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Comparing the Results of Microbiological Sterilization by Autoclaving in Different Waste Packaging Formats

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This study aimed to compare the results of microbial sterilization of infectious biohazardous waste by autoclaving in packaging formats with and without added water. Twelve strains of bacteria and fungi were studied. Three types of artificial waste were created from (1) cultures on solid media, (2) colony suspension in saline, and (3) contaminated materials. These were packed in waste bags with and without added water before autoclaving at 121°C, 15 lb square inch $^{-1}$ for 15 min under validation of quality indicators. Then, all waste types were cultured in enrichment broth, overnight prior to subculture on agar and further analysis. In waste bags without added water, *Bacillus cereus* and *Pseudomonas aeruginosa* were not completely destroyed and waste bags without added water were also spore test positive. Whereas in waste bags with added water, all microorganisms, including spores could be completely destroyed in all waste types. Therefore, the results indicate that the addition of water to waste bags can significantly enhance the efficacy of sterilization and we recommend this simple step as an additional guideline for routine laboratory autoclaving to prevent the spread of pathogens to the environment.

Keywords: Autoclave, Water, Infectious biohazardous waste, Packaging format, Spore test.

The clinical microbiology laboratory works with various kinds of medically important microorganisms. Waste containing infectious biohazardous materials must be completely sterilized. Nowadays, autoclaving or steam sterilization is the most effective and commonly used method to decontaminate these wastes. Its efficiency depends on the adequate time, temperature, and pressure in which the saturated steam is in contact with the microbial cells. However, many microbiological laboratories in Thailand, including our institution, have not taken into account these aspects in the traditional waste packaging format. Waste bags are loaded more than two thirds of their capacity, placed in double bagging and closed tightly which seem to interfere with heat transfer from steam circulation[1] This might result in incomplete sterilization and thereby cause the spread of undestroyed pathogens to the environment.

The addition of water to waste bags is greatly affecting sterilization efficacy and has been studied by Lauer *et al.* (1982). They demonstrated that water in waste bags facilitated a 20–30 times higher heat transfer than air. However, the studies of Gillespie and Gibbons (1975), Rutala *et al.* (1982) and Ozanne *et al*. (1993) showed inconsistent data. They found that the added water did not significantly improve heat-up time in containers. Therefore, to investigate the importance of water during autoclave sterilization of infectious biohazardous waste this study aimed to compare the results of autoclaving with and without added water. To our knowledge, this is the first study of the impact of waste packaging formats in Thailand

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and the results will be used to improve the guidelines for routine laboratory autoclaving.

MATERIALS AND METHODS

Microorganisms

Twelve strains of pathogens, including *Staphylococcus aureus*, Group D streptococci, *Bacillus cereus*, *Enterococcus faecalis*, vancomycin-resistant Enterococci (VRE), *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, multidrug-resistant *P. aeruginosa*, *Acinetobacter baumannii*, multidrug-resistant *A. baumannii*, and *Candida albicans* were used in this study. The microorganisms were cultured on blood agar at 37°C, 18–24 h before they were used for artificial waste creation.

Three types of artificial infectious wastes in different packaging formats

Culture on solid media

To prevent spillage of molten agar from disposable polystyrene petri dishes, twelve strains of pathogen were cultured on slant blood agar at 37°C for 18–24 h.

Colony suspension in saline

The concentrations of *B. cereus, E. coli, C. albicans* were adjusted to 10^8 cfu ml^{-1} in 0.85% normal saline by using McFarland No. 0.5. following this, serial 10-fold dilutions until to 10^0 cfu ml^{-1} were prepared.

Contaminated materials

A high inoculum of each strain on blood agar was suspended in sterile normal saline by using a sterile cotton swab. Following this process, gloves were smeared with each swab, and both swabs and gloves were transferred into a plastic bag and the bag constricted with an elastomeric ring.

To study the effect of water in the waste bags, four waste bags were used in each experimental run, two bags with 200 ml added water and two bags without added water. The double bagging polypropylene autoclave bags were loaded only 2/3 full with all waste types. In all experiments, the same kind of polypropylene autoclave bag was used (36 by 48 cm; Innova-Pack Co., Ltd.). Our study validated the efficacy of the sterilization process in each waste bag through using chemical and biological indicators. Class I chemical indicator or autoclave tape (3M™ Comply™ Steam Indicator Tapes) was attached inside and outside of each waste bag. Biological indicator or spore test (3M Attest™ no. 1262) containing *Geobacillus stearothermophilus* was placed in a glass petri dish, wrapped with a plastic bag, and then placed in the lower part of the load. Two-hundred ml of water was gently added to two of the prepared waste bags. All waste bags were constricted loosely with a single elastomeric ring and put into a vertical gravity displacement autoclave (model HV-110, Hirayama, Japan). Two bags of each packaging format were placed in the upper and lower positions of the chamber, respectively. Hereafter, we will refer to the bags by their format and position: U (upper without water), WU (upper with water), L (lower without water), and WL (lower with water). Our study used standard time, pressure and temperature recommendations at 121°C, 15 lb square $inch^{-1}$ for 15 min, and monitored the accuracy of the autoclave's time and pressure gauge via direct observation at a fixed time interval.

Enrichment process

Following the autoclaving process, all waste types were cultured in trypticase soy broth (TSB) (Lab M, England) at 37°C, overnight. To check for bacterial growth, samples of the broth were then cultured on blood agar at 37°C for 18 h and a biochemical test was used to identify the observed microorganisms.

Validation of quality control indicators

The autoclave tape and spore test were removed after the waste had been processed. The intensity of the color change of the autoclave tape was recorded immediately. The spore test was incubated at 56°C for 48 h and then read for spore survival. A positive result was determined by a change of the broth color from purple to yellow.

Biosafety approved

This study was approved by the Biosafety Committee of Thammasat University (approval no. 002/2016).

RESULTS

Comparison of different waste packaging formats to microbial sterilization and validation of quality control indicators

The collected data of the experiments is shown in Table 1. Bacterial survival was not

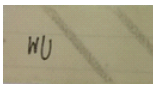
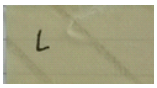
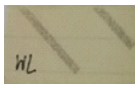
observed in infectious waste created from colony suspension in normal saline and from contaminated materials (glove and swab) following sterilization with or without added water. However, bacterial survival was still observed at 40–60% of *B. aereus* and 20–40% of *P. aeruginosa* following sterilization of agar without the addition of water to the waste bags. Furthermore, the autoclave tape attached to the inside of the bags was less dark if no water was added compared to bags with added water. The spore tests showed positive results indicating incomplete sterilization in waste bags without added water.

DISCUSSION

The effectiveness of autoclave sterilization depends on the optimal steam penetration through the biohazardous waste. Different factors can interfere with the heat transfer, including overloading the waste bag, too tightly closing the waste bag, and the type of autoclave waste bag. In Thailand, the traditional waste packaging system has a high risk of inadequate waste sterilization and might contribute to the spread of microbial pathogens in the environment. The study of Lauer *et al.* (1982) indicated that modified waste packaging with added water facilitates a better heat transfer and improves waste sterilization. However, several other studies showed conflicting results[1-3]

This study aimed to investigate whether the addition of water to autoclave bags containing biohazardous waste affected microbial sterilization. Other factors that can interfere with the heat transfer were kept constant, the same bag type was used in all experiments, bags were not filled above two thirds of their capacity and were loosely closed with a single elastomeric ring. Our results indicate the significance of the water added to the waste bags, it enhances steam circulation thereby causing the death of all microorganisms including the spores of *Geobacillus stearothermophilus*. Although, double bagging used to prevent molten agar spillage in real-life situation may be a barrier of steam admittance to the materials, our study showed that it is not a problem if sufficient water is present during the sterilization process. According to the thermal conductivity principle, the thermal conductivity of water is 20–30 times higher than

Table 1. Comparison of different waste packaging formats to microbial sterilization and validation of quality control indicators [a]

Tests	Results from different waste positions and packaging formats			
	U	WU	L	WL
Culture on solid media	G (3/5, *B. cereus*) G (2/5, *P. aeruginosa*)	NG	G (2/5, *B. cereus*) G (1/5, *P. aeruginosa*)	NG
Colony suspension in saline	NG	NG	NG	NG
Contaminated materials	NG	NG	NG	NG
Chemical indicator (Inside bag)				
Biological indicator				

[a] All results are from three to five replicates under the same conditions of the sterilization process. Abbreviations: U, upper without water; WU, upper with water; L, lower without water; WL, lower with water; G, microbial growth (positive results per number of replicates); NG, no microbial growth.

that of air and the added water significantly decreases the chances of dry pockets in the waste bag[4] Thus, the results obtained were used to develop an additional guideline for waste sterilization in routine laboratory autoclaving to prevent the spreading of harmful pathogens to the environment.

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