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# Effect of chitosan-coated iron oxide nanoparticles against Biofilm-forming potential of *Staphylococcus aureus* isolated from food of animal origin

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**Abstract**
Antimicrobial resistance (AMR) and emergence of multidrug resistant (MDR) in *Staphylococcus aureus*, is a major public health concern worldwide. *S. aureus* causes life-threatening diseases due to production of biofilm, a mechanism responsible for spread of multidrug resistance. Humans are infected with resistant foodborne bacteria via the food chain from reservoir animals. The aim of current study was to analyze the effect of chitosan coated iron oxide nanoparticles (IONP) on biofilm formation. To quantify biofilm production the Crystal Violet (CV) assay and Microtitre test assay (MTA) was used. Biofilm analysis by CV assay showed inhibition when incubated with Chitosan-coated nanoparticles. Biofilm inhibition was range from 66.17-99.25%, 68.63-98.86%, 91.41-97.66% at concentration of 500mg/mL, 1mg/mL and 4mg/mL respectively. Results indicate that chitosan coated IONP were effective in restraining biofilm formation, have potential to be developed as therapeutic agent where antibiotics are non-effective or in combination with antibiotics and against antibiotics resistant strains.

**Keywords:** Biofilm, chitoson, crystal violet, mastitis, microtitre

## Introduction

Microbial biofilms are an omnipresent component in many environments supporting life. Biofilm formation is growing and emerging concern being a great threat to human and animal health. Bacterial biofilms are able to survive the host immune response and antimicrobial therapy by slowing their growth, reducing metabolism and penetration of antimicrobial into the biofilm structure [1]. *S. aureus*, a ubiquitous microflora and community-acquired pathogen [2]. Biofilm is characteristic prevalent virulent factor involved in development of clinical infection and antimicrobial resistance. *S. aureus* is most relevant species due to virulence factors such as slime, required for biofilm formation [3]. *S. aureus* is a notorious pathogen due to its antibiotic resistance and phenotypic adaptability facilitated by development of biofilms. *S. aureus* biofilms challenge conventional anti-infective approaches and most notably antibiotic therapy [4].

Food poisoning is one of the most common food borne diseases worldwide mostly caused due to *Staphylococci* [5]. Biofilms a aggregated structured communities of bacteria referred as extracellular polymeric substances (EPS) composed of protein, DNA and polysaccharide. *S. aureus* is a highly clonal species and clonality has been shown to affect biofilm production [6]. Multicellular surface adherent biofilms formed by *Staphylococcus aureus* enables them to survive in various sources of stress, including antibiotics, nutrient limitations, heat shock, and immune responses [7].

*S. aureus* is the main cause of clinical mastitis and high somatic cell counts that reduce the quality of milk submitted to processing plants. Mostly reported cases of Mastitis are caused by biofilm-forming strains of *S. aureus* [8]. *Staphylococci aureus* have been attach to the contact surfaces in both milk and meat processing industries, form biofilms and survive on them. Their attachment to food contact surfaces in food processing plants pose a risk of contamination in milk and meat products [9]. The biofilm form of bacteria is much more resistant to disinfectants, antibiotics, and phagocytosis as compare to its free-floating planktonic counterpart which leads to potential economic losses and health problems [10]. The emergence of resistance against antimicrobial agents has led to the development of more efficient agents and new techniques for treatment of various microbial infections [11].

At the present, use of metals in the form of nanoparticles as antimicrobial agents has attained noticeable attention [12]. Nanoparticles possess increased surface areas and interactions with biological targets (such as bacteria) compared with traditional, micron particles. The nanoparticles are taken up by the phagocytes and considered as potential delivery systems in the treatment of bovine mastitis [13].
United States Food and Drug Administration has been approved the use of Chitosan as a drug carrier and bioengineering material and has good biocompatibility. Being deacetylated chitin the chitosan, poly-β (1 → 4) 2 amino 2 deoxy D glucose, is obtained from the outer shell of crustaceans. The chitosan provides various and distinctive physiological and biological properties due to its positive charge. Chitosan has great potential application in a wide range of industries including agricultural, food, cosmetic, and pharmaceutical [14]. The chitosan-coated iron oxide nanoparticles inhibit *S. aureus* biofilm formation of by decreasing biofilm biomass and number of live bacteria [2]. Magnetic iron oxide NPs is the primary choice for biological and biomedical approaches because of biocompatibility, super paramagnetic behavior, and chemical stability [15].
Treatment of biofilm-associated infections is challenging, require the development of new therapeutic strategies. As the research focus on Nanoparticle biofilm interactions is still in its early stages. Hence, this study was carried out to examine the effect of nanoparticles on antibiofilm formation potential of *S. aureus* isolates from milk and meat samples which may enter the food chain and pose potential public health hazards.

## Materials and Methods
A total of 15 samples comprising milk (n=6) from cattle suspected with Mastitis from Clinics, Madras Veterinary College, Chennai and meat (n=9; Chicken (3), Goat (1) and Sheep (5)) were collected aseptically from local vendors in sterile container, labeled and transported to laboratory for analysis.

### Isolation and Identification of *S. aureus*
The milk sample (0.5 ml) and meat samples were homogenized and inoculated in Nutrient broth and incubated at 37°C for 24 hrs. A loopful of enriched inoculum was streaked on Mannitol Salt Agar for selective isolation of *S.aureus*. Bacterial isolates which produced yellow colonies with yellow zone were presumed as *S. aureus*. The morphology was confirmed microscopically by Gram staining. The biochemical test was performed to confirm the isolates phenotypically. The tentative *S. aureus* isolates were subjected to species specific PCR by using SAS2 primer [16].

### Molecular characterization by Polymerase chain reaction (PCR)
DNA was extracted from *S. aureus* isolates using Qiagen DNA isolation kit. The isolates were subjected to genotypic confirmation and identification by PCR with published primers (Table 1).

**Table 1:** Details about Primer and PCR cycling condition

| Gene | Amplicon size (BP) | Initial denaturation | Denaturation | Annealing | Extension | Final Extension |
|---|---|---|---|---|---|---|
| SAS2-F | **894** | 94°C for 5 min | 30 cycles | | | 72 °C10 min |
| SAS2-R | | | 94 °C 30 s | 56 °C30 s | 72 °C45s | |

### Detection of biofilm production by Congo red agar method
*S. aureus* isolates were further analyzed for biofilm production on Congo red medium. The medium composed of Brain heart Infusion Broth (3.7/L), Agar (10g/L) and Congo red dye (0.8g/L) and sucrose (5gm/L) were autoclaved at 121°C for 15 minutes. The sucrose was added to autoclaved BHI agar at 55 °C using syringe filter. Congo red agar plates were inoculated with *S. aureus* isolates and incubated at 37°C for 24 to 48 hrs. Black colonies with a dry crystalline consistency indicated as positive biofilm formation while non-biofilm producing colonies remained smooth, and ranged from pink to bright red.

### Microtitre test assay (MTA)
(MTA) was used to test biofilm formation *in vitro*. Standard laboratory strain of *Staphylococcus aureus* was chosen as reference in this study. In sterile 96-well “Flat-Bottom” plates Trypticase Soy Broth (200 µL) and bacterial suspension (20 µL) was inoculated and incubated at 37 °C for 24 hrs. The supernatant was then discarded and the wells were washed three times with sterile distilled water (240 µL) and air-dried at room temperature. The culture was fixed with methanol (240 µL) for 15 min. The liquid phase was poured off and the wells were air-dried. Then, 1% crystal violet (200 µL) was added to the wells for 5 min to stain the biofilm and washed three times with distilled water, the plate was inverted and air-dried. To resolubilise the stain from the biofilm glacial acetic acid (240 µL) was added. Absorbance (570nm) was measured using microplate spectrophotometer reader (BioTek) to indicate the amount of biofilm that formed on the microtitre plate surface. Un-inoculated wells containing Trypticase Soy Broth served as blanks. Blank-corrected absorbance values for the *S. aureus* isolates were used for reporting biofilm production.

### Preparation of nanoparticles
The nanoparticles were synthesized according to standard procedure with necessary modifications [2]. The synthesized nanoparticles were characterized by Zeta potential and particle size analysis.

### Biofilm formation analysis with crystal violet staining
The inhibition of biofilm growth was analyzed using crystal violet (CV) staining method to study the effect of chitosan-coated iron oxide nanoparticles. 100 µL *S. aureus* cells at a concentration of $5 \times 10^8$ CFU/mL were seeded in 96-well microplate in triplicate. Bacterial cells were cocultured with Brain Heart Infusion broth (100 µL) supplemented with chitosan-coated iron oxide nanoparticles added with different concentrations (500 mg/ml, 1 mg/ml, 4 mg/ml). Biofilm biomass was quantified by the addition of 100 µL of 95% ethanol to each CV-stained microplate well, and the absorbance (OD-600) was determined using microplate spectrophotometer reader (BioTek). *S. aureus* without nanoparticles was set as 100%. Tandard laboratory strain of Staphylococcus aureus was chosen as reference in this study. The relative percentage of biomass for the other samples was calculated relative to that of the control and blank. All samples were incubated together in the same plate and under the same conditions.

## Results and Discussion

### Isolation and identification of *Staphylococcus aureus*

A total of 15 isolates from milk (n=6) of mastitis cases and meat (n=9) were subjected to confirmation of *S. aureus* by phenotypic and genotypic characterization.

Morphological colony character on selective Mannitol Salt Agar (MSA), Gram staining and microscopical examination was performed. All isolates grown on MSA were mannitol fermenters with yellow colonies and presumed to be *S. aureus*. Out of 15 isolates five tentative isolates (milk sample (3), chicken meat (1) and goat meat (1) sample) were confirmed as *S.aureus* isolates using selective Mannitol Salt Agar medium by phenotypic colony characters and Gram`s staining.

The *S. aureus* isolates were subjected to genotyping confirmation revealed single band amplicon of expected size (894 bp) and confirmed that four (milk (3) and chicken meat (1)) isolates as *Staphylococcus aureus*. The confirmed *S. aureus* isolates were further used to study the effect of Chitosan coated iron oxide nanoparticle.

### Biofilm formation study of *S. aureus* isolates

Biofilm characteristics of *S. aureus* were tested phenotypically for biofilm formation using Congo Red Method and Microtitre Assays method.

### Congo red agar plate method

The results of biofilm production by *S. aureus* using Congo red agar method showed black colonies with a dry crystalline consistency confirmed as positive for biofilm formation.

### Biofilm formation by microtitre test assay (MTA)

The MTA showed that most of the isolates produced biofilm in 96 well plates. The biofilm assay by MTA showed mean OD value of >0.120 for biofilm producing and <0.120 as non biofilm producing bacteria. The biofilm mean OD range varies from 0.3 to 0.8. All isolates showed high biofilm formation activity by MTA assay (Table 2).

**Table 2:** Classification of bacterial adherence by MTA method

| Mean OD values | Biofilm formation | No. of isolates |
|---|---|---|
| <0.120 | None/weak | 0 |
| 0.120-0.240 | Moderate | 0 |
| ≥0.240 | High | 4 |

### Nanoparticle characterization

The size and Zeta potential of the iron oxide nanoparticles was recorded in Horiba SZ 100 Nanopartika. The size of nanoparticle was found to be 4.8 - 6.4 nm. The average zeta potential was reported as 53.6 mV.

### Biofilm formation analysis with crystal violet staining

The effectiveness of chitosan coated iron oxide nanoparticles to inhibit biofilm production was studies by crystal violate staining assay. The nanoparticles with different concentration were co cultured with bacterial suspension and studied by CV-stained microplate well. The absorbance was determined with a plate reader.

The average of all samples reading at different concentration was taken and percentage of Biofilm inhibition was calculated by following formula.

$$\text{Biofilm inhibition (\%)} = 100-(AT-AB)/(AC-AB)$$

**AT**-Absorbance value of test sample
**AB**-Absorbance value of blank
**AC**-Absorbance value of control

Chitosan-coated iron oxide nanoparticles were able to inhibit biofilm formation by *S. aureus* at different concentrations compared to the control. Most of the samples showed biofilm growth inhibition at concentration of 500mg/mL and 4 mg/mL. Biofilm inhibition was range from 66.17-99.25%, 68.63-98.86%, 91.41-97.66% at concentration of 500mg/mL, 1mg/mL and 4mg/mL respectively. These results suggest that chitosan-coated iron oxide nanoparticles are effective in restraining bacterial colonization in biofilm (Figure 1).

Antimicrobial resistance (AMR) is currently estimated to cause more than 700,000 annual deaths worldwide, with 10 million annual deaths by 2050. The economic importance and public health risk of AMR are enormous [4]. Multiple Antimicrobial resistance (AMR) strains can be transported from animals to humans by food chain represents public health hazard due to the foodborne outbreaks. MDR bacteria due to biofilm formation potential in food animal products such as meat and milk indicating unregulated antibiotic use. Mastitis, which is caused mainly by pathogenic microorganisms, is one of the most important diseases of dairy cattle *S. aureus* biofilms impose resistance to host immune mechanisms and antimicrobial therapy with a reduced treatment efficacy and resistance development.

The antibacterial effect of nanoparticles showed that chitosan nanoparticles and copper-loaded nanoparticles could inhibit the growth of various bacteria [17] in accordance with our findings. The reduction in bacterial adhesion after chitosan treatment was lower than that of the control responsible for bacterial death and biofilm viability [18]. The inhibitory effect of nanoparticles on the existing biofilm was due to the presence of water channels throughout the biofilm. The presence of biofilm water channels (pores) for nutrient transportation, nanoparticles may diffuse through the pores and play an antibacterial function in the biofilm [19].

The smaller size of chitosan-coated iron oxide nanoparticles had an antibacterial effect contributed to their antibacterial effect compared to chitosan nanoparticles [2]. Bare iron oxide nanoparticles have been reported to exert toxic effects [20]. The adhesion of nanoparticles to negatively charged bacteria increases due to the positively charged surface of the chitosan-coated nanoparticles [21]. Therefore in this study chitosan coated nanoparticles were used to increase effectiveness against biofilm formation.

Chitosan nanoparticles formed restriction at higher concentrations (2 and 4 mg/mL) and chitosan-coated iron oxide nanoparticles are effective in restraining bacterial colonization in biofilm in accordance with our result [2]. Maximum inhibition except one isolate from meat was observed from this study due to strong biofilm production activity compared to other isolates. In lower concentrations (0.2 and 0.5 mg/ml), chitosan-coated iron oxide nanoparticles showed better restriction [2]. The findings in our study showed biofilm growth inhibition at concentration of 500mg/mL and 4 mg/mL. Chitosan coated $Fe_3O_4$ NPs were effective against antimicrobial agents and may be developed as a microbial resistant coating for biomedical devices [11]. Chitosan presented a strong antimicrobial activity not only on planktonic but also on preformed biofilms from chronic bovine mastitis that was independent of the antibiotic

resistance pattern [22]. The results showed that using Nano formulation with chitosan surface modification were effective against antimicrobial resistant foodborne bacteria.

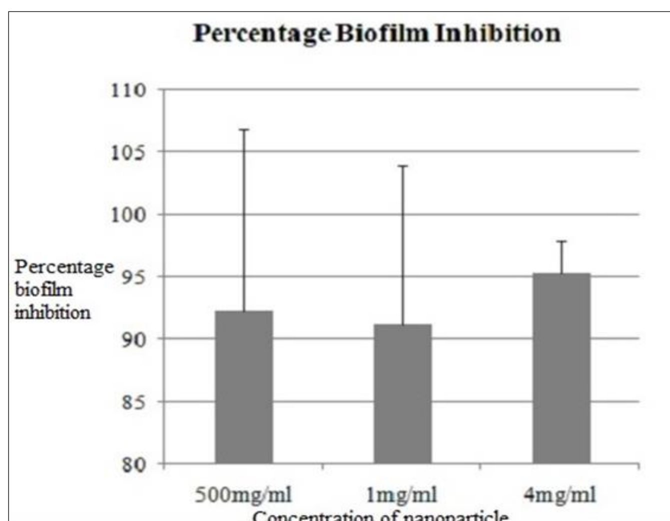


**Fig 1:** Percentage inhibition of biofilm bacterial growth by chitosan-coated iron oxide

## Conclusion

Humans are at a risk of being infected with resistant foodborne bacteria via the food chain from reservoir animals. This study showed higher activity at lower concentration of chitosan coated iron oxide nanoparticles which target antibiofilm activity of strongly adapting microorganism by biofilm formation. Good effects were found against biofilm formation using this Nano formulation. Antibiotics is the most common therapy for the treatment of bovine mastitis but increasing occurrence of resistance and presence of antibiotics residues in the milk is emerging problem. NPs are linked with toxicity and biodegradation capability but surface is modified with chitosan increases its physiological and biological properties with great potential applications. NPs coated with chitosan biomolecules have enhanced their biocompatibility gaining them approval by authorities such as the US Food and Drug Administration. This result about nanoformulation has potential to be developed as therapeutic agent in the cases where antibiotics are not effective or in combination with antibiotics and against antibiotics resistant strains.

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## References

1. Notcovich S, DeNicolo G, Flint SH, Williamson NB, Gedye K, Grinberg A *et al*. Biofilm-forming potential of *Staphylococcus aureus* isolated from bovine mastitis in New Zealand. Veterinary Science. 2018; 5(8):1-5.
2. Shi S, Jia J, Guo X, Zhao Y, Chen D, Guo Y *et al.* Reduced *Staphylococcus aureus* biofilm formation in the presence of chitosan-coated iron oxide nanoparticles, International Journal of Nanomedicine. 2016; 11:6499-6506.
3. Marques VF, Mottaa CC, Soares BS, Melo DA, Coelho SMO, Coelho IS *et al*. Biofilm production and beta-lactamic resistance in Brazilian *Staphylococcus aureus* isolates from bovine mastitis. Brazilian Journal of Microbiology. 2017; 48:118-124.
4. Bhattacharya M, Wozniak DJ, Stoodley P, Hall-Stoodley L. Prevention and treatment of *Staphylococcus aureus* biofilms. Expert Review of Anti-infective Therapy. 2015; 13(12):1499-1516.
5. Ray B, Bhunia AK. Fundamental Food Microbiology. CRC Press, Taylor and Francis Group, 2008.
6. Croes S, Deurenberg RH, Boumans ML, Beisser PS, Neef C, Stobberingh EE. *Staphylococcus aureus* biofilm formation at the physiologic glucose concentration depends on the *S. aureus* lineage. BMC Microbiology. 2009; 9:229.
7. Chung PY, Toh YS. Anti-biofilm agents: recent breakthrough against multidrug resistant *Staphylococcus aureus.* Pathogens and Disease. 2014; 7(3):231-239.
8. Petrovski KR, Heuer C, Parkinson TJ, Williamson NB. The incidence and etiology of clinical bovine mastitis on 14 farms in northland, New Zealand. New Zealand Veterinary Journal. 2009; 57:109-115.
9. Gundogan N, Ataol O. Biofilm, protease and lipase properties and antibiotic resistance profiles of *Staphylococci* isolated from various foods. African Journal of Microbiology Research. 2013; 7:3582-3588.
10. Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F. Resistance of bacterial biofilms to disinfectants: a review. Biofouling the Journal of Bioadhesion and Biofilm Research. 2011; 27:1017-1032.
11. Nehra P, Chauhan RP, Garg N, Verma K. Antibacterial and antifungal activity of chitosan coated iron oxide nanoparticles. British Journal of Biomedical Science. 2018; 75(1):13-18.
12. Allaker RP, Vargas-Reus MA, Ren GG. Nanometals as Antimicrobials. Antimicrobial Polymers*,* John Wiley and Sons Inc., 2011, 327-350.
13. Sankar P. New therapeutic strategies to control and treatment of bovine mastitis. Vet Med Open Journal. 2016; 1(2):7-8.
14. Khedri B, Shahanipour K, Fatahian S, Jafary F. Preparation of chitosan-coated Fe3O4 nanoparticles and assessment of their effects on enzymatic antioxidant system as well as high-density lipoprotein/low-density lipoprotein lipoproteins on Wistar rat. Biomedical and Biotechnology Research Journal. 2018; 2:68-73.
15. Ali A, Zafar H, Zia M, Haq I, Phull AR, Ali JS *et al.* Synthesis, characterization, applications and challenges of iron oxide nanoparticles, Nanotechnology Science and Applications. 2016; 9:49-67.
16. Shome BR, Mitra SD, Bhuvana M, Krithiga N, Velu D, Shome R *et al.* Multiplex PCR assay for species identification of bovine mastitis pathogens. Journal of Applied Microbiology. 2011; 111:1349-1356.
17. Qi L, Xu Z, Jiang X, Hu C, Zou X. Preparation and antibacterial activity of chitosan nanoparticles. Carbohydrate Research. 2004; 339(16):2693-2700.
18. Busscher HJ, Engels E, Dijkstra RJ, van der Mei HC. Influence of a chitosan on oral bacterial adhesion and growth *in vitro*. European Journal of Oral Sciences. 2008; 116(5):493-495.
19. Kalishwaralal K, Barath Mani Kanth S, Pandian SR, Deepak V, Gurunathan S. Silver nanoparticles impede the biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. Colloids Surf B Biointerfaces. 2010; 79(2):340-344.
20. Mahmoudi M, Sant S, Wang B, Laurent S, Sen T. Super paramagnetic iron oxide nanoparticles (SPIONs):

development, surface modification and applications in chemotherapy. Advanced Drug Delivery Reviews. 2011; 63(1-2):24-46.

21. Shi S, Jia J, Guo X, Zhao Y, Chen D, Guo Y *et al*. Biocompatibility of chitosan-coated iron oxide nanoparticles with osteoblast cells. International Journal Nanomedicine. 2012; 7:5593-5602.
22. Breser M, Felip EV, Bohl LP, Orellano MS, Isaac P, Conesa A *et al*. Chitosan and cloxacillin combination improve antibiotic efficacy against different lifestyle of coagulase-negative *Staphylococcus* isolates from chronic bovine mastitis. Scientific Reports. 2018; 8:5081.