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Article

# Synergy in penicillin, cephalosporin, amphenicols, and aminoglycoside against MDR *S. aureus* isolated from Camel milk

**Mahboob Ali[4+], Muhammad Avais[1*], Rakhshanda Naheed[2+], Muhammad Ameen Jamal[3+], Muhammad Sajid Hasni[1+], Mehtab Ahmad[1], Mumtaz Ali Khan[5], Sumaira Baloch[6], Aman Ullah Khan[7], Samiullah Khan[8], Amjad Islam Aqib[9]**

[+]Author's contributed equally

*[1]University of Veterinary and Animal Sciences, Lahore, Pakistan, [2]Department of Physiology, Loralai Medical College, Loralai, Balochistan, Pakistan, [3]Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China, [4]Animal Sciences Institute Livestock and Dairy Development Department Quetta, Balochistan, Pakistan, [5]Livestock and Dairy Development Department, Peshawar, KPK, Pakistan, [6]University of Balochistan Quetta, Pakistan, [7]Department of Pathobiology/ Microbiology, College of Veterinary and Animal Sciences, Jhang Campus-35200, Pakistan,*

*[8]College of Animal Science and Technology, Yunnan Agricultural University Kunming, China 650201, [9]Cholistan University of Veterinary and Animal Sciences, Bhawalpur, Pakistan 63100*

This study investigated the synergy testing of penicillin, cephalosporin, amphenicols, and aminoglycoside in the camel milk (n=768 samples), subsequently used for isolation of MDR *S. aureus* targeting *mecA* gene. Antibiotic susceptibility of *S. aureus* showed >90% isolates were sensitive to ciprofloxacin and trimethoprim and resistant against oxacillin, ampicillin, and cefoxitin. Further, 50-85% of the *S. aureus* were sensitive to gentamicin, oxytetracycline, and chloramphenicol and resistant against cefotaxime, vancomycin, and cefixime. Minimum inhibitory concentration (MIC) of cefotaxime, (C) and ampicillin (A) in combination with gentamicin (G) was reduced by 99.34% and 70.46%, respectively, while with chloramphenicol (Ch), reduction was 57.49% and 60%, respectively. In addition, the Fractional Inhibitory Concentration Index (FICI) of G+A, Ch+C and Ch+G combinations showed synergy against 80%, 60%, and 30% of MDR *S. aureus*, respectively. Similarly, C+A and Ch+G displayed indifferent interaction against 70 % and 30% of isolates, respectively, while the later showed additive interaction against 10% of MDR *S. aureus*. Altogether, our results described effective combination of gentamicin and chloramphenicol with ampicillin and cefotaxime to combat MDR *S. aureus.*

**Keywords:** MDR. *S. aureus*. Camel milk. Mono-drug trial. Fractional inhibitory concentration indices. Synergy testing.

## INTRODUCTION

*Staphylococcus aureus* is an opportunist pathogen that is responsible for numerous types of infections in animals and humans. Although, camel milk has nutraceutical properties (Aqib *et al.,* 2019) but still is more prone to *S. aureus* due to its pathogenic surges (Aqib *et al.,* 2017a). *S. aureus* isolated from camel milk were declared as multidrug resistant (Ali *et al.,* 2018), and now it is considered as one of the challenging pathogen in the century because of resistance against all kinds of antimicrobials (Kuroda *et al.,* 2001). Previously, few drugs in the form of vancomycin have been used to treat *S. aureus* infections (Worthington, Melander, 2013) but later on were not so effective. The development of esistance was due to alteration in efflux pumps and activation of various mechanisms (Johari *et al.,* 2012). Consequently, alternative and complementary drug therapies are required to cope with this multiple drug resistant (MDR) *S. aureus* (Mohtar *et al.,* 2009).

***Correspondence:** M. Avais. Department of Clinical Medicine and Surgery. University of Veterinary and Animal Sciences, Lahore. E-mail: mavais@uvas.edu.pk. A. I. Aqib. Cholistan University of Veterinary and Animal Sciences. Bhawalpur, Pakistan. E-mail: amjadwaseer@cuvas.edu.pk. Muhammad Ameen Jamal – ORCID: https://orcid.org/0000-0002-8364-6389

Combination of drugs offers many clinical advantages like minimizes toxicity induced by higher use of single drug, reduces resistance due to repetition of single drug and enhances activity of drugs that remain ineffective (Jain *et al.,* 2011). Aminoglycosides have been prescribed all over the world due to low cost and rapid and potent bactericidal activity (Mascaretti, 2003). However, continuous usage and high dose causes nephrotoxic and ototoxic effects (Drobbin, Phelan, Antonelli 2007). Ampicillin is broad spectrum beta-lactam bactericidal that inhibits final confirmation of cell wall. Hence, combination of penicillin with aminoglycoside appears to be effective due to potent bactericidal activity of later that creates fissure in outer membrane and losses proteins from bacterial contents resulting in reduction in bacterial growth (Ibezim *et al.,* 2006). Therefore, objective of this study was to evaluate wide range of antibiotics against MDR *S. aureus*, and synergy testing of selected antibiotics (penicillin, cephalosporin, amphenicols, and aminoglycoside) against MDR *S. aureus*.

## MATERIAL AND METHODS

The current study was carried out in two distant ecological zones of Pakistan having major population of camel. A total of (n=768) she-camel milk samples were aseptically collected from Punjab province (Cholistan, n=384) and Baluchistan province (Suleiman range, n=384) using convenient sampling technique (Thrusfield, 2007). The milk samples were sent to the Department of Clinical Medicine, University of Veterinary and Animal Sciences, Lahore, Pakistan for the isolation of *S. aureus* following the guidelines of Bergey's Manual of Systematic Bacteriology (Krieg *et al.,* 1984).

Initially, the mono-drug antibiotic susceptibility was conducted by Kirby Bauer disc diffusion test. The isolates resistant to more than two classes of antibiotics were declared as multiple drug resistant *S. aureus* (Hiramatsu *et al.,* 2014). The activated growth (24-48 hours) of *S. aureus* adjusted at $5\times10^8$ CFU/mL was swabbed on Mueller-Hinton agar. Antibiotic discs *viz a viz* Oxacillin (10µg), Cefoxitin (30 µg), Trimethoprim (25ug), Ciprofloxacin (5 µg), Gentamicin (10 µg), Cefotaxime (30 µg), Vancomycin (30µg), Oxytetracycline (30µg), Cefixime (5µg), Chloramphenicol (30µg), Ampicillin (10µg), Streptomycin (10 µg), Amikacin (30 µg), Enoxacin (10 µg) were aseptically applied by multichannel dispenser. Petri plates were incubated at 37˚C for 24 hours and zone of inhibition (ZOI) was measured by Vernier calipers. In order to evaluate the efficacy of antibiotics, the calculated ZOIs were compared with the standards provided by Clinical and Laboratory Standards Institute (CLSI, 2016) and then confirmed by targeting *mecA* gene (Galdiero *et al.,* 2003) (primers listed in Table I). PCR products were run on 2% agarose gel and stained as reported previously (Aqib *et al.,* 2017a). The isolates were further evaluated for the synergy of different combinations.

**TABLE I -** Primer sequence for PCR

| Gene symbol | Product size | Oligo | Primer Sequence (5'-3') |
|---|---|---|---|
| mecA | 310 | Forward | TGGCTATCGTGTCACAATCG |
| | | Reverse | CTGGAACTTGTTGAGCAGAG |

Following mono-drug trial, two antibiotics as effective and two as ineffective were selected to explore their efficacies in combination with each other. Each antibiotic, representative of its respective group i.e. gentamicin (aminoglycoside), chloramphenicol (amphenicol), ampicillin (penicillin), and cefotaxime (cephalosporin), was selected on the basis of frequent use in study areas. Three combinations: effective with effective; effective with ineffective; and ineffective with ineffective were used for combination therapy trial against MDR *S. aureus*. The range of concentration of each drug to be used for synergy testing was determined

by taking MIC as central point and concentrations were determined as follows: Lower limits; 1/16MIC, 1/8MIC, 1/4MIC, 1/2MIC; Higher Limits: 2MIC, 4MIC, 8MIC, 16MIC. In this way, 1/16MIC was taken as minimum and 16MIC was taken as a maximum concentration of each drug. The MIC calculated in mono-drug trial showed 4.3, 2.34, 28.91 and 59.38 µg/mL of ampicillin, gentamicin, chloramphenicol, and cefotaxime, respectively. According to the defined protocol, concentration range of ampicillin was set 0.27-68.80µg/mL, gentamicin 0.15-37.44 µg/mL, chloramphenicol 1.81-462.56µg/mL, and cefotaxime 3.71-950.08 µg/mL. The stock for each concentration was made separately in Eppendorf tubes. Two-fold dilutions were made and poured row wise for one drug and column wise for other drug in combination. The first well and last well were kept as positive and negative controls having bacteria in positive control and only broth in negative control. A 100 µL of activated growth of *S. aureus* having $10^5$ CFU/mL was poured on the each well except negative control. The plate was incubated for 24 hours at 37°C. The OD value was taken before and after incubation at 595nm. Minimum inhibitory concentration was measured as lowest concentration without turbidity. Fractional inhibitory concentration of drug was calculated as

FIC=MIC of drug in combination/MIC of drug alone

Fractional inhibitory concentration index (FICI) of drug's combination was calculated as:

$FICI_{A+B}=FIC_A+FIC_B$

FICI indices <0.5 indicate Strong synergism; FICI >0.5 but <1.0 partial synergism; FICI = 1.0 additive; FICI >1.0 but <4.0 indifferent, and FICI > 4.0 antagonistic (Cai *et al.,* 2007).

## RESULTS

The data indicated that 47.14% of camel milk samples were positive for subclinical mastitis, while 53.04% of these samples were positive for *S. aureus* (Figure 1). *In-vitro* antibiotic susceptibility trial of *S. aureus* isolated from camels located in different ecological zones of Cholistan & Baluchistan showed variable response against different antibiotics. Mono-drug susceptibility trial showed >90% of *S. aureus* isolates were sensitive to ciprofloxacin and trimethoprim while same percentage was resistant to oxacillin, ampicillin, and cefoxitin (Table I). The susceptibility trial further revealed 50-85% of *S. aureus* was sensitive to gentamicin, oxytetracycline, and chloramphenicol, while the same percentage showed resistant to cefotaxime, vancomycin, and cefixime. An empirical efficacy pattern estimation, based on expression of zone of inhibition, showed maximum of MDR isolates expressing lower ZOI (0-10mm) for Oxacillin, Ampicillin, Cefoxitin, Vancomycin, Streptomycin and Cefixime (Table II).

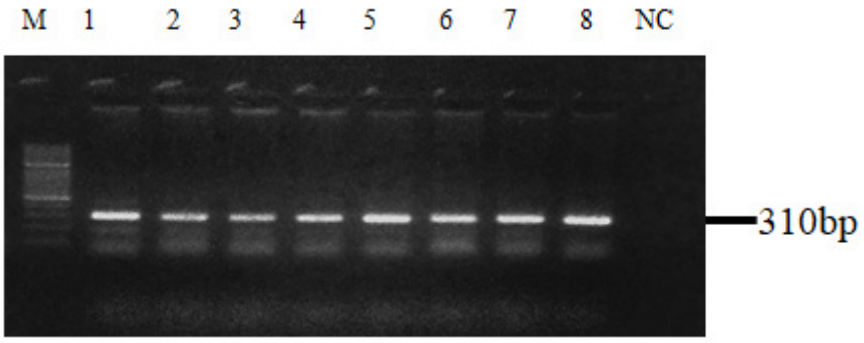


**FIGURE 1 -** PCR gel results for amplification of mecA gene of S. aureus isolated from sub-clinical camel mastitis from two distinct ecological zones. M: Marker, 1-8 indicates positive isolates at 310bp level, NC: negative control.

**TABLE II -** Percentage of *S. aureus* exhibiting zone of inhibitions against different antibiotics

| Antibiotic name | 0-10mm (%) | 11-20mm | 21-30mm | 31-40mm |
|---|---|---|---|---|
| Oxacillin (OX) | 123 (100.00) | 0 (0.00) | 0 (0.00) | 0 (0.00) |
| Ampicillin (AMP) | 90 (73.17) | 33 (26.83) | 0 (0.00) | 0 (0.00) |
| Trimethoprim (TMP) | 23 (18.70) | 0 (0.00) | 40 (32.52) | 60 (48.78) |
| Amikacin (AK) | 40 (32.52) | 66 (53.66) | 17 (13.82) | 0 (0.00) |
| Oxytetracycline (T) | 23 (18.70) | 14 (11.38) | 43 (34.96) | 43 (34.96) |
| Gentamicin (CN) | 53 (43.09) | 50 (40.65) | 20 (16.26) | 0 (0.00) |
| Ciprofloxacin (CIP) | 7 (5.69) | 37 (30.08 | 62 (50.41) | 17 (13.82) |
| Cefoxitin (FOX) | 73 (59.35) | 47 (38.21) | 3 (2.44) | 0 (0.00) |
| Vancomycin (VA) | 70 (56.91) | 46 (37.40) | 7 (5.69) | 0 (0.00) |
| Cefotaxime (CTX) | 50 (40.65) | 37 (30.08) | 29 (23.58) | 7 (5.69) |
| Chloramphenicol (C) | 17 (13.52) | 20 (16.21) | 50 (40.54) | 37 (29.72) |
| Streptomicin (S) | 52 (42.28) | 47 (38.21) | 17 (13.82) | 7 (5.69) |
| Enoxacin (EN) | 39 (31.71) | 50 (40.65) | 27 (21.95) | 7 (5.69) |
| Cefixime (CFM) | 76 (62.15) | 43 (35.13) | 3 (2.71) | 0 (0.00) |

The broth microdilution trial showed 100% inhibition of growth at different concentrations (1000, 500, 250 and 125 µg/mL) of all antibiotics against *S. aureus* (Table III). Gentamicin and chloramphenicol (at concentration of 1.953 µg/mL) inhibited 90% and 30% of *S. aureus*, respectively. The former was most effective among all antibiotics as it inhibited 40% of isolates at concentration 0.976 µg/mL. The least effective antibiotic in this trial was cefotaxime as it inhibited *S. aureus* at > 31.25 µg/mL concentration following which was ampicillin that exhibited 15.625µg/mL as least concentration inhibiting any percentage of *S. aureus*. Reduction in minimum inhibitory concentration (MIC) of cefotaxime in combination with chloramphenicol, gentamicin, and ampicillin was found to be 60, 70.46 and 44.10%, respectively (Figure 2). The MICs of ampicillin in combination with chloramphenicol, gentamicin and cefotaxime was reduced to 57.49, 99.34 and 39.99%, respectively. Chloramphenicol experienced 43.14, 38.26 and 35.18% reduction in MICs when combined with cefotaxime, ampicillin and gentamicin, respectively. The study also noted 64.96, 87.18, and 79.49% of reduction in MICs of gentamicin in combination with chloramphenicol, ampicillin, and cefotaxime, respectively.

**TABLE III -** Percentage of MDR *S. aureus* inhibited at various concentrations of selected antibiotics

| Antibiotic used | MIC (µg/mL) | Percentage isolates whose growth was inhibited at various concentrations (µg/mL) of antibiotics | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1000 | 500 | 250 | 125 | 62.5 | 31.25 | 15.625 | 7.8125 | 3.906 | 1.953 | 0.976 | 0.488 | 0.244 |
| Chloramphenicol (Amphenicols) | 4.30±1.24 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 90 | 80 | 30 | 0 | 0 | 0 |
| Gentamicin (Aminoglycosides) | 2.34±0.82 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 80 | 90 | 40 | 0 | 0 |
| Ampicillin (Penicillin) | 28.91±7.41 | 100 | 100 | 100 | 100 | 100 | 80 | 30 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cefotaxime (Cephalosporins) | 59.38±9.88 | 100 | 100 | 100 | 100 | 90 | 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

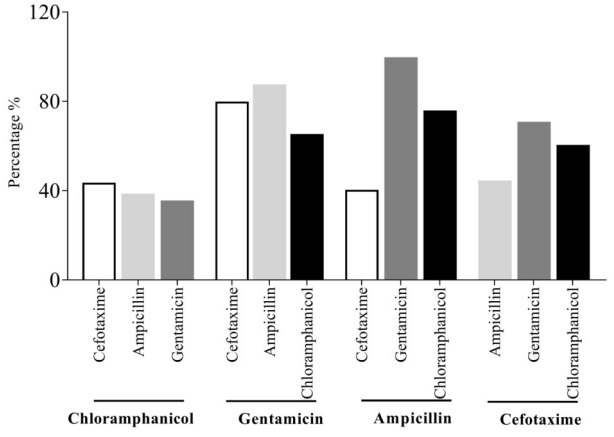


**FIGURE 2 -** Percentage reduction of minimum inhibitory concentration of different drugs in combination with each of Chloramphenicol, Gentamicin, Ampicillin, and Cefotaxime.

However, we could not find antagonistic interaction at any combination of all four classes of antibiotics against MDR *S. aureus* (Table IV). The increasing synergy was noticed in chloramphenicol & ampicillin; cefotaxime & gentamicin; and cefotaxime & gentamicin. Ampicillin (Penicillin) and Cefotaxime (Cephalosporin) combination presented 1.31±0.77 FICI against MDR *S. aureus* which was followed by chloramphenicol with ampicillin; chloramphenicol with cefotaxime; chloramphenicol with gentamicin; cefotaxime with gentamicin; and ampicillin with gentamicin expressing 0.97±0.51, 0.90±0.22, 0.73±0.31, 0.50±0.14, and 0.38±0.11 FICIs, respectively.

**TABLE IV -** Average Fractional Inhibitory Indices of different drug combinations against MDR *S. aureus* from camel milk

| Combination | Drug Class | Drug name | MIC alone | MIC in combination | FIC | FICI (FIC+FIC) |
|---|---|---|---|---|---|---|
| 1 | Amphenicols | Chloramphenicol | 4.30±1.24 | 2.04±0.34 | 0.50±0.12 | 0.90±0.22 |
| | Cephalosporin | Cefotaxime | 59.38±9.88 | 23.75±7.67 | 0.40±0.11 | |
| 2 | Amphenicols | Chloramphenicol | 4.30±1.24 | 1.83±0.52 | 0.44±0.14 | 0.97±0.51 |
| | Penicillin | Ampicillin | 28.91±7.41 | 12.29±3.49 | 0.53±0.48 | |
| 3 | Amphenicols | Chloramphenicol | 4.30±1.24 | 1.40±0.52 | 0.34±0.15 | 0.73±0.31 |
| | Aminoglycoside | Gentamicin | 2.34±0.82 | 0.82±0.30 | 0.39±0.19 | |

**TABLE IV -** Average Fractional Inhibitory Indices of different drug combinations against MDR *S. aureus* from camel milk

| Combination | Drug Class | Drug name | MIC alone | MIC in combination | FIC | FICI (FIC+FIC) |
|---|---|---|---|---|---|---|
| 4 | Penicillin | Ampicillin | 28.91±7.41 | 0.19±0.07 | 0.08±0.03 | 0.38±0.11 |
| | Aminoglycoside | Gentamicin | 2.34±0.82 | 0.30±0.11 | 8.83±2.98 | |
| 5 | Penicillin | Ampicillin | 28.91±7.41 | 17.35±7.80 | 0.69±0.48 | 1.31±0.77 |
| | Cephalosporin | Cefotaxime | 59.38±9.88 | 32.66±13.28 | 0.62±0.47 | |
| 6 | Cephalosporin | Cefotaxime | 59.38±9.88 | 17.54±6.01 | 0.30±0.11 | 0.50±0.14 |
| | Aminoglycoside | Gentamicin | 2.34±0.82 | 0.48±0.27 | 0.20±0.08 | |

(MIC in combination/MIC alone)

Interestingly, gentamicin and ampicillin with highest effective combination in that 80% of tested isolates fall in synergistic while 20% in partial synergistic category (Table V). Partial synergistic effects were found in case of chloramphenicol in combination with cefotaxime, ampicillin and gentamicin presenting 40, 70 and 30%, while cefotaxime in combination with gentamicin and ampicillin gave rise to 70 and 30%, respectively. Only chloramphenicol in combination with gentamicin proved to be having 10% of isolates falling in additive category. The indifferent category of drug combination was found 30, 30, 30, and 70% in case of chloramphenicol with gentamicin, chloramphenicol & ampicillin, gentamicin & cefotaxime, and ampicillin in combination with cefotaxime, respectively.

**TABLE V -** Percentages of synergy combinations against MDR *S. aureus* based on Fractional Inhibitory Concentration Indices

| Antibiotic combinations | Synergistic | Partial synergistic | Additive | Indifferent | Antagonistic |
|---|---|---|---|---|---|
| Chloramphenicol + Gentamicin | 30 | 30 | 10 | 30 | - |
| Chloramphenicol + Cefotaxime | 60 | 40 | - | - | - |
| Chloramphenicol + Ampicillin | - | 70 | - | 30 | - |
| Gentamicin + Ampicillin | 80 | 20 | - | - | - |
| Gentamicin + Cefotaxime | - | 70 | - | 30 | - |
| Ampicillin + Cefotaxime | - | 30 | - | 70 | - |

Synergistic effect was recorded as synergism with FICI indices <0.5; partial synergy with FICI 0.5 to <1.0; additive when FICI = 1.0; indifferent when FICI >1.00 to <4.0; and antagonistic when FICI > 4.0

## DISCUSSION

*Staphylococcus aureus* has gained considerable attention due to an increase of infections in recent years throughout the world (Chessa, Ganau, Mazzarello, 2015). Although, remarkable work has been done against *S. aureus* but already utilized antibiotics become compromised with the passage of time because of it's ability to develop MDR (Worthington, Melander, 2013). Therefore, in this study we evaluated wide range of

antibiotics against MDR, and synergy testing of selected antibiotics (amphenicols, cephalosporin, penicillin, and aminoglycoside) against MDR *S. aureus*. The results indicated poor efficacy of penicillin and cephalosporin was in line with findings of Ahmad *et al.,* (2012), that could be due to plasmid based production of beta-lactamase (Rigby, 1986). The emerging resistance against *S. aureus* isolates of camel mastitis in current study was also in agreement with the results of (Aqib *et al.,* 2017a). Increase in MDR *S. aureus* resistance was reported due to higher use of beta lactam antibiotics, and non-judicious use of antibiotics in mastitis cases. The other risk factors for spread of resistance may be unhygienic milking process, tick infestation, lack of teat dips in germicidal solution before and after milking (Aqib *et al.,* 2017b) which helps in spreading contagious *S. aureus* (Radostits *et al.,* 2007). This situation aggravates due to poor farm management (Juhász-Kaszanyitzky *et al.,* 2007), lack of awareness of farm workers, and lack of veterinary professional consultancy in specific and general ailments.

Both recent and previous studies have supported ß-lactam combination as an effective synergistic candidate against *S. aureus* (Drobbin, Phelan, Antonelli 2007, Gutmann *et al.,* 1986, Johari, Kiong 2012, Pasticci *et al.,* 2008, Tascini *et al.,* 2004). A more recent study reported 80% of isolates showing synergism due to cefaroxil (cephalosporin beta lactam) and amoxicillin (penicillin beta-lactam drug) against *S. aureus*. The previous studies also reported *in-vivo* synergy efficacy of cefotaxime in combination with ampicillin (Lapointe *et al.,* 1984). The combination of two *β*-lactam antibiotics may prove synergistic effect if the target site are different. In antibiotic combination, ampicillin act on protein binding site 3, while cefotaxime binds on site 1. In addition to this cefotaxime has potent activity of ß-lactamase inhibiting mechanism (Neu, Fu, 1980). Resistant isolates are reported to consistently produce beta-lactamases which impairs monotherapy efficacy of penicillin and cephalosporin. However, combination therapy of beta-lactam drugs (penicillin or cephalosporin) with aminoglycosides might result in reduction of resistance (Palmer, Kang, 1995). Ampicillin belonging to penicillin group and regarded as broad-spectrum beta-lactam antibiotic inhibits final stage of bacterial cell wall formation thus acts as bactericidal. Those strain that produce beta-lactamases interfere drug activity. Aminoglycosides are potent bactericidal that create fissure in outer membrane of bacterial cell wall. More specifically, the drug bind to 30S ribosomal subunit whereby inhibiting translocation of peptidul-tRNA from A to P site thus resulting mRNA misreading. This results in unavailability of proteins for bacterial growth (Ibezim, Esimone 2006).

The findings of (Perea, Torres, Borobio, 1978) contradicted the fact of antagonism between chloramphenicol (Bacteriostatic) and ampicillin (bactericidal). The antagonistic combination of bactericidal antibiotics with chloramphenicol or ampicillin against MDR *S. aureus* in current study was in line with earlier eighties' studies that reported usefulness of chloramphenicol or ampicillin combinations with other drugs to treat salmonella and shigella infections. Antagonism between bactericidal and bacteriostatic is generally known in studies conducted *in-vitro* as well as *in-vivo*. The antagonism of chloramphenicol with ampicillin might be because ampicillin activates while chloramphenicol inhibits the murein hydrolase which is responsible for bacterial lysis (Neu, Fu 1980, Tomasz, Waks, 1975). The general concept was questioned in recent studies otherwise antagonism was noticed (Perea, Torres, Borobio, 1978).

## CONCLUSION

The higher percentage of MDR. *S. aureus* from camel milk is an alarming precursor of pan-resistance in bacteria. However, combination of antibiotics from penicillin, cephalosporin, amphenicols, and aminoglycoside against MDR *S. aureus* did not show antagonistic interaction. The lease effective antibiotics (Cefotaxim and ampicillin) with most effective antibiotics (chloramphenicol and gentamicin) significantly reduced minimum inhibitory concentrations. Cefotaxime with gentamicin as well as with chloramphenicol showed synergistic interaction against all isolates while 70% of isolates responded synergistic interaction in case the latter two were combined with ampicillin. It was concluded that interaction of antibiotics could be effective to lower further resistance in *S. aureus* than to relying on single antibiotic therapy.

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## CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interests.

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