Use of a Mixture of Surrogates for Infectious Bioagents in a Standard Approach to Assessing Disinfection of Environmental Surfaces[▽]

Safaa Sabbah, Susan Springthorpe, and Syed A. Sattar*

Centre for Research on Environmental Microbiology, Faculty of Medicine, University of Ottawa,
 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5

Received 29 January 2010/Accepted 5 July 2010

We used a mixture of surrogates (*Acinetobacter baumannii*, *Mycobacterium terrae*, hepatitis A virus, and spores of *Geobacillus stearothermophilus*) for bioagents in a standardized approach to test environmental surface disinfectants. Each carrier containing 10 μ l of mixture received 50 μ l of a test chemical or saline at $22 \pm 2^\circ\text{C}$. Disinfectant efficacy criteria were $\geq 6 \log_{10}$ reduction for the bacteria and the spores and $\geq 3 \log_{10}$ reduction for the virus. Peracetic acid (1,000 ppm) was effective in 5 min against the two bacteria and the spores but not against the virus. Chlorine dioxide (CD; 500 and 1,000 ppm) and domestic bleach (DB; 2,500, 3,500, and 5,000 ppm) were effective in 5 min, except for sporicidal activity, which needed 20 min of contact with either 1,000 ppm of CD or the two higher concentrations of DB.

Disinfectant testing with a single type of organism does not represent field conditions, where bioagents or other pathogens may be mixed with other contaminants. Such an approach also cannot predict the true spectrum of microbicidal activity of a given chemical, while the identity of the target pathogen(s) is often unknown. We used a mixture of *Acinetobacter baumannii*, *Mycobacterium terrae* (15), hepatitis A virus (HAV) (4),

and the spores of *Geobacillus stearothermophilus* as surrogates for infectious bioagents, with an added soil load on disks (1 cm in diameter; 0.75 mm thick) of brushed stainless steel (AISI no. 430; Muzeen & Blythe, Winnipeg, MB, Canada), to better simulate environmental surface disinfection (1, 11). Table 1 gives details on the microbial strains, media used for their culture and recovery, and methods for preparing working

TABLE 1. Organisms in the mixture and their growth/recovery media and titers

Organism (ATCC no.)	Growth/recovery medium or host cell line	Procedure for culture and prepn of stock	Viability titer in stock
<i>Mycobacterium terrae</i> pBEN genetically modified in-house (ATCC 15755)	Middlebrook 7H11 agar, OADC, ^a and kanamycin (10 μ g/ml); incubation 20 days at $36 \pm 1^\circ\text{C}$	7H9 broth with ADC ^b and glycerol; cells washed and resuspended in deionized water (8 ml) in a Bixoux bottle (Wheaton, Millville, NJ) with glass beads (Sigma-Aldrich; 3 mm in diam; catalog no. Z143928) and stored at 4°C	3.7×10^9 CFU/ml
<i>Geobacillus stearothermophilus</i> (ATCC 12980)	Trypticase soy agar plates incubated at 56°C for 48 h	Spores heat shocked at 100°C for 45 min, washed in deionized H ₂ O, and stored at 4°C	1.5×10^8 CFU/ml
<i>Acinetobacter baumannii</i> (ATCC 19606)	Trypticase soy agar plates incubated at $36 \pm 1^\circ\text{C}$ for 24 h	Inoculated into Trypticase soy broth and incubated for 24 h at $36 \pm 1^\circ\text{C}$, broth centrifuged, and pellet resuspended in deionized H ₂ O and stored at 4°C	1.2×10^9 CFU/ml
Hepatitis A virus (ATCC VR-1402)	FRhK-4 cells (CRL-1688) infected and incubated for 6 days	Cells grown in MEM ^c with 7% (vol/vol) fetal bovine serum (Fisher; M33-500) and 1% nonessential amino acids (Gibco; 11140) at $36 \pm 1^\circ\text{C}$, monolayers infected and incubated at $36 \pm 1^\circ\text{C}$ for 7 days in medium with no antibiotics, flasks frozen and thawed (thrice), cell lysate centrifuged, and supernatant aliquoted for storage at -80°C	8×10^8 PFU/ml

^a OADC, oleic acid-albumin-dextrose-catalase.

^b ADC, albumin dextrose-catalase.

^c MEM, minimal essential medium.

* Corresponding author. Mailing address: Centre for Research on Environmental Microbiology, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5. Phone: (613) 562-5800, ext. 8314. Fax: (613) 562-5452. E-mail: ssattar@uottawa.ca.

[▽] Published ahead of print on 16 July 2010.

stocks. The quantitative carrier test (QCT) method, ASTM standard E-2197 (1), was used to test the organisms singly and in a mixture. Each 200 μ l of the inoculum contained 34 μ l each of the four organisms, 40 μ l of bovine mucin, 14 μ l of yeast extract, and 10 μ l of bovine serum albumin stocks.

Disinfectants tested were peracetic acid (PAA; 500 and 1,000 ppm), chlorine dioxide (CD; 500 and 1,000 ppm), and domestic bleach (DB; 2,500, 3,300, and 5,000 ppm). Buffered saline (pH 7.2) was the control fluid, eluent, and diluent. Hard water (400 ppm CaCO_3) was the diluent for disinfectants (1).

Each disk received 10 μ l of the inoculum, dried and covered with 50 μ l of test substance, or saline at $22 \pm 2^\circ\text{C}$. At the end of the contact time, each disk was eluted in a neutralizer and the eluates were assayed (1, 9, 11, 12). The neutralizer consisted of 1% dextrose (Difco), 0.7% lecithin (Alfa Aesar), 0.25% sodium bisulfite (J. T. Baker), 0.1% sodium thioglycolate (Sigma), 0.6% sodium thiosulfate (Analar), 0.2% L-cysteine (Sigma), 0.5% tryptone (Oxoid), and 0.1% Tween 80 (Bioshop) in buffered saline (pH 7.2). In each experiment, three control and three test carriers were used, and all experiments were repeated thrice. The performance criteria for the tested substances were $\geq 3.0 \log_{10}$ reduction in PFU of the virus and $\geq 6.0 \log_{10}$ reductions in the CFU for the other three organisms. When the mixture of test organisms was used, the components were separated by first passing the mixture through a membrane filter (0.22- μm pore diameter) to retain all the organisms except the virus. The filtrate was subjected to plaque assays for HAV in FRhK-4 cells. For the three bacteria, separate filters were placed on appropriate agar plates (Table 1) and incubated.

The data for 5-min contact are given in Table 2. All levels of the disinfectants tested met the criterion for *M. terrae* and *A. baumannii* when tested individually or in mixture. Only 1,000 ppm of PAA was effective against the spores. Both levels of PAA were ineffective against HAV, while the other disinfectants could reduce its titer between 3.5 and 4 $\log_{10}$. Only 1,000 ppm of PAA could consistently meet the criterion for sporidicidal activity after 10 min (data not shown). Extending the contact time to 20 min allowed both levels of PAA and DB to meet the criterion for sporidicidal activity, while 500 ppm of CD failed to do so; CD at 1,000 ppm barely met the criterion when tested alone against the spores but could not do so in the mixture (Fig. 1).

The study showed the feasibility of testing liquid chemicals against a mixture of suitable surrogates for infectious bioagents. This approach allowed standardized and simultaneous assessment of the spectrum of microbicidal activities of the test formulations under identical conditions that better simulate field conditions and that can be readily adapted to test foams and gaseous chemicals on other carrier materials. The surrogates selected covered the spectrum of microbicide resistances of all currently known classes of infectious bioagents.

A. baumannii is among the more environmentally stable and microbicide-resistant vegetative bacteria known (7, 13). *M. terrae* represented pathogens with generally higher resistance to microbicides (3) and possibly drug-resistant *Mycobacterium tuberculosis* and category C agents (6). HAV, a small, nonenveloped virus known for its stability and microbicide resistance (9), represented select agents (CBW, biological weapons classification, 2001 [http://www.selectagents.gov/Select%20Agents%20and%20Toxins%20

TABLE 2. Reductions by the test formulations in 5 min at $22 \pm 2^\circ\text{C}$ when tested against each organism individually and in a mixture

Disinfectant (concn [ppm])	<i>M. terrae</i>		<i>A. baumannii</i>		<i>G. steuthornophilus</i>		Hepatitis A virus	
	Individual	Mixture	Individual	Mixture	Individual	Mixture	Individual	Mixture
Peracetic acid (500)	8.18 $\pm$ 0.19	7.33 $\pm$ 0.16	7.19 $\pm$ 0.03	6.33 $\pm$ 0.03	4.03 $\pm$ 0.08	4.45 $\pm$ 0.98	Not tested	0.30 $\pm$ 0.01
Peracetic acid (1,000)	8.18 $\pm$ 0.19	7.33 $\pm$ 0.16	7.19 $\pm$ 0.03	6.33 $\pm$ 0.03	8.03 $\pm$ 0.28	7.21 $\pm$ 0.59	0.58 $\pm$ 0.22	0.68 $\pm$ 0.09
Chlorine dioxide (500)	8.18 $\pm$ 0.19	7.72 $\pm$ 0.21	7.22 $\pm$ 0.03	6.37 $\pm$ 0.13	1.47 $\pm$ 0.45	0.69 $\pm$ 0.05	4.30 $\pm$ 0.18	3.97 $\pm$ 0.19
Chlorine dioxide (1,000)	8.18 $\pm$ 0.19	7.72 $\pm$ 0.21	7.22 $\pm$ 0.03	6.37 $\pm$ 0.13	3.07 $\pm$ 0.09	1.27 $\pm$ 0.05	4.30 $\pm$ 0.18	3.97 $\pm$ 0.19
Domestic bleach (2,500)	8.18 $\pm$ 0.19	7.72 $\pm$ 0.21	7.22 $\pm$ 0.03	6.37 $\pm$ 0.13	0.27 $\pm$ 0.03	0.25 $\pm$ 0.02	4.41 $\pm$ 0.23	3.97 $\pm$ 0.29
Domestic bleach (3,500)	8.18 $\pm$ 0.19	7.72 $\pm$ 0.21	7.22 $\pm$ 0.03	6.37 $\pm$ 0.13	0.27 $\pm$ 0.03	0.25 $\pm$ 0.02	4.41 $\pm$ 0.23	3.45 $\pm$ 0.09
Domestic bleach (5,000)	8.18 $\pm$ 0.19	7.72 $\pm$ 0.21	7.22 $\pm$ 0.03	6.37 $\pm$ 0.13	0.28 $\pm$ 0.01	0.25 $\pm$ 0.02	4.41 $\pm$ 0.23	3.97 $\pm$ 0.29

FIG. 1. Reductions of *G. stearothermophilus* spores by the test formulations after 20 min of contact, individually and in a mixture at $22 \pm 2^\circ\text{C}$.

List.html]) and also food- and waterborne pathogens listed as biothreats (2, 10). The spores of *G. stearothermophilus* may be more resistant to oxidizing chemicals than the spores of *Bacillus anthracis* (8); their thermophilic nature made them safer to handle and easy to separate from the mixtures.

The disinfectants were selected for their commercial availability and broad-spectrum and relatively rapid action (5, 14). The last criterion excluded all but oxidizers because other common active agents are limited as microbicides and/or require hours of contact for sporicidal action.

For PAA tests, the recovery of infectious HAV in the absence of any viable spores is somewhat anomalous but not surprising. While we do not believe HAV to be more resistant than bacterial spores, the small size of the virus in the dried inocula likely afforded it significant protection. Compared to HAV, the mycobacterium proved more susceptible to all the disinfectants tested. This highlights a serious weakness in the traditional rankings of disinfectant susceptibility, where mycobacteria are often considered more resistant than nonenveloped viruses (5, 14).

In the initial trials with the mixtures, the titer of *A. baumannii* dropped sharply; using virus pools without antibiotics resolved the issue. The ability of *A. baumannii* to grow on 7H11 agar and thus interfere with the recovery of *M. terrae* was addressed by replacing the standard strain of *M. terrae* with one containing a kanamycin resistance gene (15). Incorporation of enough kanamycin in 7H11 suppressed the growth of *A. baumannii* while allowing the mycobacterium to grow.

Using a mixture of surrogates in QCT not only proved fea-

sible but also highlighted the need to review certain long-held concepts about the relative sensitivities of classes of pathogens to disinfectants. The details reported should allow extension of the work to CL-3 and possibly CL-4 agents to confirm that the results obtained with the carefully chosen surrogates are indeed applicable to various classes of infectious bioagents.

This study was supported by a grant (04-00118RD) from the CRTI program of Defense R&D Canada.

REFERENCES

1. ASTM. 2009. Standard quantitative disk carrier test method to determine the bactericidal, virucidal, fungicidal, mycobactericidal and sporicidal activities of liquid chemical germicides; standard E-2197. Annual book of ASTM standards. ASTM International, West Conshohocken, PA.
2. Boone, S. A., and C. P. Gerba. 2007. Significance of fomites in the spread of respiratory and enteric viral diseases. *Appl. Environ. Microbiol.* **73**:1687–1696.
3. Falkinham, J. O., III. 2009. Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. *J. Appl. Microbiol.* **107**:356–367.
4. Mbithi, J. N., V. S. Springthorpe, and S. A. Sattar. 1990. Chemical disinfection of hepatitis A virus on environmental surfaces. *Appl. Environ. Microbiol.* **56**:3601–3604.
5. McDonnell, G., and A. D. Russell. 1999. Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev.* **12**:147–179.
6. Morse, S. A., R. B. Kellogg, S. Perry, R. F. Meyer, D. Bray, D. Nicholson, and J. M. Miller. 2003. Detecting biothreat agents: the laboratory response network. *ASM News* **69**:433–437.
7. Peleg, A. Y., H. Seifert, and D. L. Paterson. 2008. *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin. Microbiol. Rev.* **21**:538–582.
8. Rogers, J. V., C. L. K. Sabourin, Y. W. Chol, W. R. Richter, D. C. Rudnicki, K. B. Riggs, M. L. Taylor, and J. Chang. 2005. Decontamination assessment of *Bacillus anthracis*, *Bacillus subtilis*, and *Geobacillus stearothermophilus* spores on indoor surfaces using a hydrogen peroxide gas generator. *J. Appl. Microbiol.* **99**:739–748.
9. Sattar, S. A., V. S. Springthorpe, O. Adegbenrin, A. A. Zafer, and M. Busa. 2003. A disc-based quantitative carrier test method to assess the virucidal activity of chemical germicides. *J. Virol. Methods* **112**:3–12.
10. Sattar, S. A. 2007. Hierarchy of susceptibility of viruses to environmental surface disinfectants: a predictor of activity against new and emerging viral pathogens. *J. AOAC Int.* **90**:1655–1658.
11. Springthorpe, V. S., and S. A. Sattar. 2003. Quantitative carrier tests (QCT) to assess the germicidal activities of chemicals: rationale and procedures. Centre for Research on Environmental Microbiology, Ottawa, ON, Canada.
12. Springthorpe, V. S., and S. A. Sattar. 2005. Carrier tests to assess microbicidal activities of chemical disinfectants for use on medical devices and environmental surfaces. *J. AOAC Int.* **88**:182–201.
13. van den Broek, P. J., T. J. K. van der Reijden, E. van Strijen, A. V. Helmig-Schurter, A. T. Bernards, and L. Dijkshoorn. 2009. Endemic and epidemic *Acinetobacter* species in a university hospital: an 8-year survey. *J. Clin. Microbiol.* **47**:3593–3599.
14. Weber, D. J., and W. A. Rutala. 2006. Use of germicides in the home and the healthcare setting: is there a relationship between germicide use and antibiotic resistance? *Infect. Control Hosp. Epidemiol.* **27**:1107–1119.
15. Zafer, A. A., Y. E. Taylor, and S. A. Sattar. 2001. Rapid screening method for mycobactericidal activity of chemical germicides that uses *Mycobacterium terrae* expressing a green fluorescent protein gene. *Appl. Environ. Microbiol.* **67**:1239–1245.