Full Length Article

IN-VITRO ANTIBACTERIAL EFFECT OF COMBINING VITAMIN C WITH ENROFLOXACIN AGAINST QUINOLONE RESISTANT *Escherichia coli* (EXPEC) ISOLATED FROM BROILER CHICKEN

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ABSTRACT

Avian pathogenic E. coli (APEC) is the causative agent of colibacillosis in broiler chicken and various antibacterials used to control the infection may lead to the development of multidrug-resistant (MDR) and pan drug-resistant bacteria. Further, antibacterial resistant bacteria also emerge as a result of inappropriate use of antibacterials in chicken, reducing the efficacy of antibacterials used for medical purposes. Keeping this in view, this study was conducted to evaluate the in vitro antibacterial property of vitamin C against resistant bacteria when used along with enrofloxacin. A total of 60 liver swabs collected from the field were used in this study. E. coli were isolated using selective media and were genotypically confirmed by amplification of Adk *gene. The resistant genes (*qnrA*,* qnrB *and* qnrS*) were detected through PCR. E. coli isolates were subjected to antimicrobial screening against enrofloxacin and vitamin C (L- ascorbic acid) both by qualitative method and quantitative method. Kirby-Bauer disc diffusion revealed enrofloxacin resistance in 83.33 percent of samples. The MIC of enrofloxacin against resistant isolates was 90.25 µg/ml, but when vitamin C was administered, the MIC was non-significantly lowered to 86.16 µg/ml in the macro broth dilution method. It was also found that vitamin C alone exhibited antibacterial activity at a concentration of ≥ 5 mg/ml. Hence, it is likely that vitamin C may improve antibacterial effects of enrofloxacin in poultry.*

Keywords: Antimicrobial resistance, Ascorbic acid, Broiler Chickens, Colibacillosis, Enrofloxacin

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INTRODUCTION

Colibacillosis caused by avian pathogenic *E. coli* (APEC) is one of the

leading causes of economic loss, accounting for 20% mortality and a 20% drop in bird growth rate (Dho-Moulin and Fairbrother, 1999). Though *E. coli* is a commensal in both animal and human intestinal tracts, some pathogenic virulent strains can cause disease, making them a potential source for infections in humans such as urinary tract infection, which can lead to cystitis, pyelonephritis, uro-sepsis, meningitis, septicemia, enteritis, and other complications (Kaper *et al.,* 2004). Antibiotics are routinely used in chickens to treat a variety of bacterial infections, as well as to prevent infection and enhance growth. As a result of the antibiotic selection pressure, resistance is developed in both commensal and pathogenic *E. coli*. Emergence of multidrug resistant (MDR)) organisms has become major health threat to both humans and animals. The prevalence of MDR in avian pathogenic *E. coli* (APEC) was found to be 81% and among them 45% were capable of forming biofilm (Goudarztalejerdi *et al*., 2021).

Fluroquinolones are the commonly used antibacterial agents against *E. coli* infection in humans as well as in poultry and this has led to development of resistance (Zhao *et al*., 2005). Lack of stewardship in antimicrobial use in poultry is making, antimicrobial resistance (AMR) a potential health threat. Various approaches followed to overcome the AMR include vaccination against diseases, immunomodulation, replacing antibiotics with pro and prebiotics in feeds, combination therapy and use of engineered bacteriophages and various natural compounds as antibacterial resistance breakers (Rahal and Kumar, 2021).

Vitamin C is a nutritional supplement with antioxidant property and is used as an adjuvant in cancer therapy. Apart from this it is also found to have antibacterial property in *in-vitro* studies (Verghese *et al.,* 2017a). Present study was carried out to evaluate the effect of vitamin C (L- ascorbic acid) as antibiotic resistance breaker along with enrofloxacin, a veterinary specific fluoroquinolone.

MATERIALS AND METHODS

Study area and sample collection: Swabs were taken from dead broiler birds aseptically from various integrated poultry farms in Tamil Nadu and transported to laboratory under refrigerated condition

Isolation of bacteria: Collected liver swabs were enriched with nutrient broth and incubated at 37˚ C overnight. From that a loopful of broth was streaked on MacConkey agar and incubated at 37˚C for 18 hours. The dry, pink donut shaped presumptive *E.coli* colonies from MacConkey were sub cultured on Eosin Methylene Blue (EMB) agar and incubated overnight at 37˚ C.

Identification of bacteria: *E.coli* identification was done based on colony characteristics on selective media MacConkey and EMB agar, gram staining and biochemical tests such as methyl red, VogesProskauer, Indole production and citrate utilization tests

Antimicrobial sensitivity screening: Isolated *E. coli* samples were screened for antimicrobial susceptibility against enrofloxacin, L- ascorbic acid and combination of both by Kirby-Bauer disc diffusion method. Enrofloxacin 15 mcg from HiMEDIA™ and Vitamin C

(L- ascorbic acid) from SRL Chem was used in this study. Vitamin C stock solution was prepared by diluting 10 mg of L- ascorbic acid in 1 ml of sterile distilled water. Sterile discs were impregnated with 15 µl of vitamin C stock solution and air dried for 30 minutes. For screening of antimicrobial activity of combination of vitamin with antibiotic, enrofloxacin discs were impregnated with 8 and 15 µl of vitamin C stock solution and used after 30 minutes of air drying. In quantitative screening, broth macrodilution method was followed as per CLSI M07, 2018 guidelines for determining MIC of enrofloxacin and its combination with ascorbic acid against extra intestinal pathogenic *E. coli.* Mueller Hinton broth (HiMEDIA™) was used for determining MIC. Stock solution of enrofloxacin (Sigma Aldrich) was prepared by dissolving 10 mg of enrofloxacin powder with 400 µl of 0.1 N NaOH and volume was made into 1 ml using sterile distilled water. Equal volume of enrofloxacin (10 mg/ml) and vitamin C (10 mg/ml) were added to first tube and then serial two- fold dilution was carried out.

DNA extraction: A single bacterial colony from overnight grown bacterial culture on nutrient agar was picked up using sterilized metal loop and suspended in 150 µl of nuclease free water (NFW). The suspension was boiled at 100˚C in water bath for 10 minutes and then chilled immediately at - 20˚C for 5 minutes. Then cooled suspension was centrifuged at 10000 rpm for 2 minutes. The supernatant containing DNA template was transferred into a sterile 1 ml Eppendorf tube was used for PCR reactions.

Polymerase Chain Reaction: 20 µl of reaction mixture for resistance genes was prepared by adding 10 µl of PCR red dye master mix, 1 µl forward primer and 1 µl reverse primer of respective resistant genes, 2 µl DNA template and 6 µl of NFW. The primer sequences for identification of *E. coli* and their resistant genes are depicted in table 1. The cyclical conditions of polymerase chain reaction for *Adk*, *qnrA, qnrB* and *qnrS* genes are given in table 2 and 3.

Statistical analysis: MICs of enrofloxacin alone and its combination with ascorbic acid was statistically analyzed using Mann Whitney test (GraphPad Prism6 software).

RESULTS AND DISCUSSION

The phenotypic (Fig.1) and biochemical analysis showed 80 per cent and 60 per cent prevalence respectively for *E. coli.* The genotypic prevalence was found to be 60 per cent (Fig. 2). The results were found to be similar to earlier studies reported by Ramasamy *et al.* (2021). When these isolates were tested for antimicrobial resistance to enrofloxacin, 83.33 percent were found to be resistant, while 5.56 percent showed intermediate susceptibility (Fig. 3), which is similar to the findings of Wang *et al.* (2010). Widespread use of enrofloxacin to treat illnesses including colibacillosis and chronic respiratory disease (Grakh *et al*. 2020) could possibly be the reason for this.

The genotypic validation of the quinolone resistance in isolated *E.coli* was done through polymerase chain reaction. The resistance genes in *E.coli* of poultry origin were 34.3 per cent of *qnrS and* 15.62 per cent of *qnrB* for enrofloxacin (Fig. 4 & 5) and, there was no expression of *qnrA*

Table 1. Nucleotide sequences for primers used in this study

Genes	Primer sequence (5' to 3')	Size (bp)	Reference
Adk	F: ATTCTGCTTGGCGCTCCGGG R: CCGTCAACTTTCGCGTATTT	590	Wirth *et al*., 2006
qnrA	F: TCAGCAAGAGGATTTCTCA R: GGCAGCACTATTACTCCCA	627	Wang *et al*., 2004
qnrB	F: GATCGTGAAAGCCAGAAAGG R: ACGATGCCTGGTAGTTGTCC	469	Gay *et al*., 2006
qnrS	F: ACGACATTCGTCAACTGCAA R: TAAATTGGCACCCTGTAGGC	417	Gay *et al*., 2006

Table 2. PCR cyclical conditions for *Adk* gene

Steps	Temperature	No. of cycles
Initial denaturation	95 ˚ C for 2 minutes	1
Denaturation	95˚ C for 1 minutes	30
Annealing	54 ˚ C for 1 minute	
Extension	72 ˚ C for 1 minute	
Final extension	72 ˚ C for 5 minutes	1

Table 3. PCR cyclical conditions for resistant genes

Genes Targeted	Initial Denaturation	Denaturation	Annealing	Extension	Final extension
qnr (A)	94˚C for 5 minutes	94˚ for 45 seconds	48˚C for 45 seconds	72˚C for 60 seconds	72˚C for 5 minutes
		Repeated for 32 cycles			
qnr (B)	94˚C for 5 minutes	94˚C for 45 seconds	55˚C for 45 seconds	72˚C for 1 minute	72˚C for 5 minutes
		Repeated for 32 cycles			
qnr (S)	94˚C for 5 minutes	94˚C for 45 seconds	53˚C for 45 seconds	72˚C for 1 minute	72˚C for 5 minutes
		Repeated for 32 cycles			

gene. The resistances to fluoroquinolones developed due to spontaneous mutation in QRDR (Quinolone Resistance Determining Region) in chromosome such as *gyrA, gyrB, parC* and *parE* would alter the binding affinity to antibiotics. Other than mutation in QRDR, altered drug permeation and presence of plasmid-mediated quinolone resistance (PMQR) determinants such as *qnrA, qnrS, qnrB, qnrC, qnrD* and *qnrVC* also contribute to quinolone resistance. In Enterobacteriaceae species, coexistence of mutation in QRDR and transferrable PMQR determinants contribute to resistance (Hooper and Jacoby, 2015) (Kotb *et al.,* 2019).

The MIC of enrofloxacin against resistant isolates in this study was 90.25µg/ml with a range of 2.44 to 156.25 µg/ml (Table 4). This is consistent with the findings of Seo and Lee, (2021) that fluoroquinolones resistant *E. coli* had high MIC of ≥ 128 µg/ml against enrofloxacin and they also reported that these highly resistant isolates had mutations in *gyrA* and *parC* gene in chromosome.

Table 4. MIC of enrofloxacin and vitamin C

Group	MIC (µl/ml) (Mean± SE); (n=32)
Enrofloxacin	90.25 ± 18.11
Enrofloxacin + Vitamin C	86.16 ± 16.58
P value	0.923^{NS}

In broth macro dilution method vitamin C exhibit antimicrobial property at concentration ≥ 5 mg/ml against *E. coli*. Vitamin C did not exhibit any antibacterial property in disc diffusion test even in increasing concentrations; and also it did not improve the zone of inhibition of enrofloxacin. When the enrofloxacin was used along with vitamin C against ExPEC isolates the MIC of enrofloxacin was non-significantly decreased to 86.16µg/ml from 90.25 µg/ml. A study by Verghese *et al.* (2017b) for the combined effect of ascorbic acid and ciprofloxacin on uropathogenic *E. coli,* did not reveal any synergistic effect with ciprofloxacin. However, it produced concentration dependent inhibition against *E. coli* when used alone. Further studies are required to confirm the ability of vitamin C to decrease the MIC of enrofloxacin with large sample size.

CONCLUSION

Combining vitamin C with enrofloaxicin non-significantly decreased the MIC of enrofloxacin against *E. coli.* Further as observed in broth macro dilution method, vitamin C alone also exhibited some antibacterial activity at concentration ≥ 5 mg/ml against *E. coli*. Further studies are required to explore the possibility of combining vitamin C with enrofloxacin to overcome the problem of AMR in poultry.

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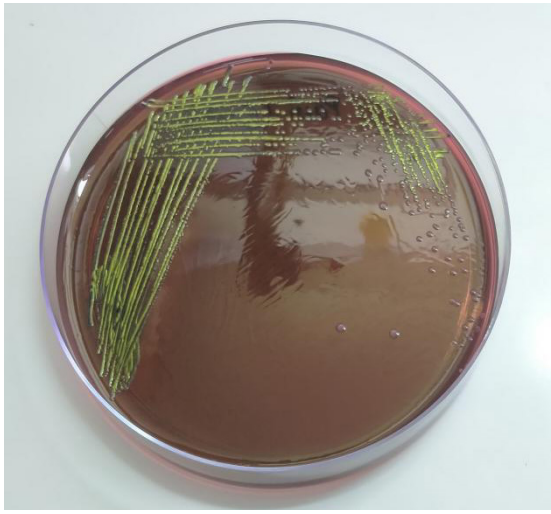

Fig. 1. *E. coli* in EMB agar (green metallic sheen colonies)

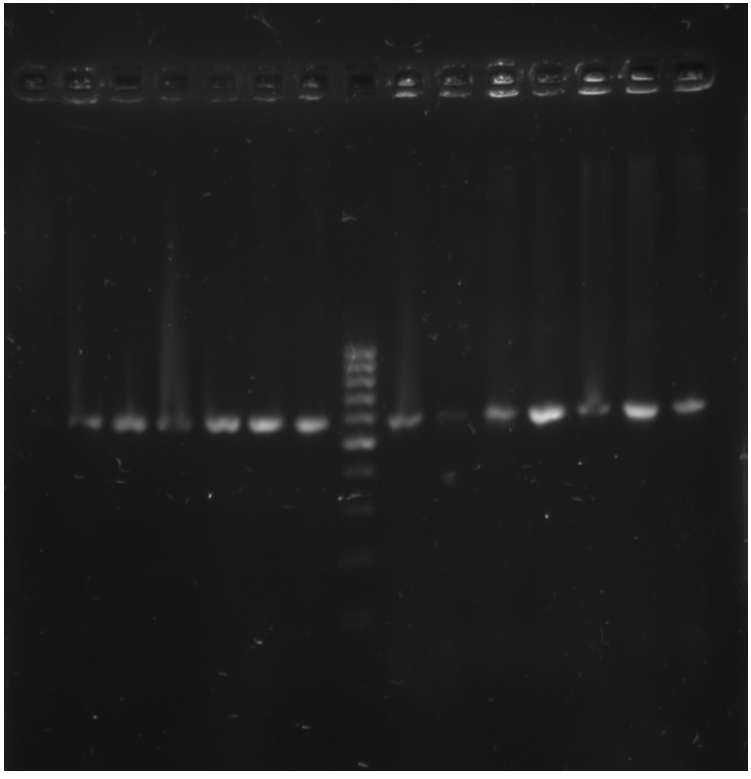

Fig. 2. PCR for *E. coli* (*Adk* gene) confirmation

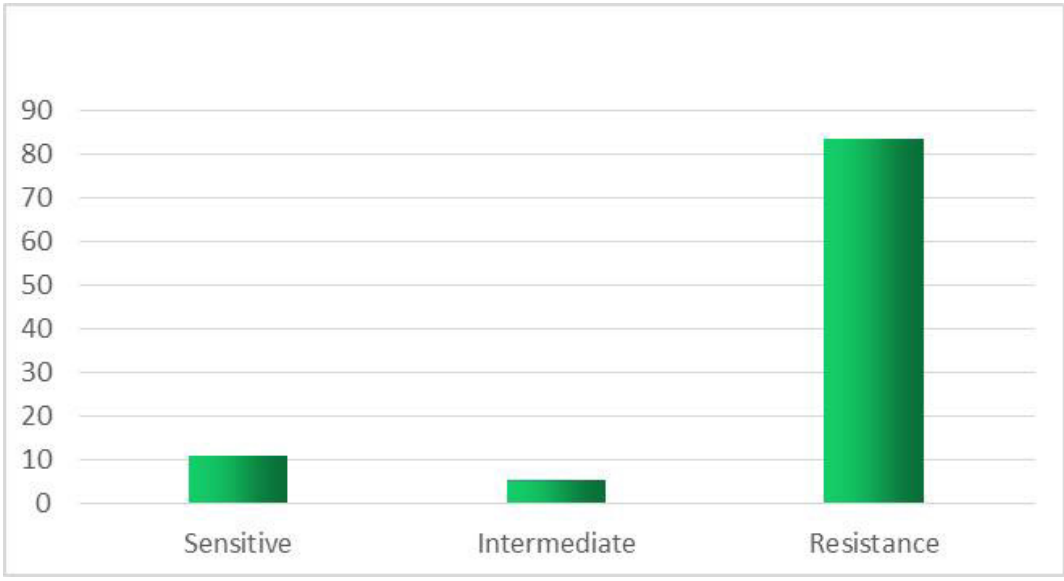

Fig. 3. Antibiotic resistance pattern of *E. coli* against enrofloxacin

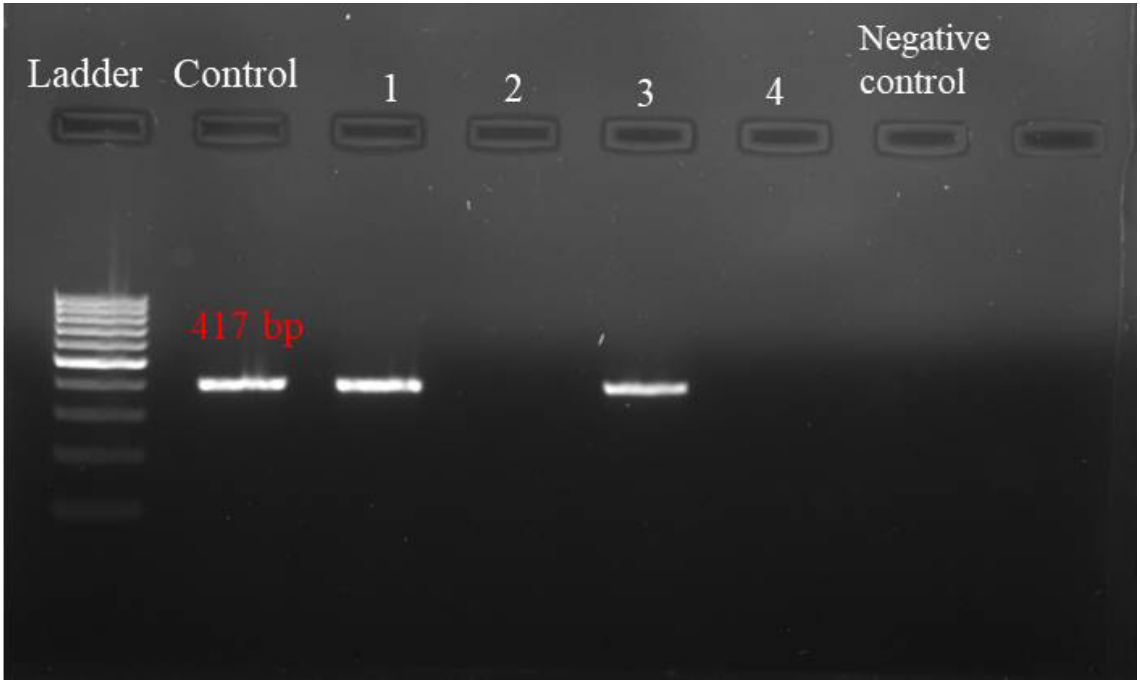

Figure. 4. Agarose gel electrophosed *qnrS* gene PCR

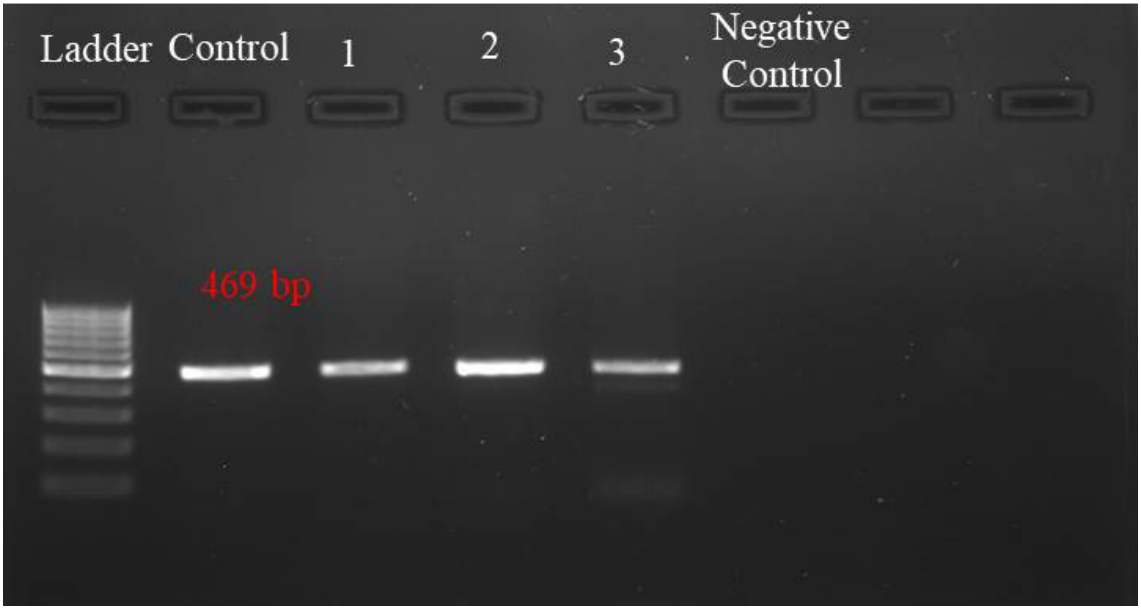

Fig. 5. Agarose gel electrophosed *qnrB* gene PCR

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