The Effect of Electrolyzed Oxidative Water Applied Using Electrostatic Spraying on Pathogenic and Indicator Bacteria on the Surface of Eggs

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ABSTRACT Research was conducted to compare the effectiveness of electrolyzed oxidative (EO) water applied using an electrostatic spraying system (ESS) for killing populations of bacteria that are of concern to the poultry industry. Populations of pathogenic bacteria (*Salmonella typhimurium*, *Staphylococcus aureus*, and *Listeria monocytogenes*), and the indicator bacterium *Escherichia coli* were applied to eggs and allowed to attach for 1 h. EO water completely eliminated all *Salmonella typhimurium* on 3, 7, 1, and 8 out of 15 eggs in Repetitions (Rep) 1, 2, 3, and 4, respectively, even when very high inoculations were used. EO water completely eliminated all *Staphylococcus aureus* on 12, 11, 12, and 11 out of 15 eggs in Rep 1, 2, 3, and 4, respectively. EO water completely eliminated all *Listeria monocytogenes* on 8, 13, 12, and 14 out of 15 eggs in Reps 1, 2, 3, and 4, respectively. EO water completely eliminated all *Escherichia coli* on 9, 11, 15, and 11 out of 15 eggs in Reps 1, 2, 3, and 4, respectively. Even when very high concentrations of bacteria were inoculated onto eggs (many times higher than would be encountered in industrial situations), EO water was found to be effective when used in conjunction with electrostatic spraying for eliminating pathogenic and indicator populations of bacteria from hatching eggs.

(*Key words*: *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*)

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INTRODUCTION

Research has demonstrated that the hatchery is one of the most important areas, within a vertically integrated poultry company, in which *Salmonella* control is essential. Studies have associated the presence of *Salmonella* in the hatchery with contamination among broiler flocks. In a survey conducted in Denmark from 1992 to 1995, Christensen et al. (1997) discovered that a 180-kb plasmid found in 88% of hatchery isolates was also present in 69% of the flocks, and that the hatchery was the primary source of this particular *Salmonella* spp. among the flocks.

Bailey et al. (1998) reported that, although very few fertile eggs entering the hatchery are contaminated with *Salmonella*, the spread of *Salmonella* from these eggs to other chicks hatching in the same cabinet may be extensive. Olesiuk et al. (1969) studied the dissemination of *Salmonella typhimurium* in fertile hatching eggs and found that only 3 of 5,527 (0.0005%) eggs were positive. Over several years of sampling, Wilding and Baxter-Jones (1985) found that only 1 in 10,000 (0.0001%) hatching eggs were contaminated with *Salmonella*. Sampling in commercial hatcheries has indicated that 5 to 9% of day-old chicks may be colonized with *Salmonella* (Lahellec and Colin, 1985; Jones et al., 1991), which is a large increase from 0.0001 to 0.0005% prevalence in hatching eggs. Moreover, *Salmonella* may be isolated from a variety of sources within the broiler hatchery. Cox et al. (1990) found that 71% of eggshells, 80% of chick conveyor belts, and 74% of paper pad trayliners contained *Salmonella* spp. These studies demonstrate the necessity to reduce the presence of *Salmonella* on eggshells entering the hatchery and to control cross-contamination from chick to chick during hatching.

Eliminating *Salmonella* from the surfaces of hatching eggs and preventing cross-contamination during hatching usually involves the application of formaldehyde gas or fogging hydrogen peroxide into the hatching cabinet. An electrostatic spray-charging system has been developed, which results in a 1.6- to 24-fold increase in spray deposition over conventional application methods, such as commercial fogging (Law and Lane, 1981). Thus, electrostatic spraying may be an appropriate means of applying sanitizers in the hatchery environment because the sanitizer is distributed more effectively over the surface of eggs and equipment than can be accomplished with commercial foggers. Moreover, because dust and dander (moving around the inside of the hatching cabinet

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Abbreviation Key: EO = electrolyzed oxidative acidic; ESS = electrostatic spraying system; Rep = repetition; ORP = oxidation/reduction potential.

due to rapid air movement) are responsible for cross-contamination with *Salmonella*, electrostatic spraying should be able to disinfect these fomites more effectively, as the sanitizer droplets are attracted to the oppositely charged dust and dander.

Electrolyzed oxidative (EO) water has been shown to be a nontoxic and inexpensive sanitizer. Research has demonstrated that EO water was able to eliminate 8.88 $\log_{10}$ cfu (Kim et al., 2000b) and all viable *Escherichia coli* O157:H7 after 30 s of exposure (Kim et al., 2000a). Venkitanarayanan et al. (1999a) observed that exposing *E. coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* to EO water for 5 min resulted in a 7 $\log_{10}$ reduction in all three pathogens. Exposure for 10 min completely eliminated these bacteria. EO water has also been shown to be effective for eliminating pathogens that are attached to surfaces. EO water reduced *E. coli* O157:H7 and *Listeria monocytogenes* populations, firmly attached to cutting boards, by 4–5 $\log_{10}$ cfu/100 cm^2 (Venkitanarayanan et al., 1999b). These reports suggest that EO water would be an effective means of eliminating pathogenic bacteria from broiler hatching eggs.

The purpose of this research was to determine the effect of EO water applied using electrostatic spraying on *Salmonella typhimurium*, *Staphylococcus aureus*, *L. monocytogenes*, and *E. coli* on eggshell surfaces. The hypothesis was that EO water in combination with ESS would be an effective means of eliminating pathogenic bacterial populations from eggshell surfaces.

MATERIALS AND METHODS

Pathogenic Bacterial Isolates

Salmonella typhimurium, *L. monocytogenes*, *Staphylococcus aureus*, and *E. coli* were obtained from the United States Department of Agriculture, Agricultural Research Service's (USDA-ARS) Poultry Microbiological Safety Unit laboratory. These isolates were originally collected from commercial broiler carcasses. Each isolate was assayed for Gram reaction, cytochrome oxidase activity, and production of catalase and was identified using either the Vitek,[2] Biolog,[3] or Micro-ID[4] rapid identification methods.

EO Water Preparation

A solution of EO water was prepared by electrolysis of a 20% saline solution made with tap water. The final pH and oxidation-reduction potential of this solution were 2.1 and 1,150, respectively. Due to electrolysis of the saline solution, small concentrations of antimicrobial substances were produced including chlorine ions (8 ppm free chlorine), chlorine dioxide, ozone, and hydrogen peroxide. It is believed that the combination of very low concentrations of these compounds in an acidic environment is the mechanism of action for EO water.

Egg Preparation

Eggs were collected from layer chickens housed at The University of Georgia, Poultry Research Center. After collection, the eggs were washed with a commercially available chlorine-based sanitizer and allowed to dry. Each egg was then rinsed thoroughly three times with sterile deionized water to remove any residual sanitizer that may have remained from the washing process.

Egg Inoculation

An inoculation solution was prepared by placing 0.1 mL of an actively multiplying pure bacterial culture (incubated 24 h in brain heart infusion broth[5] at 35 C) into 200 mL of sterile 1% peptone broth. The bacterial cultures used were *Salmonella typhimurium*, *Staphylococcus aureus*, *L. monocytogenes*, and *E. coli*. Eggs were individually dipped into the inoculum and allowed to dry under a laminar flow hood for 1 h. This procedure provided time for the bacteria to attach to the surface of the egg.

Electrostatic Spraying of Eggs

Each egg was placed into a clean egg flat and positioned in an electrostatic spraying chamber. Tap water (2 repetitions) or EO water (4 repetitions) was sprayed onto the eggs using two electrostatic spray nozzles for 15 s each hour for 24 h. After treatment, the eggs were allowed to dry, under a laminar flow hood for 1 h. In addition, 2 eggs were dipped in each bacterial isolate, allowed to dry, and stored for 24 h in an enclosed chamber with 96% humidity as a control.

Neutralization of Sanitizer

Each control and treated egg was cracked using a sterile blade and the contents were removed. Eggshells and membranes were placed into 25 mL of sterile 1% peptone broth[5] containing 3% Tween 80,[6] 0.3% lecithin,[6] and 0.1% histidine[6] to neutralize the sanitizers.

Microbiological Evaluation

One milliliter of this mixture was placed into 9 ml of sterile BHI,[5] which acts as a growth medium for conducting impedance or conductance assays, and vortexed. One ml of this mixture was placed into a Bactometer module well in duplicate. Samples were monitored using the Bactometer Microbial Monitoring System M128.[2] All of the bacterial isolates tested were monitored at 35 C. All samples were monitored for 48 h using impedance except for *E. coli*, which was monitored using conductance.

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[2] BioMérieux Vitek, Inc., Hazelwood, MO.
[3] Biolog, Inc., Hayward, CA.
[4] Organon Teknika Corporation, Durham, NC.
[5] Becton Dickinson-Difco, Franklin Lakes, NJ.
[6] Sigma-Aldrich Corp., St. Louis, MO.

TABLE 1. Number of bacteria (cfu/mL) inoculated onto the surface of eggs

Bacterium	Repetition 1	Repetition 2	Repetition 3	Repetition 4
Salmonella typhimurium	>1,000,000	371,000	620,000	>1,000,000
Staphylococcus aureus	820	9,350	1,070,000	719,600
Listeria monocytogenes	225	200	15,350	62,300
Escherichia coli	70,140	67,300	47,500	1,415,400

Statistical Analysis

The experimental design was a 4 × 4 × 2 of replication, bacterial type, and treatment (EO water and controls). All microbiological analyses were conducted in duplicate. Data were analyzed after averaging the duplicates. Results were analyzed using the general linear models procedure of SAS software (SAS Institute, 1994). Treatment means were separated using Fisher's least significant difference option (SAS Institute, 1994). All values reported as significant were analyzed at the $\alpha = 0.05$ level.

RESULTS AND DISCUSSION

Bacterial proliferation requires the use of nutrients such as carbohydrates, proteins, or lipids. As bacteria break down and utilize these nutrients, they release charged by-products, such as lactic acid and acetic acid (Cady, 1974). As charged metabolites accumulate, the conductance and capacitance of the growth medium increases, and impedance decreases. A significant and dramatic shift in the electrical component of the medium occurs when bacterial populations reach a threshold of 10^6 to 10^7 cells/ml (Firstenberg-Eden, 1983). The time required for this shift to occur is called the detection time. Detection time is dependent on the initial concentration of bacteria, the rate at which bacteria in the sample reproduce, the temperature, and the test medium used (Richards et al., 1978; Silley and Forsythe, 1996). Using electrical methods, highly contaminated samples would be detected first. For example, a sample that initially contains 10^5 organisms would require fewer cell divisions to reach the 10^6 detection threshold than a sample that initially contains only 10^1 bacteria. Thus, detection time is inversely proportional to the initial bacterial level in the sample. If impedance or conductance detection times are significantly increased when bacterial populations are exposed to a chemical sanitizer, then the sanitizer had an inhibitory effect on the proliferation of the bacterium or group of organisms. In addition, if no detection time is recorded in 48 h, then it is assumed that the organism was deactivated or injured beyond repair by the sanitizer, as it was unable to multiply under optimal growth conditions.

In this study, significant differences in bacterial inhibition by EO water were observed between replicates. For each replicate, different concentrations of bacteria were used, and the oxidation/reduction potential (ORP) of the EO water evolving from the electrostatic spray nozzle head varied within and between replicates. Thus, the differences observed between replicates may be attributed to application of high numbers of bacteria in some instances and fluctuation in ORP values. Fluctuation in ORP at the nozzle head may be attributed to the charge of the liquid coming out of the nozzle, the air speed of compressed air carrying the sanitizer, or the size of the liquid droplet coming from the nozzle. None of these variables is associated with the sanitizer, but they are able to be controlled by adjustments to the electrostatic spray nozzle system, especially if this system is to be used in an industrial setting.

Log_{10} colony-forming units of bacteria per milliliter of inoculum exposed to EO water are presented in Table 1. It should be noted that, in some cases, very high concentrations of bacteria were challenged in this study to determine the effect of the sanitizer on high numbers of actively growing pathogens and indicator populations of bacteria.

Impedance and conductance detection times (h), and $\log_{10}$ cfu estimations for pure cultures of *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, and conductance detection times (h) for *E. coli* on eggs that have been treated with tap water (two replicates) or EO water (four replicates) using electrostatic spraying and control eggs that were not treated are presented in Tables 2 and 3, respectively. EO water completely eliminated all *Salmonella typhimurium* on 3 (20%), 7 (46.7%), 1 (6.7%), and 8 (53.3%) eggs of 15 tested in Repetitions (Rep) 1, 2, 3, and 4, respectively. In all Rep, for the sanitizer to eliminate *Salmonella typhimurium* on an egg completely, a minimum of a 5 $\log_{10}$ reduction would be required. In Rep 4, when 53.3% of eggs were negative for *Salmonella typhimurium*, 6 $\log_{10}$ *Salmonella typhimurium* were killed. In addition, for eggs that remained positive, the number of *Salmonella typhimurium* remaining were significantly reduced by a minimum of 4 $\log_{10}$ when compared to control eggs.

EO water was able to completely eliminate *Staphylococcus aureus* on 12 (80%), 11 (73.3%), 12 (80%), and 11 (73.3%) eggs of 15 tested in Rep 1, 2, 3, and 4, respectively (Table 2). In Rep 3 and 4, for the sanitizer to eliminate *Staphylococcus aureus* on an egg completely, a minimum of a 6 $\log_{10}$ and a 5 $\log_{10}$ reduction would be required, respectively. In addition, for eggs that remained positive, the number of *Staphylococcus aureus* remaining were significantly reduced by a minimum of 3 $\log_{10}$ when compared to control eggs.

For *L. monocytogenes*, EO water completely eliminated all bacteria on 8 (53.3%), 13 (86.7%), 12 (80%), and 14 (93.3%) eggs of 15 tested in Rep 1, 2, 3, and 4, respectively (Table 2). In Rep 3 and 4, for the EO water to eliminate *L. monocytogenes* on an egg completely, a minimum of a 4 $\log_{10}$ reduction would be required. In addition, for eggs that remained positive, the number of *L. monocytogenes* remaining were significantly reduced by a minimum of

TABLE 2. Impedance or conductance detection times (DT, h) for eggs coated with *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* populations and treated with tap water and electrolyzed oxidative acidic (EO) water applied with electrostatic spraying, or no treatment (controls)

		Repetition 1			Repetition 2			Repetition 3			Repetition 4		
Sanitizer used	Bacterium	Recovery[1]	Average DT[2]	Control DT[3]	Recovery	Average DT	Control DT	Recovery	Average DT	Control DT	Recovery	Average DT	Control DT
Tap water	*Salmonella typhimurium*	10/10	6.44	—[4]	10/10	6.34	—	—	—	—	—	—	—
EO water	*Salmonella typhimurium*	12/15	7.2^a	2.35^b	8/15	14.6^a	5.07^b	14/15	9.4^a	7.1^b	7/15	8.2^a	5.2^b
EO water	*Staphylococcus aureus*	3/15	11.2^a	3.9^b	4/15	8.2^a	4.2^b	3/15	13.8^a	3.6^b	4/15	10.7^a	5.6^b
EO water	*Listeria monocytogenes*	7/15	8.2	8.5	2/15	9.6^a	7.6^b	3/15	10.7^a	6.5^b	1/15	19.4^a	5.4^b
EO water	*Escherichia coli*	6/15	6.05^a	1.05^b	4/15	16.0^a	5.35^b	0/15	$>48.0^a$	8.7^b	4/15	7.1^a	5.0^b

[a,b] Numbers with no common superscripts differ significantly ($P \leq 0.05$).

[1] Number of eggs that produced a detection time out of the number evaluated (DT = time required for bacterial populations to reach 10^6 cfu/mL; no DT indicates no bacteria survived).

[2] Average time required for bacteria that survived treatment to multiply to 10^6 (longer DT = fewer bacteria).

[3] Average time required for control eggs dipped in each bacterium that were not exposed to sanitizer to multiply to 10^6.

[4] Samples were not evaluated.

TABLE 3. Log_{10} cfu/mL estimations[1] from impedance or conductance detection times (DT, h) for eggs coated with *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* populations and treated with tap water and electrolyzed oxidative acidic (EO) water applied with electrostatic spraying, or no treatment (controls)

		Repetition 1			Repetition 2			Repetition 3			Repetition 4		
Sanitizer used	Bacterium	Recovery[2]	Average $\log_{10}$	Control $\log_{10}$	Recovery	Average $\log_{10}$	Control $\log_{10}$	Recovery	Average $\log_{10}$	Control $\log_{10}$	Recovery	Average $\log_{10}$	Control $\log_{10}$
Tap water	*Salmonella typhimurium*	10/10	4.6	—	10/10	4.6	—	—	—	—	—	—	—
EO water	*Salmonella typhimurium*	12/15	4.0	7.5	8/15	>0.1	5.5	14/15	2.4	4.1	7/15	3.3	5.4
EO water	*Staphylococcus aureus*	3/15	>0.1	5.9	4/15	2.2	5.6	3/15	>0.1	6.1	4/15	0.1	4.4
EO water	*Listeria monocytogenes*	7/15	5.0	4.9	2/15	4.4	5.3	3/15	3.8	5.9	1/15	>0.1	6.4
EO water	*Escherichia*	6/15	4.0	8.8	4/15	>0.1	4.7	0/15	0	1.4	4/15	3.0	5.0

[1] Impedance or conductance detection times were subjected to analyses using line equations from established calibration curves for each bacterial species, and $\log_{10}$ estimations were generated.

[2] Number of eggs that produced a detection time out of the number evaluated (DT = time required for bacterial populations to reach 10^6 cfu/mL; no DT indicates no bacteria survived).

1 $\log_{10}$ (Rep 2) or 2.2 $\log_{10}$ (Rep 3 and 4) when compared to control eggs, except in Rep 1.

EO water completely eliminated all *E. coli* on 9 (60%), 11 (73.3%), 15 (100%), and 11 (73.3%) eggs of 15 tested in Rep 1, 2, 3, and 4, respectively (Table 2). In all Rep, for the sanitizer to eliminate *E. coli* on an egg completely, a minimum of a 4 $\log_{10}$ reduction would be required. In Rep 4, when 73.3% of eggs were negative for *E. coli*, 6 $\log_{10}$ *E. coli* were killed. In addition, for eggs that remained positive, the number of *E. coli* remaining were significantly ($P \leq 0.05$) reduced by a minimum of 2 $\log_{10}$ when compared with control eggs. In Rep 3, EO water performed especially well by eliminating all *E. coli* on all eggs, even when a concentration of 47,500 cfu/mL were used.

These data are promising in that EO water is nontoxic and can be consumed as produced. Moreover, this sanitizer is environmentally friendly and is not harmful to humans. Because *Salmonella* testing is part of the USDA-Food Safety and Inspection Service (FSIS) Pathogen Reduction Final Rule (USDA-FSIS, 1996), and *Salmonella* is spread throughout the hatchery environment, leading to cross-contamination and eventual contamination of the product, this sanitizer should prove effective as a means of treating hatching eggs. Currently used hatchery sanitizers (formaldehyde gas and glutaraldehyde) are noxious to humans and chicks, and may pose a serious health risk. Thus, a sanitizer that does not harm chicks, is inexpensive to produce, and is effective would be a useful tool for the poultry industry.

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