Effectiveness of Five-day-old 10% Bleach in a Student Microbiology Laboratory Setting

NANCY GOODYEAR

ABSTRACT

In the student laboratory, 10% bleach (sodium hypochlorite) is used to disinfect benches before and after work, and when a spill occurs. Centers for Disease Control and Prevention (CDC) Guidelines recommend diluting bleach daily. In a student laboratory setting, the organisms in use are known and may include only selected standard bacteria. Diluting bleach daily is time consuming and wastes bleach. We hypothesized that 10% bleach stored in low density polyethylene (LDPE) wash bottles would maintain sufficient chlorine concentration to be effective against the organisms used in the student laboratory for five days, so that bleach could be diluted weekly instead of daily. Approximately 3 x 10^6 CFU of each bacterium were spotted to a laboratory bench surface in duplicate and allowed to air dry. One spot was individually cleaned with five-day old 10% bleach following the same protocol as student laboratories. The second spot was uncleaned and sampled as a control. Contact plates containing D/E Neutralizing agar were touched to the spots, incubated overnight at 35°C and examined for growth. An uninoculated spot was also sampled as a background control. A total of 22 different organisms were tested, representing the major groups of organisms used in the student laboratories. All organisms tested were eliminated by the five-day old bleach. All uncleaned spots showed dense growth. The background control had no growth. Reducing the dilution of bleach to once a week rather than daily will save time and money, which can then be devoted to more teaching and curriculum responsibilities, while still maintaining laboratory safety.

ABBREVIATIONS

LDPE-low density polyethylene, CDC-Centers for Disease Control and Prevention, CFU-colony forming units, TSA-tryptic soy agar, TSB-tryptic soy broth

INDEX TERMS

Disinfection, education, microbiology, sodium hypochlorite

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Nancy Goodyear, PhD, MLS(ASCP)^{CM}, University of Massachusetts Lowell, Lowell, MA

Address for Correspondence: Nancy Goodyear, PhD, MLS(ASCP)^{CM}, 3 Solomont Way, Suite 4, University of Massachusetts Lowell, Lowell, MA 01854, 978-934-4427, Nancy_Goodyear@uml.edu

INTRODUCTION

Sodium hypochlorite, a chlorine-releasing agent, is a very common disinfectant in healthcare and laboratory settings and has a broad range of antimicrobial activity.[1,2] The Centers for Disease Control and Prevention (CDC) Guideline for Disinfection and Sterilization in Healthcare Facilities recommends preparing 10% bleach daily in order to ensure adequate disinfection of infectious agents.[1] This is standard practice in clinical settings; however, this recommendation is based on the need to kill all potential pathogens, including non-enveloped viruses, in patient care facilities. In the student laboratory setting only known bacteria and yeast are in use, and there may not be any patient samples in use.

The Department of Clinical Laboratory and Nutritional Sciences at the University of Massachusetts Lowell offers multiple laboratory sections of Basic and Clinical Microbiology and Pathology (a required course for students majoring in Medical Laboratory Science, Clinical Sciences, Nutritional Sciences, Nursing, Community Health, and Environmental Health), and Medical Microbiology, required for Medical Laboratory Science majors. In the fall semester of 2011 there were seven sections of Basic and Clinical Microbiology enrolling more than 100 students. In the spring semester of 2012 there were two sections of Basic and Clinical Microbiology and Pathology plus two sections

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of Medical Microbiology.

For all laboratory sections, laboratory benches are disinfected with 10% bleach before and after work, and when spills occur. Diluted bleach is stored in translucent laboratory wash bottles on the bench tops for easy access by students. Student workers, under the oversight of staff, are responsible for refilling the bottles with diluted bleach. If bleach is diluted daily, leftover bleach in the wash bottles must be discarded. This process is time consuming and wastes bleach.

In addition, bleach is a well-known respiratory irritant and is strongly linked to occupational asthma in healthcare workers, even with exposures limited to once or twice a week.[3,4] The process of diluting bleach daily exposes staff or students repeatedly, increasing the risk of asthma or other respiratory irritation.

Rutala, et al[5] showed that bleach diluted to 5% in tap water at pH 8, stored for 30 days at room temperature in polyethylene wash bottles contained 83% of the original free chlorine levels. The remaining free chlorine was adequate to kill *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella choleraesuis*.

Our hypothesis was that five-day old 10% bleach stored at room temperature in polyethylene wash bottles would effectively kill the bacteria used in the student labs, using the standard student laboratory procedure for disinfecting bench surfaces.

MATERIALS AND METHODS

Materials and Bacterial Cultures

Clorox® Regular Bleach (The Clorox Company, Oakland, CA) was purchased from a local retailer and diluted to 10% with tap water five days before the experiments were performed. The diluted bleach was stored in a translucent low density polyethylene wash bottle (Thermo Fisher Scientific, Inc, Pittsburgh, PA) at room temperature as it would be in the student laboratory.

Bacterial stock strains were obtained from the American Type Culture Collection (Manassas, VA) or from Lowell General Hospital, Lowell, MA (see Table 1). Stock cultures were maintained on TSA (Difco™ Tryptic Soy Agar, Becton, Dickinson and Co, Sparks, MD) plates. Overnight growth in TSB (Bacto™ Tryptic Soy Broth, Becton, Dickinson and Co, Sparks, MD) was used for bench inoculation for most organisms. *Streptococcus gallolyticus* and *Pasteurella multocida* were grown in TSB supplemented with 10% bovine calf serum (CELLect® Calf Bovine Serum, MP Biomedicals, LLC, Solon OH). The quantity of bacteria used was estimated using a Thermo Spectronic 20D+ spectrophotometer (Thermo Spectronic, Rochester, NY). D/E Neutralizing agar (Difco™ D/E Neutralizing Agar, Becton, Dickinson and Co, Sparks, MD) was used to determine growth after cleaning bench surfaces with 10% bleach.

Disinfection Testing Procedure

The procedure was modified from standard surface disinfection protocols.[6,7] In brief, a clean laboratory bench surface was rinsed with water to remove any remaining bleach or other disinfectant and sectioned into four inch x four inch squares using laboratory tape. An overnight broth culture of each bacterium was standardized spectrophotometrically to a 1 McFarland (0.257 A, ~3 x 10^8 CFU/mL). Approximately 3 x 10^6 CFU of each bacterium in 10 μL were spotted to a laboratory bench surface in duplicate and allowed to air dry. One spot per bacterium was individually cleaned with five-day old 10% bleach following the student laboratory protocol (bleach squirted onto surface, wiped with a paper towel and allowed to air dry). The second spot was uncleaned and was sampled as a positive control. Contact plates containing D/E Neutralizing agar were touched to the spots, incubated overnight at 35°C in either air or CO_2 and examined for growth. An uninoculated spot was also sampled as a background control.

Neutralization Test Procedure

Neutralization tests were performed to confirm that each organism would grow on D/E Agar and to confirm that the disinfecting activity of the bleach was neutralized and did not continue to kill or inhibit organisms after the contact time on the bench had ended. The procedure followed was modified from a standard disinfectant neutralization protocol.[8] In brief, the laboratory bench was prepared as described above, but no bacteria were spotted. After treating the bench surface with bleach, D/E plates were touched to the surface. Each organism was subcultured to the D/E plate after it was touched to the treated laboratory bench. The plates were incubated overnight and

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examined for growth. The presence of growth indicated the neutralization of the bleach, allowing the organism to grow. This also confirmed the ability of each organism to grow on D/E agar, which does not support the growth of some fastidious organisms such as *Neisseria gonorrohoeae*.

RESULTS

All organisms listed in Table 1 were eliminated by the five-day old bleach. All uncleaned spots showed dense growth. The background control had no growth. The neutralization tests showed that the D/E agar effectively neutralized the activity of bleach and that each of the organisms listed was able to grow on the D/E agar.

Table 1. Organisms tested and results.

Organism	ATCC #	Uncleaned CFU	Cleaned CFU
Staphylococcus aureus	6538	TNTC	0
Staphylococcus epidermidis	12228	TNTC	0
Rothia mucilaginosa	20258	TNTC	0
Enterococcus faecalis	29212	TNTC	0
Streptococcus pyogenes	19615	TNTC	0
*Streptococcus gallolyticus**	49147	TNTC	0
Escherichia coli	29214	TNTC	0
*Klebsiella pneumoniae**	13883	TNTC	0
Enterobacter aerogenes	13048	TNTC	0
Proteus vulgaris	49132	TNTC	0
Serratia marcescens	8100	TNTC	0
Salmonella enteriditis	13076	TNTC	0
Shigella sonnei	9290	TNTC	0
Alcaligenes faecalis	35655	TNTC	0
Pseudomonas aeruginosa	9027	TNTC	0
*Pasteurella multocida**	12945	TNTC	0
*Listeria monocytogenes**	7644	TNTC	0
Bacillus subtilis	6633	TNTC	0
Saccharomyces cerevisiae	2601	TNTC	0
Candida albicans	10231	TNTC	0
Nocardia asteroides	19247	TNTC	0
Mycobacterium smegmatis	14468	TNTC	0

ATCC: American Type Culture Collection; CFU: Colony Forming Units; TNTC: Too Numerous To Count

* Organisms kindly provided by the microbiology laboratory at Lowell General Hospital, Lowell, MA.

DISCUSSION

In patient care settings, 10% bleach is commonly used for cleaning and disinfection. Diluting bleach daily is appropriate in direct patient care settings because any infectious agent may be present, including those that are most resistant to disinfection such as non-enveloped viruses and mycobacteria. The highest level of disinfection is required. This practice is also commonly followed in settings such as the student laboratory where there are no patient samples and the infectious agents in use are well known. In student laboratories, only a small volume of diluted bleach may be used each day, but it is difficult to dilute the exact amount needed. This results in the disposal of leftover one-day old bleach as bottles are emptied and refilled. While bleach is not generally expensive, student laboratories budgets are small and any cost savings is helpful. In addition, there is a labor cost in gathering bleach bottles, emptying them, preparing a fresh dilution, refilling the bottles, and redistributing them to the laboratories.

The stability of diluted bleach depends on a number of factors: pH, exposure to light, initial concentration, and the presence of organic soil such as blood.[1,5] Organic soil such as blood decreases effectiveness due to chemical reactions with the free chlorine and/or the creation of a physical barrier between the organism and the disinfectant. In a student microbiology laboratory the most likely organic soil that would be mixed with organisms in a spill would be liquid culture media or a reagent such as rabbit plasma. Human blood contamination is possible in the case of a student injury, but less likely. These experiments were performed using bacteria grown in liquid media, modeling a real student laboratory situation.

Another factor in effectiveness is contact time. The CDC guidelines recommend a wet contact time of at least 10 minutes.[1] Wet contact time refers to the amount of time the surface remains visibly wet. However, for routine cleaning of benches in the student laboratory before and after work, students squirt an unmeasured quantity of bleach onto the bench surface, wipe the surface with a paper towel, and leave. The benchtops will dry well before 10 minutes has passed, usually within one minute. Dried bleach residue remains on the surface, but this is not in compliance with the recommended wet contact time.

The active form of diluted bleach is hypochlorous acid (HOCl); the concentration is generally measured as free available chlorine. Hypochlorous acid breaks down rapidly into hypochlorite ion (OCl^-), which has little antimicrobial activity.[2,9] This reaction occurs most rapidly at high pH (>9). The mechanism of action of sodium hypochlorite is not fully understood. It is

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believed that chlorine inhibits specific biochemical pathways and causes protein denaturation and modification of amino acids within the bacterial cell, which ultimately destroys it.[1,2] Some research has shown that chlorine-releasing agents significantly damage bacterial DNA.[10]

Undiluted household bleach contains between 5.25% (52,500 ppm) and 6.15% (61,500 ppm) sodium hypochlorite. Therefore 10% bleach will contain at least 5,000 ppm free available chlorine. In the absence of organic soil, diluted bleach will kill organisms at as low as five ppm free available chlorine for many vegetative bacteria, and as high as 5,000 ppm for *Clostridium difficile* spores (10 minute contact time).[1,2] Rutala et al[5] showed that after 30 days, a 5% dilution of bleach stored at room temperature in similar containers retains 83% of free available chorine, while a 50% dilution retained 47%. (In Rutala's study a 10% bleach dilution was not tested.) If a 10% dilution starts at 5,000 ppm, even if only 47% is retained, more than 2,000 ppm will remain, well beyond the level necessary to kill typical bacteria and yeast used in a student laboratory.[1]

An alternative to liquid bleach sometimes used in clinical settings is pre-packaged bleach wipes. The bleach percentage in these wipes is generally less than 1%, while still maintaining a minimum 5,000 ppm. In addition, stabilizers are added to preserve the effectiveness of the bleach for a longer period of time. The manufacturer's stated wet contact time for common bacteria is 30 seconds; the contact time for *Candida albicans* and *Mycobacterium bovis* is three minutes.[11] One limitation of pre-packaged wipes is that if the container is not closed properly or they are not used quickly enough the wipes could dry out, resulting in insufficient wet contact time. Although a viable alternative, the cost of pre-packaged wipes is likely prohibitive in a student laboratory when compared to the cost of household bleach.

In our experiments, each organism tested grew on the D/E agar and was completely eliminated by the five-day old bleach. D/E agar is a standard medium known to neutralize the activity of sodium hypochlorite. This medium is used to ensure that the activity of the bleach did not continue during the incubation period. The organisms tested were chosen from the list of organisms used in the Basic and Clinical Microbiology and Pathology and Medical Microbiology laboratories. The range of organisms tested included representatives of most major groups of organisms: Gram-positive cocci in chains and clusters, enteric Gram-negative rods, non-fermenting Gram-negative rods, fastidious Gram-negative rods, Gram-positive rods, yeast, and mycobacteria.

A limitation of this study is that additional organisms that may be used in a student laboratory, such as *Neisseria gonorrhoeae* and *Haemophilus influenzae*, will not grow on D/E agar and therefore couldn't be tested with this experimental design. In addition, organic soils such as blood or other liquid media were not tested, only TSB. If patient samples or other organisms are in use, or for clean up of spills, freshly diluted bleach may be more appropriate.

Reducing the dilution of bleach to once a week rather than daily will save time and money, which can then be devoted to more teaching and curriculum responsibilities, while still maintaining laboratory safety. We have shown that a wide range of organisms commonly used in student laboratories are killed by 10% bleach that has been stored in translucent wash bottles for five days following routine bench cleaning procedures.

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Search Committee Chair
Department of Medical Laboratory and Radiation Science
University of Vermont
302 Rowell, 106 Carrigan Drive
Burlington, VT 05405
paula.deming@uvm.edu
(802) 656-2506

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