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PROOF

RESEARCH PAPER

Revolutionizing Technique to Control *Mycoplasma gallisepticum* in Hatching Eggs Using Zinc oxide, Antibiotic nano-particles

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Abstract

Zinc oxide (ZnO), Flumequine, and Tiamulin nanoparticles were designed to target *Mycoplasma gallisepticum* (MG) without adversely affecting egg-hatching potential. 3,000 eggs were selected from 3,500 of Hy-Line breeder flock which were divided into 5 equal groups (600 eggs each) as detailed below: Group A (infected by MG and sanitized by using ZnO nanoparticles); Group Bf (infected by MG and sanitized by using Flumequine nanoparticles); Group Bt (infection by MG and sanitized by Tiamulin nanoparticles); Group C (infected by MG, serving as the positive control); Group D (uninfected and untreated, serving as the negative control). The findings revealed a statistically significant reduction ($P<0.05$) in colony-forming units per millilitre (CFU/mL) of MG and Total Bacterial Colony Count (TBCC) in groups: A, Bf and Bt after 5 days of treatment as compared to group C. The treatments exhibited notable bactericidal properties while allowing for normal embryo development, hatching, and mortality rates. Eggs treated with these antibacterial agents experienced a significant decrease ($P < 0.001$) in embryonic mortality during incubation. ZnO NOs, Flumequine, and Tiamulin nanoparticles exhibit promise in preventing *Mycoplasma gallisepticum* -induced hatchery infection.

Introduction

In the poultry sector, maintaining the health of hatching eggs is critically important, as the intrusion of microorganisms through the eggshell can lead to significant repercussions, including detrimental impacts on embryo development, hatchability, and the health of chicks post-hatching (Oliveira *et al.,* 2022; Yousef *et al*., 2023). Additionally, such microbial contamination can facilitate the spread of infections within the growing flock, thereby worsening the situation (Adame and Ameha, 2023). Nevertheless, insufficient use of sanitizers may not offer adequate protection (Tebrün *et al.,* 2020; Oliveira *et al*., 2022).

Once bacteria penetrate the egg, they may produce toxins that directly threaten the embryo's health (Jan and Baron 2017; El-Saadony *et al*., 2022). Insufficient application of sanitizers allows pathogens to breach the eggshell pores and disrupt the embryo (Tebrün *et al,* 2020; Faria *et al.,* 2014; Jan. and Baron, 2016.). Among the various pathogens, *Mycoplasma gallisepticum* (MG) is a prominent contributor to air sacculitis and chronic respiratory disease (CRD) in chickens (Qoraa *et al*., (2023). A particular concern is the capacity of MG infections to spread both vertically and horizontally, which significantly affects breeding flocks (Marouf *et al.,* 2020; Marouf *et al*., 2022). A crucial strategy for interrupting the cycle of bacterial infections in the chicken production chain is the sanitation of hatchability (Olsen *et a*l., 2017; Motola *et al*., 2023). The industry encounters considerable challenges regarding the disinfection of hatching eggs, as the disinfectant

must effectively act on the egg's surface while ideally penetrating its pores without jeopardizing embryo viability, hatchability, or the overall performance of the chicks post-hatching (Silva *et al.,* 2008; Steiner, 2020; Ferguson *et al*., 2020).

The primary objective was to assess innovative techniques and new biocidal materials to achieve effective sanitization without harming embryos, with the broader goal of aiding in the containment and prevention of diseases within the poultry sector.

Materials and Methods

Ethics statement

The animal experiment was conducted in strict accordance with and adherence to the relevant policies regarding animal handling as mandated under international, national, and/or institutional guide lines for the care of animals and was approved by the Research Ethical Committee at the National Research Centre, Cairo, Egypt.

Hatching Eggs and Experimental Design

Based on molecular and serological analysis of the breeders' flocks, the hatching eggs were taken the same day from a 50-week-old Hy-Line breeder flock at Sharqia Farms, Al-Sharqia Governorate, Egypt that was free of Mycoplasma. Consistent eggshell thickness and porosity were guaranteed throughout all experimental trials thanks to this simultaneous collection. With an average egg weight of 63.2 grams, this flock showed exceptional reproductive characteristics, including 82% hatchability, 18.3% embryo mortality, and 85.2% fertility.

This research took place from June 2023 to December 2024 at Depart. of Veterinary Hygiene, Faculty of Veterinary Medicine, and Faculty of Agriculture at Cairo University. The study employed nest eggs that showed no signs of contamination and had smooth shell surfaces. Following that, the eggs were carefully evaluated, with 20 eggs allocated for microbial load counting, while another 20 were reserved for MG counts prior to disinfection assessment. In a randomized controlled trial (RCT), a completely randomized design was implemented for the selected 3,000 eggs, which were divided into 5 equal groups (600 eggs each) as detailed below: 3,000 eggs were selected from Hy-Line breeder flock which were divided into 5 equal groups (600 eggs each) as detailed below: Group A (infected by MG and sanitized by using ZnO nanoparticles); Group Bf (infected by MG and sanitized by using Flumequine nanoparticles); Group Bt (infection by MG and sanitized by Tiamulin nanoparticles); Group C (infected by MG, serving as the positive control); Group D (uninfected or untreated, serving as the negative control).

Pre-disinfection

The process of bacterial sampling commenced after the eggs were allowed to air dry for one and a half hours in an incubator located in Shandong, China, which has the capacity for 1000 eggs and features automatic egg handling and manipulation (Musgrove *et al*., 2005).

Egg Inoculation

Strain utilized

The MG strain utilized is catalogued under the Gen-Bank identifier MZ826700 and features a 26 bp DNA linear BCT as of September 30, 2021.

Egg Infection

The eggs were subjected to a warming procedure lasting 2 to 3 hours at a temperature of 37°C. Following this, four test groups designated A, Bf, Bt and C were immersed for 5 minutes in a bacterial MG suspension, which was kept at a low temperature of 5°C and contained MG at a rate of 10^5 CFU per millilitre, as described in the research conducted by Hafez *et al.* (1995). The strain was propagated on pleuropneumonia-like organism (PPLO) media in accordance with the methods specified by Naylor *et al*. (1992). The concentration of CFU was assessed following the standard protocols established by Rodwell and Whitcomb, (1983) to ensure a concentration of 10^5 CFU/mL of the MG strain for inoculation into the experimental groups. After allowing the eggs to air-dry at room temperature for 60 minutes, they were transported to the animal testing facilities (to be penetrating them, as noted in earlier research by Russell, (2003).

Application of Antimicrobial Agents

1. ZnONPs

Zinc oxide Nano-particles (Zn O, 99%, APS =15-20 nm (TEM)) Molecular Weight 81.39 Morphology: Sphere, (USA) was used at 0.015 and 0.025 mg/mL.

Minimum inhibitory concentration (MIC) tests were conducted to assess the efficacy of ZnONPs against MG. The antimicrobial properties of the NPs were evaluated using the micro-dilution method on 96-well micro-plates (Tajik *et al*., 2014; Bajaj *et al*., 2017; CLSI rules, 2018).

A stock solution was prepared by placing and suspended 1 mg of ZnONPs powder in 100 mL of sterile saline and then subsequently diluted and introduced into wells containing the bacterial culture. The test wells were adjusted to achieve a final bacterial concentration of 1.5 x 10^8 CFU/mL, with NPs at a rate of 0.5 and 1 µg/mL (Herigstad *et al.,* 2001). Nano-emulsion (MIC50 was recorded at 150 µg/mol) has emerged as a promising delivery mechanism for ZnONPs (spherical in shape and measure 15 nm in diameter) (Sharma *et al*., 2010).

2. Antibiotics (Flumequine and Tiamulin NPs) (Antibiotic NPs)

The MIC50 of Flumequine NPs was 50 µg/mol and Tiamulin, was 0.0009 µg/mL. The antibiotics were produced in the form of NPs using the Nano Spray Dryer (Buchi B-191 HP, Netherlands), with Flumequine being sprayed at a concentration of 150 µg/mL and Tiamulin at a concentration of 0.0009 µg/mL.

Procedures

The cold fogging technique, which employs an ultralow volume (ULV) or cold fogger (1.6 Litter ULV Cold Fogger 110 V Atomizer Machine Fogger Nano Portable Disinfection Mist Sprayer), was implemented. This fogger features a tank capacity of 1.6 litter and is calibrated to produce droplets ranging from 20 to 40 nm in size. The procedure commenced with the placement of eggs onto a clean, open plastic tray, followed by the activation of the ultra-fogger for duration of 6 minutes for fogging. The eggs were then subjected to an additional 20 minutes of exposure to the fog (Motola *et al*., 2020; Tebrün *et al.,* 2020). After treatment, the eggs were placed under a laminar flow hood (Lam-systems, Berlin-Germany) for a full hour to ensure proper drying. The environment in which the eggs were sprayed maintained a relative humidity (RH) of 20% and a temperature of 24°C.

Sampling and Microbiological Analysis

Analysis of Eggs involving Groups A to D, a total of 20 eggs was tested following disinfection procedures. Microbial sampling was conducted on the 5th, 10 th, and 18 th days of incubation. To mitigate any potential bias related to egg positioning, samples were consistently taken from designated locations on the trolleys and trays across all groups. A rigorous sampling protocol was implemented to ensure adequate representation from each group while preventing cross-contamination. Each group of eggs was sampled and transported separately to preserve the integrity of the testing process (Motola *et al*., 2023).

Total Bacterial Colony Counts

The analysis of total bacteria and Mycoplasma enumeration was carried out using established methodologies (Russell, 2003; Fasenko *et al.,* 2009); Haščk *et al.,* (2015).

Mycoplasma Colony Counts

Mycoplasma counts were assessed by placing 0.1 mL of the diluted samples onto Mycoplasma agar plates supplemented with G (Oxoid, Dardilly, France). These plates were incubated for 7 to 20 days at 37°C in a 5% CO_2 environment, facilitating the enumeration of Mycoplasma counts (Razin, 2012). Moreover, post-hatching, microbiological samples were collected from the lung, trachea, and air sacs for Mycoplasma isolation.

Incubation and Measurements

Enterobacteriaceae contamination, surface samples were microbiologically analysed prior to restocking the incubators (Tebrün *et al*., 2020). Following this, eggs from each designated group were placed in the same setter under controlled conditions: a temperature of 37.6°C, relative humidity of 60%, and a rotation of 45° every hour for the duration of 18 days. Upon completion of the incubation period, the eggs were transferred to a Hatcher set to maintain a temperature of 36.5°C and relative humidity of 65%, with each egg housed in individual boxes to ensure accurate identification of each hatched chick (Tebrün *et al*., 2020).

Early embryo mortality

Early embryo mortality was calculated from days 1 to 6 of incubation Mid-term embryo mortality from days 7 to 18 of incubation Late embryo mortality was assessed by dividing the number of dead embryos from days 19 to 21 of incubation by the total number of eggs initially set, minus both early and mid-term dead embryos, with this ratio reported as a percentage. Eggs that did not show signs of early embryonic mortality were classified as infertile. The fertility rate was calculated by determining the proportion of fertilized eggs relative to the total number of eggs initially set, expressed as a percentage.

Hatchability

Furthermore, hatchability percentages were assessed to measure the effectiveness of the hatching process. The hatchability of the set eggs was calculated by finding the ratio of the number of chicks that hatched to the total number of eggs initially set, expressed as a percentage (Hrnčár *et al*., 2021).

Organs weights of hatching eggs

Thirty chicks, selected for their alignment with the average body weight of their respective groups, were chosen for further analysis. The procedure commenced with the euthanasia of these chicks, after which samples were obtained from the liver, heart, lung and air sacs for microbiological examination. Following the dissection of the birds, the weights of essential organs, including the heart, liver, spleen, and *Bursa of Fabricius*, was carefully recorded (Rizk *et al*., 2022).

Statistical Analysis

The data collected and the subsequent analyses were conducted using Microsoft Excel 2016 and Statistical Package for Social Sciences software, version

25.0 (SPSS Inc., Chicago, IL). The statistical analysis employed a fixed effect model to evaluate the effectiveness of electrostatic and cold fog disinfection methods in combating MG contamination in hatching eggs. The model was specifically designed to test the effects of the disinfection treatment on different groups. The normality of the data was assessed through the Kolmogorov–Smirnov test. Both descriptive and inferential statistics, including analysis of variance (ANOVA), were employed to present and interpret the results. Post hoc testing using the least significant difference (LSD) method was performed to determine significant differences between treatment means. Mortality, fertility, hatchability, and hatched fertile egg data were analysed using Chi-square (X^2) to determine the effect size (Φ) and significant differences. For each statistical test, a significance level of less than 0.05 was considered statistically significant, following the guidelines presented in Campbell and Swinscow (2011).

Results and Discussion

Effect of ZnONPs Flumequine and Tiamulin NPs on *M. gallisepticum* colony count (MCC) and total bacterial colony count (TBCC)

A statistically significant reduction ($P < 0.001$) in colony-forming units per millilitre (CFU/mL) of total bacterial colony count (TBCC) and MCC in group A, Bf and Bt from 4 $\log_{10}$ to 0 log at the 5^{TH} d of post-treatment. In group D, TBCC revealed no significant reduction on the day 18^{TH} of incubation (log 3), without any reduction in group (C) (log 5.86). The treatments exhibited notable bactericidal properties while allowing for normal embryo development, hatching, and mortality rates. Eggs treated with these antibacterial agents experienced a significant decrease ($p < 0.001$) in microbial load, as showed in Table 1. In other meaning, Group D showed variable TBCC logs (ranged: 2.4- 5) and consistently showed no Mycoplasma growth (uninfected and untreated) while Group D showed highly Mycoplasma growth. The utilization of innovative ZnONPs and antibiotics NPs, applied through ultra-cold fogging, has shown encouraging results in decreasing bacterial population (M.gallisepticum and total bacterial colony count) in infected eggs. Groups A (ZnONPs) and Bf and Bt (Antibiotic NPs) exhibited significantly lower rates of of bacterial population, and diminished *M gallisepticum* colony counts when compared to the untreated infected Group C. (CEVA, 2008; Olsen *et al*., 2017). ZnONPs have demonstrated significant potential across various applications, particularly in the fight against antibiotic resistance (Padmanabhan *et al*., 2019; Bhunia *et al.,* 2021). Furthermore, ZnONPs exhibit biocompatibility with avian cells and have proven effective as antimicrobial agents. Dastjerdi and Montazer, 2010). Recently, the antibacterial properties of ZnONPs have been examined against *Klebsiella pneumonia,* it was determined that the effective dose for inhibiting growth of the ZnONPs was 5 µg/ml (B)

Effect of ZnONPs and antibiotics NPs *on M. gallisepticum* infected fertile eggs in relation to hatchability %

In ZnONPs and antibiotics NPs treated infected eggs (groups: A, Bf, Bt). were revealed normal hatchability %. Table 2, highlights the impact of MG-infected eggs on the hatchability of fertile eggs. Groups A, Bf and Bt were exhibited the highest hatchability (84.6, 83.4 and 83%, respectively), whereas, Group C (MG infected but did not treat) exhibited the lowest values (hatchability: 35%). Notably, the hatchability of fertile eggs was markedly lower in Group D (81%) than in the other disinfected groups (A, Bf, Bt). The analysis of hatchability revealed chi-square statistics, with 4 degrees of freedom and a statistically significant association ($p < 0.001$) with effect size (0.471).

Impact of MG fertile eggs infection on Embryo Mortality

In this study, the results indicated an impact of MG contamination on embryo mortality in the different treatment groups. Group C, which was contaminated but untreated, exhibited significantly greater mortality rates during the late (65%) incubation periods than did the treated groups, Group A (15.4%), Group Bf (16.6%), Group Bt (17%), Group C, the negative control, showed comparatively higher mortality percentages than the treated groups (A, Bf and Bt) across the incubation periods (as showed in Tables 3a and 3b). To evaluate the effectiveness of the treatment methods across the 5 different groups, a chi-square test of independence was performed (Table, 3b). The analysis of cumulative mortality (1−21 d) resulted in a chi-square statistic of, with 4 degrees of freedom. The associated p-value was 0.00001, below the alpha level of 0.05, suggesting a statistically significant association between group and treatment efficacy.

Additionally, the effect size was calculated using Phi (F), which was found to be 0.508. While this effect size is statistically significant, it is strong in magnitude. The same trends were observed for the early, middle, and late mortality percentages (Olsen *et al.,* 2017). The treatments exhibited notable bactericidal properties while allowing for normal embryo development, hatching, and mortality rates. Eggs treated with these antibacterial agents experienced a significant decrease ($p < 0.001$) in embryonic mortality. In other meaning, Group D showed variable TBCC logs (ranged: 2.4-5) and consistently showed no Mycoplasma growth (uninfected and untreated) while Group D showed highly Mycoplasma growth.

Effect of *M. gallisepticum* infected fertile hatching eggs on the weight of the internal organs of hatched chicks

The average weights of the heart, liver, spleen, bursa, and chicks in each group. Group (Table 4).

Table1. Log_{10} Colony Count of *M. gallisepticum* (MCC) and Total bacterial colony count (TBCC) in the infected egg and control groups during the incubation period and in day-old chicks

Group	Group A		Group Bf		Group B		Group C		Group D	
	*MCC	ᶜTBCC	MCC	TBCC	MCC	TBCC	MCC	TBCC	MCC	TBCC
Before infection	4.04	3.3	4.02	3.22	4. 03	3.3	4.03	2.4		2.4[c]
After infection	4.07	3.2	4.06	3.4	4.06	3.26	4.0 6	3.3	Not-	3.3[c]
5th d of incubation	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	4.45	4	Infected	2[c]
10th d of incubation	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	5.61[b]	5		2[c]
18th d of incubation	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	5.86[b]	4.8		2.2[c]
Day-old Chick	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	5.45[b]	3.8		2[c]

a,b,c: The mean values within the rows marked with different superscript letters are significantly different (P<0.05): *M. gallisepticum* colony count; ᶜ: Total bacterial colony count

Table 2. Effect of *M. gallisepticum* infected hatching eggs on hatchability rates

Empty Cell	Hatchability		X^2 (df)	P	Phi
Groups	Hatched%	Non-hatched%	1,328.907 (4)	0.0001	0.471
A	84.6	15.4			
Bf	83.4	16.6			
Bt	83.0	17.0			
C	35.0	65.0			
D	81.0	19.0			

Groups are significantly different (p< 0.0001)

Table 3. Effect of *M. gallisepticum* infected hatching eggs on Cumulative mortality embryo mortality

Cumulative mortality (1– 21d)[1]			X^2 (df)	P	Phi
Group	Dead %	Alive %			
A	15.4	84.6	1,547.170 (4)	0.0001	508
Bf	16.6	83.4			
Bt	17.0	83.0			
C	65.0	35.0			
D	19.0	81.0			

Table 4. Effect of *M. gallisepticum* infected hatching eggs on the weight of the internal organs (Heart, liver, spleen, and bursa) of day-old chicks.

Group	Group A	Group_Bf	Group_Bt	Group	Group D	P value
Heart	1.43 ± 0.005[a]	1.25 ± 0.003[b]	1.27 ± 0.012[b]	0.92 ± 0.004[d]	1.03 ± 0.004[c]	0.001
Liver	7.71 ± 0.007[a]	6.51 ± 0.006[b]	6.46 ± 0.016[b]	4.30 ± 0.062[d]	4.92 ± 0.016[c]	0.001
Spleen	0.12 ± 0.0[a]	0.12 ± 0.002[a]	0.12 ± 0.000[a]	0.08 ± 0.005[b]	0.10 ± 0.012[ab]	0.001
Bursa	0.28 ± 0.003[a]	0.26 ± 0.005[a]	0.27 ± 0.005[a]	0.19 ± 0.011[b]	0.20 ± 0.014[b]	0.001
Ck. weight	30.12 ± 0.327[a]	28.57 ± 0.485[ab]	28.47 ± 0.485[ab]	22.12 ± 0.888[c]	26.78 ± 0.564[b]	0.001

The mean values within the rows marked with different superscript letters are significantly different (P<0.05). The values are presented as the mean ± SE

A displayed significantly higher organ weights than did the other groups (heart: 1.43 g, liver: 7.71 g, spleen: 0.12 g, bursa: 0.28 g), while Group C exhibited the highest mortality and lowest organ weights (heart: 0.92 g, liver: 4.30 g, spleen: 0.08 g, bursa: 0.19 g). The reduction in organ weights, particularly in the heart and liver, among the infected groups underscores the potential physiological consequences of MG infection on embryonic development (Motola *et al.,* 2023).

ZnONPs have demonstrated significant potential across various applications, particularly in the fight against antibiotic resistance (Padmanabhan *et al*., 2019; Bhunia *et al*., 2021). Furthermore, ZnONPs exhibit biocompatibility with avian cells and have proven effective as antimicrobial agents. Dastjerdi and Montazer, 2010). Recently, the antibacterial properties of ZnONPs have been examined against Klebsiella pneumonia, it was determined that the effective dose for inhibiting growth of the ZnONPs was 5 µg/ml. Antibiotic NPs represent a promising Trojan horse approach to bypass mechanisms of antibiotic resistance. The development of a wide range of Antibiotic NPs with multiple targets is on the rise, thanks to advancements in nanotechnology (Sirelkhatim *et al*., 2015; Fatima *et al.,* 2020).

Conclusions

Perspectives notwithstanding the encouraging results, this study has certain limitations. The research concentrated on a specific strain of MG; therefore, additional studies involving various strains from different geographical regions are necessary to confirm the universal efficacy of the disinfection methods employed. Results revealed a statistically significant reduction in colony-forming units of MG and Total Bacterial Colony Count in treated groups. The treatments exhibited notable bactericidal properties while allowing for normal embryo development, hatching, and mortality rates

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