The Effect of Microaerosolized Hydrogen Peroxide on Bacterial and Viral Poultry Pathogens[1]

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ABSTRACT The effect of microaerosolized H_2O_2 on bacterial and viral poultry pathogens was investigated. Bacterial cultures and viruses were dried on sterile glass Petri dishes and subjected to direct and indirect 5% (H_2O_2) microaerosol mist. In the trials using *Escherichia coli* and *Staphylococcus aureus*, there was complete inactivation following exposure to H_2O_2. Using *Salmonella typhimurium*, indirect exposure resulted in only partial inactivation whereas direct exposure to H_2O_2 gave complete inactivation. For the viruses studied, 5% H_2O_2 microaerosol mist completely inactivated infectious laryngotracheitis virus. Newcastle disease virus, infectious bronchitis virus, and avian influenza virus showed reduced infectivity but were not completely inactivated. Avian reovirus susceptibility varied with the method of exposure and infectious bursal disease virus was highly resistant. The use of 10% H_2O_2 mist, however, resulted in total inactivation of infectious bursal disease virus.

The effect of 10% H_2O_2 on equipment and selected materials representative of a hatcher or poultry house was investigated. A solar cell calculator, a thermostat containing a microswitch, and samples of uncoated steel, galvanized steel, and uncoated aluminum were subjected to 10 fumigation cycles. No damage was detected in the calculator and the thermostat. Both the uncoated steel and the galvanized steel showed signs of oxidation. The aluminum did not show signs of oxidation.

(*Key words*: pathogens, hatchery, microaerosolization, hydrogen peroxide, disinfection)

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INTRODUCTION

A 3 to 6% concentration of H_2O_2 is very effective in inactivating certain human viral pathogens (Zolotarskaia *et al.*, 1975; Mentel *et al.*, 1977; Podoplekina *et al.*, 1986; Sporkenback *et al.*, 1987; Pepose *et al.*, 1989; Tyler *et al.*, 1990). It has been reported that H_2O_2 is also effective in inactivation of bacteria (Fox and Kosikowski, 1962; Yoshpe-Purer and Eylan, 1968; Toledo *et al.*, 1973; Wardle and Renninger, 1975; Favero, 1983). The disinfectant mode of action of H_2O_2 has been shown to result from the formation of a free hydroxyl radical that causes oxidation of membrane lipids, nucleic acids, and other cell components (Turner, 1983).

Recent utilization of H_2O_2 disinfection in the poultry industry has proven effective against bacteria, yeasts, and molds when aerosolized onto hatching eggs (Anonymous, 1991; Sheldon and Brake, 1991). Alternative application procedures to microaerosolization have also proven effective (Rickloff, 1988; Klapes and Vesley, 1990).

Concern over the use of formaldehyde and restrictions regarding its use have

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provided a need for an alternative to help maintain biosecurity in intense poultry rearing areas. The purpose of this study was to test the effectiveness of H_2O_2 delivered by microaerosolization against poultry viral and bacterial pathogens in the hope that it may be an efficacious replacement for formaldehyde fumigation.

MATERIALS AND METHODS

Bacteria

Bacterial strains tested were obtained from American Type Culture Collection (ATCC).[4] The following isolates were used: 14028 *Salmonella typhimurium*, 6538 *Staphylococcus aureus*, and 11229 *Escherichia coli*. Bacterial cultures were grown for 48 h in nutrient broth at 37 C.

Chicken Embryos

Specific-pathogen-free (SPF) fertile eggs were obtained from a commercial source[5] and were incubated at 37.6 C until 10 d of age when they were then used for the isolation of viruses that had been exposed to H_2O_2. Additional eggs were maintained until hatch at which time kidneys were aseptically removed for cell culture (Schat and Purchase, 1989).

Damage Assessment Materials

The following devices and materials were obtained from commercial sources: solar cell calculator,[6] a temperature controller containing a microswitch,[7] samples of uncoated steel sheet, galvanized steel sheet, and uncoated aluminum sheet.

Hydrogen Peroxide

Certified (American Chemical Society) 30% H_2O_2 was obtained from a distributor of scientific and chemical supplies.[8] Hydrogen peroxide was adjusted to concentrations of 5 and 10% in double deionized water.

Indirect Fluorescent Antibody Test

Primary chick kidney cell cultures were infected with virus inocula recovered from treated and untreated glass Petri dishes. Following incubation, cells were fixed to the plate with acetone by the method of Pursell and Cole (1976). Primary antibody produced in chickens to avian reovirus Strain S1133 and infectious bursal disease virus Strain D-78 was diluted 1:10 in PBS. Twenty-five microliters of this dilution were added to each well and allowed to incubate for 30 min at 37 C, followed by washing 3× with carbonate bicarbonate buffer (pH 9.0) containing .05% Tween 20. Twenty-five microliters of a 1:100 dilution of rabbit anti-chicken antibody conjugated with fluorescein isothiocyanate[9] in PBS were added to each well, incubated, and washed as described above. Plates were then examined for fluorescence using a light microscope equipped with an epifluorescent attachment. Wells were scored as positive or negative based on the presence or absence of apple green fluorescence from infected cells.

Microaerosolization Equipment

A microaerosolization test panel[10] consisting of two spray nozzles was provided with air by an oilless air compressor to generate H_2O_2 microaerosol. Air was filtered to .9 μ.

Test Chamber

The test chamber consisted of a 30-m^3 room equipped with a high efficiency particulate air (HEPA) filter.

Viruses

The poultry viruses used were as follows: GB-Texas Newcastle disease virus (NDV) Lot 82-3,[11] Massachusetts 41 infectious bronchitis virus (IBV) Lot 80-1,[11] infectious laryngotracheitis virus (ILT) Lot 83-2,[11] and TY/WIS/66 H9N2 influenza

[4]American Type Culture Collection, Rockville, MD 20852.
[5]HYVAC Inc., Gowrie, IA 50543.
[6]Sharp Electronics Corp., Mahwah, NJ 07430.
[7]Penn 9205, Johnson Controls, Goshen, IN 46526.
[8]Fisher Scientific, Fair Lawn, NJ 07410.
[9]Pel-Freez Laboratories, Rogers, AR 72757.
[10]Atomizing Systems, Ho-Ho-Kus, NJ 07423-1433.
[11]National Veterinary Services Laboratory, USDA/APHIS, Ames, IA 50010.

virus (FLU).[12] In addition, a cell-culture-adapted avian reovirus Strain S1133[13] and infectious bursal disease virus vaccine strain D-78[14] were tested.

Experimental Design

One hundred microliters of a 48-h culture of bacteria, or .5 mL of stock virus was spread on a 25.8 cm^2 area in the bottom of each sterile 100-mm glass Petri dish and allowed to dry in a vertical flow hood for 30 min. Plates were placed in three positions in 10 replications in the test room. Some plates were subjected to direct H_2O_2 exposure by placing them on top of the Petri dish lid with the coated surface facing up. Indirect exposure was accomplished by either turning the plate over with the coated surface toward the floor supported on the edge of the plate lid or by leaning the plate bottom against the wall with the coated surface toward the wall. One liter of either plain double deionized water or 5 or 10% solution of H_2O_2 was then microaerosolized for approximately 30 min into the room using a single nozzle operating at 2.8 kg/cm^2 and .114 m^3/min. Thirty minutes of contact time was allowed, followed by venting the room for 30 min. The plates were removed from the room and returned to the laboratory for reisolation attempts.

Attempts to recover bacteria were made using PBS only, whereas viruses were recovered in PBS solution supplemented with 15 mg/L of catalase (2,600 units/mg)[15] in addition to antibiotics due to the sensitivity of the cell culture system to H_2O_2. The use of the catalase in recovering viruses was an adaptation of experiments with bacteria done by Sheldon and Brake (1991). Bacterial suspensions were serially diluted in PBS, and 100 μL was plated on selective media. Plates were incubated at 37 C for 24 h and colonies counted. Virus viability and titer was determined by inoculation of primary chick kidney cells, or 10-d-old SPF embryonated eggs depending upon virus type (Schat and Purchase, 1989).

Equipment and materials representative of that which might be found in hatcheries, or poultry houses were exposed to 10 fumigation cycles of 10% H_2O_2. The backs of two solar cell calculators were removed to expose the circuit board and were leaned keyboard up at approximately a 10-degree angle to the inside wall of a poultry isolator with the door open. Two thermostats were hung within the direct path of the microaerosol. Samples of uncoated steel, galvanized steel, and uncoated aluminum were also exposed to direct contact with the microaerosol.

Statistical Analysis

The logarithm of bacterial colony-forming units +1 and the percentages of positive eggs or chick kidney culture wells were analyzed by one-way analysis of variance within each bacterial species or virus strain to compare exposures. Each plate was a replication. The 5% level LSD was used to separate means. The observed percentages of positive eggs or cell culture wells and the geometric mean of colony-forming units are presented in the tables.

RESULTS

Inactivation of Bacteria

Trials using *E. coli* and *S. aureus* resulted in total inactivation using both indirect and direct exposure to 5% H_2O_2 microaerosol (Table 1). Using *S. typhimurium*, floor direct and wall exposure resulted in a complete inactivation, whereas indirect floor exposure resulted in only a 4 $\log_{10}$ reduction.

Inactivation of Viruses

Virus titers for the six poultry viruses used in the study determined for the stock virus and after drying on glass Petri dishes are summarized in Tables 2 and 3. Exposure of ILT virus to 5% H_2O_2 microaerosol resulted in complete inactivation of the virus by all exposure methods. Infectious bronchitis virus was completely inactivated by floor direct and indirect wall exposure. Indirect floor exposure of IBV showed a

[12]Originally obtained from Charles W. Beard, Athens, GA 30605.

[13]Originally obtained from Louis Van der Heide, Storrs, CT 06268.

[14]Intervet, Inc., Millsboro, DE 19966.

[15]Sigma Chemical Co., St. Louis, MO 63178-9916.

TABLE 1. Reduction of colony-forming units of bacteria subjected to direct and indirect 5% hydrogen peroxide microaerosol[1,2]

Treatment	*Salmonella typhimurium*	*Staphylococcus aureus*	*Escherichia coli*
	(cfu/mL[1,2])		
Untreated control	$1.06 \times 10^{6.0}$ [a]	$4.26 \times 10^{4.0}$ [a]	$2.03 \times 10^{5.0}$ [a]
Water control	$3.5 \times 10^{6.0}$ [a]	$6.28 \times 10^{5.0}$ [b]	TNTC[3]
Floor direct	0[b]	0[b]	0[b]
Floor indirect	$7.34 \times 10^{2.0}$ [b]	0[b]	0[b]
Wall	0[b]	0[b]	0[b]

[a,b]Means in the same group (bacteria) with no common superscript differ significantly ($P < .05$).

[1]Geometric mean of bacterial colonies recovered from plates.

[2]Values represent the means of 10 plates per treatment with H_2O_2 and 5 plates per control.

[3]TNTC = too numerous to count.

significant reduction, with some residual activity in only 12% of the inoculated eggs. Newcastle disease virus was completely inactivated by the floor indirect exposure, 8% of the eggs were positive by wall exposure and 12% of the eggs were positive with direct floor exposure, both significant reductions. Influenza virus (FLU) was not completely inactivated, but did show significant decreases from the controls for each exposure position. Direct floor exposure was the most effective with only 4% of the eggs testing positive for hemagglutinating activity, this being followed by 12 and 20% for wall and indirect floor exposure respectively, all three treatments causing a significant reduction from the controls (Table 2).

Reovirus S1133 and IBDV Strain D78 were evaluated for virus activity using an indirect fluorescent antibody test in primary chick kidney cells. The reovirus was completely inactivated by the indirect floor and wall treatment, with 20% of the plates testing positive for the direct floor exposure, a significant reduction from the controls (Table 3). Infectious bursal disease virus was significantly reduced by only wall exposure when using 5% H_2O_2, however exposure to 10% H_2O_2 resulted in complete inactivation by all treatments (data not shown).

The effect of fumigation on calculators was measured by turning them on and determining their ability to perform simple mathematical calculations. No physical or functional damage was detected. Temperature controls were checked by measuring the resistance between microswitch contact points before and after exposure. A change in resistance was not detected. Steel and

TABLE 2. Inactivation of egg grown avian infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILT), influenza virus (FLU), and Newcastle disease virus (NDV) using 5% microaerosolized hydrogen peroxide

Treatment	Infectious bronchitis virus	Infectious laryngotracheitis virus	Influenza virus	Newcastle disease virus
	(% positive eggs[1])			
Positive control	100[a]	100[a]	100[a]	100[a]
Floor direct	0[b]	0[b]	4[c]	12[b]
Floor indirect	12[b]	0[b]	20[b]	0[b]
Wall	0[b]	0[b]	12[bc]	8[b]
Stock titer	$10^{5.0}$	$10^{6.0}$	$10^{6.0}$	$10^{6.0}$
After drying titer	$10^{3.6}$	$10^{4.8}$	$10^{5.0}$	$10^{5.8}$

[a–c]Means within a column with no common superscript differ significantly ($P < .05$).

[1]Values represent five tests utilizing five eggs each.

TABLE 3. Inactivation of cell culture propagated avian reovirus Strain S1133 and infectious bursal disease virus (IBDV) Strain D-78 with 5% microaerosolized hydrogen peroxide

Treatment	Avian reovirus	Infectious bursal disease virus
	(% positive tests[1])	
Positive control	100[a]	100[a]
Floor direct	20[b]	87[a]
Floor indirect	0[b]	73[a]
Wall	0[b]	33[b]
Stock titer	$10^{4.3}$	$10^{6.6}$
After drying titer	$10^{3.3}$	$10^{4.5}$

[a,b]Means within a column with no common superscript differ significantly ($P < .05$).

[1]Values represent five tests utilizing three replicate cell culture wells.

aluminum samples were visually inspected for damage. Both the uncoated steel and galvanized steel showed evidence of oxidation. The aluminum was not noticeably affected.

DISCUSSION

Results show that the poultry bacterial pathogens tested were significantly reduced in number upon exposure to 5% microaerosolized H_2O_2. These results parallel earlier studies by Sheldon and Brake (1991) and Anonymous (1991). Poultry viral pathogens tested against 5% microaerosolized H_2O_2 showed varying sensitivities. Infectious laryngotracheitis virus was completely inactivated by all treatments, which corresponds to the findings of Pepose *et al.* (1989) with other herpesviridae. Influenza, NDV, and IBV were highly susceptible although not completely inactivated. These findings correspond to those reported earlier for the human counterpart viruses (Mentel *et al.*, 1977). Reovirus S1133 was completely inactivated by the indirect treatments but not by the direct floor treatment. Infectious bursal disease virus showed a significant drop in only the wall treatment with 5% H_2O_2 but was totally inactivated with 10% H_2O_2. These viruses are both unenveloped and are well known for their ability to resist chemical inactivation (Benton *et al.*, 1967; Rosenberger and Olson, 1991).

Hydrogen peroxide appears to be effective in the inactivation of a number of poultry bacterial and viral pathogens using a 5% concentration, and highly effective using 10%. A microaerosol vapor nozzle system facilitates the uniform delivery of the disinfectant with minimal labor input. Hydrogen peroxide is economical and environmentally safe. However, there may be lasting effects on structural metals through accelerated surface oxidation, as was seen with repeated exposure using 10% H_2O_2. Partially protected electronic equipment show no adverse effects after limited exposure but may be damaged by repeated long-term exposure. The field use of H_2O_2 offers promise of effective control of a number of poultry pathogens but may require precautionary measures to limit exposure of electronics and metals.

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