ABSTRACT Five disinfectant chemicals were tested individually for effectiveness against low pathogenic avian influenza virus (LPAIV), A/H7N2/Chick/MinhMa/04, on hard, nonporous surfaces. The tested agents included acetic acid, calcium hydroxide, sodium carbonate, sodium hydroxide, and a powdered laundry detergent without bleach. Multiple common chemicals including acetic acid (1 and 3%), sodium hydroxide (2%), and calcium hydroxide (1%) effectively inactivated LPAIV on a metal surface. The laundry detergent without bleach, sodium carbonate (4%), and the lower concentration of sodium hydroxide (1%) were not able to consistently inactivate LPAIV on hard, nonporous surfaces.

INTRODUCTION

The risk of an avian disease outbreak is always a concern for the poultry industry. Highly pathogenic avian influenza virus (AIV) H5N1 emerged in Hong Kong in 1996 and 1997. Between 2003 and 2009, sixty-two countries, including countries in Asia, Europe, Africa, and parts of the Middle East, have had cases of H5N1 avian influenza in domestic poultry and wild birds (World Organisation for Animal Health, 2009). By January 2009, there have been 403 confirmed human cases of highly pathogenic AIV with 254 deaths in Asia, the Middle East, and Africa (World Health Organization, 2009).

Avian influenza virus is a lipid-enveloped, negative-sense, single-stranded RNA virus of the family Orthomyxoviridae and genus Influenzavirus A (Swayne and King, 2003). Avian influenza is transmitted horizontally between birds and infected birds can shed large amounts of the virus via aerosol respiratory droplets and feces. Anything coming in contact with respiratory secretions or feces of infected birds can become contaminated, including poultry house surfaces and farm equipment. To properly control and eradicate virus during an outbreak, it is necessary to effectively disinfect all surfaces contaminated with potentially infectious material. Lipid-enveloped viruses have been found to stay infective on hard, nonporous surfaces, up to 14 d in the case of human immunodeficiency virus (Terpstra et al., 2007). Postoutbreak response plans need to consider both disinfectant availability and effectiveness.

Evaluation of virus inactivation is a critical component of disinfectant testing. The US Environmental Protection Agency (EPA) considers a disinfectant agent to be effective if the product can demonstrate complete inactivation of the virus at all dilutions while at least 4 logs of virus particles per milliliter must be recovered from the nonvirucidal treated control carrier (US EPA, 1981). In vivo testing relies on the H surface glycoprotein to bind to receptors on a variety of mammalian and avian erythrocytes if the virus is active. This produces hemagglutination, or clumping of cells that is visible to the naked eye (Swayne et al., 1998).

Organic and inorganic acids are highly virucidal and have differing modes of action. Inorganic acids are effective against viruses with susceptibility to lower pH environments, whereas organic acids lower the pH and inactivate viruses by the interaction of lipophilic structures with membranes of enveloped viruses (De Benedictis et al., 2007). Acetic acid (vinegar) is a chemically simple carboxylic acid considered to be an alternative or “green” disinfectant because it is less harsh than many other common disinfectant compounds. Acetic acid can inactivate selected foodborne pathogens, such as Listeria monocytogenes, Salmonella Enteritidis, Salmonella Sonnei, and Yersinia sp. (Medina et al., 2007), but showed inadequate disinfection efficacy for other bacteria, including Pseudomonas aeruginosa and Salmonella Choleraesuis (Rutala et al., 2000).
Sodium hydroxide (caustic soda) along with calcium hydroxide (lime) and sodium carbonate (washing soda) are members in the group of alkali chemicals. Alkalis act as disinfecting agents by means of protein denaturation and have the added benefit of being effective in the presence of organic material (Agriculture and Resource Management Council of Australia and New Zealand, 2000). Sodium hydroxide has been shown to be effective at inactivating lipid-enveloped viruses such as human immunodeficiency virus and pseudorabies virus and against nonenveloped viruses such as porcine circovirus type 2 and minute virus of mice (Boschetti et al., 2003; Terpstra et al., 2007; Martin et al., 2008).

Calcium hydroxide (lime) is commonly used to alter soil pH, stabilize animal wastes, and reduce odor emissions and is readily available in agricultural regions. As a disinfectant, calcium hydroxide has been shown to reduce pathogen levels, particularly viruses, in slurries and wastewater treatment processes (Turner and Burton, 1997).

Sodium carbonate has been used with laundry detergent as a water softener by preventing magnesium and calcium ions in hard water from bonding with the laundry detergent. Sodium carbonate can be used as a disinfectant against Orthomyxoviridae species and also may be used as an antiviral by raising the pH when added to washing water (Agriculture and Resource Management Council of Australia and New Zealand, 2000).

Disinfectants are required to be EPA-registered with respect to specific pathogens. Individual companies rarely choose to have common chemicals such as acetic acid EPA-registered. In an emergency situation, with appropriate scientific data, an exemption can be granted. The purpose of this research was to evaluate the efficacy of common, commercially available chemicals and detergents to inactivate low pathogenic AIV (LPAIV) on hard, nonporous surfaces.

MATERIALS AND METHODS

An experiment was conducted to evaluate the virus inactivation ability of selected chemicals, soaps, and commercial detergents. The chemicals in the study included acetic acid (1 and 3%), sodium carbonate (4%), sodium hydroxide (1 and 2%), calcium hydroxide (1%), and powdered laundry detergent without bleach (2, 4, and 6 g/L). Nonporous 14-gauge galvanized steel coupons (A & H Metals Inc., Newark, DE, 2.2 cm × 2.2 cm × ~0.2 cm) were triple-washed, thoroughly dried, and sterilized at 121°C for 30 min before being inoculated with AIV. The virus was allowed to dry on the materials at room temperature before treatment for 10 min with common, commercially available soaps, detergents, chemicals, or disinfectants. After treatment, the fluids from the disinfectant were collected, diluted, and inoculated into 9- to 11-d-old specific-pathogen-free (SPF) White Leghorn embryonated chicken eggs (SPAFAS Inc., Norwich, CT), which were incubated for 5 d and candled daily for mortality. At 5 d postinoculation, egg fluids were collected and checked for hemagglutination activity (HA) as a sign of viral growth in the eggs.

The LPAIV strain A/H7N2/Chick/MinhMa/04, isolated from a commercial broiler flock during the 2004 Delmarva outbreak, was used as a low-embryo-passage challenge virus. A seed stock of A/H7N2/Chick/MinhMa/04 was prepared from the sixth chorioallantoic sac passage in SPF embryonated eggs and was titrated as described (Swayne and King, 2003). The seed stock was determined to have a titer of $10^7.8$ 50% egg infective dose (EID$_{50}$/0.1 mL and used for all tests at such titer. To increase the organic load of the tested sample, 5% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO) was mixed with virus.

Six agents at various concentrations were tested individually for effectiveness against LPAIV for a 10-min contact time. Test combinations included the following: 1 and 3% acetic acid, 1% calcium hydroxide, 4% sodium carbonate, 1 and 2% sodium hydroxide, and 2, 4, and 6 g/L of powdered laundry detergent without bleach.

The test agents were diluted into 400 mg/L of hard water solution on the day of use. The hard water solution was prepared by combining 40 mL of hard water (1 mg calcium carbonate/mL) solution with 60 mL of double-distilled (deionized) water per 100 mL of 400 mg/L of calcium carbonate solution produced.

Acetic acid solution was prepared by diluting United States Pharmacopeia-grade acetic acid to produce 1 and 3% solutions of acetic acid. The solutions were made by combining 2 and 6 mL, respectively, of acetic acid with 198 and 194 mL of prepared 400 mg/L of calcium carbonate hard water solution.

Calcium hydroxide (1%), sodium carbonate (4%), and sodium hydroxide (1 and 2%) solutions were individually prepared by combining by weight to volume with reagent-grade calcium hydroxide, sodium carbonate, and sodium hydroxide anhydrous crystals, respectively, into 200 mL of prepared 400 mg/L of calcium carbonate hard water solution.

One conventional laundry detergent without bleach, (Tide, Proctor and Gamble, Cincinnati, OH) was tested individually at multiple concentrations (2, 4, and 6 g/L). To prepare the laundry detergent test solutions, the specified weight of powdered laundry detergent was mixed into a solution of 1,000 mL of prepared 400 mg/L of calcium carbonate hard water solution.

Phosphate-buffered saline was prepared by adding 8.5 g of sodium chloride, 1.18 g of dibasic sodium phosphate, and 0.22 g of monobasic sodium phosphate to 1 L of double-distilled water. Cold, sterile PBS was used to dilute a penicillin-streptomycin antibiotic solution (Lonza Walkersville Inc., Walkersville, MD) 10,000 units/mL and 10,000 μg/mL, respectively. This PBS antibiotic mixture was used for all necessary dilutions.

Fourteen metal coupons were coated with 0.1 mL of the virus-5% FBS mixture. Additionally, 6 metal coupons were placed on the foil to be used as chemical compound cytotoxic control. This coupon set was
coated with 0.1 mL of the prepared cytotoxic control solution consisting of 1.9 mL of PBS containing 0.5× antibiotic solution (above) combined with 0.1 mL of FBS to produce a solution of 5% FBS.

All coupons were allowed to dry for approximately 1.5 h at room temperature. Once dry, 6 coupons were placed into 6-well, flat-bottom, nontissue culture plates (BD Biosciences, Franklin Lakes, NJ). Two plates, A and B, were used for each material. The remaining 2 coupons were placed in a separate plate to serve as the virus control group. An additional 6-well plate containing 6 metal coupons coated with cytotoxic control solution served as the chicken embryo cytotoxic control.

Each disinfecting agent was prepared as specified above. Two milliliters of each solution was placed into each of the 6 wells of the treatment plates and cytotoxic control plate. Each plate was then gently agitated on a horizontal plate shaker for 10 min at room temperature. After agitation, each coupon was scraped with a pipette and the fluid was aspirated from the well and jetted back onto the coupon 3 times to dislodge virus from the coupon. The fluids from the 6 wells of the plate were pooled into a single tube and the 2 plates, A and B, were analyzed separately. The virus control plate was handled in a fashion similar to the other test plates; however, 2.0 mL of PBS was used to dislodge virus from the coupons rather than the disinfecting agent. The virus control fluid was pooled into a single tube, resulting in a virus control for each material.

The pooled fluid from the coupons was diluted by making three 10-fold serial dilutions, resulting in dilutions from 10⁻¹ to 10⁻³. The positive controls were diluted using six 10-fold serial dilutions, resulting in dilutions from 10⁻¹ to 10⁻⁶. The cytotoxic control was diluted once, resulting in a 10⁻¹ dilution. Difco D/E Neutralizing Broth (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for the first dilution for each group to inactivate the chemical compounds in question, with subsequent dilutions occurring in PBS. Virus recovery attempts were made using each dilution.

Virus recovery was performed by inoculating 0.2 mL of the test solution into 9- to 11-d-old embryonated SPF chicken eggs, which were incubated for 5 d and candled daily for viability. Embryo mortality observed within the first day postinoculation was considered nonspecific and discarded. Recorded mortalities between d 2 and 5 postinoculation were held at 4°C for subsequent screening. At 5 d postinoculation, all remaining embryos were place at 4°C for 24 h. Chorioallantoic fluid (CAF) was collected from each egg and tested for HA activity as described (Swayne and Halvorson, 2003). Briefly, chicken red blood cells (CRBC) were obtained by mixing whole blood with 50% Alsever’s solution, centrifuging at 1,000 × g for 5 min, and washing the cells 3 times in PBS. A final dilution was made in PBS to produce a solution to contain approximately 25% CRBC. The HA tests were performed by combining 60 μL of CAF from 1 egg and 36 μL of CRBC. The mixture of CAF and red blood cells was gently agitated for 3 to 5 min at room temperature. A positive result indicated the presence of the LPAIV strain A/H7N2/Chick/MinhMa/04 in the CAF sample tested. The HA test was performed on each egg individually.

A neutralizing index (NI) of virus inactivation was used to evaluate the efficacy of each agent (Lombardi et al., 2008). This method was a modification of the classical avian serological virus-neutralization test (Swayne et al., 1998). This NI of virus inactivation (equation [1]) was determined by subtracting the log₁₀ titer (hereafter simply titer) of the recovered virus from the disinfectant-treated plates (tᵣ), from the titer of the positive control plate (tₚc; equation [1]). Inactivation of AIV was considered effective when NI ≥ 2.8, the positive control titer was ≥ 4.0, and there was no recoverable virus from any treated coupon. No recoverable virus provides a titer of <1.2 via the method of Reed and Muench (1938):

\[
NI = tᵣ - tₚc. \quad [1]
\]

RESULTS AND DISCUSSION

A summary of the recovered virus titer, positive control titer, and NI for the common chemicals are shown in Table 1 and NI are shown in Figure 1. The NI values were used as one of the primary means of determining if a disinfection agent was effective. The NI value, however, is heavily dependent on the positive control titer for a given test. A NI value ≥2.8 alone does not directly indicate effectiveness in the inactivation of AIV if the positive control titer is high. Strong positive control titers of ≥5.5 EID₅₀/mL were recovered for each of the treated coupons in this experiment. Viral recovery from the treated surfaces must be taken into account when determining whether or not a disinfectant is effective. To be effective, there must be no viral recovery (no HA can be seen). When the EPA criteria for effectiveness are applied, multiple common chemicals including 1 and 3% acetic acid, 2% sodium hydroxide, and 1% calcium hydroxide effectively inactivated LPAIV on a metal surface, whereas laundry detergent without bleach, 4% sodium carbonate, and the lower concentration of sodium hydroxide (1%) were not effective.

Acetic acid 1% and 3% had the highest NI values (≥5.0) of the chemicals tested for both metal coupons. A strong positive control of 10⁶.² EID₅₀/mL for metal contributed to the high NI values.

Calcium hydroxide 1% was effective at inactivating LPAIV on metal surfaces. There was a strong recovered virus titer of 10⁶.⁰ EID₅₀/mL for the metal positive control resulting in the high NI values for both metal A and B of (≥4.8). No positive HA was observed on treated metal A and B surfaces.

A 4% test solution of sodium carbonate (4%) was not effective at inactivating LPAIV on hard, nonporous
surfaces. Because positive HA activity was observed on treated metal A and B surfaces, a 4% sodium carbonate solution is not recommended for disinfection of LPAIV from hard, nonporous surfaces.

Sodium hydroxide showed a concentration effect. Sodium hydroxide (2%) was effective at inactivating LPAIV on nonporous surfaces. Sodium hydroxide (1%), however, failed to meet EPA standards for AIV inactivation despite a NI value above the 2.8 minimum. Sodium hydroxide 1% showed inconsistent results between groups metal A and metal B. The metal positive control virus recovery titer was 10^6.0 EID_{50}/mL. Virus from the metal A treatment group was inactivated, whereas the virus from the metal B treatment group was not fully inactivated because positive HA activity was observed on treated metal B surfaces. Sodium hydroxide 2% was effective at inactivating LPAIV on metal surfaces. The NI values for metal groups A and B (≥4.8) were high due to the strong positive control titer of 10^6.0 EID_{50}/mL. No positive HA was observed on the metal A and B treated surfaces tested with sodium hydroxide 2%. This suggests that a minimum concentration of 2% is required for reliable inactivation of LPAIV on hard, nonporous surfaces.

The commercial powdered laundry detergent was shown to be ineffective at completely disinfecting LPAIV at any test concentration. At each test concentration, virus was recovered from both sets of treated coupons. The metal positive control titer was satisfactory for the test at 10^6.0 EID_{50}/mL; however, virus was recovered from all concentrations of the detergent. The results, shown in Table 1, show a decrease in virus recovery titer and an increase in NI values as the concentration of powdered laundry detergent increases (Figure 2). Although all of the tested concentrations had positive HA, the efficacy of the detergent appeared to be concentration-dependant. As the concentration of the detergent increased, there was less viral recovery. These results suggest that concentrations higher than 6 g/L may be effective at inactivation of LPAIV on hard, nonporous surfaces; however, further testing is required to confirm.

Cytotoxic controls were collected for each chemical. There was 100% survivability of all the cytotoxic controls except for high first day mortality in the sodium carbonate 4% and 1 lesion found in the sodium hydroxide 2%. However, when repeated in 6 additional eggs, there was 100% survivability and no visible lesions, indicating any egg death was not due to any cytotoxic effect due to the presence of the chemical agent. All cytotoxic control eggs were negative for HA activity and no visible lesions were seen on the embryos.

Table 1. Summary of recovered virus titer, positive control titer, and neutralization indices (NI) for the common chemicals tested

<table>
<thead>
<tr>
<th>Chemical compound</th>
<th>Coupon set</th>
<th>Recovered virus [\log_{10}] titer</th>
<th>Positive control [\log_{10}] titer</th>
<th>NI</th>
<th>Virus recovered from coupons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid, 1%</td>
<td>A</td>
<td>6.2</td>
<td>&lt;1.2</td>
<td>5.0</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.2</td>
<td>&lt;1.2</td>
<td>5.0</td>
<td>No</td>
</tr>
<tr>
<td>Acetic acid, 3%</td>
<td>A</td>
<td>6.2</td>
<td>&lt;1.2</td>
<td>5.0</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.2</td>
<td>&lt;1.2</td>
<td>5.0</td>
<td>No</td>
</tr>
<tr>
<td>Calcium hydroxide, 1%</td>
<td>A</td>
<td>6.0</td>
<td>&lt;1.2</td>
<td>4.8</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.0</td>
<td>&lt;1.2</td>
<td>4.8</td>
<td>No</td>
</tr>
<tr>
<td>Detergent, 2 g/L</td>
<td>A</td>
<td>5.5</td>
<td>2.1</td>
<td>3.4</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.5</td>
<td>1.4</td>
<td>4.1</td>
<td>Yes</td>
</tr>
<tr>
<td>Detergent, 4 g/L</td>
<td>A</td>
<td>5.5</td>
<td>1.4</td>
<td>3.0</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.5</td>
<td>2.0</td>
<td>3.5</td>
<td>Yes</td>
</tr>
<tr>
<td>Detergent, 6 g/L</td>
<td>A</td>
<td>5.5</td>
<td>1.3</td>
<td>4.2</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.5</td>
<td>1.4</td>
<td>3.0</td>
<td>No</td>
</tr>
<tr>
<td>Sodium carbonate, 4%</td>
<td>A</td>
<td>7.4</td>
<td>&lt;1.2</td>
<td>4.8</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7.4</td>
<td>&lt;1.2</td>
<td>4.8</td>
<td>No</td>
</tr>
<tr>
<td>Sodium hydroxide, 1%</td>
<td>A</td>
<td>6.0</td>
<td>&lt;1.2</td>
<td>4.8</td>
<td>No</td>
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<tr>
<td></td>
<td>B</td>
<td>6.0</td>
<td>&lt;1.2</td>
<td>4.8</td>
<td>No</td>
</tr>
<tr>
<td>Sodium hydroxide, 2%</td>
<td>A</td>
<td>6.0</td>
<td>&lt;1.2</td>
<td>4.8</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.0</td>
<td>&lt;1.2</td>
<td>4.8</td>
<td>No</td>
</tr>
</tbody>
</table>

Figure 1. Neutralization indices for common chemicals and their ability to inactivate low pathogenic avian influenza virus on metal surfaces. Neutralization index values are shown for individual replicates. Solid bars indicate chemicals that met the Environmental Protection Agency criteria for inactivation including a \[\log_{10}\] 2.8 reduction in titer and no positive virus recovery.
The results of this experiment show that for an AIV outbreak in commercial poultry facilities, dried virus can be effectively disinfected from hard, nonporous surfaces by commonly available chemicals (acetic acid, calcium hydroxide, sodium hydroxide) at the proper concentrations. These disinfectants are readily available and are ideal for poultry growers. After depopulating an infected flock, the sooner surfaces and equipment can be decontaminated, the sooner the flock can be replaced, which avoids further possible financial losses. Additionally, chemicals such as acetic acid and other alternative chemicals are ideal for quick, effective, and safe decontamination because they are relatively mild chemicals. Many disinfectants, such as some aldehydes, hypochlorites, and stronger inorganic acids, can be corrosive to many surfaces. Additional testing is required to discover the virucidal efficacy of other alternative chemicals.

ACKNOWLEDGMENTS

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REFERENCES


