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Bactericidal effect of silver nanoparticles on methicillin resistant *Staphylococcus. aureus* (MRSA) isolated from bulk milk tanks

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Abstract

Recently, silver nanoparticles (Ag-NPs) have been widely used in various applications as antimicrobial agents, anticancer, diagnostics, biomarkers, cell labels, and drug delivery systems for the treatment of various diseases. The present study investigated the bactericidal effect of Ag-NPs againstmethicillin resistant *Staph. aureus* (MRSA) isolated from bulk milk tanks. A total of 105 bulk milk tank samples were collected from cow's, buffalo's and mixed milk of both (35 milk samples of each). The mean total staphylococcal count were $4.2x10^3 \pm 6.1x10^2$, $3.8x10^3 \pm 5.9x10^2$ and$1.1x10^4 \pm 7.6x10^3$. MRSA strains were identified in 8, 4 and 11 isolates out of 10, 11 and 15 positive *Staph. aureus* strains isolated from cow's, buffalo's and mixed milk with an incidence of 22.9%, 11.4% and 31.4% out of all samples respectively. MRSA isolates were only sensitive to vancomycin at percentage of 82.6% while, the resistance pattern of MRSA isolates revealed that they resists to most antibiotics used (multidrug resistant strains (MDR). Ag-NPs solution was prepared, identified by Transmission Electron Microscopy (TEM) in nano size ranged from 18.1 nm: 29.3 nm and examined for bactericidal activity against MRSA by using well diffusion assay. The results of the mean values of inhibition zone of using 30%, 60% and 90% concentrations of Ag-NPs were18.1±0.31, 21.3±0.27 and 23.3±0.29, respectively. Also, the statistical analysis showed highly significant differences in bactericidal effect of different concentrations of Ag-NPs on MRSA strains.

Keywords: *Silver nanoparticles, MRSA, bulk milk tanks.*

Introduction

Bacterial resistance to most conventional antibiotics has become a clinical and public health problem. Infections due to multidrug-resistant microorganisms, such as mthicillinresistant *Staph. aureus* (MRSA) considered a challenge which ought to be controlled due to its high treatment coasts, therapeutic failure and death **(Silva and Lincopan, 2012 and Cantas *et al.,* 2013)**. MRSA has been identified as an emerging pathogen in livestock animals that is readily transferable to humans in contact with livestock. Also, it has been identified as a mastitis causing pathogen in dairy cows that can be isolated from bulk tank milk **(Spohr*et al.,* 2011)**. MRSA's medical importance is attributed to the mortality and high morbidity rate of its infections and for being the main cause of nosocomial infections worldwide **(Bustos-Martinez *et al.,* 2006)**. The incidence of MRSA in some Asian countries reached 70% to 80% out of all *Staph. aureus* isolates **(WHO 2004)**.

A lot of investigations targeted to seek out the alternatives methods for MRSA infections treatment and among several compounds used for this target, silver nanoparticles (Ag-NPs) considered a most important promising new bactericidal agent that would be helpful to control this drug-resistant bacteria **(Ayala-Núñez *et al.,* 2009)**. The silver nanoparticles are environmentally friend, non-toxic, act as an antibacterial agent that could be linked to broad-

spectrum activity, with the decrease in propensity to become bacterial resist in comparing with other antimicrobial agents **(Quinteros *et al.,* 2016 and Meena *et al.,* 2018)** and Ag-NPs exhibits low toxicity to mammalian cells **(Li *et al.,* 2011)**. So, Ag-NPs have been applied to a wide range of products, the most important current use is as antimicrobial agents to prevent infection, such as in burns, traumatic wound dressings, diabetic ulcers, coating of catheters, dental works, scaffold, and medical devices **(Kim *et al.,* 2007; Law *et al.,* 2008 and Rai *et al.,* 2009)**. Ag-NPs are also used to get hygienic products including water purification systems, linings of washing machine, dishwashers and refrigerators. **(Silver *et al.,* 2006 and Rai *et al.,* 2009).**

The current investigation aimed to evaluate the prevalence of MRSA strains specially those of multidrug resistant features in milk samples that may constitute a public health hazard to consumers. Also, to evaluate *in vitro* antimicrobial efficacy of the Ag-NPs against MRSA isolated from bulk tank milk.

Materials and Methods

1- Sample collection:

A total of 105 bulk milk tank samples which were collected from cow's, buffalo's and mixed milk of both (35 milk samples of each) from dairy farms in different localities in (Qalyubia province, Egypt). The samples were collected in sterile separate containers, labeled and carried on ice tank to be transferred with a minimum delay to the laboratory for bacteriological examination.

2- Enumeration, Isolation and identification of *Staph. aureus*: according to FDA (2001):

3- Isolation of MRSA on MRSA agar base (7420acumedia) according to National Committee for Clinical Laboratory Standards "NCCLS" (1997): Positive *S. aureus* isolates were inoculated onto MRSA Agar Base (7420) for identification of MRSA strains. MRSA Agar Base is composed of Beef Extract and Acid Hydrolysate of Casein, providing nitrogen, vitamins, carbon, and amino acids. Starch is added to absorb any toxic metabolites produced with Agar as the solidifying agent. The high concentration of sodium chloride enhances growth of *Staph. Aureus* and Oxacillin is added to determine if the particular strain of *Staph. aureus*is oxacillin resistant.

4- Antimicrobial sensitivity testing of MRSA according to **Tiwari *et al.*,(2009):**

Mueller- Hinton agar plates were overlaid with the saline suspension of strains (turbidity equal 0.5 McFarland standard) and antibiotic discs of Cephalexin (30µg), Colistin, Erythromycin (15 µg), Gentamycin (10 µg), lincomycin (2mcg), Neomycin S. (30mcg), Oxacillin (1µg), Penicillin G. (10U), streptomycin (10 µg), Tetracyclin (30 µg), Trimethoprim (1.25µg) and vancomycin (30µg) (Difco Laboratories and BioMerieux, France). The antimicrobial susceptibility test was applied according to the guidelines stipulated by National Committee for Clinical Laboratory Standards **"NCCLS" (2004)**.

5- **Synthesis of silver nanoparticles**:

Silver nitrate (Ag NO_3) crystal (ACS Ag NO_3 F.W. 169.87 Gamma laboratory chemicals, assay: 99%). Stable Ag-NPs<100 nm were synthesized according to **Vigneshwaran *et al.* (2006)** and the solutions were identified and obtained from the Chemistry Department, Faculty of Science, Azhar University, Assiut branch, Egypt. While, the size of Ag-NPs was measured by Transmission Electron Microscopy (TEM) Model JEOL-JEM-100CX II in the Electron Microscopy Unit, Assiut University, Egypt. (size of AgNps ranged from 18.1-29.3 nm).

6- In vitro testing of silver nanoparticles antibacterial activity:

The isolated MRSA strains were used to assess the antibacterial activity of Ag-NPs by the well diffusion method on Mueller- Hinton agar **"NCCLS" (2004)**. The isolates were propa-

gated in BHI broth and incubated at 37°C for 24 h. The growth density was adjusted to the turbidity of a 0.5 McFarland standard by adding sterile saline to achieve a strain concentration of approximately $1x10^5$ colony forming cfu/mL. 0.1 ml of the inoculated broth was streaked into the Mueller- Hinton agar plates. 100µL of different concentration of silver nanoparticle 30%, 60%, and 90% from the original powder was poured on each well. After 24 hours incubation the various levels of zone of inhibition was measured and interpreted according to **(Rajeshkumar and Malarkodi, 2014)**.

7– Statistical analysis:-

The prevalence of *Staph.aureus* and MRSA were calculated and compared by using the Microsoft Excel Spreadsheet. Evaluation of antibacterial effects of different concentrations of silver nanoparticles against MRSA isolates were represented as max., min. and means ± standard error and were analyzed using "f" test. P values higher than 0.05 were considered significant.

Results and Discussion

Table (1). Prevalence of total *Staphylococcus aureus* count in the examined bulk milk tank samples (N=35):

Samples	Positive samples		Count of c.f.u/g.		
	No.	%*	Min.	Max.	mean±S.E.
Cow's milk	10	28.5	$2.1x10^2$	$8.2x10^3$	$4.2x10^3±6.1x10^2$
Buffalo's milk	11	31.4	$1.1x10^2$	$7.6x10^3$	$3.8x10^3±5.9x10^2$
Mixed milk	15	42.8	$1.1x10^2$	$2.1x10^4$	$1.1x10^4±7.6x10^3$

*The percent calculated according to total number of samples

Table (2). Incidence of (MRSA) strains in relation to the total isolates of *Staph. aureus* (N=35):

Types of samples	No. of positive *Staph. aureus* samples	%*	No. of (MRSA) strain positive samples	%*
Cow's milk	10	28.5	8	22.9
Buffalo's milk	11	31.4	4	11.4
Mixed milk	15	42.8	11	31.4
Total	36	35.2	23	21.9

*The percent calculated according to number of total samples

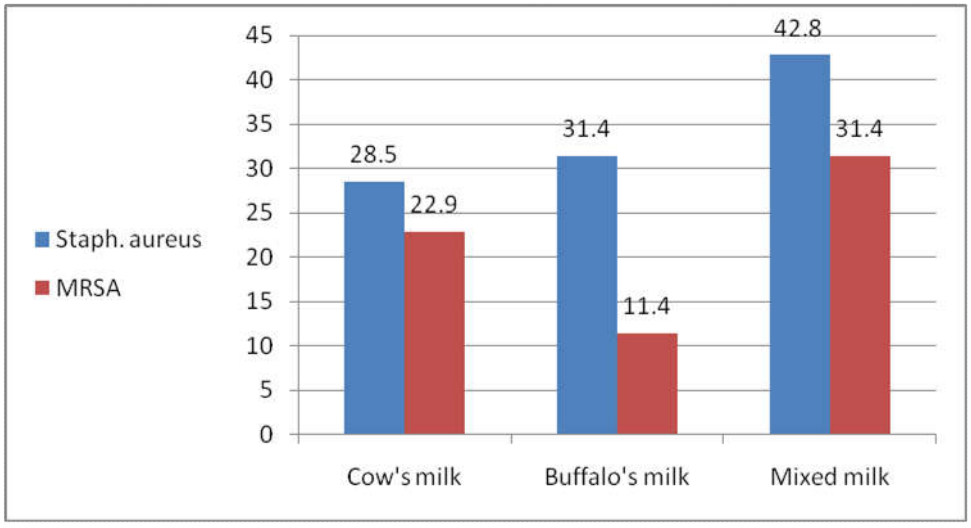

Fig. (1): The prevalence of *Staph. aureus* and MRSA strains isolated from bulk milk tank samples

Table (3). Antibiogram of *(*MRSA*)* isolated from bulk milk tank (n=23)

Antibiotic discs	MRSA (23)		
	No.	%*	Anti-biogram susceptibility
Cephalexin	11	47.8	I
Colistin	0	0	R
Erythromycin	4	17.4	R
Gentamycin	3	13	R
lincomycin	0	0	R
Neomycin S.	0	0	R
Oxacillin	0	0	R
Penicillin G	0	0	R
Streptomycin	2	8.7	R
Tetracyclin	1	4.3	R
Trimethoprim	4	17.4	R
Vancomycin	19	82.6	SS**

*% calculated according to number of tested MRSA strains
** The mean diameter of inhibitory zone=17.3±0.51
ss= sensitive R= resistant I= intermediate

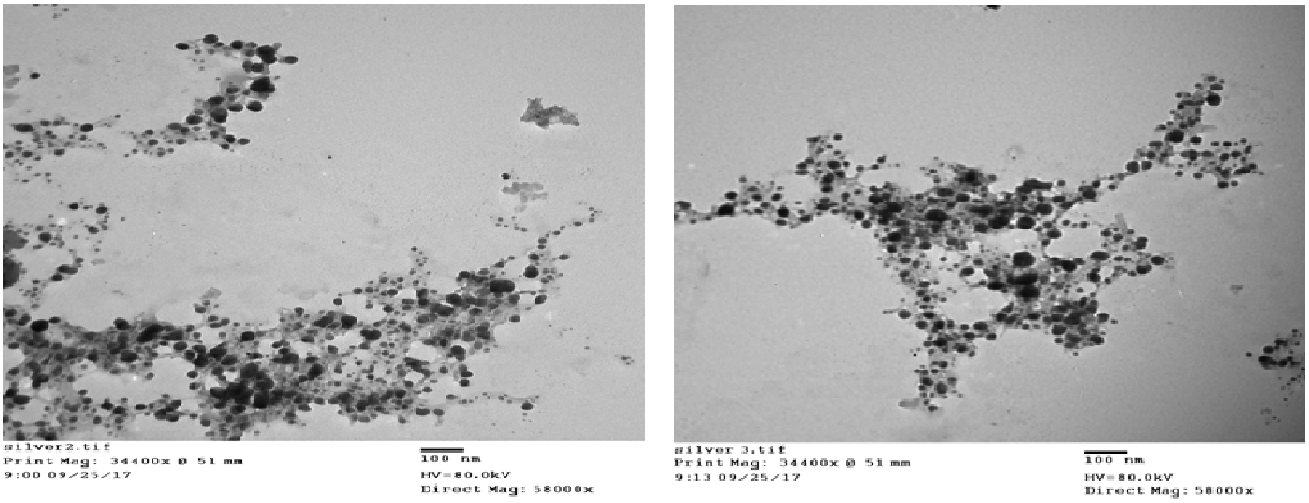

Photo. (1): Transmission Electron Microscopy (TEM) image of Ag-NPs with spherical shapes (sizes ranged from 18.1 nm to 29.3 nm).

Table (4). Antibacterial effects of silver nanoparticles against MRSA isolates

Silver nanoparticles concentrations/100 µL	Inhibition zone by mm.			F	P
	Min.	Max.	Mean ± SE		
30 µg (30 %)	15	20	18.1±0.31	75.2	< 0.001
60 µg (60 %)	18	25	21.3±0.27		
90 µg (90 %)	21	25	23.3±0.29		

Highly Significant = *P*< 0.001

Discussion

One of the most common agents in bacterial food poisoning outbreaks is *Staph. aureus* **(Pelisser *et al.,* 2009)**. It considered normal inhabitant in healthy mucous membranes and the skin of humans and many animals, and causes one of the most common types of chronic mastitis **(Bassam *et al.,* 2014)**. Recently, MRSA infections in dairy sector was increased, including 2 outbreaks of infection in veterinary teaching hospitals in North America **(O'Mahony *et al.,* 2005 and Rich *et al.,* 2005).** So the present study focused on the prevalence of *Staph. aureus* with special reference to MRSA isolated from cow's, buffalo's and mixed milk. Table (1) revealed that the highest prevalence of *Staph. aureus*was in mixed milk samples (42.8%)followed by buffalo's milk (31.4%) and then cow's milk (28.5%). These results nearly agreed with **Abo -Shama (2014)** who isolated *Staph. aureus* in 36.7% and 46.4% from cow's and buffalo's milk from Sohag Governorate, Egypt, respectively. Also, the obtained results were similar to that previously observed in Ethiopia by**Mekonnen *et al.,* (2011)** (39.5%) and also in Sudan by **Zakary *et al.,* (2011)** (40%). Higher results were recorded by **Hundera *et al.,* (2005)** (52.8%), **Abera *et al.,* (2010)** (43.1%), **Lingathurai and Vellathurai, (2011)** (61.70%) and **Ganai *et al.,* (2016)** who isolate *Staph. aureus* in 60% and 52% from cow's and buffalo's milk, respectively.

The study (table 1) showed mean count ± standard error (S.E.) of staphylococcal count were $4.2x10^3 \pm 6.1x10^2$, $3.8x10^3 \pm 5.9x10^2$ and $1.1x10^4 \pm 7.6x10^3$ for cow's, buffalo's and mixed milk samples, respectively. Nearly similar result recorded by **Riekerink *et al.*, (2010) and Nádia *et al.,* (2012)** but lower than **Bahout and Moustafa (2006)** and **Hussien *et al.,* (2013)**. The variation among results may be due to the level of hygienic practices in different dairy farms with special reference to biosafety practices adopted. Improving the hygienic conditions of the milking environment and/or utensils may reduce the prevalence of *staph. aureus* in milk and prevent its transmission to humans **(Abo-Shama, 2014).**

MRSA agar is helpful in screening and rapid detection of methicillin and oxacillin resistance *Staph. aureus* due to the addition of oxacillin (6 mg/L) during the preparation of the media. (MRSA) was first recognized in the 1980's as a major clinical and epidemiological problem (**Murray *et al*, 1995)**. Hospitals are still facing this problem tell today. MRSA Agar was developed to detect the presence of *staph. aureus* harboring the *mec*A gene (classic resistance) .**("NCCLS" 1997)**

Table (2) and Fig.(1) revealed that the incidence of MRSA isolates from cow's, buffalo's and mixed milk samples were 22.9%, 11.4% and 31.4% respectively, and the total prevalence was 21.9%. These results agree with that reported by **Haran *et al.,* (2012)** where MRSA isolates were 21.9% from all milk samples while, **Antoci *et al.,* (2013)** evaluated the prevalence and molecular traits of MRSA among dairy farms in the province of Ragusa, South-Eastern Sicily, it was about 44% of bulk tank milk samples and **Ammar *et al.,* (2016)** isolate 30 MRSA strains from milk samples in Egypt. The variation of results reflect the policy of massive antibiotics administration among different regions and farms, the more antibiotics are used, the greater the antibiotic resistant bacteria. Recently, it has been pointed out, that MRSA in dairy herds are not only observed as intra-mammary infections but can also be isolated from healthy calves fed with whole milk and other body sites of dairy cows. The importance of this colonization as a source of infection of the mammary glands has not been determined so far **(Kreausukon *et al.,* 2011)**. Furthermore, the cross contamination of MRSA between dairy cattle and farm workers has been reported, despite the fact that *Staph. aureus* is commonly associated with bovine mastitis, MRSA was detected in milking associated environmental samples such as milk cup, floor, fence, and ventilation instrument of the milking parlor. Also, all MRSA isolates from the farm environment were genetically

identical to those of milk isolates **(Lim *et al.*, 2013).**

It is worrisome to be found that the resistance of MRSA strains extent to resist many antibiotics. As shown in Table (3) shown that MRSA isolates were resistant to all antibiotic tested except vancomycin and with intermediate action for cephalexin defined such strains as multidrug resistant.In the case of MRSA, multiple drug resistance was common and only a few antibiotics were active against these isolates. Erythromycin, Gentamycin, streptomycin and Trimethoprim were the antibiotics to which the resistances were observed. MRSA strains were sensitive to vancomycin in percentages 82.6% so it consider as a drug of choice for treatment of MRSA infections. The sus-ceptibility of the isolates observed in this study to vancomycin and resistance to many antibiotics agreed with data reported by **Aydin *et al.*, (2011)**. MRSA strains have been observed to be multi-drug resistant, such as Aminoglycosides, Macrolides, Lincosamides, Streptogramins, Tetracyclines, etc., which are often used in the treatment of mastitis **(Kumar *et al.*, 2010)**. These multidrug resistant MRSA that may contaminate milk and milk products considered as a potential risk to consumers. Therefore, control measures during processing of these products and judicious application of effective antibiotics should be adopted to overcome this critical situation and avoid the risk of human infection **(Gopal and Divya, 2017)**.

Due to the growing number of outbreaks caused by MRSA and the development of antibiotic resistance, it becomes essential to investigate other antibacterial agents alternative for treatment of MRSA infections. Among the range of compounds whose bactericidal activity is being investigated, Ag-NPs rise as a promising new antibacterial agent that could be helpful to confront drug resistant *Staph. aureus*. Furthermore, recently, the field nanotechnology represents a modern and innovative approach to develop new formulations based on metallic nanoparticles with antimicrobial properties **(Kotb and Sayed, 2015).**
As shown in photo (1) morphological structure and distribution of synthesized silver nanoparticles were characterized at high magnifications done by TEM and showed the well dispersed predominantly spherical shape of silver nanoparticles and also it confirms that the synthesized silver nanoparticles were in nanosize ranged from 18.1 nm to 29.3 nm.

Table (4) illustrated the mean values of inhibition zone for MRSA growth by Ag-NPs at concentrations of 30%, 60% and 90% were 18.1±0.31, 21.3±0.27 and 23.3±0.29, respectively and the statistical analysis showed highly significant differences in bactericidal activity of different concentrations of Ag-NPs on MRSA strains, That mean the efficacy of Ag-NPs were differ according to its concentrations in addition it was highest in concentration of 90µl. Similar results were recorded by **Kazemi *et al.*, (2014), Quinteros *et al.*, (2016)** and **Scandorieiro *et al.*, (2016)** who used Ag-NPs against MRSA at different concentrations nearly to those used in this study. Also, lower concentrations of Ag-NPs 4µg/mL at average size 11nm recorded bactericidal effect against MRSA by **Yuan *et al.*, (2017) and Sharifi-Rad *et al.*, (2014)** whose reported that the minimum inhibitory concentrations of AgNPs against methicillin-resistant *Staph. aureus* Was found to be 5.6 µg/mL. Higher mean value (31.25±0.26) of bactericidal effect at nearly same size of Ag-NPs against MRSA recorded by **Kotb and Sayed (2015)**. Higher concentrations of Ag-NPs (100 µL) with complete inhibition of MRSA studied by **Abdel Rahim and Mohamed (2015)**. **Rastogi *et al.*, (2011) and Paredes *et al.*, (2014)** showed that MRSA was more sensitive to AgNP and that could be attributed to the structural of the external cell wall of gram-positive bacteria (as MRSA) lack of a lipopolysac-charide layer and it has a cell wall consti-tuted by multiple layers of peptidoglycan and teichoic acid. These acids are chains of negatively charged glycerin. MRSA are susceptible to compounds positively charged by means of electrostatic interactions.

However, many mechanisms of action were suggested for Ag-NPs antimicrobial activity but not completely clear. Also, the strong antimicrobial effect of Ag-NPs may be due to attachment to cell receptors and inhibi-tion of important metabolic enzymes resulting in disrup-tion of microbial cell reproduction and respiration **(Abdel Rahim and Mohamed, 2015)**.

Pal *et al.*, (2007) and Ayala-Núñez *et al.*, (2009) demonstrated that physicochemical properties of noble metal nanocrystals are influenced by size and defined that the bactericidal and antiviral properties of silver nanoparticles are size dependent and that the only nanoparticles that present a direct interaction with the bacteria or virus preferentially have a diameter of 1–30 nm. A smaller size implies the ability to reach structures that otherwise is not available for bigger nanoparticles. Also, at this sizes it eliminate bacteria but keeping human cells alive and that because the bacteria have a larger surface area-to volume ratio than eukaryotic cells, which allows for rapid uptake and intracellular distribution of nutrients and excretion of wastes. This characteristic is achieved by having a rigid cell wall composed of peptidoglycan **(Lok *et al.*, 2006)**. For that reason, at the same concentration, silver nanoparticles would be preferentially absorbed and accumulated by bacteria, thus exerting its antibacterial effect without significantly damaging human cells. In addition, silver nanoparticles have been found to bound and disturb bacterial cell membrane activity **(Sondi and Salopek-Sondi, 2004)**.

Conclusion and Recommendation

According to the results of MRSA isolated strains from cow's, buffalo's and mixed milk indicate bad hygienic measures for milk production and lead to public health hazards. From the obtained results it is recommended to use silver nanoparticles as antibacterial against MRSA as well it could be a good alternative for cleaning and disinfection of equipment and surfaces in food-related environments as well as in silver-incorporated food packaging. Thereby, there is a need for an appropriate study for using silver nanoparticles in cleaning and disinfection of equipment and in food packaging.

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