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Use of Ultrasonic Energy in Assessing Microbial Contamination on Surfaces

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Ultrasonic tanks were evaluated for their ability to remove viable microorganisms from various surfaces for subsequent enumeration. Test surfaces were polished stainless steel, smooth glass, frosted glass, and electronic components. The position of contaminated surfaces in relation to the ultrasonic energy source, distance of the ultrasonic source from the test surfaces, and temperature of the rinse fluid were some of the factors which influenced recovery. Experimental systems included both naturally occurring microbial contamination and artificial contamination with spores of *Bacillus subtilis* var. *niger*. The results showed that ultrasonic energy was more reliable and efficient than mechanical agitation for recovering surface contaminants. Conditions which increased the number and percentage of microorganisms recovered by ultrasonic energy were: using a cold rinse fluid, placing the sample bottle on the bottom of the ultrasonic tank, and facing the contaminated surfaces toward the energy source. It was also demonstrated that ultrasonic energy could be effectively used for eluting microorganisms from cotton swabs.

The ability to remove and enumerate precisely viable microorganisms from a variety of surfaces has been one of the conditions imposed upon the aerospace industry by the National Aeronautics and Space Administration (NASA) with its requirement that spacecraft destined to impact Mars be sterile. This requirement has presented a unique problem to the microbiologist. Conventional methods for assessing surface contamination were designed to detect large numbers of microorganisms on surfaces associated with water, milk, or food products, and have not changed appreciably in recent years. Little consideration has been given to surfaces containing small numbers of microorganisms. The problems encountered in removing and enumerating microbial contaminants on spacecraft and components, and the four basic procedures employed for recovering microorganisms from surfaces, viz., swab-rinse technique, agar contact procedure, rinsing technique, and in situ plating [i.e., direct surface agar plate (DSAP)] have been described previously (12).

With the development of industrial "clean rooms" (any enclosed area where there is control over particulates in the air), especially laminar flow clean rooms, for the assembly and testing of spacecraft required to be dry-heat sterilized, a totally new standard of cleanliness has evolved which surpasses the previously accepted standards of the hospital operating room. Employment of clean rooms has so markedly reduced the number and types of microbial contaminants which accumulate on surfaces (7) that detection and enumeration have necessitated reevaluation of established methods and the formulation of new techniques to assure the proper evaluation of contamination on surfaces of spacecraft and components.

One approach to this problem has been the use ultrasonic energy to recover microbial contaminants from surfaces (12). This was an application of techniques developed over many years by biologists interested in the physiological and biological effects of ultrasonic energy on bacterial cells (5, 8, 15). More recently, ultrasonic therapy (2, 11, 13) in physical medicine has gained favorable reception both in the United States and in Europe. Hospitals utilize ultrasonic energy for cleaning surgical instruments (14). The dairy industry uses it for cleaning milk-contact surfaces (9), and, experimentally, for homogenization and pasteurization of milk and milk products (3, 4, 6, 10). The uses of ultrasonic energy in the aerospace industry are numerous.

Puleo, Favero, and Tritz (12), using a probe-type system, studied the feasibility of employing

ultrasonic energy for recovering viable microorganisms from surfaces. They showed that insonation (exposure to ultrasonic energy) was an efficient and precise technique for quantitating the microbial contaminants on surfaces. However, only one sample at a time could be assayed when an ultrasonic probe was used. Consequently, further investigations were undertaken with ultrasonic tanks to remove microbial contaminants from various types of surfaces for subsequent enumeration and to determine the effect of several physical parameters on the efficiency of recovery.

MATERIALS AND METHODS

Strips of stainless steel [1 × 1 or 1 × 2 inches (2.5 × 2.5 or 2.5 × 5 cm), 24 gauge type 302, no. 4 finish], smooth glass (1 × 2 inches), and frosted glass (1 × 1 inch) were used as the test surfaces. Each strip was washed and sterilized as previously described (12). A suspension of *Bacillus subtilis* var. *niger* spores in ethyl alcohol (95%) was used for these tests. Two methods of inoculating the strips were employed: (i) The spore suspension was diluted in sterile buffered distilled water (*p*H 7.2), and 0.1 ml was spread evenly over the surface of each strip, allowed to air-dry for 2 hr in a laminar-flow clean bench, and stored overnight. (ii) By means of air-blast atomization, an aerosol of spores in ethyl alcohol droplets was introduced into a specially designed chamber [11 × 16 × 22 inches (27.9 × 40.6 × 55.9 cm)] where it was maintained by a fan while the ethyl alcohol evaporated. When the fan was turned off, the spores deposited uniformly on strips placed on the bottom of the chamber. The parameters for achieving the desired deposition were developed empirically. The strips were air-dried for 2 hr in a laminar-flow clean bench. Initial testing showed that the mean level of contamination in the aerosol chamber could be reliably controlled.

In most tests, after drying, half of the strips were placed in a preheated oven (120 C) for 20 min to produce a "worst case" condition by having the spores "baked" onto the surfaces.

For recovery studies involving mechanical agitation, each strip was placed in a 4-oz (113 ml) bottle containing 50 ml of sterile 1% peptone water and mechanically agitated on a Kahn shaker (270 oscillations per min) for 20 min. Depending on the spore concentration used, duplicate 5-ml portions of the peptone water were then plated with Trypticase Soy Agar (TSA, BBL), or the entire 50-ml portion was plated with 50 ml of double-strength TSA. The strip was rinsed gently with sterile distilled water and plated directly by overlaying with molten TSA. Plates were incubated at 32 C for 48 hr.

Ultrasonic tanks (Branson Instruments, Inc., Stamford, Conn.) having power outputs of 150 or 300 w and a frequency of 25 kc/sec were used for insonation studies. The tank fluid was an aqueous solution of 0.3% (v/v) polyoxyethylene sorbitan monooleate (Tween 80). The temperature of the tank fluid was held between 25 and 32 C, and the level was maintained at least 1 inch (2.5 cm) above the level of the liquid in the test bottles. Each strip was placed in a 4-oz bottle containing 50 ml of sterile 1% peptone water and insonated for a 12-min period. Duplicate 5-ml portions were plated with TSA, or the entire 50 ml was plated with 50 ml of double-strength TSA, depending on the spore concentration used. The strip was rinsed gently with sterile distilled water, and plated directly by overlaying with molten TSA. Plates were incubated at 32 C for 48 hr.

An estimate of the number of spores inoculated onto each surface was obtained by comparing the results of plate counts made at the time the strips were inoculated with the sum of the number of spores recovered after insonation and the number remaining on the strip. However, pretreatment of inoculated surfaces with dry heat decreased the viable count 30 to 50%. Consequently, the second method was used for calculating percentage of recovery to maintain uniformity. Bottles containing contaminated strips were placed on the bottom of the tank with the inoculated surface facing the transducers. In some experiments, the inoculated surfaces were intentionally faced away from the transducers.

Several experiments were performed in which the test surfaces (capacitors) were contaminated by human handling. Each series of components was handled by one person, and assays were performed immediately. The components were assayed in the same manner as spore-inoculated strips, except that they were not subjected to a heat treatment.

The relative efficiency of ultrasonic energy and mechanical agitation for eluting microorganisms from cotton swabs was tested. The standard cotton swab (swab rinse) method (1) was employed for removing viable spores from metal surfaces (2 × 2 inches) artificially contaminated with airborne spores of *B. subtilis* var. *niger* in an aerosol chamber.

Similar tests were used to enumerate natural contamination at randomly selected sites on floors of a laboratory. In these tests, insonation time was 12 min, and mechanical agitation time was 10 min.

The observed differences were tested for significance by use of the Student *t* test.

RESULTS

When ultrasonic energy was employed to recover spores from seeded surfaces, efficiency of recovery was proportional to the length of time the surfaces were insonated (Table 1). Values shown in the table represent the mean of five determinations.

Comparisons made between insonation and mechanical agitation showed that significantly more spores were recovered from surfaces of stainless steel and frosted glass by insonation than by mechanical agitation (Table 2). In most cases, the per cent recovery also was significantly higher with insonation than with mechanical agitation.

Significantly more naturally occurring airborne contaminants were recovered from stainless-steel strips by insonation than by mechanical agitation (Table 3).

Table 4 shows the comparative efficiency of in-

TABLE 1. *Comparative recovery of Bacillus subtilis var. niger spores from various surfaces by insonation*

Test	Surface	Pretreatment	Avg no. of microorganisms[a]	Percentage of spores recovered after being insonated for		
				4 min	8 min	12 min
1[b]	Stainless steel	None	5,130	74	90	99
		Heat	2,995	74	91	99
	Glass	None	4,527	93	98	99
		Heat	1,010	58	85	98
	Frosted glass	None	4,678	84	94	99
		Heat	1,713	70	88	97
2[b]	Stainless steel	None	655	77	94	97
		Heat	176	69	89	99
	Glass	None	620	76	91	94
		Heat	336	57	80	83
	Frosted glass	None	698	77	89	95
		Heat	396	74	89	90
3[c]	Stainless steel	None	85	—	—	98
		Heat	18	—	—	95
	Glass	None	67	—	—	99
		Heat	6	—	—	74
	Frosted glass	None	62	—	—	100
		Heat	13	—	—	100

[a] Mean of five samples.
[b] Three consecutive 4-min treatments.
[c] One 12-min treatment.

TABLE 2. *Comparison of recovery of spores of Bacillus subtilis var. niger from various surfaces by mechanical agitation and insonation*

Test	Surface	Crit rion	Pretreat-ment	Mechanical agitation[a]	Insonation[b]	Probability[c]
A	Stainless steel	Microorganisms recovered	None	416[d]	512[d]	<0.001
		Per cent recovery		96.7	99.0	>0.3
	Stainless steel	Microorganisms recovered	Heat	150	193	<0.02
		Per cent recovery		94.9	91.8	>0.3
B	Stainless steel	Microorganisms recovered	None	112	170	<0.001
		Per cent recovery		85.0	99.4	<0.001
	Stainless steel	Microorganisms recovered	Heat	99	136	<0.001
		Per cent recovery		93.7	99.9	<0.001
C	Frosted glass	Microorganisms recovered	None	112	510	<0.001
		Per cent recovery		26.1	94.9	<0.001
	Frosted glass	Microorganisms recovered	Heat	29	317	<0.001
		Per cent recovery		11.5	96.3	<0.001

[a] Twenty-minute treatment.
[b] Twelve-minute treatment.
[c] $P < 0.05$ based on Student t test considered significant.
[d] Each figure represents mean value of 13 samples.

sonation and mechanical agitation in recovering naturally occurring contaminants from electronic components. In absolute numbers, more colony-forming units were obtained with insonation, and per cent recovery was significantly higher.

The importance of the distance between the energy source and the test surfaces for the recovery of spores was indicated by the markedly higher recovery from surfaces in sample bottles placed on the bottom of the ultrasonic tank next to the

TABLE 3. *Recovery of naturally occurring airborne contaminants from stainless-steel strips*

Test	Procedure	Microorganisms recovered[a]	Probability[b]	Per cent recovery	Probability[b]
1	Mechanical agitation[c]	197	<0.001	98.5	<0.001
	Insonation[d]	486		99.6	
2	Mechanical agitation	441	<0.01	99.3	>0.20
	Insonation	859		99.0	

[a] Mean of 13 samples.
[b] $P < 0.05$ based on Student t test considered significant.
[c] Five-minute treatment.
[d] Twelve-minute treatment.

TABLE 4. *Recovery from electronic components of naturally occurring microbial contaminants resulting from human handling*

Test	Procedure	Microorganisms recovered[a]	Probability[b]	Per cent recovery	Probability[b]
1	Mechanical agitation[c]	334	>0.10	98.6	<0.05
	Insonation[d]	461		99.3	
2	Mechanical agitation	32	<0.02	87.0	<0.02
	Insonation	72		96.0	

[a] Mean of 12 samples.
[b] $P < 0.05$ based on Student t test considered significant.
[c] Twenty-minute treatment.
[d] Twelve-minute treatment.

source than from others suspended 2.5 cm above the bottom (Table 5).

The position of the inoculated surface with respect to facing toward (down) or away (up) from the ultrasonic energy source (transducers) also was a critical factor. The number of microorganisms and per cent recovery were significantly higher when the inoculated surface faced the energy source (Table 6).

When the standard cotton swab (10) method was employed for enumerating viable spores, consistently higher numbers were recovered with insonation than with mechanical agitation, and coefficients of variation were lower (Table 7). Similar results were found when swabs were used to enumerate natural contaminants on floors (Table 8).

Three experiments, two with stainless-steel strips and one with smooth glass, compared cold (4 C) rinse fluid with rinse fluid at room temperature (25 C), and revealed that in all cases the per cent recovery of spores in cold rinse fluid was significantly higher than in warm rinse fluid. The average number of spores recovered also was greater in two of three cases. These preliminary findings prompted controlled experiments in which the temperatures of both the rinse fluid and the tank solution were varied. The results with both stainless-steel and smooth glass were in agreement with the preliminary findings (Table 9). With the rinse fluid at 4 C and the tank solution at 25 C, both the average number of spores recovered and the average per cent recovery were higher than with any other combination tested. In most cases, the differences were considered significant.

Suspensions of *Staphylococcus aureus*, *Pseudomonas alcaligenes*, *Escherichia coli*, and natural microbial contaminants resulting from hand-

TABLE 5. *Comparative recovery of Bacillus subtilis var. niger spores from surfaces placed onto or suspended from the ultrasonic tank bottom*

Surface	Pretreatment	Microorganisms recovered[a]		Probability[d]	Per cent recovery		Probability[d]
		Suspended[b]	Nonsuspended[c]		Suspended	Nonsuspended	
Stainless steel	None	248	392	<0.001	77	97	<0.02
	Heat	111	238	<0.01	54	96	<0.01
Glass	None	274	372	>0.1	74	92	>0.1
	Heat	176	236	<0.05	67	98	<0.001

[a] Mean of five samples.
[b] Strip bottles suspended 1 inch from tank bottom.
[c] Strip bottles resting on tank bottom.
[d] $P < 0.05$ based on Student t test considered significant.

TABLE 6. *Recovery of microbial contaminants by insonation from stainless-steel strips in two positions*

Test	Criterion	Position		Probability[c]
		Up[a]	Down[b]	
1[d]	Microorganisms recovered	382[f]	591[f]	<0.01
	Per cent recovery	99.4	99.9	<0.01
2[e]	Microorganisms recovered	472	540	<0.02
	Per cent recovery	82	88	<0.01

[a] Contaminated surface facing away from source of ultrasonic energy.

[b] Contaminated surface facing source of ultrasonic energy.

[c] $P < 0.05$ based on Student t test considered significant.

[d] Naturally occurring airborne contaminants collected on 1 × 2 inch stainless-steel strips.

[e] Aerosols of *Bacillus subtilis* var. *niger* spores air-dried on 1 × 2 inch stainless steel strips.

[f] Each figure represents mean of 13 samples.

TABLE 7. *Comparative techniques for recovering spores of Bacillus subtilis var. niger from cotton swabs*

Test	Procedure	Spores recovered per 4 inch^2 [a]	Coefficient of variation (%)	Probability[b]
1	Mechanical agitation	4,376	9.8	<0.001
	Insonation	5,648	8.1	
2	Mechanical agitation	3,640	22.0	>0.10
	Insonation	4,188	4.1	
3	Mechanical agitation	604	19.5	<0.001
	Insonation	856	7.2	
4	Mechanical agitation	392	35.6	<0.02
	Insonation	584	10.1	

[a] Mean of six samples.

[b] $P < 0.05$ based on Student t test considered significant.

washings were insonated for up to 24 min to determine whether the ultrasonic treatments were detrimental to microorganisms. Figure 1 shows that no reduction occurred under the conditions employed, indicating that ultrasonic treatments had no lethal effect on the bacterial cells. In some cases, a slight increase in count occurred, probably due to clumps of cells being broken into smaller aggregates.

DISCUSSION

Ultrasonic energy can be employed effectively to remove microbial contaminants from surfaces for enumeration. Recovery from surfaces with high or low levels of contamination was accurate and reliable (Table 1).

More microorganisms were consistently recovered by insonation from surfaces of stainless steel and frosted glass than by mechanical agitation. Similar findings were observed when electronic components were used. This phenomenon was attributed to clumps of microorganisms being broken into smaller aggregates of cells. Therefore, the values derived from insonated samples more accurately represent the actual number of microorganisms. When per cent recovery rather than total microorganisms recovered was used as the criterion for comparison, insonation in most tests showed significantly higher per cent recoveries than did mechanical agitation. In no instances were significantly more microorganisms recovered by mechanical agitation than by insonation.

Highest recovery rates were obtained when the contaminated surfaces faced the ultrasonic energy source. These results confirm earlier observations with an ultrasonic probe (12).

Although the temperature of the rinse fluid in the sample bottle appeared to influence recovery rates, the reason that more microorganisms were recovered when the rinse fluid was 4 C and tank solution was 25 C than with any other temperature combination cannot be explained, but there is a practical application for the phenomenon. In

TABLE 8. *Comparative techniques for recovering microorganisms from cotton swabs*

Test	Procedure	Microorganisms recovered per 4 inch^2 [a]	Coefficient of variation (%)	Probability[b]
1	Mechanical agitation	132[c]	73.0	<0.001
	Insonation	284	70.7	
2	Mechanical agitation	72	87.2	>0.5
	Insonation	84	54.8	

[a] Natural contamination on floor of laboratory.

[b] $P < 0.05$ based on Student t test considered significant.

[c] Each figure represents mean value of 20 samples.

TABLE 9. *Effects of varying temperatures of rinse fluid and ultrasonic tank solution on recovery of spores of Bacillus subtilis var. niger from surfaces*

Surface	Temp of rinse fluid (C)	Temp of tank solution (C)	Avg no. of spores recovered[a]	Probability[b]	Avg per cent recovery	Probability[b]
Stainless steel	4	25	416	—	92.1	—
	4	4	318	<0.001	79.7	<0.01
	25	25	394	>0.20	89.5	>0.20
	25	4	316	<0.001	82.0	<0.001
Smooth glass	4	25	389	—	94.9	—
	4	4	354	>0.05	85.6	<0.01
	25	25	350	<0.01	88.6	<0.02
	25	4	354	<0.05	89.2	>0.05

[a] Mean of 15 samples.

[b] Probability factors are for comparison with rinse fluid at 4 C and ultrasonic tank solution at 25 C. $P < 0.05$ based on Student t test considered significant.

assessing levels of microbial contamination on spacecraft or on stainless-steel strips exposed to spacecraft assembly and test areas, assays are made for bacterial spores. This is done by heating the rinse fluid from the sample to a temperature high enough to kill vegetative microorganisms, but not spores. When a large number of samples is being assayed, some may remain at room temperature for 30 to 40 min before being heat-shocked, and during this time it is probable that some of the spores germinate and lose their heat resistance. This could reduce the number of colony-forming units and thus affect the accuracy of the assay. Since the germination of spores is retarded by low temperatures, and higher recoveries were obtained when cold rinse fluid was used, this should serve not only to improve the overall efficiency of the assay but also to increase the sensitivity of the technique for enumerating bacterial spores.

Although ultrasonic energy appeared to be an efficient recovery technique, investigation needs to be done in other areas; e.g., a problem which must be resolved is a method by which ultrasonic units used in environmental microbiology can be standardized. Three possible ways would be by microbiological, chemical, or physical tests. Ideally, a physical method would be best, since it would eliminate sampling variations that may occur in a microbiological system, or differences between technicians using a chemical method. Chemical (*Cavitation Activity Measuring Procedure*, Ultrasonic Manufacturers Association, Inc., New Rochelle, N.Y.) and physical methods (cavitation meters) are available which use cavitation activity as the basis for the test, but both leave much to be desired with respect to reproducibility. Studies are presently in progress to evaluate these methods and to determine whether removal of microorganisms from surfaces can be correlated to a standard measurement of cavitation activity.

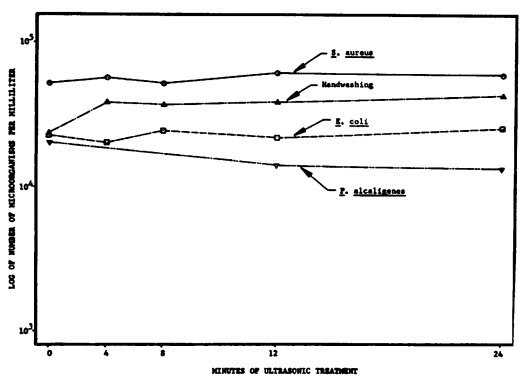

FIG. 1. *Effect of ultrasonic energy on suspensions of Staphylococcus aureus, Pseudomonas alcaligenes, Escherichia coli, and natural contamination resulting from hand washings.*

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