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Microwave Sterilization of Plastic Tissue Culture Vessels for Reuse

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A simple protocol has been developed for recycling plastic tissue culture vessels. The killing properties of microwaves were used to decontaminate plastic tissue culture vessels for reuse. Nine bacterial cultures, four gram-negative and five gram-positive genera, including two *Bacillus* species, were used to artificially contaminate tissue culture vessels. The microwaves produced by a "home-type" microwave oven (2.45 gHz) were able to decontaminate the vessels with a 3-min exposure. The same exposure time was also used to completely inactivate the following three test viruses: polio type 1, parainfluenza type 1 (Sendai), and bacteriophage T4. The recycling procedure did not reduce the attachment and proliferation of the following cell types: primary chicken and turkey embryo, HEp-2, Vero, BGMK, and MK-2.

One of the major disadvantages of using plastic tissue culture vessels has been lack of reusability. This situation imparts a high cost per sample to tissue culture experiments. With increasing oil prices, the cost of plastic tissue culture vessels is bound to rise, thus increasing the already high cost of tissue culture. It would be advantageous to develop a recycling technique for tissue culture equipment which would be effective, rapid, and relatively inexpensive.

Cleaning of plastic tissue culture vessels presents few problems for most uses (M. R. Sanborn, unpublished results). A detergent wash followed by proper rinsing usually removes residual materials and provides a clean surface for reseeding cells. Resterilization of the material poses the greatest problem for laboratories not equipped with gas sterilization equipment. Gas sterilization with ethylene oxide, even though effective, requires considerable time for degassing before use. Gas sterilization equipment is also relatively expensive.

Recent reports have shown microwaves to be effective in the decontamination of food products (1, 2) and various materials in clinical microbiological laboratories (4). Microwaves have also been used for the fixation of fetal as well as surgical and autopsy material (5, 9). Furthermore, and more important to this study, Patterson and Bulard have shown that microwaves are capable of fixing cells in tissue culture (7, 8).

We have expanded these studies and have applied the fixation and killing properties of microwaves to the recycling of plastic tissue culture vessels. We have found that microwaves generated from small "home-type" microwave ovens are effective in sterilizing plastic tissue culture vessels rapidly and inexpensively.

MATERIALS AND METHODS

A locally purchased Kenmore (model 99601) microwave oven operating at 2.45 gHz with the power selector on HI was used throughout the experiments. Exposure times were controlled by the built-in digital timer.

Tissue culture vessels purchased and tested included the following: 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.), 25-cm^2 tissue culture flasks (Lux Scientific Corp., Newbury Park, Calif.), Tissue Culture Cluster 24 dishes and 60- by 15-mm dishes (Costar, Cambridge, Mass.), and 850-cm^2 Tissue Culture Roller Bottles (Falcon Plastics, Oxnard, Calif.).

Sterile vessels were contaminated with 0.2 to 5.0 ml of broth cultures (>10^6 cells per ml) of the following organisms: *Escherichia coli, Pseudomonas fluorescens, Klebsiella pneumoniae, Proteus vulgaris, Sarcina lutea, Corynebacterium equi, Bacillus alvei, Bacillus globigii*, and *Streptococcus faecium*. The contaminating broth was shaken in the vessels to ensure contact of the organisms with the vessel walls. Broth was poured out of the vessels just before microwave treatment. No attempt was made to dry the inside of the vessels. Contaminated vessels were subjected to microwave irradiation for 0, 15, 30, 60, 120, 180, 240, 300, and 600 s. After irradiation, the contaminated surface of the vessel was overlayed with nutrient agar. The vessels were then incubated at 37°C for 48 h. Each container was then observed for colony formation and scored for the presence or absence of bacterial growth. Nonsterile used vessels were washed with detergent, Alconox, and rinsed with deionized

water from a Barnstad Nano-pure water system. After overnight drying, the vessels were subjected to microwave irradiation as described above. Each vessel then received a sample of tissue culture medium and was incubated at 37°C for 48 h. Vessels were then scored for contamination by observing the medium for a pH shift and by microscopic observation with Gram stains. Further experiments were conducted to determine if used tissue culture flasks could be washed, sterilized with microwave irradiation, and reused. Used flasks were washed with a test tube brush and detergent to remove attached cells and were rinsed four times with deionized water. After a 2-h drying period, the flasks were submitted to microwave irradiation as described above. Flasks were then seeded with the following cell types: Primary chicken embryo, primary turkey embryo, HEp-2, Vero, BGMK, and MK-2. The ability of these cells to attach and form monolayers was compared with that of control cells grown on new flasks. All cells were seeded at a cell density of 10^6 cells per ml. The avian cells were maintained in Eagle basal medium (BME) with 5% calf serum and 100 μg of streptomycin and 100 U of penicillin per ml. Cell lines were maintained in minimal essential medium (MEM) with 10% calf serum and 100 μg of streptomycin and 100 U of penicillin per ml.

Three virus suspensions, polio type 1, parainfluenza type 1 (Sendai OSU-T), and phage T4, were subjected to microwave irradiation for the time sequences given above. Polio and Sendai viruses were suspended in 1 ml of Hanks balanced salt solution; the T4 phages were in a nutrient broth lysate. After treatment, the suspensions were plated out in standard plaque assays by using the following virus-host systems: polio-BGMK cells, Sendai-primary chicken fibroblast cells, and phage T4-*E. coli* B.

Polio virus dilutions were plated in triplicate onto monolayers of BGMK cells by using a 0.1-ml volume per 60-mm culture dish. The virus was allowed to attach for 1.5 h at 37°C before the addition of a 4-ml agar-MEM overlay. The agar overlay was prepared by mixing 2× MEM with 2% agar (Difco Laboratories, Detroit, Mich.) at 45°C. Neutral red was added to a final concentration of 1:12,000. Plates were incubated at 37°C for 48 h before the plaques were counted.

The OSU-T strain of Sendai virus was isolated at Oklahoma State University, Stillwater, Okla., by M. R. Sanborn. This particular strain of Sendai virus plaqued either turkey or chicken fibroblast cells without the addition of exogenous trypsin. In the Sendai virus inactivation experiments, dilutions of the virus were plated in triplicate onto monolayers of chicken embryo fibroblast cells by using a 0.2-ml volume per 60-mm culture dish. The virus suspensions were allowed to attach for 1 h at 4°C before the addition of 4 ml of the first agar overlay. The first agar overlay was prepared by mixing 2× BME with 1.5% Noble agar (Difco) at 45°C. Plaques of Sendai OSU-T were developed by the addition of a duplicate agar overlay (containing 1:12,000 neutral red) 48 h postinfection. Sendai OSU-T plaques were visible at 60 h postinfection.

Treated phage T4 suspensions were diluted, and 0.1-ml samples were mixed with 5 ml of soft agar containing nutrient broth, 0.5% NaCl (wt/vol), 0.75% agar (Difco), and 10^6 *E. coli* B cells per ml. The soft agar mixtures were plated onto support agar made of nutrient agar and 0.5% NaCl. Triplicate plates were made for each dilution of each treatment. Plates were incubated at 37°C until plaques developed.

RESULTS

Before sterilization experiments, samples of plastic tissue culture vessels were tested to determine if they could withstand the microwave process. All vessels could withstand times up to and, in most cases, exceeding 10 min. However, it was necessary to include a small beaker of water (250 to 500 ml) in the microwave oven as a heat sink. It was also necessary to let the glass bottom of the microwave oven cool between runs to prevent melting of the vessels.

A problem was encountered with vessels with black caps, such as the Lux 25-cm^2 tissue culture flask. The black caps retained heat and tended to melt the tops of the flasks at treatment times of over 5 min. This problem was solved by autoclaving the caps separately and by covering the mouth of the flasks with plastic wrap during the microwaving process. Soft, colored caps, such as the Corning orange caps and the Falcon blue caps (roller bottles), presented no problems during the microwaving process.

The results of the vessel contamination experiments are shown in Table 1. All vessels were rendered sterile by 3 min of microwave treatment.

Primary chicken embryo, primary turkey embryo, HEp-2, Vero, BGMK, and MK-2 cells have all been successfully grown in the recycled tissue culture vessels. No difference can be observed between the control cells grown in sterile nonrecycled tissue culture flasks and those grown in the recycled microwave-sterilized flasks (Fig. 1).

Figure 2 shows one trial with nonsterile, used tissue culture flasks. After microwave treatment, sterile tissue culture medium was added, and the flasks were incubated for 48 h at 37°C. After 2 min, the flasks were rendered sterile, as can be seen by the absence of turbidity.

The three virus types were inactivated within 3 min of microwave treatment, as shown in Fig. 3.

DISCUSSION

Our data indicate that microwave irradiation is a practical method for the sterilization of plastic tissue culture vessels. It is of particular value for laboratories which could benefit from the reuse of plastic vessels. We have found that, for the vessels tested, all commercially available tissue culture equipment is reusable after microwave sterilization.

The killing times for the organisms tested generally agree with those reported by Latimer and Matsen (4), Goldbrith and Wang (3), and

TABLE 1. Microwave sterilization time of tissue culture equipment contaminated with selected organisms

Organism	Growth after the following times (s)[a]								
	0	15	30	60	120	180	240	300	600
Escherichia coli	+	+	+	+	−	−	−	−	−
Pseudomonas fluorescens	+	+	+	+	−	−	−	−	−
Klebsiella pneumoniae	+	+	+	+	−	−	−	−	−
Proteus vulgaris	+	+	+	+	+	−	−	−	−
Streptococcus faecium	+	+	+	+	+	−	−	−	−
Sarcina lutea	+	+	+	+	−	−	−	−	−
Corynebacterium equi	+	+	+	+	+	−	−	−	−
Bacillus alvei	+	+	+	+	+	−	−	−	−
Bacillus globigii	+	+	+	+	+	−	−	−	−

[a] Power selector on high. +, Growth of the organism; −, no growth of the organism.

Culkin and Fung (2). In the case of the *Bacillus* species, the cultures did contain free spores and sporulating cells. It is interesting to note that the killing times for these organisms in our system were not much different from those of the other organisms tested. We have, however, tested spore suspensions of the two *Bacillus* species and have found these species to be much more resistant to microwave irradiation, particularly when dry.

The viruses tested were inactivated within the killing times determined for the bacterial strains. The viruses represented enveloped and naked particles as well as both RNA- and DNA-type virions. Phage T4 was selected as the representative of the DNA viruses simply because it was available and our laboratory does not routinely deal with DNA animal viruses. From the data presented and what is known about viral stability, it is not likely that DNA animal viruses would exceed the 3-min inactivation time to any great extent.

In considering the reuse of tissue culture vessels, it should be noted that the normal level of contamination, after washing, is much lower than that of our test contaminated vessels. Therefore, our sterilization times should be adequate for normal reuse. Low levels of contamination by spores normally would not present a problem if they escaped inactivation, since most

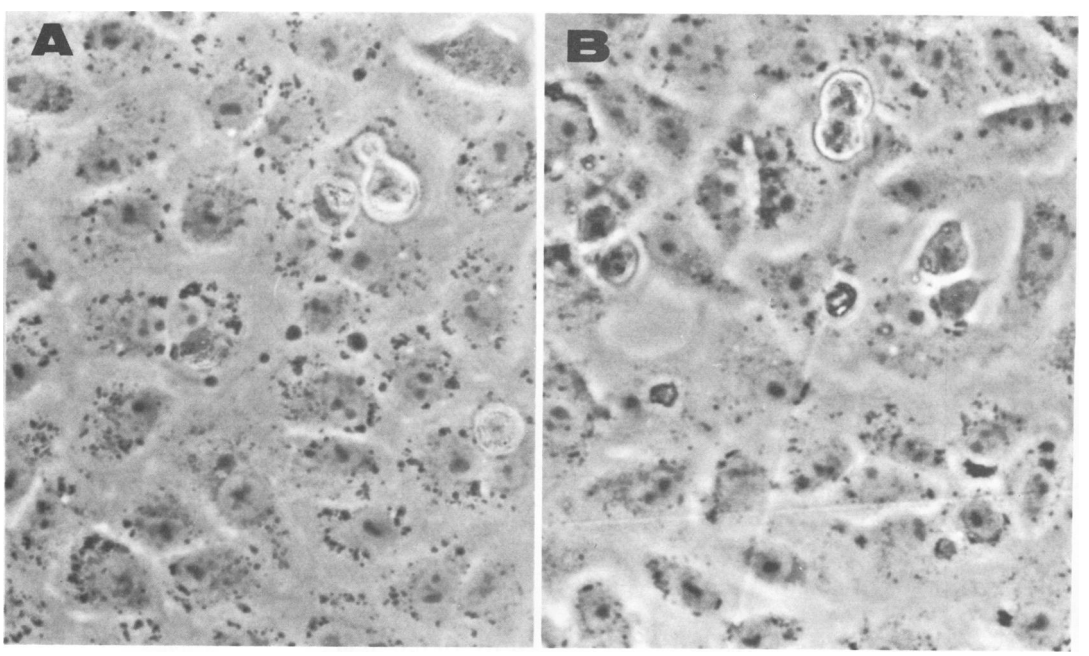

FIG. 1. Representative photomicrograph of HEp-2 cells grown on (A) a sterile control flask and (B) a microwave-sterilized recycled flask. Cells were seeded at 10^6 cells per ml. Photographs were taken after 48 h of incubation under MEM as described in the text.

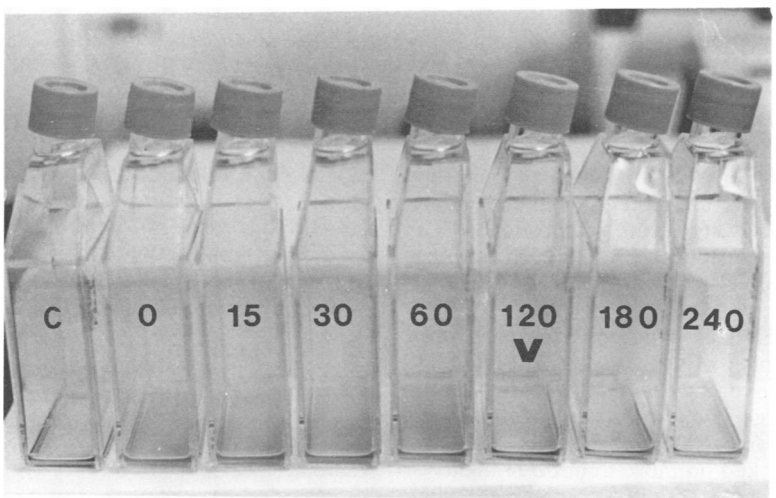

FIG. 2. Recycled tissue culture flasks after microwave treatment and incubation with MEM for 24 h at 37°C. Flask C represents a sterile nontreated control. All other flasks were treated with microwave irradiation. The numbers on the flasks denote the seconds of microwave exposure. The V indicates the first flask showing no turbidity and hence no growth from contaminating organisms.

tissue culture media contain adequate antibiotics to prevent these organisms from growing.

The nonsterile flasks, washed for reuse, were naturally contaminated with fungi. It was noted that fungal contamination was also eliminated with 180 s of microwave irradiation. No attempt was made to identify these contaminants, and no antifungal agents were included in the culture medium.

Recently, concern has been raised about cross-contamination of tissue culture lines with other cell lines (6). This concept becomes a problem when one contemplates the reuse of tissue culture vessels. We feel that even if residual cells escape the washing process, the microwave sterilization process will kill them. In support of this contention, Patterson and Bulard (7, 8) have shown tissue culture cells to be adequately fixed for fluorescent-antibody staining after only 12 s of microwave irradiation. The likelihood of tissue culture cells surviving microwave irradiation times of several minutes is nil.

We have been unable to detect any difference in the growth of the cells routinely grown in our laboratory in recycled tissue culture vessels (Fig. 1). Apparently, the washing and microwave processes do not damage the attachment properties of the plastics to any great extent.

At present, we do not reuse our flasks more than four times. After the third cycle through the microwave oven, the plastics begin to show a yellowing effect, indicating that the plastic may be breaking down.

From our experience, we have generated the following general protocol for the reuse of plastic tissue culture vessels. First, all culture medium is poured into a disposal container for autoclaving. Vessels containing virus-infected cells or other pathogenic agents are decontaminated by microwave treatment for 10 min. Caps on the flasks are loose at this time. The vessels are washed with a detergent, followed by four washes with deionized water. The vessels are allowed to dry overnight. Caps on flasks are loosened about one turn before sterilization. If the caps are of the black type, they are autoclaved and

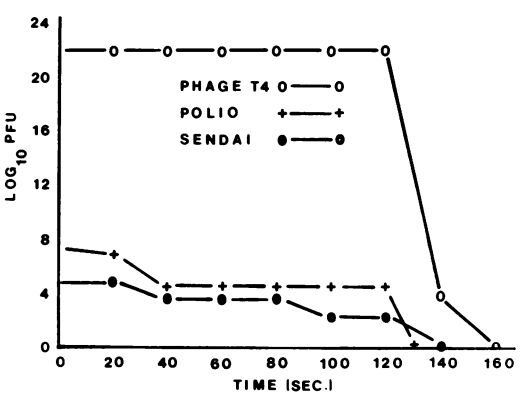

FIG. 3. Inactivation curves for three representative viruses after microwave treatment. Volumes (1 ml each) of the virus suspensions were treated in the microwave oven for each of the times shown. The treated samples were then plated onto their respective host cells, as described in the text. Control samples are shown at zero time. All virus samples were inactivated within 180 s of exposure.

added to the flasks after microwave treatment. Tissue culture equipment is then placed in the microwave oven along with a flask of water (250 to 500 ml), which serves as a heat sink. We have selected a 10-min treatment time for the vessels. This ensures that all areas within the oven receive the proper irradiation time. Home-type microwave ovens are noted for their uneven heating. If one is sterilizing several loads, it is best to let the inside of the oven cool before adding the second load to prevent the plastics from reaching too high a temperature.

The chamber sizes of microwave ovens vary. Thus, the number of vessels per load may also vary. In our oven, we are able to recycle as many as 20 large 75-cm^2 tissue culture flasks per load. Flasks are usually placed on end, and dishes are stacked no more than three high.

Certain cautions should be observed when using this protocol. First, we do not at this time recommend treating tissue culture vessels with large volumes of liquid in them. The liquid may begin to boil, generating heat, which may melt the vessels. This is true for small culture dishes with volumes as low as 2 ml. If antibiotics are not routinely used in culture medium, sterilization times may need to be extended to assure the killing of spores. The data of Latimer and Matsen may serve as a guide for adjusting killing times for spores (4). They found that a 5-min exposure inactivated *Bacillus stearothermophilus* spore strips, whereas it took 11 min to kill 1.3×10^9 *Bacillus subtilis* spores suspended in 5 ml of distilled water. Granulated detergent should be completely dissolved before use to prevent it from acting as an abrasive and scratching the surface of the plastic. We have found that if washing of the vessels is performed too vigorously, the test tube brush may scratch the surface of the plastic. Such a scratch is seen in Fig. 1B. These scratches do not seem to affect cell attachment but will show up in micrographs. Completely nonmetallic brushes are available, and their use might prevent this problem.

We have found that microwave irradiation is a rapid and economical way to sterilize plastic tissue culture vessels for reuse. By recycling three to four cases of 75-cm^2 flasks, one can pay for a more than adequate microwave oven. This feature will become more prominent as the price of plastic equipment increases.

ACKNOWLEDGMENTS

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