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RESEARCH ARTICLE

# Response of Single and Mixed Culture Bacteria of *S. aureus* and *E. coli* of Camel Mastitis against Antibiotics and Tungsten Oxide Nanoparticles

Saima Talib[1], Maham Fatima[1]*, Muhammad Usama[2], Tasleem Kausar[1], Shumaila Noreen[1]

[1]Department of Zoology, The Government Sadiq College Women University, Bahawalpur-63100, Pakistan.
[2]School of Life Sciences, Anhui Agriculture University, Hefei-230036, China

*Corresponding author: mahammalikiub@gmail.com

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## ABSTRACT

Mastitis is a serious issue responsible for declining udder health and milk production of camel. *S. aureus* and *E. coli* are the major pathogens responsible for mastitis. The study comprised of isolation of *S. aureus* and *E. coli* from camel mastitis milk. Camel milk samples (n=200) were obtained from different areas and subjected to a series of biochemical tests for the isolation and identification of these microbes. Tungsten oxide nanoparticles were coupled with ciprofloxacin antibiotic through chemical synthesis process. Both individual and mixed cultures of both bacteria were subjected to antibacterial susceptibility trial against wider range of antibiotics. These bacterial cultures were then subjected to evaluate their response against nanoparticles coupled antibiotics using well diffusion assay. Both parametric and non-parametric tests were applied to analyze data obtained in this study. The results revealed variable response in that at some instances, mixed bacteria showed higher zone of inhibition (ZOI) compared to the single bacterial culture, and vice versa against different antibiotics. Over all, penicillin and doxycycline showed highest ZOI against bacterial cultures. Ciprofloxacin showed higher efficacy than nanoparticle-coupled ciprofloxacin against single bacterial cultures. The efficacy of products was found concentration dependent against all bacterial cultures. The study thus concluded non-conventional responses of bacteria against antibiotics and nanoparticle coupled antibiotics reflecting that mixed bacterial cultures should be considered for drug trial in order to get a comprehensive view of antimicrobial resistance. Moreover, alternative to antibiotics thought to be opted and for this in vivo and field trials are required. Coupled with all steps of development of nanobiotics equally effective on single and mixed bacterial culture may help achieve optimum health and production.

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## INTRODUCTION

Mastitis (Inflammation of mammary gland) is the cause of many challenges in dairy industry. These challenges are related to profit, quality and quantity of milk, food security and animal welfare (Hogeveen *et al*., 2011). Mastitis causes serious problems in udder tissue and effect milk production. It also has adverse effect on humans as it decreases the nutritional value of milk because of subclinical mastitis (Gurjar *et al*., 2012). Subclinical mastitis

also decreases the reproductive efficacy of animal. There are many microorganisms that cause mastitis in dairy animals. These microbes increase their numbers in the teat lesions and skin of mammary gland. Among these pathogens *E. coli, Staphylococcus aureus, Streptococcus agalactiae and Klebsiella pneuminiae* are the major cause of mastitis in dairy animals (Kabelitz *et al*., 2021). *Staphylococcus aureus* is the most pathogenic bacteria in *Staphylococci* species that is associated with diseases and considered as most important danger to veterinary medicine (Javed *et al*., 2021; Sarwar *et al*., 2021). Subclinical mastitis is manifested by zero physical change in the health of udder. The only change is the increase in the number of somatic cells in the milk. The conversion of subclinical mastitis into clinical mastitis can be prevented by adopting right solution for subclinical mastitis.

Many researches showed that mastitis is not a single pathogen disease; rather it is caused a variety of pathogen percentages. *Enterobacteriaceae* family is responsible for 62.07% subclinical mastitis. Among these, *E. coli* is responsible for 25.86%. In same manner, *Staphylococci* family is responsible for 87.93% subclinical mastitis. Among these, *S. aureus* is responsible for 36.20% subclinical mastitis. Another research showed that 66% mastitis is caused by multiple bacteria (Abdennebi *et al*., 2020). Another research showed that 11.10% mastitis is caused by multiple bacteria (Bradley, 2002).

In the current situation, 5R concept should be used that includes refinement, responsibility, replacement, reduction and review of antimicrobial use. Alternative products are required to decrease the antibiotic use or replacing them. Nanoparticles are the important option in this regard. They have synergistic effect with antibiotics and serve as carrier for antibiotics. (Ahmed *et al*., 2022). Recent researches showed that WO3-X Nano dots showed very strong bactericidal activity against a wide range of bacteria that includes *S. aureus, E. coli, B. subtilis and P. multocida* (Matharu *et al*., 2020)

Therefore, this research is organized to determine the susceptibility of antibiotics against single and mixed culture of two important bacteria *(S. aureus* and *E. coli)* responsible for mastitis in camel and effectiveness of different types of nanoparticles in the treatment of mastitis against these bacteria.

## MATERIALS AND METHODS

**Collection of Samples:** 200 milk samples were collected from different areas of Bahawalpur region and Cholistan desert. After collecting the samples Surf field mastitis test was performed on each sample to check the presence of subclinical mastitis. The samples that were positive for surf field mastitis test were shifted to Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Punjab, Pakistan. Collected sample category involves any sample falling in subclinical mastitis +1 category (Muhammad *et al*., 2010). Single sample was considered from an animal having positive subclinical mastitis for two or more than two teats. Samples from all teats were pooled together to make a single sample.

**Bacterial Isolation:** For the isolation of the both bacteria (*S. aureus* + *E. coli*), Firstly, samples were put on sterile nutrient broth in falcon tubes and then put in incubator at 37°C for 24 hours. On the next day centrifugation done at 6000rpm for 15 minutes. After that to start the further process, the sterile swabs were taken and soaked these swabs into the sedimentary material of all samples one by one for further swabbing on blood agar. For swabbing into the blood agar the agar was poured into the petri plates and let these plates to cool down for 5 to 10 minutes. Swabbing was done by swabs (sterile swabs) and then put these plates again in incubator at 37°C for 24 hours. On next day bacterial colonies were observed in the plates. Then these Specific colonies were picked with sterile steel loop. It was streaked on different selective media like *Staphylococcus aureus* (Mannitol salt agar) and *E. coli* (MacConkey agar), and put these plates in incubator for 24 hours at 37°C. Pink colonies were observed on MacConkey agar and opaque yellow were seen on the Mannitol salt agar. For further confirmation different biochemical tests were performed with positive control. These tests included (urease, catalase, Gram staining, indole, coagulase, citrate, methyl red and CAMP test). Following directions of Bergey's manual of determinative bacteriology, confirmation of the targeted bacteria done with the help of Bergey's manual of biochemical test results and specific growth. (Holt, 1994).

**Antibiotic Susceptibility Test (Disk Diffusion Test):** Nine antibiotics (penicillin, enrofloxacin, spiramysin, tylosin, amoxicillin, doxycycline, lincomycin, florfenicol, and neomycin) were checked against both bacteria (*Escherichia coli* and *staphylococcus aureus*) separately and in mix culture. The antibiotics were selected on the basis of their use and importance in human as well as veterinary medicine and their presence in the market stores. On the basis of instructions provided by Clinical and Laboratory Standard Institute, Kirby Bauer's disc diffusion method used (CLSI, 2021). 1-1.5 × $10^8$ CFU/mL of fresh growth of each bacterium were prepared and then swabbed on sterile Mueller Hinton agar. Antibiotics discs are placed with the help of the sterile spatula on the Mueller Hinton agar plates at equal distance aseptically. Then placed in

incubator at 37°C for 24 hours. On the next day plates were observed and measured the zones of each antibiotic against both bacteria (CLSI, 2021).

**Agar Well Diffusion Method:** To perform the well diffusion test, cultures of *Escherichia coli* and *staphylococcus aureus* individually prepared and was adjusted to the 1–1.5 × $10^8$ CFU/mL to get the turbidity of culture 0.5 McFarland. The mix culture of the both bacteria was achieved by mixing 1/2:1/2 ratio of both bacteria, to get the final adjustment at the 1–1.5 × $10^8$ CFU/ml. To achieve 1mL (0.5 McFarland) half of an *E. coli* and half of *S. aureus* solution was mixed. Petri plate were prepared by adding Mueller-Hinton agar. The culture of the both bacteria individually and mix culture was uniformly swabbed on Mueller-Hinton agar. On sterile Mueller-Hinton agar, wells (6–8 mm) were created at equal intervals using a well borer. Culture of both bacteria alone and mix swabbed on the plates. The plates were then incubated for 24 hours at 37 °C after adding tungsten oxide nanoparticles alone and tungsten coupled with ciprofloxacin were added to the wells (15µL). On the next day, zones were measured using a Vernier calliper against both mix culture and *S. aureus* and *E. coli* alone (Anwar *et al*., 2020).

**Statistical Analysis:** The obtained data was analyzed through descriptive statistics in case of univariate information, while prevalence was calculated as per formula described by (Thrusfield, 2007). Minitab was applied to analyze data at 5% probability.

## RESULTS

**Antibiotic susceptibility test:** The current study showed the different patterns of resistance, intermediate susceptibility and sensitivity against these nine antibiotics (penicillin, enrofloxacin, spiramysin, tylosin, amoxicillin, doxycycline, lincomycin, florfenicol, and neomycin). To check the susceptibility pattern disc diffusion method was used against these strains of bacteria

***E. coli*:** In case of *E. coli* 20% isolates showed resistance against Amoxicillin, Spiramycin, Lincomycin and Florfenicol. However, 70% isolates showed the sensitivity against Florfenicol, 60% showed against the Lincomycin and the Spiramycin and 50% against the Neomycin. 40% isolates showed the intermediate sensitivity. 30 % isolates showed the resistance against the Tylosin, Doxycycline and Enrofloxacin.

***S. aureus*:** While in case of *S. aureus* 60% samples were sensitive against the Neomycin and Doxycyclin. 20% were resistance against the Florfenicol and Tylosin and 40% were seen against the Penicillin. 25% isolates showed the intermediate sensitivity against the Enrofloxacin, Doxycycline, Amoxicillin and Spiramycin.

**Mix culture:** Against mix culture of both bacteria the 30% resistance against the Lincomycin, Enrofloxacin and 60% sensitivity against the Neomycin and Amoxicillin was observed, while 20% showed the intermediate sensitivity against Neomycin and Penicillin. While 40% isolates also showed the resistance against the penicillin, 25% against the Florefenicol and 20% against the Spiramycin (Table 1; Fig. 1).

**Table 1:** Comparison of zones of inhibition against antibiotics

| Antibiotics | *E. coli* (%) | | | *S. aureus* (%) | | | Mix culture (*S. aureus* + *E. coli*) (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | S | I | R | S | I | R | S | I | R |
| Neomycin | 50 | 30 | 10 | 60 | 10 | 30 | 60 | 20 | 20 |
| Amoxicillin | 40 | 40 | 20 | 50 | 25 | 25 | 60 | 10 | 30 |
| Penicillin | 30 | 30 | 40 | 30 | 30 | 40 | 40 | 20 | 40 |
| Enrofloxacin | 30 | 40 | 30 | 45 | 25 | 30 | 45 | 25 | 30 |
| Lincomycin | 60 | 20 | 20 | 70 | 20 | 10 | 35 | 35 | 30 |
| Doxycyclin | 40 | 30 | 30 | 60 | 25 | 15 | 50 | 40 | 10 |
| Spiramycin | 60 | 20 | 20 | 40 | 25 | 35 | 50 | 30 | 20 |
| Florfenicol | 70 | 10 | 20 | 50 | 30 | 20 | 60 | 15 | 25 |
| Tylosin | 40 | 30 | 30 | 45 | 35 | 20 | 55 | 30 | 15 |

S= sensitive; I= intermediate; R= resistance

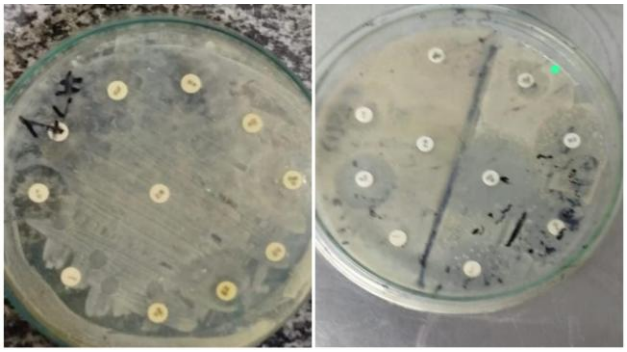

**Fig. 1: A**ntibiotic susceptibility to *S. aureus* and *E. coli* alone and their mix culture

### Agar well diffusion test

***S. aureus, E. coli* and mix culture:** The current study showed the significant difference ($p$<0.05) between different treatment groups against *S. aureus* in term of mean zone of inhibition. The highest men zone of inhibition was seen in case of CW at each concentration, while the lowest zone of inhibition was seen in case of C at each concentration. $WO_3$ showed the zone of inhibition (9.667 ±1.528) at 0.5 ug/mL Table 2. In case of *E. coli* at 10ug, CW showed the highest mean zone of inhibition (26.333 ± 1.528) and C showed the lowest zone of inhibition (19.00 ± 1.00). The lowest zone of inhibition was seen at 0.25 ug/mL (9.67 ± 2.08) Table 3. Same pattern was seen in case of the mix culture of bacteria Table 4, Fig. 2.

**Table 2:** Comparison of zone of inhibition of S. aureus against different treatment groups at each concentrations

| Groups | Concentrations (ug) | | | |
|---|---|---|---|---|
| | 10ug/mL | 1 ug/mL | 0.5 ug/mL | 0.25 ug/mL |
| Ciprofloxacin (C) | 18.33 ±2.08$^{b}$ | 15.67± 2.08$^{b}$ | 11.67± 2.89$^{ab}$ | 9.67± 2.08$^{a}$ |
| Tungsten oxide ($Wo_3$) | 16.667± 1.155$^{b}$ | 13.00± 1.00$^{b}$ | 9.667± 1.528$^{b}$ | 7.00± 1.00$^{a}$ |
| ciprofloxacin+$Wo_3$ (CW) | 27.67± 2.52$^{a}$ | 23.00± 1.73$^{a}$ | 17.67± 3.51$^{a}$ | 14.33± 5.13$^{a}$ |

C= ciprofloxacin; $Wo_3$ = Tungsten oxide; CW= ciprofloxacin+$Wo_3$.Different superscripts within column showed the significant difference ($p$<0.05).

**Table 3:** Comparison of zone of inhibition of *S. aureus* against different treatment groups at each concentrations

| Groups | Concentrations (ug) | | | |
|---|---|---|---|---|
| | 10ug/mL | 1 ug/mL | 0.5 ug/mL | 0.25 ug/mL |
| Ciprofloxacin (C) | 19.00± 1.00$^{b}$ | 15.333± 1.528$^{b}$ | 11.333± 1.155$^{a}$ | 9.00± 1.00a$^{b}$ |
| Tungsten oxide (Wo3) | 17.333± 1.528$^{b}$ | 14.333± 1.155$^{b}$ | 10.00± 1.00$^{b}$ | 6.333± 1.528$^{b}$ |
| ciprofloxacin+Wo3 (CW) | 26.333± 1.528$^{a}$ | 21.00± 1.73$^{a}$ | 16.333± 1.528$^{b}$ | 10.667± 1.528$^{a}$ |

C= ciprofloxacin; $Wo_3$ = Tungsten oxide; CW= ciprofloxacin+$Wo_3$. A significant difference ($p$<0.05) shown by the different superscripts in columns.

**Table 4:** Comparison of zone of inhibition of mix culture against different treatment groups at each concentrations

| Groups | Concentrations (ug) | | | |
|---|---|---|---|---|
| | 10ug/mL | 1 ug/mL | 0.5 ug/mL | 0.25 ug/mL |
| Ciprofloxacin (alone) | 15.667± 1.528$^{b}$ | 12.333± 1.155$^{b}$ | 9.333 ± 0.577$^{a}$ | 6.000 ±1.000$^{a}$ |
| Tungsten oxide (Wo3) | 13.667± 1.528$^{b}$ | 10.667± 1.155$^{b}$ | 7.00 ± 2.00$^{a}$ | 5.333 ± 1.528$^{a}$ |
| ciprofloxacin+Wo3 | 21.667± 1.528$^{a}$ | 18.000± 1.000$^{a}$ | 11.00 ± 3.61$^{a}$ | 8.000 ± 1.000$^{a}$ |

C= ciprofloxacin; $Wo_3$ = Tungsten oxide; CW= ciprofloxacin+$Wo_3$. . A significant difference ($p$<0.05) shown by the different superscripts in columns.

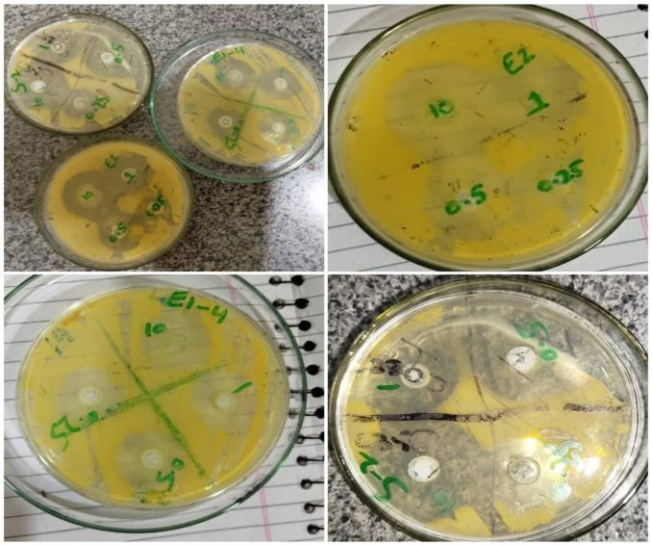


**Fig. 2:** Effect of different Nanoparticle concentrations *on S. aureus* and *E. coli.*

## DISCUSSION

The pathogens used in this research are important bacteria for human and animal health. *S. aureus* becomes very common bacteria in the cattle milk (Javed *et al.*, 2021), this bacterium can also be found in other animals rather than the cattle (Sarwar *et al*., 2021). In contrast to the results of the current study (Abakar *et al.*, 2022) observed 30% prevalence of *E. coli* and 40% prevalence of *S. aureus*. 85.7% prevalence of *E. coli* was found in another research (El-Mohandes *et al*., 2022). In Punjab, district Muzaffargarh has 35%, Lahore has 30% and other regions of Punjab has 40% subclinical mastitis cases (Ali *et al*., 2011). This difference in the prevalence of subclinical mastitis in the different regions of the same country might be because of age, health status of animal, parity, presence of other diseases, physiological status, season and hygiene of animals. A large number of protection mechanisms has been adopted by the pathogen to prevent from physical and chemical effects of antibiotics. Glycocalyx, an exopolysaccharide is produced by *E. coli* that helps in the attachment to the epithelial lining and diminishes the efficacy of antibiotics (Rosini *et al*., 2015). *E. coli* prevents itself by the production of an endotoxin that is a lipopolysaccharide. When bacteria proliferate, it starts inflammatory reaction. Inflammatory mediators and leukocytes also move to that space to diminish the infection. So, the mastitis caused by *E. coli* is associated with host more than that of its pathogenesis (Gilbert *et al*., 2013). The development of disease also depends upon the nutritional status, environmental factors and transition stage of the animal (Cheng *et al*., 2020). Previous research described the antibacterial action of tungsten nanoparticle against pathogens like *S. aureus* and *E. coli*. (Syed *et al*., 2010). Another research showed that nanoparticle (tungsten oxide) showed very effective antimicrobial response against both bacteria, *Escherichia coli* (gram negative) and *staphylococcus* (gram positive) (Wayne *et al*., 2019). Nanoparticle coated antibiotics show highest Minimum Inhibitory Concentration Value against *S.aureus* and *E. coli*. Similar to our results, other researches define the antibacterial capability of WO3-X nanodots that is because of their photocatalytic properties and membrane stress (Acosta *et al*., 2019).

## Conclusion

The research showed that the mastitis is caused by different bacteria rather than single bacteria. In vitro research described that penicillin, doxycycline, enrofloxacin and florfenicol are effective drugs in case of mastitis. The least effective antibiotics against mastitis bacteria includes neomycin and spiramycin. Ciprofloxacin and Nanoparticle-10 are most effective against mastitis pathogens. While WO3 and Nanoparticle-0.25 are least effective. Mixed culture of bacteria is more susceptible to antibiotics and nanoparticles than separate bacteria at

some points. At the same time, single bacteria showed many folds more susceptibility than mixed culture. To find the reason behind this phenomenon, researches at molecular level is required. In order to determine the perfect dose of these antibiotics and nanoparticles, in vivo study and field trials are necessary.

**Conflict of Interest:** No

**Authors' contribution:** ST executed the experiments. MF Write up work. MU write up work. MU analyzed the data.TK and SN revised the manuscript.

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