In vitro effect of zinc oxide nanoparticles on Staphylococcus aureus isolated from Layer Chickens
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Abstract
This study aimed to evaluate the antibacterial activity of zinc oxide nanoparticles (ZnO-NPs) on Staphylococcus aureus (S. aureus) isolated from layer chickens using minimum inhibitory concentration test (MIC). Samples (n=120) from the hock joints and livers of layers were collected under aseptic conditions from diseased and freshly dead chicken layers from different private farms in Assyut Governorate. Twenty-nine isolates of Staphylococci were recovered from 120 collected samples, with a prevalence rate of 24.16%. Nineteen (65.5 %) of these 29 isolates were coagulase negative and ten (34.5 %) were coagulase positive. The coagulase positive strains (CPS) were further analyzed using PCR. Our PCR results confirmed that these isolates are S. aureus as they encoded (clfA gene), while 6 isolates only were Methicillin Resistant Staphylococcus Aureus (MRSA) as they harbored mecA gene. Nine isolates of the demonstrated S. aureus displayed variable antibiotic sensitivity patterns in MIC assays against ZnO-NPs and some selected antibiotics. Our data showed that, ZnO-NPs have the potential antibacterial effect against majority of the isolates compared to other tested antibiotics.

Keywords: Staphylococcus aureus, sensitivity tests (MIC), layer chicken, zinc oxide nanoparticles.

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Introduction

In poultry, the disease conditions associated with staphylococcosis are a worldwide problem and vary with the site and route of infection in hatchery and poultry farms, and can infect the bones, joints, tendon sheaths, skin, sternal bursa, navel, and yolk sac through breakage of the skin and mucosal membrane of the birds, especially immunocompromised ones which are often more prone to staphylococcal infections. Once in the host, *S. aureus* invades the metaphyseal area of the nearest joint, which leads to osteomyelitis and become localized within that joint (Onaolapo et al., 2017). However, if *S. aureus* invades the bloodstream, it causes systemic infection in multiple organs, thereby causing economic losses, which accrued as a result of decreased weight gain, decreased egg production, lameness, mortality, and condemnation at slaughter (Andreasen, 2003). Resistance to antibiotics is an increasing concern worldwide and is associated mainly with uncontrolled usage of antimicrobials for treatment or as growth promoters in food animals (Barber et al., 2003). *S. aureus* resistant to methicillin is referred to as methicillin-resistant *S. aureus* (MRSA). Resistance to methicillin is mediated by the meca gene, which encodes penicillin-binding protein 2a, (PBP2a) (Chambers, 2001 and Chambers, 1997) and carry insertion sites for mobile genetic elements that facilitate acquisition of resistance determinants to other antibiotics, because of its multidrug resistance pattern, MRSA is considered a public health concern (Malachowa and Deleo, 2010).

Compared with other metal oxide nanoparticles, ZnO NPs has comparatively inexpensive and relatively less toxic property exhibit excellent biomedical applications particularly as antibacterial agent [Xiong, 2013; Zhang and Ziong, 2015; Kim et al., 2017 and Mishrat et al., 2017]. Zinc oxide nanoparticles (ZnO-NPs) have been reported as an antimicrobial agent and of all the metal oxide nanoparticles studied so far exhibited the highest toxicity against pathogenic microorganisms, ZnO-NPs are such combinations that their purity is very high, their toxicity and pollution is very rare (Sangeetha et al., 2012). ZnO-NPs antibacterial activity directly correlates with their concentration and its activity is size dependent as reported by several studies, however, this dependency is also influenced by concentration of NPs (Sirelkhatim et al., 2015 and Wang et al., 2016). ZnO-NPs application as antimicrobial agent have been attributed to the induction of oxidative stress due to the formation of reactive oxygen species (ROS), that the formation of ROS is the main antibacterial mechanism of ZnO nanoparticles (Zhang et al., 2007 and Espitia et al., 2012), also to the membrane disruption as a result of accumulation of ZnO-NPs therein, and internalization of nanoparticles followed by the release of antimicrobial ions (Zn$^{+2}$) (Sirelkhatim, et al 2015, Raghunath and Perumal, 2017). Scanning electron microscopical (SEM) and transmission electron microscopical (TEM) images characterize the size and shape of ZnO-NPs and also demonstrated that ZnO-NPs firstly damage the bacterial cell wall, then penetrate, and finally accumulate in the cell membrane. They interfere with metabolic functions of the microbes causing their death. All the characteristics of the ZnO-NPs depend on their particle size, shape, concentration, and exposure time to the bacterial cell (Siddiqi et al., 2018). This study aimed to determine the antibiotic resistance profile of *S. aureus* and *MRSA* recovered from layer chicken
and to evaluate the antibacterial activity of ZnO-NPs against \textit{S. aureus} isolates.

**Materials and Methods**

**Collection of chicken samples**

A total number of 120-layer chicken samples obtained from 5 chicken farms in Assiut governorate. Samples were collected from hock joints and livers under aseptic conditions from diseased and freshly dead chickens from different private farms from different regions in Assiut Governorate, Egypt. Liver samples were collected from diseased and freshly dead birds while joint samples were obtained from birds suffering from lameness. The samples were then transferred to laboratory in an icebox.

**Isolation and identification**

The collected samples were inoculated into Tryptic soy broth (TSB) containing 70mg/ml Na Cl and incubated at 37 °C for 24 h. A loopful of the inoculated broth was sub cultured on Baird Parker agar medium at 37 °C for 48 h. The typical suspected colonies of being staphylococci were sub cultured on Mannitol salt agar which used as selective as well as differential medium for isolation and identification of Staphylococci and tentatively identified according to morphological features, gram staining, catalase test, coagulase test (in tubes) according to the methods of Sullia and Santharan (1998) and Quinn et al. (2004).

**Polymerase Chain Reaction (PCR) for identification of clfA and mecA genes**

The coagulase positive staphylococcal isolates were tested for both molecular confirmation and the presence of both \textit{clfA} and \textit{mecA} genes in the Reference Laboratory for Veterinary Quality Control on Poultry Production in Animal Health Research Institute, Dokki, Giza, Egypt, as follow:

**DNA extraction:** DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer’s recommendations.

**PCR:** Primers used were supplied from Metabion (Germany) and listed in table (1).

| Target gene | Primers sequences (5’ - 3’) | Amplified segment (bp) | Primary denaturation | Secondary denaturation | Annealing | Extension | Final extension | Reference |
|-------------|-----------------------------|------------------------|---------------------|------------------------|-----------|----------|----------------|----------|
| \textit{mecA} | GTA GAA ATG ACT GAA CGT CCG ATA A | 310 | 94°C 5 min. | 94°C 30 sec. | 50°C 30 sec. | 72°C 30 sec. | 72°C 7 min. | McClure et al., 2006 |
| CCA ATT CCA CAT TGT TTC GGT CTA A | | | | | | | |
| \textit{clfA} | GCAAAAATCCAGCACA ACAAGAAAACGA | 638 | 94°C 5 min. | 94°C 30 sec. | 55°C 45 sec. | 72°C 45 sec. | 72°C 10 min. | Mason et al., 2001 |
| CTTGATCTCCAGCCAT AATTGGTGG | | | | | | | |
**PCR amplification:**

Primers were utilized in a 25-µl reaction containing 12.5µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1µl of each primer of 20 pmol concentration, 4.5µl of water, and 6µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

**Analysis of the PCR Products:**

The products of PCR were analyzed using 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20µl of the products were loaded in each gel slot. A GelPilot 100bp DNA Ladder (Qiagen, Germany, GmbH) and generuler 100bp ladder (Fermentas, Thermo, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

**Determination of minimum inhibitory concentration (MIC):**

MICs of antibiotics were evaluated using the broth microdilution method in Mueller-Hinton broth (MHB) with an initial inoculum of 5x10^8 cells in non-treated polystyrene microtiter plates CC7672-7596; (CytoOne) in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2007). Nine *S. aureus* were prepared in phosphate-buffered saline (PBS) until a McFarland standard of 0.5 was achieved. The solution was subsequently diluted 1:300 in Mueller-Hinton broth (MHB) to reach a starting inoculum of 5x10^5 colony-forming units (CFU/mL). Bacteria were then transferred to a 96-well microtiter plate. Antibiotics were added (in triplicate) to wells in the first row of the microtiter plate and then serially diluted along the vertical axis. Zinc oxide nanoparticles were diluted in 0.5 ml HCL (70%) and 0.5 ml distilled water, the plates were incubated at 37°C for 22–24 hours before the MIC was determined. MIC was defined as the lowest concentration which inhibited the visible growth of bacteria.

**Synthesis of the ZnO-NPs**

Zinc oxide nanoparticles synthesis were performed at Physics Department, Faculty of Science, Assiut University using procedures published by Othman et al. (2017, 2018).

**Characterization of ZnO-NPs:**

ZnO-NPs were characterized by SEM to find out the surface morphology of synthesized zinc oxide nanoparticles and TEM image of synthesized ZnO-NPs was carried out to find out exact particle size of synthesized ZnO-NPs. These images were captured in The Electron Microscopy (EM) unit at Assiut University. (Figs 1, 2)

![Fig 1: Scanning Electron Microscope (SEM) image of synthesized ZnO nanoparticles. Direct magnification: 50,000 x. HV=15 KV. SEM studies shows zinc oxide is in pure form and particles are white colored nanoparticles.](image)
Results

Prevalence of bacterial isolates:

A total of 29 staphylococcal isolates were recovered from 120-layer samples (106 livers and 14 joints). The incidence of *staphylococci* in liver and joint samples was 24.52% and 21.42%, respectively. Ten of the 29 isolates (34.5%) were coagulase positive *staphylococci* (CPS) and 19 (65.5%) were coagulase negative *staphylococci* (CoNS) as demonstrated in table (2).

Table (2): Shows the prevalence of *staphylococci* in examined organs of layer chickens.

| Examined organs | No. of examined organs | No. of the isolates | Percentage (%) | No. of coagulase positive *staphylococci* | Percentage (%) | NO. of coagulase negative *staphylococci* | Percentage (%) |
|-----------------|------------------------|---------------------|----------------|------------------------------------------|----------------|------------------------------------------|----------------|
| Liver           | 106                    | 26                  | 24.52          | 8                                        | 30.8           | 18                                        | 69             |
| Joint           | 14                     | 3                   | 21.42          | 2                                        | 66.7           | 1                                        | 33             |
| Total           | 120                    | 29                  | 24.16          | 10                                       | 34.5           | 19                                        | 65.5           |

PCR results of detecting clfA and mecA genes in CPS isolates:

In this approach, PCR was used to confirm *S. aureus* strains among 10 tested CPS isolates and to simultaneously detect methicillin resistant strains (MRSA). This assay targeted the *clfA* and *mecA* genes. Out of the 10 tested CPS strains, 10 were confirmed to be *S. aureus* as *clfA* gene was found, while 6 isolates only confirmed to be MRSA as they have *mecA* gene as shown in figures (3, 4).
Minimum inhibitory concentration (MIC):

Results revealed that the nine isolates demonstrated multiple antibiotic resistance patterns against most used antibiotics (table 3). Ciprofloxacin showed the lowest MIC against most of the *S. aureus* isolates, while ampicillin, lincomycin and colistin
demonstrated the highest MIC against most *S. aureus* isolates. While some of the tested isolates demonstrated resistance against most tested antibiotics with an MIC $>256$, however, ZnO-NPs showed the highest antibacterial effect against most multidrug resistant *S. aureus* isolates with an MIC ranging from 16-64 μg/mL, as compared to all tested antibiotics.

**Table (3):** MIC (μg/mL) of selected antibiotics and ZnO-NPs against nine *S. aureus* isolates.

| Antibacterial agent | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  |
|---------------------|----|----|----|----|----|----|----|----|----|
| Ampicillin          | 7256 | 64 | 256 | 128 | 128 | 128 | 128 | 128 | 128 |
| Streptomycin        | 64 | 64 | 256 | 128 | 128 | 128 | 128 | 128 | 128 |
| Neomycin            | 128 | 64 | 256 | 64 | 128 | 128 | 128 | 128 | 128 |
| Ciprofloxacin       | 128 | 64 | 256 | 64 | 4 | 4 | 4 | 4 | 4 |
| Lincomycin          | 128 | 128 | 256 | 256 | 256 | 256 | 256 | 256 | 256 |
| Oxytetracycline     | 128 | 128 | 256 | 256 | 256 | 256 | 256 | 256 | 256 |
| Amphenicin          | 128 | 64 | 256 | 64 | 64 | 256 | 256 | 256 | 256 |
| Florfenicol         | 128 | 64 | 256 | 64 | 64 | 256 | 256 | 256 | 256 |
| Colistin            | 128 | 128 | 256 | 256 | 256 | 256 | 256 | 256 | 256 |
| ZnO-NPs             | 32 | 16 | 32 | 32 | 16 | 16 | 16 | 16 | 16 |

**Discussion**

*The S. aureus* infection has become an increasingly grave problem in industrialized poultry farming, and create major human and animal health problems globally (Lowder et al., 2009). The relatively high rate of isolation of *S. aureus* (34.5 %), in the present study, from layer chickens comes in accordance with previous findings of Mamza et al. (2010) and Mamza et al. (2019) who recorded 35% and 31.5% carriage of *S. aureus* in layer chickens, Otalu et al. (2011) who recorded 38.5% prevalence of *S. aureus* from layer farms and Bounar-Kechih et al. (2018) who reported 42% in laying hens. However, Onaolapo et al., (2017), isolated *S. aureus* at higher prevalence (51.2%) from broilers and layers.

The virulence of *S. aureus* is accompanied by the presence of virulence genes (*clfA* and *clfB*) that encode proteins with affinity to fibrinogen i.e., clumping factors A and B (Momtaz, 2013 and Erfan and Marouf, 2015). The last authors detected *clfA* virulence gene in 76.82% and 100% of their samples, respectively. These findings simulate our PCR results where all coagulase positive isolates (100%) demonstrated *clfA* gene. However, six isolates out of ten (60%), in the present study, confirmed to be MRSA as they have *mecA* gene. The current high prevalence of *mecA* gene could be the cause of multi-drug resistance of the MRSA strains. These results agree with those of Bounar-Kechih et al. (2018) who recorded 57% MRSA in laying hens and at the same time they found that MRSA strains isolated from poultry showed cross-resistance to aminoglycosides, macrolides, sulphonamides, fluoroquinolones and cyclins.

In the current study, the patterns of antibiotics resistances to the commonly used antibiotics in poultry practice against *S. aureus* comes in agreement with those of Otalu et al. (2011), Onaolapo et al., (2017) and Mamza et al. (2019), who reported multiple resistances across families of antibiotics to isolates of *S. aureus* isolated from chickens. These multiple drug resistances can limit therapeutic options in cases of *S. aureus* infections. ZnO is generally recognized as safe substance by the US Food and Drug Administration (Rasmussen et al., 2010). As an alternative antibacterial, ZnO-NPs were used in this study against *S. aureus* isolated from layer chickens to evaluate its antibacterial activity. We found that all obtained isolates were highly sensitive to ZnO-NPs compared to all used antibiotics in the present investigation. ZnO-NPs sizing less than 100nm (33.7, 43.1 and 51.9 nm) effectively inhibited the growth of all *S. aureus* isolates, a result which concord to that found by Yamamoto (2001) and...
Raghupathi et al. (2011), who believed that ZnO-NPs with smaller size (higher specific surface areas) showed the highest antibacterial activity against *S. aureus* and *E. coli*. Zhang et al. (2007), reported that ZnO-NPs of smaller sizes can easily penetrate into bacterial cell membranes due to their large interfacial area, thus enhancing their antibacterial efficiency. This has been also confirmed by Iram et al. (2015) who found that higher concentrations and ≤50 nm size of ZnO-NPs have efficiently inhibited the growth of Gram-positive organism such as *S. aureus*. MIC of 1 mg/mL was the concentration that efficiently inhibited the growth of *S. aureus* in our study, which agrees with what has been reported by Reddy et al. (2007). The last authors recorded concentration 1 mg/mL of ZnO-NPs for *S. aureus* and 3.4 mg/mL for *E. coli*. However, Emami-Karvani and Chehrazi, (2011) found that 1.5 and 3.1 mg mL⁻¹ inhibited *S. aureus* and *E. coli*, respectively.

**Conclusions**

The current study provides a foresight into the role of ZnO-NPs as an effective alternative antibacterial treatment and control for *S. aureus* infections.

**Competing interest**

The authors have declared that no competing interest exists.

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