Schmitt and Strohmayer, Bruckberg, Germany) were placed in the stomachs of the dead mice.

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Test 1. We placed 15 to 20 carcasses in an autoclavable plastic bag (300 × 200 mm, Brand, Wertheim, Germany) and placed the filled bag inside a IVC (Sealsafe, Tecniplast). We distributed 15 heat sensors among 5 IVC cages, each containing 1 bag of carcasses. Sterilization was set at 134 °C for 10 min.

Test 2. We placed 5 to 7 cadavers well separated inside a single IVC cage containing 300 g of aspen bedding (Tapvei, Harjumaa, Estonia); 18 heat sensors implanted in dead mice were distributed among 4 separate IVC cages. Sterilization was set to 134 °C for 20 min.

Test 3. We collected approximately 10 kg of carcasses in an autoclavable plastic bag (capacity, 30 L; Greiner Bio-One, Frickenhausen, Germany) and placed the filled bag inside a stainless steel sterile container (XXL Endo Container, Aesculap, Tuttlingen, Germany); 13 heat sensors inside mouse carcasses were distributed among 6 bags and 3 containers. A program for liquids with a preheating time of 6 h and sterilization at 121 °C for 60 min was used. A temperature probe in a 5-L polypropylene vacuum bottle (Nalgene, Thermo Fisher Scientific, Waltham, MA) filled with 5 kg of water was used to assure accurate preheating of the material prior to sterilization time. Temperature measurement was done once for each set-up.

Tests 4 through 7. In a second set of experiments (tests 4 through 7), biologic indicators for solutions (ProAmp biologic indicator, BAG Health Care, Licht, Germany) and porous goods (ST/DA, Simicon, Munich, Germany) containing 10^6 *Geobacillus stearothermophilus* spores were placed inside the peritoneal cavity of mouse carcasses. After autoclaving, these biologic indicators were incubated at 55 °C according to the manufacturer's instructions (48 h for BAG-ProAmp; 7 d for Simicon ST/DA). A positive control provided by the manufacturer was included.

For tests 4, 5, and 6, the 'solid and porous goods' program was set to sterilization at 134 °C for 15 min. In test 4, 20 to 30 carcasses were collected in a disposable bag (300 × 200 mm, Brand) and placed inside an IVC; 5 spore vials for solutions and 5 for porous goods were included. In test 5, we placed 5 mouse carcasses well separated in an IVC cage, with 5 spore vials for solutions and 5 for solid goods. In test 6, we collected 1 to 2 kg of carcasses in a disposable bag (capacity, 30 L, Greiner Bio-One). We inserted biologic indicators into the peritoneal cavity of 2 mouse carcasses per bag; a maximum of 5 filled bags were placed inside a stainless steel container (XXL Endo Container, Aesculap). A maximum of 6 containers were placed in the autoclave chamber and autoclaved in a single run.

Test 7. Test 7 used the same set-up as for test 6, except the program for autoclaving liquids (sterilization at 121 °C for 60 min) was run.

The spore tests were repeated twice for tests 4 and 5, once for test 6, and 8 times for test 7.

Results

In a first setting, we tested a default procedure (named 'cadavers') by using 15 to 20 carcasses in an autoclavable plastic bag placed inside an IVC cage (Figure 1 A). Heat sensors revealed a marked difference in temperature inside the carcasses compared with the autoclave chamber. The temperature inside the carcasses did not reliably reach the necessary 121 °C even though the chamber temperature reached 134 °C (Figure 1 B and C). The temperature ranged from a minimum of 105.8 °C to a maximum of 135.3 °C (Table 1, test 1).

We suspected that either the number of carcasses was too large for the autoclave program or the preheating duration was too short. Therefore we assessed the inactivation of individual mouse carcasses by using the 'dirty cages' program: preheating for 1 h, followed by sterilization at 134 °C for 20 min. The heat sensors were introduced into the stomachs of the mouse carcasses; 5 to 7 carcasses were placed inside an IVC (Figure 2 A). The dirty cages program achieved the necessary core temperature of 121 °C and maintained it for 25 min. The temperature ranged from 134.0 to 135.3 °C (Figure 2 B and Table 1, test 2).

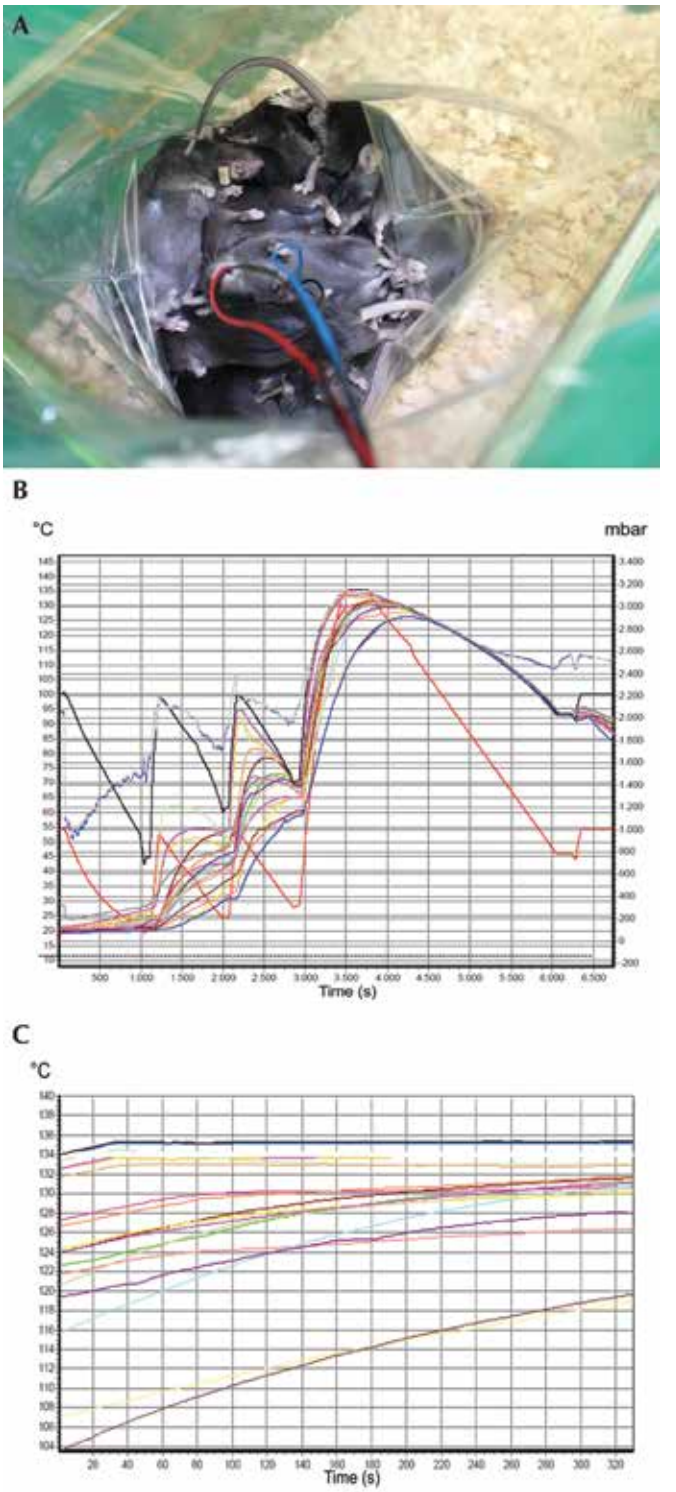

Figure 1. (A) Autoclaving mouse carcasses in bags, with heat sensors placed in the stomachs of carcasses. (B) Procedure: 'cadavers'; sterilization: 134 °C, 10 min. (C) Variation in temperature inside the mouse carcasses at the working temperature (134 °C).

Table 1. Lowest and highest temperatures (°C) according to heat sensors placed in stomachs of carcasses

	Test 1		Test 2		Test 3	
	Lowest	Highest	Lowest	Highest	Lowest	Highest
1	122.75	126.40	134.01	135.17	121.47	123.18
2	108.27	118.94	135.02	135.37	121.45	123.15
3	133.52	134.22	134.70	135.23	121.48	122.71
4	128.39	130.75	134.53	135.14	121.49	122.69
5	117.92	130.79	135.06	135.34	121.05	123.30
6	125.23	131.62	135.23	135.39	121.54	122.65
7	132.84	134.49	135.22	135.62	121.60	123.22
8	125.47	129.96	134.87	135.30	121.66	122.36
9	132.74	133.05	134.96	135.31	122.12	122.71
10	125.11	131.08	134.53	135.19	121.94	122.69
11	105.78	119.68	134.70	135.24	122.06	122.77
12	133.70	133.99	134.96	135.25	121.01	121.81
13	120.39	128.14	135.07	135.40	121.54	122.16
14	127.71	131.79	134.95	135.33		
15	122.72	130.53	134.43	135.24		
16			135.39	135.53		
17			134.39	135.36		
18			135.47	135.58		

However, autoclaving individual mouse carcasses is only suitable for small numbers of carcasses. In a routinely operating biocontainment unit, several hundreds of carcasses might need to be decontaminated at once. We therefore tested a modified program for liquids on bags containing about 1 kg of carcasses in stainless-steel containers (Figure 3 A); as done for the first setting (Figure 1 A), heat sensors were placed into carcass stomachs. A period of 6 h was required to reach and maintain the required sterilization temperature of 121 °C for 60 min at the center of the waste, resulting in a total procedure duration of 8 to 10 h. The temperature inside the carcasses ranged from 121.0 to 123.3 °C (Figure 3 B and Table 1, test 3).

Applying these results, we designed 2 new autoclave programs for the sterilization of mouse carcasses. One was a program for solid and porous goods, with sterilization at 134 °C for 15 min, which we modified for the sterilization of a few mouse carcasses together with other waste material. The other program was the modified program for liquids described for test 3, which can be used for larger numbers of carcasses. We validated these programs by using biologic indicators: spore vials for solutions and porous goods were implanted into the peritoneal cavities of dead mice. All spores were negative for carcasses that were placed separate from each other (Table 2, test 5). However, when we attempted to sterilize bags of carcasses by using the program for porous and solid goods, 10 of the 20 spore indicators for solutions and 11 of the 20 indicators for porous and solid goods were positive for growth (Table 2, test 4).

Because German authorities require biologic validation of the decontamination of all waste material every 6 mo,[2] we regularly validate our autoclave programs. Thus, we can provide data regarding spore vials placed inside mouse carcasses in large bags of carcasses and stainless-steel containers autoclaved by using the program for liquids for 8 validation procedures to date. Each of the spore vials showed negative growth of spores (Table 2, test 7). However, by using the same setup but with the program for porous and solid goods, all 9 spore vials were positive, indicating ineffective sterilization (Table 2, test 6).

Discussion

None of the programs recommended by the autoclave manufacturer reliably maintained the necessary temperature of 121 °C for 20 min inside mouse carcasses, and spore growth was positive. Heat sensors revealed marked temperature differences between autoclave chamber and various mouse carcasses (Table 1). The time to achieve sterilization temperatures inside the carcasses was considerably longer than for typical laboratory autoclavable goods. Moreover, the heat was not distributed homogeneously throughout the material.

Sterilization of solid and porous goods in an autoclave is achieved by steam reaching every surface and entering through all pores of the material. Sterilization of liquids is achieved through heat transmission to the center of the solution. Steam likely was prevented from reaching every surface of carcasses by the disposable bag and dense packing. In addition, body cavities cannot be evacuated by fractional prevacuum, thus preventing steam from entering the carcasses. Furthermore, the insulating properties of mouse fur and subcutaneous fat tissue inhibit even heating throughout the entire carcasses. Therefore, we recommend monitoring sterilization efficacy from inside carcasses.

Decontamination of a standard-use bag containing approximately 1 kg of carcasses cannot be ensured by using a standard program for autoclaving waste. For sterilizing a few mouse carcasses, a modified dirty cage procedure, which includes a prolonged preheating phase and remains at 134 °C for 15 min, is suitable. In this scenario, steam can reach all body surfaces, and the body mass is small enough that the sterilization temperature can be maintained for a sufficient time at the center of the carcass. Processing a few, well separated carcasses inside dirty cages and waste might be a suitable procedure for small numbers of carcasses or facilities that lack the equipment or space to store refrigerated carcasses.

The WHO recommends monitoring sterilization efficacy by using a 'worst-case' load.[13] Packing approximately 10 kg of mouse carcasses in autoclavable plastic bags, which then are placed in stainless steel containers, can serve as such. Many animal facilities might have to sterilize large amounts of carcasses,

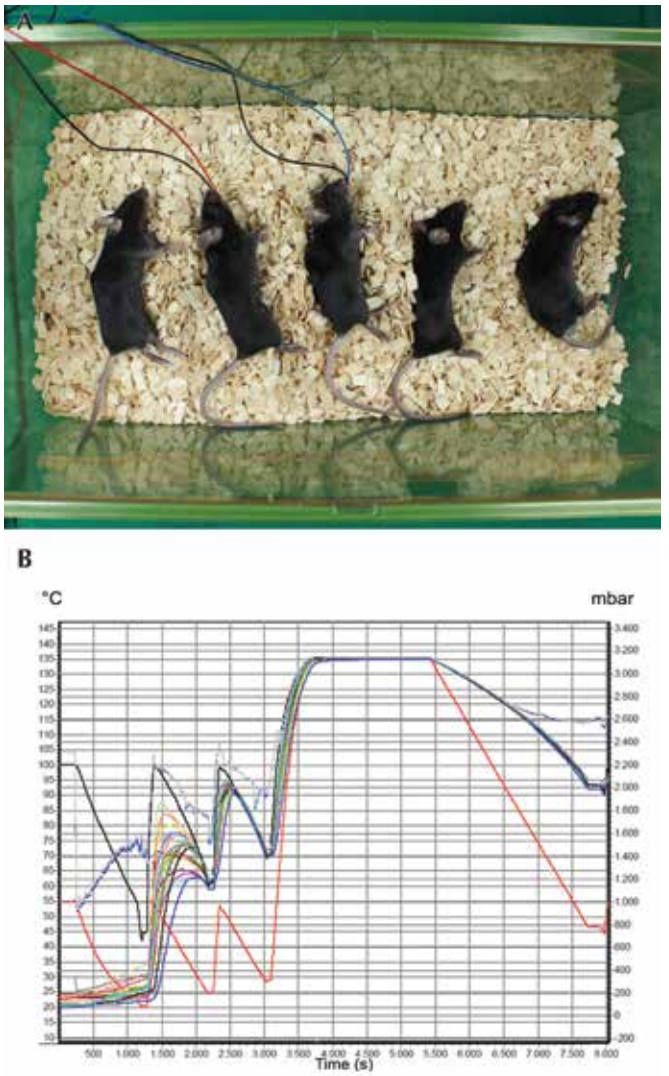

Figure 2. (A) Autoclaving well separated mouse carcasses. (B) Procedure: 'dirty cages'; sterilization: 134 °C, 20 min.

which are collected and stored in disposable bags. These bags cannot be opened completely to allow the steam to reach every surface, because doing so would lead to the accumulation of fluid inside the container used. Therefore, sterilization temperature needs to be achieved by heating the material from the periphery to the center. Because mammalian bodies are approximately 70% water, it seems reasonable that they should be treated as liquids for efficient sterilization. Consequentially, we tried an autoclave program designed for liquids for processing larger numbers of carcasses. By these means, a more homogeneous temperature distribution was achieved in the material requiring decontamination, and the biologic indicators confirmed appropriate sterilization.

Only by using biologic indicators such as *G. stearothermophilus* spores can efficient sterilization be validated and documented. Placing a single reference temperature probe in a 5-L polypropylene vacuum bottle filled with water was necessary to control preheating to the sterilization temperature. This fluid-filled container might not precisely mimic mouse carcasses but was sufficient to achieve sterilization temperatures in our setting as well as in a previously described validation procedure.[10]

Given our validation results, we recommend placing carcasses in an autoclavable plastic bag inside a stainless steel

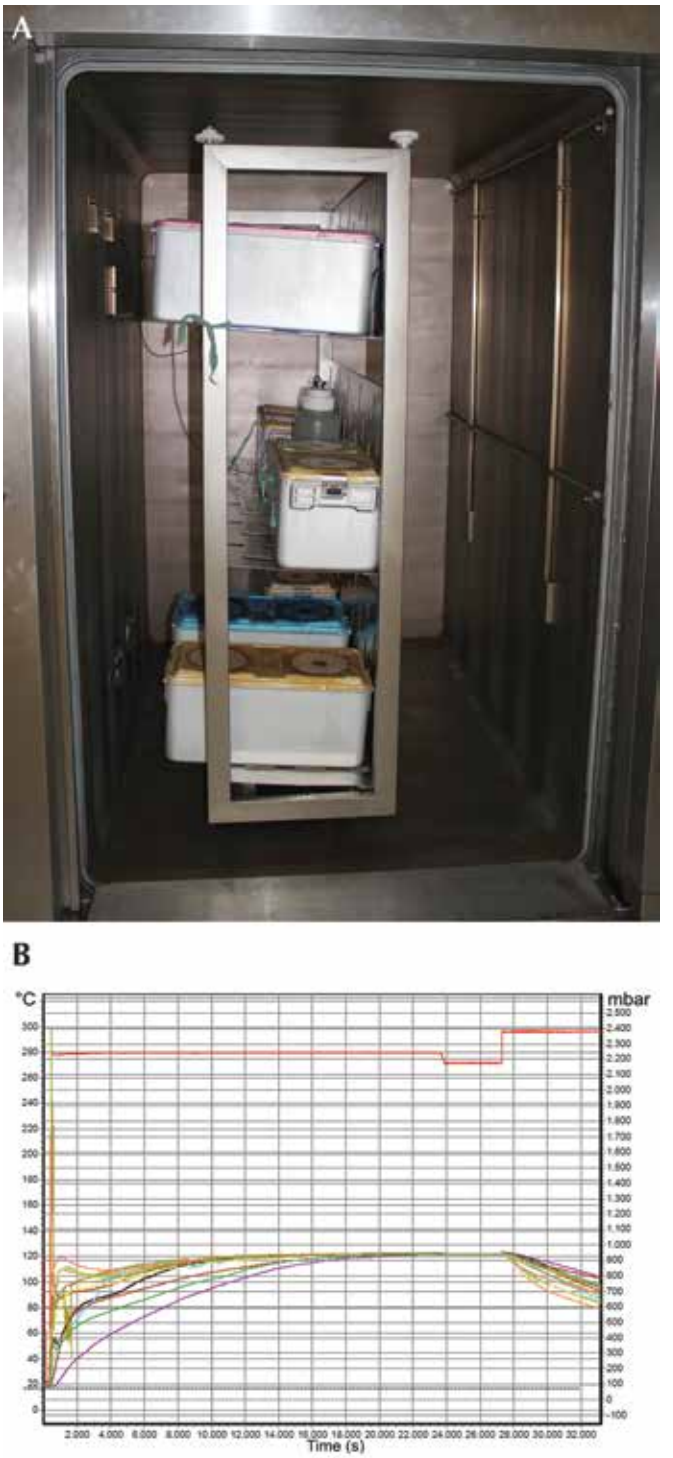

Figure 3. (A) Autoclaving mouse carcasses in boxes. (B) Procedure: 'liquids'; sterilization: 121 °C, 60 min.

container and using a program for liquids with a 6-h preheating period prior to the sterilization phase of 121 °C for 60 min. It is important to use a prolonged sterilization time of 60 min to ensure sterilization of the biohazard waste material.[10] Even though this method is time- and energy-consuming, we consider it the method of choice for an animal facility working as a quarantine or biocontainment unit under ABSL2 or ABSL3 conditions, where large numbers of carcasses must be sterilized effectively through a routine procedure. However, the protocol we described here can only serve as a guideline

Table 2. Details regarding autoclave setup and results of biologic indicator (spore) tests

	Test no.			
	4	5	6	7
Material to be decontaminated	15-20 carcasses	5-7 carcasses	10 kg of carcasses	10 kg of carcasses
Packing	Disposable bags in IVC	IVC	Disposable bags in stainless steel boxes	Disposable bags in stainless steel boxes
Type of program	Solid and porous goods	Solid and porous goods	Solid and porous goods	Liquids
Sterilization temperature	134 °C	134 °C	134 °C	121 °C
Sterilization time	15 min	15 min	15 min	60 min
Autoclave cycle no.	1/2	1/2	1	1/2/3/4/5/6/7/8
No. of positive spore tests for solutions[a]	2/8	0/0	9	0/0/0/0/0/0/0/0
No of negative spore tests for solutions[a]	8/2	5/5	0	9/10/12/10/8/10/15/16
No of positive spore tests for solid goods[a]	4/7	0/0	not tested	not tested
No of negative spore tests for solid goods[a]	6/3	5/5	not tested	not tested

[a]Data regarding spore tests are given according to autoclave cycle no.

for other facilities. Validation of each autoclave program in the facility-specific setting is necessary. In particular, the size and make of the autoclave, the containers used, and the number and size of carcasses to be sterilized need to be taken into account.

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