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Efficacy of silver nanoparticles and activated electro-chemical water as poultry disinfectants against *Salmonella entriditis*

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ABSTRACT

Salmonellae are commonly found in the environment and there are many instances throughout the grow-out phase in which birds can come into contact with Salmonella and other pathogens. Laboratory trial and other two separate field trials were conducted to evaluate the efficacy of various disinfectants on the isolated Salmonella entriditis when applied to poultry house floors, as well as an innovative trial also, carried out to evaluate the efficacy of same disinfectants when they contained Ag nanoparticles. The results revealed that (1) The following disinfectants without Ag nanoparticles: white wash, phenuique, formalin, iodophors and Envirolyte-Anolyte (1/1000) for disinfection of floor plots significantly impacted Salmonella populations ($P < 0.05$) (2 ;3 ; 3 ; 3 ; 5 log10 reduction, respectively) but unfortunately, failed to kill all the populations. While, Envirolyte-Anolyte (1/500) significantly reduced the population of S. entenidis with a complete reduction of the population. (2) White wash and iodophores containing Ag nanoparticles showed highly significant ($P<0.05$) reduction of Salmonella populations in floor after disinfection process (5; 4 log10 reduction, respectively). Interestingly, Salmonella populations completely destroyed when exposed to phenuique and formalin containing Ag nanoparticles in field trial. This may be due to the ubiquitous nature of Ag nanoparticles, which are able to enhance the disinfectant power.

Keywords: *Salmonella, Ag nanoparticles, Envirolyte-Anolyte, Disinfectants.*

INTRODUCTION

Salmonella is an infectious agent involved in millions of cases of human disease all over the world (Vestby *et al.*, 2010). Contaminated feed and feed ingredients with *Salmonella* is a well-known problem and several authorities, as well as the feed industry, are using large resources in the fight against *Salmonella* (Veldman et al. 1995; Davies and Wray 1997; Lunestad et al., 2007; Anon. 2008; Vestby et al., 2010).

Zoonotic potential of same microbial agents like *Salmonella spp*. or *E. coli* present a special epidemiological problem in poultry breeding (Pavlović et al. 1988; Ilić et al., 1989). Those agents pose a permanent risk for human health, mostly to people who work with poultry or to the consumers who eat contaminated poultry meat or eggs (Anderton, 1989; Mayhall, 1996). *Salmonella Enteritidis* is one of the primary serovars involved in human *salmonellosis* (Anonymous, 2008).

A high population of pathogenic bacteria in the poultry house contributes to a decline in wellness of the flock and increased levels of pathogens recoverable on carcasses entering the processing plant. The spread of pathogens to processing equipment can therefore increase the chances of a contaminated product entering the consumer market (Payne et al., 2005).

Salmonella are commonly found in the environment (Murray, 1991) and there are many instances throughout the grow-out phase in which birds can come into contact with *Salmonella* and other pathogens (Reiber et al., 1990) These pathogens are able to survive for extended periods of time in the environment and can be commonly found in the litter on which the birds live. Large amounts of feces that are deposited in the litter can lead to increases in pathogen populations in the bird's environment.

To eradicate this infectious agent it is necessary to disinfect poultry houses properly before and after chicken arrival and during the production period, especially if health problems occur (Vukičević, 1989 and Böhm, 1997).

Disinfectant efficacy is often tested against laboratory bacterial suspensions (Parkinson, 1981; Bloomfield et al., 1991). However, this approach may not always prove to simulate commercial production conditions, thus, making it difficult to determine the true effectiveness of the disinfectant. Disinfectants that are effective against bacterial suspensions may have a reduced effect against bacteria that adhere to surfaces (Mosteller and Bishop, 1993). Moreover, the increase in incidence of antimicrobial resistance among pathogenic bacteria has made the search for new antimicrobials inevitable.

In the current situation, one of the most promising and novel agents are the nanoparticles (Rai and Bai, 2011) and the unique physiochemical properties of electrochemical stimulated water has led to the upsurge in the research on novel disinfectants.

Therefore, this study was carried out as a field trial to test a novel strategy and identify new disinfectant agents to control microbial infections in poultry production.

MATERIALS AND METHODS

In the period 2010-2012 we performed examination at 20 broiler poultry farms. The isolation of *Salmonella spp*. from poultry houses was done by taking swabs from various parts of poultry houses (floor and litter).

Laboratory trial and other two separate field trials were conducted to evaluate the efficacy of various disinfectants on the isolated *Salmonella entriditis* when applied to poultry house floors, as well as an innovative trial also, carried out to evaluate the efficacy of same disinfectants when they contained Ag nanoparticles (AgNps).

Prevalence of Salmonella

Prevalence of *Salmonella* was determined in accordance with the Payne et al., (2005) and ISO 6579 (2002) (Microbiology of foods and animal feeding stuff-horizontal method for detection of *Salmonella spp.)* with the addition of the drag swab (floors and litter) as a medium for sample collection. Briefly, BPD samples were incubated at 37°C for 24 h, and then 0.5 mL was transferred into10 mL of tetrathionate broth and 0.1 mL was transferred into 10 mL of Rappaport Vassiliadisbroth followed by 24-h incubation at 42°C. Both broths were then streaked onto xylose lysine tergitol 4 (XLT4), brilliant green sulfa, and modified lysine iron agar plates and incubated at 37°C for 24 h. Suspect colonies were inoculated onto triple sugar iron agar and lysine iron agar slants and incubated at 37°C for 24 h. *Salmonella* confirmation was performed with polyvalent O antiserum Negative controls were used for all plating procedures to ensure that the media had been properly sterilized

Diagnostic poly and monovalent *Salmonella "0" and "H"* antisera for serological identification of *Salmonella. (*Mast *Salmonella* diagnostic antisera*).* Serological identification of *Salmonella* isolates was carried out according to Popoff, (2001).

Evaluation of the Efficacy of the Disinfectants

Disinfectants

White wash

Prepared through using slaked lime Ca $(OH)_2$ (prepared by mixing quick lime with water) diluted 20% suspension using water *Formalin* (40% formaldehyde gas in water).A product of Pharmaceutical Chemical Company, Egypt. The preparation was used to provide formaldehyde concentration of 4%.

Iodine anti germ (Iodophor)

Iodophor preparation containing 12% iodophor concentrate and detergent carrier (A product of Kemate Egypt). The preparation was used in a concentration of 1 % in water.

Phenuique

An emulsified coal tar disinfectant. Product from (Morgan Company). The preparation was used in a concentration of 3% in water.

Ag nanoparticles

Silver oxide-nanoparticles - (AgNs) stock solution was prepared by adding 0.5 and 1 mg of 15 nanometer diameter AgNPs powder (IBU-tec / Nanotechnology, 15 nm in diameter)

Envirolyte-Anolyte (Env)

Envirolyte-Anolyte (1\500) [pH 2.5-3.5, ORP>1150mV, C_{active} ~500mg/l]. It contains various mixed oxidants predominantly hypochlorous acid and sodium hypochlorite (HClO, ClO_2, $HClO_3$, $HClO_4$, H_2O_2, O_2, ClO^-, ClO_2^-, ClO_3^-, O^-, HO_2^-, OH^- - working substances, pH from 2.0 to 8.5, 1\500 = 2 mg /L active chlorine, 1\1000 = 1mg /L active chlorine).

Sampling procedure

Stock culture of *the isolated Salmonella entriditis* was used for the disinfection assays. Bacteria were harvested by centrifugation at 1,900 x gravity for 10 min and were washed twice with 10 mL of sterile distilled water (DW); the final pellet was then resuspended in 5 mL of sterile DW. A 1–2-mL quantity of this stock solution was added to 5 mL of sterile DW to attain a final working solution of 1–2 x 10^7 colony-forming units/mL. The starting concentrations of bacteria were the same as the concentration in the working solution, which served as the control.

Field trials

Experimental test units were 1-ft^2 floor plots randomly blocked with a 1-ft^2 space between each experimental plot. Two treatments were carried out to evaluate the efficacy of the disinfectants without and when they contained Ag nanoparticles. Each disinfectant was prepared according to the manufacturers' recommendations using distilled water and tested twice as a disinfectant only and containing Ag nanoparticle 10% (v\v). Each disinfectant was applied to 10 plots as a coarse spray at a low application rate of 125 mL/plot. The rate was chosen due to its ability to create a good surface coverage. Ten untreated plots, receiving no disinfectant, served as the negative control.

Statistical analysis

Statistical analysis of the data was performed using the Statistical Analysis System version 9.2 (SAS Institute, Cary, North Carolina). A paired t-test was implemented, and differences between means were considered to be significant at P-values less than 0.05.

RESULTS AND DISCUSSION

With pathogen reduction becoming increasingly important to both consumers and integrators, it is very crucial to examine the various strategies to reduce these pathogens on the final processed carcass. Should a sanitation program be an effective method for reducing food-related pathogens at the grow-out level, then proper implementation of disinfectant use could be important for reducing carcass contamination. If pathogens can be reduced in the bird's environment, contamination on the exterior of the bird should be reduced, followed by a reduction in pathogenic bacterial populations at the processing plant and on the finished product (Payne et al., 2005).

Prevalence of Salmonellae

The obtained results showed that 4 *Salmonella species* were isolated from 20 broiler poultry houses. Prevalence of *Salmonella spp.* was 60 % in open broiler houses whether raised Cobb, Ross or Sasso breeds .But it was 40 % in closed broiler houses (Sasso).

The most prevalent *Salmonella* serovars in broilers were *S. entenidis* (25%) *S.typhimuiurium* (15 %), *S.*, *S.infantis* (5%) and *S. kenlucky* (5%). *Salmonellae* had been isolated from poultry litter by many authers (Humphrey and Lanning, 1988; Poppe *et* al., 1991; Pieskus et al., 2008; Dhanarani *et al.*, 2009; Andreatti-Filho et al., 2009).

In Egypt, *Salmonellae* were isolated from 5.3% of litter samples collected from Kafr El-Sheikh province, the only identified one of the collected samples was *S. enteritidis* (Mohammed *et al.*, 1999). Trawinska et al. (2008) isolated *S. typhimurium* from geese, broiler chickens and reproductive laying hens, *S. enteriditis* proved the most commonly reported serovar in poultry isolated by Trawinska et al., (2008).

Effect of disinfection programs on Salmonellae of infected poultry house (after the disinfection process).

The obtained data in Table 1, revealed that 35 % of visited broiler houses used phenuique, formalin and iodophors program and 35 % of farms also, used formalin and iodophors program for disinfection of poultry houses. While 30 % of these farms used white wash and spraying formalin.

Table 1. Prevalence of *Salmonellae* of the examined poultry farms

Disinfectants	Number of farms	No. of +ve *S.entriditis* and percentage (%)	Log_{10} after disinfection	Log_{10} before disinfection
Phenique, Formalin, and Iodophors (G1)	7	2(28.57)1	1.5*3	2*7
Whitewash and Formalin (G2)	6	2(33.33)2	2*2.5	1.5*7
Formalin and Iodophor (G2)	7	1(14.28)3	1.4*2	2*7
Total	20	5(76.18)		

1: Two isolates S.entriditis and one isolate typhimuiurium

2: Two isolates S.entriditis and one isolate typhimuiurium and one isolate S.infantis

3: One isolate S.entriditis ,one isolate typhimuiurium and one isolate S. kenlucky

For all the three disinfectant program effects (phenuique, formalin and iodophors; formalin and Iodophors; white wash and formalin), significantly impacted *Salmonella* populations ($P < 0.05$) (4.5; 5; 2.8 $\log_{10}$ reduction, respectively) but unfortunately, failed to kill all the populations,

In one hand, the obtained data in Table 2 , revealed that the following disinfectants without Ag nanoparticles: white wash, phenuique, formalin, iodophors and anolyte (1/1000) for disinfection of floor plots significantly impacted *Salmonella* populations ($P < 0.05$) (2; 3; 3; 3; 5 $\log_{10}$ reduction, respectively) but unfortunately, failed to kill all the populations. While, Env (1/500) significantly reduced the population of *S. entenidis* with a complete reduction of the population. Layton, (2006) that Clorox spray was most effective of all the disinfectants in eliminating *Salmonella* as measured by zone of inhibition after being incubated for 24 and 48 hours. After the exposure to the chlorine solution, the count of *S. entenidis* was reduced by 0.92 to 2.35 log cycles (Al-Mohizea, 1995).

Table 2. Effect of disinfection programs on *Salmonellae* of infected poultry house (after the disinfection process)

Disinfectants	T.C.C.(Log_{10}) of floor after disinfection	
	Without [a]	Plus Nanopart.[b]
Whitewash	1.5 x 5	1 x 2*
Phenique	5.6 x 4	0**
Formalin	1.4 x 4	0**
Iodophors	2 x 4	1.4 x 3*
Envirolyte-Anolyte(1/500)†	0	----
Envirolyte-Anolyte	1.5 x 2	----

125 mL/ft2 application rate per plot (surface coverage). N^2 =10 plots per disinfectant. (Common usage level of500 gal/16,000 ft2). Control 3 x 10^5, 1: 1.5x10^7; 2: 2.5 x10^7:3: 1.4 x 10^8

Each disinfectant was prepared according to the manufacturers' recommendations using distilled water and tested twice as a disinfectant only and containing Ag nanoparticle 10% (v\v). It contains various mixed oxidants predominantly hypochlorous acid and sodium hypochlorite ($HClO$, ClO_2, $HClO_3$, $HClO_4$, H_2O_2, O_2, ClO^-, ClO_2^-, ClO_3^-, O^-, HO_2^-, OH^- - working substances pH from 2.0 to 8.5, †:2mg /L active chlorine, ‡:1mg /L active chlorine. *:($P < 0.05$), **:($P < 0.01$)

Although the present study did not examine the exact mechanism of action, we believe that the bactericidal effect of Envirolyte-Anolyte against bacteria was due to the combined action of hydrogen ion concentration, oxidation-reduction potential and dissolved chlorine. Envirolyte-Anolyte is a strong acid, but it is different to hydrochloric acid or sulphuric acid. These acids have a strong degree of ionization, and when oxidation occurs, H^+ is used and new H^+ is generated (Iwasawa *et al.*, 1993). In case of Env, no new H^+ is generated because it is produced by electrolyses only of the saline solution. On controlling food borne pathogens, aqueous chlorine dioxide (ClO_2) was most effective in reducing *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* at concentration of 15 ppm (Wu and Kim, 2007). Indeed, some chemical byproducts formed when chlorine is used for reducing microorganisms in food processing are considered as mutagenic or carcinogenic (Richardson et al., 1998). Researchers have focused on chlorine dioxide (ClO2) as an alternative sanitizer since it has 2.5 times the oxidation capacity of chlorine and is less reactive to organic compounds (Benarde et al., 1967; Richardson et al., 1998; Beuchat et al., 2004; Han et al., 2004; Lee et al., 2004; Sy et al., 2005).

Formalin is widely use at 5% strength as a general disinfectant, but it needs contact time to be effective (Williams ,1980) but, best reduction in total bacterial count could be obtained with 10% formalin solution followed by creolin 3% while lower efficiency was recorded with iodophors (Mohamed ,1990). Huber, (1977); Williams (1980); Ka-oud (1986); Sainsbury (2000) and Mandel et al, (2005) recommended using the following disinfectants, formalin, iodophors, and phenique for disinfection of poultry houses and the most common disinfectant is formalin, due to it is cheap and available in market.

Disinfection does not always guarantee elimination of the problem-causing bacteria and high level of disinfection is required to prevent contamination of the next flock. In addition, sublethal concentrations of disinfectant may even cause organisms to enter a viable but non culturable state or develop antimicrobial resistance.

On the other hand, white wash and iodophores containing Ag nanoparticles showed highly significant ($P < 0.05$) reduction of *Salmonella* populations in floor after disinfection process (5; 4 $\log_{10}$ reduction, respectively). Interestingly, *Salmonella* populations completely destroyed when exposed to phenuique and formalin containing Ag nanoparticles in field trial 2. This may be due to the ubiquitous nature of Ag nanoparticles, which are able to enhance the disinfectant power. The mechanism of antibacterial effect of silver nanoparticles has been reported in the literature (Sondi and Salopek-Sondi, 2004), which suggests that the particles are bactericidal.

Several mechanisms have been proposed to explain the inhibitory effect of silver nanoparticles on bacteria. It is assumed that the high affinity of silver towards sulfur and phosphorus is the key element of the antimicrobial effect. Due to the abundance of sulfur-containing proteins on the bacterial cell membrane, silver nanoparticles can react with sulfur-containing amino acids inside or outside the cell membrane, which in turn affects bacterial cell viability. It was also suggested that silver ions (particularly Ag+) released from silver nanoparticles can interact with phosphorus moieties in DNA, resulting in inactivation of DNA replication, or can react with sulfur-containing proteins, leading to the inhibition of enzyme functions

(Gupta ,1998; Matsumura et al.,2003). The general understanding is that Ag nanoparticle of typically less than 20 nm diameters get attached to sulfur-containing proteins of bacterial cell membranes leading to greater permeability of the membrane, which causes the death of the bacteria (Morones et al., 2005).

CONCULSION

The unique physiochemical properties of the nanoparticles combined with the growth inhibitory capacity against microbes has led to the upsurge in the research on nanoparticles and their potential application as antimicrobials.

Envirolyte-Anolyte (1/500) significantly reduced the population of *S. enteriditis* with a complete reduction of the population. But at concentration of 1/1000 produced a 4 $\log_{10}$ reduction in bacterial growth. Envirolyte-Anolyte (1/500) was very effective against *S. enteriditis* killing all bacteria in poultry houses.

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