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# Ascorbic acid enhances ciprofloxacin antibacterial activity *in vitro* against isolates of *Escherichia coli* from subclinical mastitis cases of buffaloes

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**Abstract**
*Escherichia coli* is one of the major bacteria along with *Staphylococcus aureus* and Streptococci that are encountered in mastitic milk samples obtained from subclinical mastitis cases in India. Ciprofloxacin is an antimicrobial fluoroquinolone, which is effective against *Escherichia coli*. As antioxidants like selenium, vitamin, E and Vitamin C have become adjunct therapeutic agents in the cases of mastitis along with antibacterial agents, the present study was aimed to find out the *in vitro* pharmacodynamic interaction of ascorbic acid with ciprofloxacin against *E. coli* isolated from mastitis milk samples of buffaloes. *E. coli* were isolated from the milk from buffaloes of sub clinical mastitis cases after clinical diagnosis. Cultural and biochemical tests were performed to identify the isolated organisms as *E. coli* from milk. Further DNA template isolated from the organism is subjected for polymerized chain reaction (PCR) for the specific primers Eco 2083 and Eco 2745 to confirm the identity of the organism. Minimum inhibitory concentration (MIC) of ciprofloxacin, ascorbic acid alone and combination of both compounds in serial dilution against clinical isolates of *E. coli* was performed using microplate method. Ciprofloxacin alone has shown MIC of 0.03 µg.mL$^{-1}$ against *E. coli* whereas ascorbic acid alone has no antibacterial activity. MIC of ciprofloxacin has been reduced to two fold (0.015µg.mL$^{-1}$) when it was diluted in 8:2 to 4:6 ratio with ascorbic acid (0.1mg/ml) and thus indicating ascorbic acid potentiating the antibacterial activity of ciprofloxacin.

**Keywords:** e. coli, ascorbic acid, ciprofloxacin, MIC

## Introduction
Mastitis is one of the major health problem in livestock which causes productive and economic loss to the dairy farmers. The common bacteria responsible for the production of subclinical mastitis are *Staphylococcus. aureus*, *Streptococus sps* and *E. coli*. Among the different types of subclinical mastitis. Coliform mastitis is the common one, as the organism is ubiquitos.

The commonly used antibiotic in the treatment of coliform mastitis is ciprofloxacin, a fluoro quinolone antibiotic. Even though specific antimicrobial agents are available for the organism, the success rate in the treatment of coliform mastitis is low. The development of resistance is one which may hinder the activity of antimicrobial agent in producing its pharmacological effect.

It is documented that oxidative damage plays a role in the pathophysiology of mastitis which makes the usage of antioxidants in the treatment of mastitis. In addition to the regular antibiotic usage, the antioxidants like vitamin A, Vitamin E, Selenium, Vitamin C are also being used in the treatment of mastitis (Feng Li Yang and Xiao Shan Li, 2015) [5]. Recent development in the treatment of microbial infections includes use of antioxidants along with antibiotics.

Ascorbic acid is commonly available antioxidant which posseses various pharmacological activities. The isolates (*Pseudomonas aureginosa*) P22 andP24 showed MICs of tetracycline (TC) less than the breakpoint of 16 µg mL (NCCLS 2000) [7]. When given in combination with ascorbic acid the MIC of tetracycline was reduced to 25% MIC. (Luciana Cursino; *et al* 2005) [6].

It was also reported intramuscular administration of l-ascorbic acid in adjunct with intramammary infusion of ampicillin and cloxacillin enhanced the rate of recovery from mastitis (Rakesh Ranjan *et al*., 2005) [9].

Studies conducted in the 1970s reported that megadoses of ascorbic acid in combination with antimicrobials inhibited *P. aeruginosa* growth (Rawal *et al*., 1974) [10]. Some of the studies on ascorbic acid reported the synergistic interaction with antibiotics in acute bacterial diseases, and considerably broadened the activity spectrum of the antibiotics (Cathcart, 1991) [4]. More recently, high doses of ascorbic acid incombination with antibiotics were shown to inhibit the growth of *Helicobacter pylori* in vitro as well as in vivo (Zhang *et al*., 1997; Tabak *et al*., 2003) [15, 13]. The β-lactamase activity in *Enterobacter cloacae* decreased when the bacterium was grown in the presence of ascorbic acid (Shoeb *et al*., 1995) [12]. Ascorbic acid shows synergistic interaction with ampicillin against *Staphylococcus aureus* (Akhilandeswari, 2013). Based on the above reported results on ascorbic acid, it is revealed that ascorbic acid has an ability to potentiate the antibacterial activity of antibiotics.

Some of the studies on drug interaction with ascorbic acid reveals that the ascorbic acid reduced the antibacterial activity. Heatley's method revealed that in the presence of ascorbic acid the antibacterial efficacy of substances was reduced from 9.6 to 40.7% and 10.1 to 45.1% in *Staphylococcus aureus* and *Escherichia coli*, respectively. It has been reported that the rate of survival of bacteria in the presence of ofloxacin as well as tetracycline with ascorbic acid produced a statistically significant increase in log10 of CFU/ml in *S. aureus* as well as *E. coli.* (Belicová A *et al*, 2000). This has prompted us to study the effect of ascorbic acid on *E. coli* in the presence of Ciprofloxacin with an aim to determine the pharmacodynamic interaction.

In this study we determined the ascorbic acid effect on antibacterial activity of Ciprofloxacin against *E. coli* isolated from mastitis milk sample.

## Materials and Methods

**Drugs and Chemicals:** Ciprofloxacin (1mg,ml), ascorbic acid (0.1mg/ml), Dimethyl sulphoxide (DMSO), Mueller Hilton broth (MHB), Mueller Hilton Agar(MHA), Tryptic Soya broth(TSB)

**Isolation of the organism:** The milk samples were collected from buffaloes with subclinical mastitis. Each sample was enriched in tryptic soya broth and incubated at $37^0$ C for 24 hours. Each inoculum was streaked on Mac Conkey Agar, a differential media and pink colored colonies appeared after incubation at $37^0$ C for 24 hours. A single colony was then picked and streaked on Nutrient agar slant. The cultural characteristics of isolates were confirmed by streaking the pure culture on Eosin Methylene Blue Agar (EMB). The EMB agar showed greenish metallic sheen with black centered colonies.

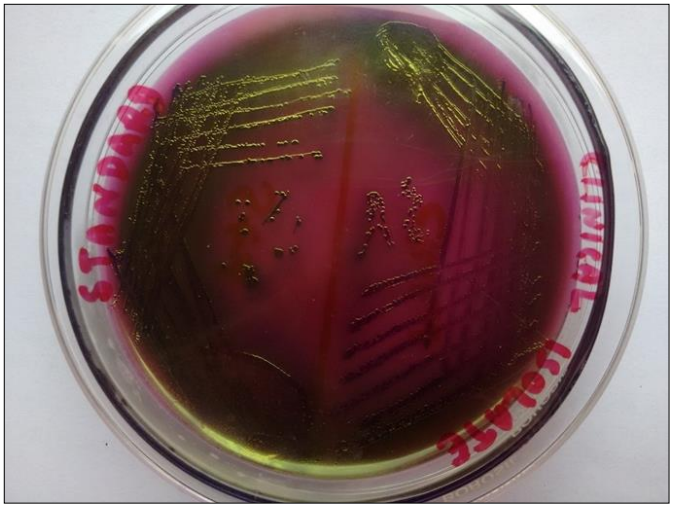

**Fig 1**

**Biochemical tests:** Biochemcal tests IMViC showed ++**--** for the bacteria which is an indicative of *E. coli*

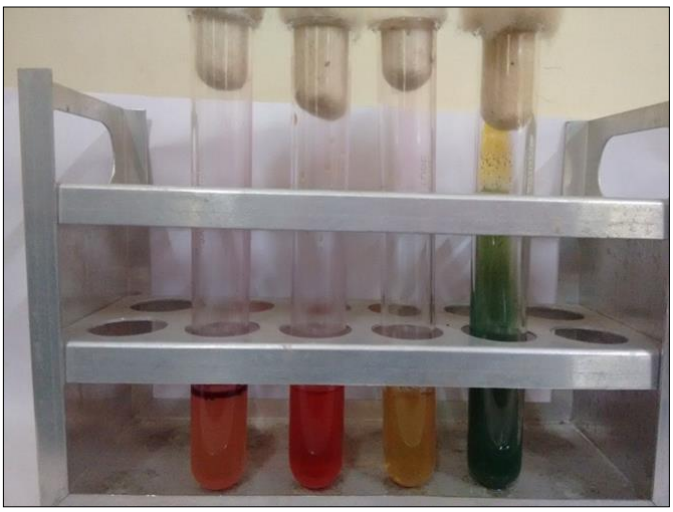

**Fig 2**

**Identification of isolated bacteria by PCR**

**Isolation of bacterial DNA:** The 18hr culture was inoculated in TSB and incubated at 37 °C for 18 hrs. After incubation 2ml of the bacterial culture was centrifuged at 5000rpm for 10 min. The pellet of bacterial cell mass was collected. The DNA template from bacterial cell mass was isolated by high salt method of DNA extraction (Aravindakshan *et al*; 1977) [2] with suitable modifications

The DNA template isolated was confirmed with primers Eco 2083 GCT TGA CAC TCA ACA TTG AG AND Eco 2745 GCA CTT ATC TCT TCC GCA TT (Riffon *et al*, 2001) [11].

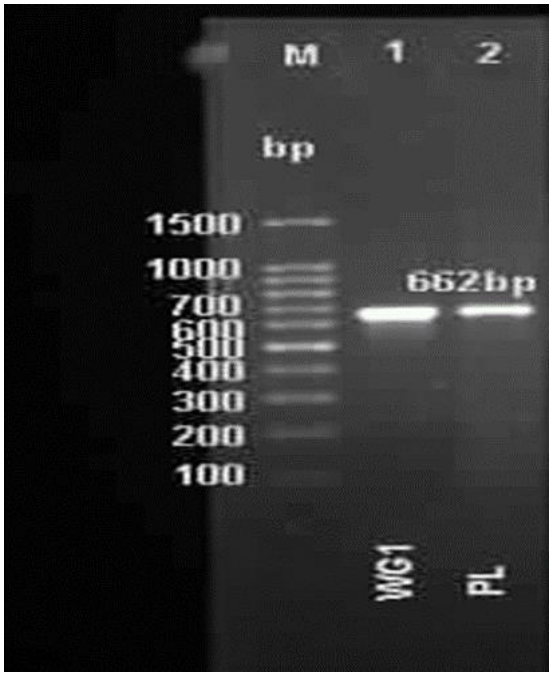


**Fig 3**

**Evaluation of antibacterial activity**

**Microtitre plate test:** A sterile 96 well flat bottomed plastic tissue culture plate with a lid was taken and filled 100 µl of MHB to all wells. 100 µl of the ciprofloxacin was added to the first well and serially diluted. The ciprofloxacin and ascorbic acid in different ratios such as 80:20,60:40,40:60 were serially diluted in MHB which were compared with the ciprofloxacin control i.e A & B. In 12$^{th}$ row first four wells were kept as broth controls and next four wells were kept as culture controls. The 18hr bacterial culture was taken and adjusted to 0.5 Mc Farl and standard.10µl of the bacterial culture was added uniformly to all wells and incubated at 37$^{0}$ C for 18- 24 hrs. The absorbance was taken at 660nm and the bacterial growth was confirmed by development of blue colour with the addition of Nitro Blue Tetrazolium.

**Results**

Ciprofloxacin at the dose rate of 10µg/ml showed the MIC 0.03 µg/ml. MIC values of Ciprofloxacin with as ascorbic acid in the ratios of 80:20, 60:40 and 40:60 were 0.015µg/ml,0.01 µg/ml and 0.015 µg/ml respectively.

**Table 1**

| ***E. coli*** | **Ciprofloxacin MIC µg/ml** | **Ciprofloxacin + ascorbic acid 80 µg 60 µg 40 µg** | | |
|---|---|---|---|---|
| *E. coli*clinical isolate | 0.03 | 0.015 | 0.01 | 0.015 |

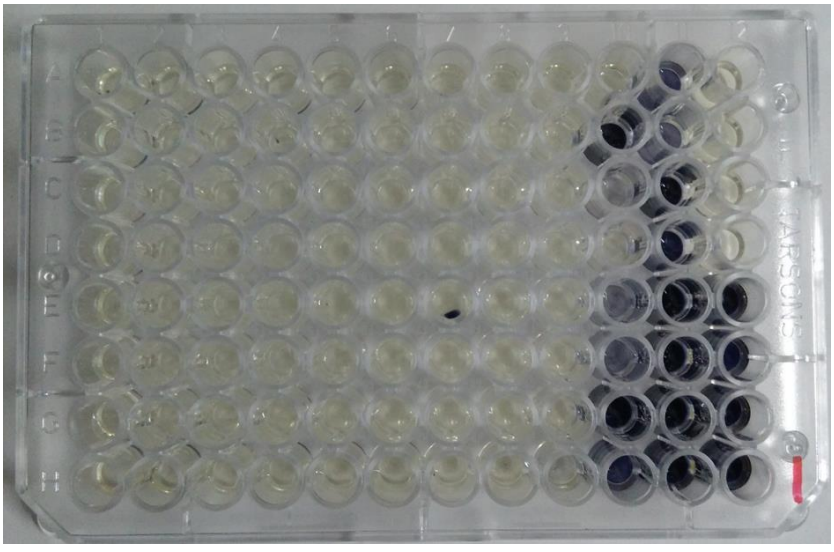

**Fig 4**

Ciprofloxacin has shown 0.03 µg/ml as MIC against clinical isolates of *E. coli* whereas MIC were lowered to 0.015 and 0.01 µg/mlwhen it was combined with ascorbic acid in the ratios of 80: 20, 60: 40 and 40: 60 concentrations

## Discussion

Ciprofloxacillin, one of the widely used quinolone group of antibiotics prescribed for infections due to *E. coli*. It produces its antibacterial activity by inhibiting the DNA synthesis and cell division (Vila, 2005. Increased rate of application of ciprofloxacin has resulted in its resistance to *E. coli* and resistance to one generation of fluoroquinolone may also confer resistance to other quinolones.

Recent trend of using combination of antibiotics with natural compounds may have the possibility of overcoming the resistance developed by the bacteria to antibiotics. In this study we used the combination of ascorbic acid with ciprofloxacin to study the drug interaction.

Ascorbic acid or Vitamin C have several pharmacological effects such as anti-oxidant, anti-bacterial, anti-viral, food preservative etc. In addition to its antioxidant with nature, it also exhibits a pro-oxidant effect which generates reactive free radicals and induces cytotoxicity. (Putchala MC *et al*, 2013) [8]. In the present study the MIC values of ciprofloxacin and ascorbic acid were measured alone and in combination at different levels. The MIC of ciprofloxacin alone is 0.015 µg/ml. The combination of ciprofloxacin with ascorbic acid in 80: 20 showed MIC of 0.015µg/ml. The combination of ciprofloxacin with ascorbic acid in 40:60 showed MIC of 0.015µg/ml the combination of ascorbic acid along with ciprofloxacin maintained the same levels of MIC even after 2 fold dilution of the ciprofloxacin. The ascorbic acid as such doesn't show any antibacterial activity against *E. coli*. The antibacterial activity of Ciprofloxacin is maintained even at low concentration in the presence of ascorbic acid. Based on the above results it is concluded that the sensitivity of ciprofloxacin against *E. coli* has enhanced in the presence of ascorbic acid which may be due to its prooxidant activity or by altering the membrane permeability. The ascorbic acid may increase the diffusion of the drug in to the bacteria ther by increasing the intacellular concentration. So that the ciprofloxacin antibacterial activity is maintained even at reduced dosages.

## Conclusion

The results of the present study indicates the positive interaction between ascorbic acid and ciprofloxacin. After thorough investigation on the exact mechanism of action of ascorbic acid on *E. coli* along with ciprofloxacin it can be used for therapeutic application. As the combination will reduce the quantum of ciprofloxacin has to be administered as per the dosage regimen and it may reduce the side effects as well as development of resistance.

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