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In vitro evaluation of antibacterial effect of silver nanoparticles with antibiotics on *Staphylococcus aureus* isolated from bovine mastitis

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ABSTRACT

A study was undertaken on *'**In vitro*** **evaluation of the antibacterial effect of silver nanoparticles with antibiotics on** ***Staphylococcus aureus*** **isolated from bovine mastitis**' in high yielding cross breed jersey cows from different areas of Balangir, Odisha during the period January 2018 to June 2018. Based on history, clinical sign and MCMT, 187 samples of milk collected from high yielding cross breed jersey cows (11 acute, 4 chronic,84 apparently healthy and 88 subclinical). Milk sample were inoculated on nutrient broth and incubated at 37 ˚C. All the 88 samples were subjected to gram staining. Based on staining chacteristics 41 samples were identified as *S. aureus*. Further the growth was streaked on nutrient agar plate for finding cultural characteristics and morphology. Further to identify pathogenic strain culture were inoculated in Blood Agar (BA) and Mannitol Salt Agar (MSA). The isolated were subjected to tests 16S rRNA gene amplification by PCR along with gene sequencing to identify 31 strains as *Staphylococcus aureus*. All bacterial isolates were tested *in vitro* for their sensitivity to different antibiotics commonly used in veterinary practices. Disk diffusion test and Macro dilution were performed to determine Minimum Inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC). Fractional Inhibitory Concentration (FIC) index was calculated to determine the interaction between a combination of Ag-NPs and Ampicillin. 41 strains of *Staphylococcus aureus* were isolated from 88 samples and 31 samples were confirmed through PCR among them randomly two strains were sequence and found that 96% similarity with *Staphylococcus aureus*. MIC of ampicillin of most of the strains was in between range 1.0-1.5mg while MIC of ampicillin in combination with 5µg silver nanoparticles reduces the range of ampicillin to 0.50-1.00mg. According to FIC index, ampicillin and Ag-NPs had synergistic effect in 90% in isolated strain and additive effect in 10% strain. It could be concluded that a combination of Ag-NPs with ampicillin showed synergistic antibacterial properties with reduction the range of MIC of ampicillin on *Staphylococcus aureus* isolated from Bovine Mastitis.

I. INTRODUCTION

Bovine mastitis is the most prevalent disease complex in dairy cattle causing a colossal economic burden on milk producers all over the world. Loss due to bovine mastitis occurs in form of production loss, unsuitability of milk for marketing and human consumption, cost of medicine and veterinary attendances and culling of animals. The global monitoring loss due to mastitis is put at 140 billion US$, whereas, in India, it is more than Rs6053.21crores per year (Radostits*et al.*,1994; Dua *et al.*, 2001)

Mastitis is predominantly treated by antibiotic(s), thus antibiotic resistance is increasing among mastitis causing strains (Park *et al.*, 2012). Vancomycin was considered the drug of choice to overcome MRSA infection but later on became intermediate or resistant and MRSA could be vancomycin resistant (Cui *et al.*, 2000)Furthermore, various control programmes have been used to combat the problem variable degree of efficacy (Capurro *et al.*, 2010). Extensive use of antimicrobial agents contributes to the development and rapid spread of bacterial resistance. However, the resultant negative impact is decrease in antibiotic efficacy in both human and veterinary medicine (Schwarz *et al.*, 2001)Although several new antibiotics were developed in the last few decades, none have improved activity against multidrug-resistant bacteria (Mohanty *et al.*, 2012). Hence, the emergence of antibiotic resistance in dairy cows due to*S. aureus*is a serious concern. It narrows down the treatment possibilities and alters the spectrum of antibiotics used in veterinary hospitals. Therefore, it is important to develop alternative and more effective therapeutic strategies especially, to treat resistant form of bacteria. The nanoparticles were used successfully for the delivery of therapeutic agents against chronic diseases (Zhang *et al.*, 2008, Hong *et al.*, 2008). Silver is a more toxic metal to bacteria than many other elements in decreasing order of their antibacterial effects such as Ag >Hg >Cu>Cd>Cr >Pb>Co>Au>Zn>Fe (Zhao and Stevens., 1998) Silver has a strong antimicrobial potential, which has been used since the ancient times. But with the advent of antibiotics progress, the medical applications of silver as antimicrobial were declined (Castellano *et al.*, 2007; Chen and Schluesener., 2008). Silvernanoparticles having a size in the range of 10–100 nm showed strong bactericidal against both Gram-positive and Gram-negative bacteria (Morones *et al.*,2005). The high surface area to volume ratio and unique chemiophysical properties of various nanomaterials are believed to contribute to effective antimicrobial activities (Muhling *et al.*, 2009). Silver is one of non-toxic and safe antimicrobial agent to the body and kills many harmful microorganisms (Dowling *et al.*, 2003).Silver nanoparticle (Ag-NPs) have recently been synthesized and shown to exhibit antimicrobial activity against several species of bacteria including *S. aureus* (Dung *et al.*, 2009). There is synergistic activity between Ag-NPs and Ciprofloxacin (Namasivayam *et al.*, 2011). It also recorded that Ag-NPs increase thebactericidal activity of certain antibiotic such as amoxicillin, erythromycin, vancomycin, clindamycin and penicillin (Graves *et al.*, 2015). Therefore, the bactericidal metals have an advantage over the conventional antibiotic which often causes the selection of antibiotic-resistant microorganism. The use of modern technology and therapeutic properties of silver nanoparticle have a requirement that had already been proven and it seems necessary. Limited *in vitro* studies to access antimicrobial properties against *S. aureus* has been conducted on Ag-NPs alone(Brett *et al.*, 2006;Kim *et al.*,2007;Guzman *et al.*, 2012) and in combination with suitable antibiotics, ampicillin(Brown *et al.*,2012), ciprofloxacin(Fayaz *et al.*, 2010; Namasivayam *et al.*,2011)with better results.

II. MATERIALS AND METHODS

The present study was carried out in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology (OUAT), Bhubaneswar during the period from January 2018 to May 2018. Based on history, clinical sign and MCMT, 187 samples of milk collected from high yielding cross breed jersey cows (11 acute, 4 chronic,84 apparently healthy and 88 subclinical). Milk sample were inoculated on nutrient broth and incubated at 37 ˚C. All the 88 samples were subjected to gram staining. Based on staining chacteristics 41 samples were identified as *S. aureus*. Further the growth was streaked on nutrient agar plate for finding cultural characteristics and morphology. Further to identify pathogenic strain culture were inoculated in Blood Agar (BA) and Mannitol Salt Agar (MSA). The isolated were subjected to tests 16S rRNA gene amplification by PCR along with gene sequencing to identify 31 strains as *Staphylococcus aureus*. All bacterial isolates were tested *in vitro* for their sensitivity to different antibiotics commonly used in veterinary practices. Disk diffusion test and Macro dilution were performed to determine Minimum Inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC). Fractional Inhibitory Concentration (FIC) index was calculated to determine the interaction between a combination of Ag-NPs and Ampicillin.

Table1. Minimum Inhibitory concentration of ampicillin against *S. Aureus* isolated from bovine mastitis

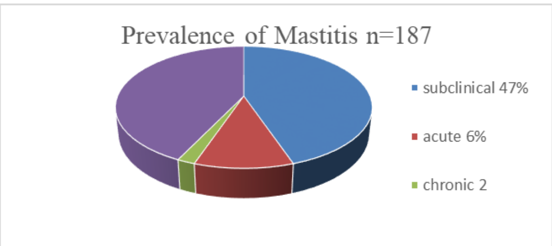
Test tube no	Ampicillin (µl)	Bacterial Culture(µl)	Nutrient Broth (µl)	Final Volume (µl)
1	10 (0.5mg)	50	940	1000
2	15 (0.75mg)	50	935	1000
3	20 (1mg)	50	930	1000
4	25 (1.25mg)	50	925	1000
5	30 (1.5mg)	50	920	1000
6	35 (1.75mg)	50	915	1000
7	-	50	950	1000

Table.2: Minimum Inhibitory concentration of ampicillin in combination with silver nanoparticles against *S. aureus*isolated from bovine mastitis

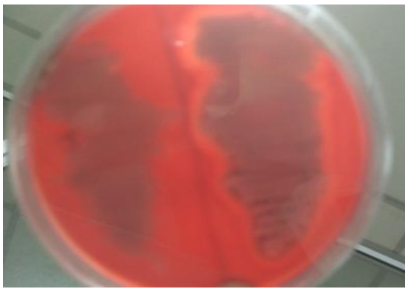
Test tube no	Ampicillin (µl)	Bacterial Culture(µl)	Nutrient broth(µl)	Silver Nano (µl)	Final Volume(µl)
1	30 (1.5mg)	50	670	250	1000
2	25(1.25mg)	50	675	250	1000
3	20 (1mg)	50	680	250	1000
4	15(0.75mg)	50	685	250	1000
5	10(0.5mg)	50	690	250	1000
6	-	50	950	-	1000

III. RESULT:

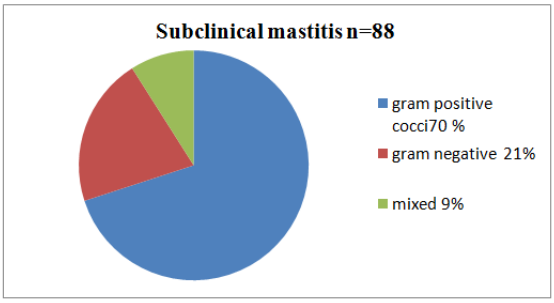
A total number of 187 milk samples were collected randomly from high yielding cross breed jersey. Based on history, clinical sign and MCMT, 88 samples detected as subclinical, 11samples as acute, 4 samples as chronic mastitis and remaining 84 samples were apparently healthy.Ours study limited to subclinical mastitis as the prevalence of subclinical mastitis was more. All the 88 sub clinically detected samples were subjected to Gram staining. Among the 88 samples,only 62 sample shows gram positive cocci, 18 samples shows gram negative and 8 samples with mixed infection. Among all the positive gram cocci, 41 samples were identified as *Staphylococcus spp.* based on colonies morphology. Further all the 41 identified samples were inoculated into nutrient agar and incubated overnight and studied the colony morphology.These isolates were grown over the selective media (MSA and BA), only 31 samples could show the positive results for *S. aureus*. These 31 isolates were further subjected to PCR by Molecular identification by 16s rRNA gene which confirmed 31 strains to be *S. aureus*. Out of these only two isolates were selected randomly and sent to Eurofins Genomics India Pvt. Ltd. Bangalore, India for gene sequencing of 16S rRNA gene which were found to have 96% matching with *S. aureus* accessory no H9KDAGWK015 and H9KHM80R015. After that ABST, MIC and MBC test were performed and finally, the synergistic action was determined by calculating the FIC index.



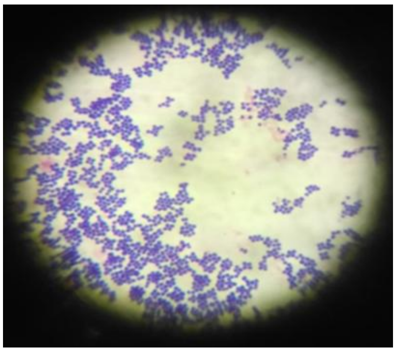
Distribution of bovine mastitis in Bolangir district during 2018



Growth on Blood Agar *S. aureus*



Association of different bacterial pathogen in subclinical mastitis



Gram stainingof *S. aureus*

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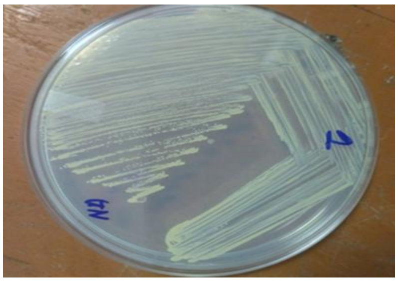
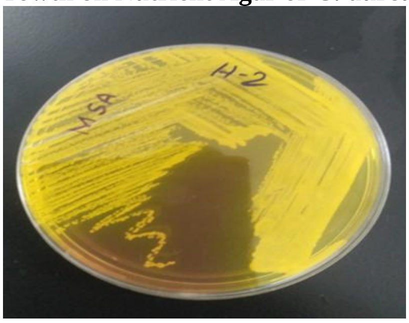
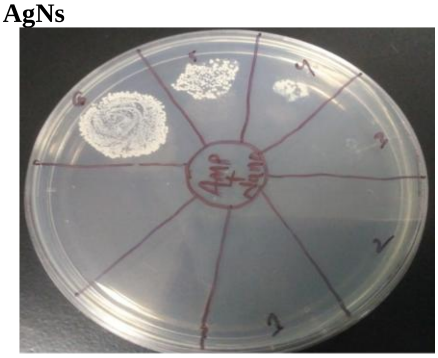


Growth on Nutrient Agar of *S.* aureus



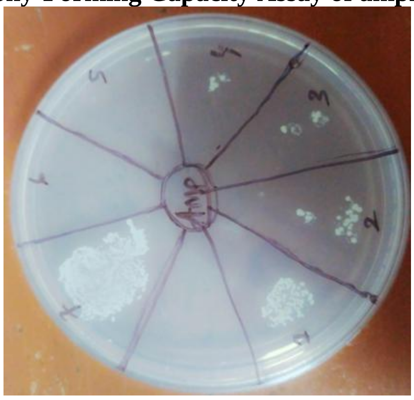
Growth on MSA *S. Aureus*

Colony-Forming Capacity Assay of ampicillin with AgNs



1) Growth at 1.5mg/ml of ampicillin and 5µg/ml of Ag-NPs 2) Growth at 1.25mg/ml and 5µg/ml of Ag-NPs. 3) Growth at 1mg/ml and 5µg/ml of Ag-NPs. 4) Growth at 0.75 mg/ml and 5µg/ml of Ag-NPs. 5) Growth at 0.5mg/m and 5µg/ml of Ag-NPs & 6) Positive control. The isolates were inhibited at the concentration of 1.0mg/ml and 5µg/ml of Ag-NPs at 0.5 McFarland's standard where no visible bacterial growth was observed in nutrient agar plates.

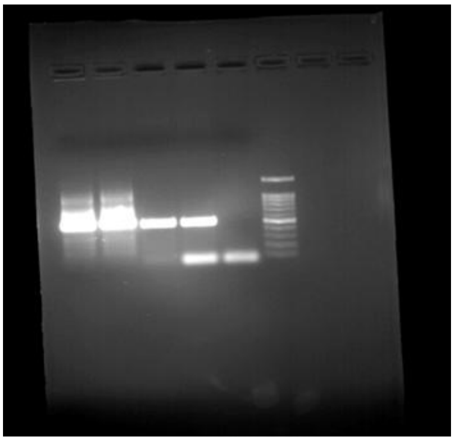
Colony-Forming Capacity Assay of ampicillin



1) Growth at 0.5mg/ml of ampicillin. 2) Growth at 0.75mg/ml. 3) Growth at 1mg/ml. 4) Growth at 1.25 mg/ml. 5) Growth at 1.5mg/ml. 6) Growth at 1.75mg/ml. & 7) Positive control. The isolates were inhibited at the concentration of 1.5mg/ml at 0.5 McFarland's standard where no visible bacterial growth was observed in nutrient agar plate

Results of the interaction of silver nanoparticles and ampicillin S. aureus isolated from bovine mastitis.

Strain	MIC ampicillin (A)	MIC of Ampicillin in combination with Ag-NPs(5µg) (B)	FIC: MIC of ampicillin and silver nanoparticle (B)/MIC of ampicillin alone(A)	Interaction
1	30µl(1.5mg)	15µl(0.75mg)	0.50	**Synergistic**
2	25µl(1.25mg)	15µl(0.75mg)	0.60	**Synergistic**
3	30 µl(1.5mg)	15µl(0.75mg)	0.66	**Synergistic**
4	25µl(1.25mg	20µl(1.0mg)	0.80	**Synergistic**
5	20µl(1.0mg)	20µl(1.0mg)	1.00	**Additive**
6	30µl(1.5mg	20µl(1.0mg)	0.66	**Synergistic**
7	25µl(1.25mg	15µl(0.75mg)	0.60	**Synergistic**
8	30µl(1.5mg	10µl(0.50mg)	0.33	**Synergistic**
9	25µl(1.25mg	15µl(0.75mg)	0.60	**Synergistic**
10	25µl(1.25mg	15µl(0.75mg)	0.60	**Synergistic**



Electrophoresis of PCR products of 16S rRNA gene
1: positive control, **2-4**: PCR products of expected size of 500bp, **5:** Negative control
6- Ladder 100bp

IV. DISCUSSION

An important increase of scientific researches for nanostructured products development in the last years has been verified in Veterinary Medicine, especially using antimicrobials actives. Conventional synthetic and natural antimicrobial substances are being tested, and have shown excellent results against multi-resistant microorganisms and bacteria strains that are normally hard to eliminate by using the conventional treatment, like *Brucella, Mycobacterium bovis, Staphylococcus aureus, Salmonella, Ehrlichia, Anaplasma; Rhodococcus equi.* (Mc Millan *et al*., 2011)Indiscriminate use of antibiotics in the treatment and/ or control of mastitis not only pave ways towards antimicrobial resistance but also leads to drug residue in the milk and milk product - a major public's health hazards now. Although a variety of treatment and prevention protocols have been developed over the years, success rates have been variable and a true solution to the problem has not been in practice. According United States Government's National Nanotechnology Initiative, nanotechnology is defined as: "Research and technology development at the atomic, molecular and macromolecular levels at the scale of approximately 1 - 100 nanometres range. Nanoparticles systems include functionalized fullerenes and carbon nanotubes, liposomes, iron oxide nanoparticles, polymeric micelles, dendrimers, nanoshells, polymeric nanospheres, nanobins, quantum dots, polymer-coated nanocrystals and metallicnano (Troncarelli *et al*.,2013). Several *in vitro* studies of silver nanoparticles already have been undertaken to answer the resistant strain of *Staphylococcus aureus* isolated from bovine mastitis with silver nanoparticles alone and in combination with antibiotics promising results. Hence, in order to overcome the antibiotic resistance present studies was undertaken using silver nanoparticle as an alternative with a hypothesis to reduce the dose of antibiotic used along with an increase in the zone of efficacy. However, despite genetic upgradation and modern methods of livestock rearing, production per capita has remained low (Ramesh *et al*., 2014). One of the reasons for this discouraging statistics could be mastitis, especially of the subclinical type.

The early isolation and identification of the mastitis causative agents are very important in their prevention, treatment, and control point of view. Screening of milk samples from 187 cows by MCMT revealed that 47 % cows to be affected with subclinical mastitis, 6% with acute mastitis, 2% with chronic mastitis and 45% apparently healthy.Further positive samples were subjected to gram staining and growth on a nutrient agar plate to recover *S. aureus*. Out of which, 47% (41/88) samples were *staphylococcus spp.* which also in accordance with Almaw *et al*. (2007) who suggested that, mastitis as a disease of multifactorial agents involving a wide range of microbes including *S. aureus, S agalactiae, S. epidermidis, Brucella melitensis, Corynebacterium bovis, Mycoplasma, Pseudomonas aeruginosa, E. coli* and *Klebsiella pneumonia*. *S. aureus* is the predominant organism.Thirty-one (35%) samples were confirmed by growth on selective media. As gram staining and growth on selective media are not enough for identification of bacteria, PCR was conducted and finally, 31 samples (35%) were confirmed as *S. aureus.* According to CLSI standardized disk diffusion testing of 31 isolates intermediate, susceptible and resistant to ampicillin were found in 41, 51 and 8%, respectively.

In the present study, ampicillin was taken as a test antibiotic in combination with Ag-NPs because Ampicillin, being a broad spectrum antibiotic, used most predominantly in the field condition for the treatment of bovine mastitis also Damaging effect on the cell wall of bacteria is the common mechanism of action with ampicillin as well as Ag-NPs Nanoparticles from different sizes (7 to51 nm), concentration (3-100µg/ml), shape (Round, spherical, irregular) and sources (biosynthesis or chemical synthesis) were used against *S. aureus*. In the present trail, nanoparticles were procured from commercial source having the size of 10nm TEM with a concentration of 20 microgram per litre.MIC and MBC of ampicillin alone and along with Ag-NPs was done for all the 10 isolates by standard broth dilution (tube) method. MIC of ampicillin of 10 different strains was fallen in the range of 1.0mg/ml (20µl)-1.5mg/ml (30µl) which were in agreement with Jennifer *et al* who recorded MIC of the ampicillin 0.03-128 mg/lit against *S. aureus.*Shahverdi *et al.* (2007); Birla *et al.*(2009) and Fayaz *et al.*(2010) studied the interaction between Ag-NPs and drugs and suggested that drugs at concentrations lower than the MIC value should be used to know the synergistic action between Ag-NPs and drugs and hence ampicillin with concentration 1.5mg/ml and lower than 1.5mg/ml concentration were used along with Ag-NPs in this studies. MIC of ampicillin in combination with silver nanoparticles (5µg) reduced the MIC range of ampicillin to the range of 0.50 (10µl) - 1mg (20µl) indicating the synergistic action of silver nanoparticle with ampicillin which are in accordance with Rajawat *et al.* (2012) who investigated that the ability of ampicillin to lyse the cell wall of bacteria was increased when it combined with Ag-NPs that led to increasing concentration of these antibiotic in the site of infection. Reduced doses of ampicillin when used along with Ag-NPs are in accordance with Smekalova *et al*. (2015) who recorded that the combination of Ag-NPs with antibiotics lowers the concentrations of both antibiotics and silver required to kill the bacteria.Further, the concentration of silver nanoparticles used in the study (5µg/ml), that showed high antimicrobial activity has low cytotoxicity Salomoni *et al.* (2015).According to FIC index, ampicillin along with silver nanoparticles had synergistic effects to a tune of 90% of the isolated strains. In addition, ampicillin and Ag-NPs had additive effects in 10% of the isolated strains. The high rate of synergistic interaction and low level of cytotoxic suggested the use of their combination for the treatment of mastitis caused by *S. aureus.* Growing evidence shows that Ag-NPs are an alternative to antibiotics because the doses that exhibit an antimicrobial effect are also cytocompatible. The studies on the combined use of Ag-NPs with ampicillin can help to reduce the problem of toxicity by reducing the doses of ampicillin used and also avoid the potential for development of resistance and above all, strongly enhance the microbicidal effects of ampicillin. Further, studies are needed to highlight the efficiency of Ag-NPs against other bacterial isolates and others antimicrobial agent. Detailed systematic approach is needed to unveil the mechanism of this synergistic effect. Also, use of Ag-NPs combined with antibiotic(s) against resistant form of strains isolated from the clinical biosamples with toxicity of nanoparticles towards human cell before proposing their potential as antimicrobial agent need to be evaluated in greater interest of the public. Undoubtedly, the *in vitro* results of the study surfaced encouraging antibacterial activity of Ag-NPs, further investigation is essential before clinical application.

V. CONCLUSION

Microbiological tests coupled with molecular characterization of bacterial isolates from milk samples confirmed association of *Staphylococcus aureus* in 31.0 per cent cows with subclinical mastitis and Silver nanoparticle of 10nm TEM with a concentration of 20 microgram per litre with ampicillin reduced the MIC on *Staphylococcus aureus* isolated from bovine mastitis.

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