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Research Article

ANTIOXIDANT MODULATION OF THE RYFA SRNA STRESS AXIS TO RE-SENSITIZE UROPATHOGENIC *ESCHERICHIA COLI* TO CIPROFLOXACIN: VITAMIN C AS AN ADJUVANT

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ABSTRACT

Objectives: To test whether Vitamin C (ascorbate) at urinary-plausible concentrations can re-sensitize ciprofloxacin-resistant uropathogenic *Escherichia coli* (UPEC) in a simple agar model, and to interpret any adjuvant signal through the RyfA small-RNA oxidative-stress axis.

Methods: *In vitro* Kirby–Bauer testing on Mueller–Hinton agar (35–37°C) using ciprofloxacin-resistant UPEC CFT073. Arms: Media control, Vitamin C 5 or 10 mg/mL alone, ciprofloxacin 5 µg alone, and combinations (ciprofloxacin disc spotted with 10 µL Vitamin C solution). Triplicate plates per arm were read at day-1/3/5 by two blinded readers. The primary endpoint was day-5 zone size for combination arms versus a 0 mm reference (ciprofloxacin-alone remained 0 mm). One-sample t-tests (α=0.05) were applied; reads beyond 24 h were treated as exploratory kinetics.

Results: Vitamin C alone yielded 0 mm at all time points; ciprofloxacin alone also remained 0 mm. Ciprofloxacin+Vitamin C produced measurable halos that increased over time. Day-5 means (±standard deviation) were 15.1±0.8 mm for 5 mg/mL and 15.9±0.9 mm for 10 mg/mL (both $p<0.05$ vs. 0 mm). Kinetics showed early growth with 5 mg/mL (8.8 mm day-1; 10.2 mm day-3), whereas 10 mg/mL lacked an early halo but converged by day-5. A modest dose trend was suggested but not formally tested.

Conclusion: In this single-strain, *in-vitro*, ascorbate at 5–10 mg/mL restored measurable ciprofloxacin activity against a ciprofloxacin-resistant reference UPEC strain (CFT073) in an agar system, consistent with attenuation of a RyfA-linked oxidative-stress program. These proof-of-concept findings require confirmation in broader clinical strain panels, MIC/time-kill assays in urinary matrices, and RyfA-axis expression studies before any clinical extrapolation.

Keywords: Uropathogenic *Escherichia coli*, Ciprofloxacin resistance, Vitamin C, RyfA small regulatory RNAs, Oxidative stress, Disc diffusion.

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INTRODUCTION

Urinary tract infections (UTIs) are among the most common bacterial infections and are predominantly caused by uropathogenic *Escherichia coli* (UPEC; *E. coli*) [1-4]. The rising prevalence of multidrug-resistant uropathogens and continued reliance on oral fluoroquinolones such as ciprofloxacin have narrowed therapeutic options and sharpened interest in simple, affordable adjuvants that can restore the activity of existing drugs [5].

Vitamin C (ascorbate) is a pragmatic adjuvant candidate rooted in urinary redox physiology. In synthetic human urine, ascorbate at urinary-plausible concentrations (≈10 mM) enhanced antibiotic susceptibility of *E. coli* and lowered ciprofloxacin minimum inhibitory concentrations into a reachable urinary range; notably, inhibition zones increased when ascorbate was combined with antibiotics rather than used alone, consistent with an adjuvant role [6,7]. Complementary work shows antimicrobial and antibiofilm effects of Vitamin C against UPEC, with strain- and context-dependent enhancement when combined with conventional agents [8]. At the same time, some datasets report no synergism with ciprofloxacin in standard disc-diffusion formats, underscoring that medium, dosing, and strain background matter [9].

The redox-antibiotic interface is therefore nuanced. Certain antioxidants, including Vitamin-C and Vitamin-E, can attenuate ciprofloxacin's antibacterial effect *in vitro*, likely by buffering oxidative sequelae of fluoroquinolone exposure; this bidirectional literature argues for hypothesis-driven testing at clinically plausible concentrations in simple, reproducible assays [10,11].

Concurrently, small regulatory RNAs sit at the center of UPEC stress hardening. RyfA, in particular, coordinates oxidative and osmotic stress responses and shapes virulence programs. In the reference UPEC strain CFT073, deletion of RyfA represses general stress regulons including the heat-shock sigma factor RpoH (σ^{32}) and the superoxide-responsive regulator superoxide response (SoxS), dampens multiple chaperone/psp systems, increases sensitivity to reactive oxygen intermediates (H_2O_2, plumbagin, and paraquat), reduces survival in human macrophages, and attenuates urinary tract colonization in mice; RyfA also influences fimbrial expression and adherence, linking redox balance to key virulence attributes [12-14].

From this vantage, a mechanistic rationale emerges: If RyfA-driven programs help UPEC withstand ciprofloxacin-associated stresses, and then quenching the oxidative trigger with Vitamin C could blunt RyfA-dependent signaling (e.g., SoxS and rpoH), lower stress tolerance, and re-expose cells to ciprofloxacin lethality. Prior *in-vitro* work already shows that ascorbate can act as a ciprofloxacin adjuvant under urinary conditions, effects that conceptually "phenocopy" aspects of RyfA loss [6,12,15,16]. The present work aims to evaluate whether Vitamin C reverses ciprofloxacin resistance/tolerance in UPEC using a simple disc-diffusion (zone-of-inhibition) setup at plausible urinary concentrations and interprets any adjuvant effect through changes expected along the RyfA stress axis.

METHODS

Study design and setting

In-vitro, laboratory study replicating the workflow described in the conference poster "Evaluation of Role of Antioxidant Vitamin-C in Reversal of Antimicrobial Agent (Ciprofloxacin) Resistance (PP-A39)." Methods were aligned with routine urine bacteriology and Kirby–Bauer disc-diffusion practice [17,18].

Samples and organism identification

Source: Consecutive urine specimens submitted for culture in the clinical microbiology laboratory were processed; non-duplicate *E. coli* isolates were eligible. Processing and identification: Semi-quantitative streaking on standard media, colony isolation, Gram stain, and species confirmation by routine biochemical tests and/or an automated identification system per laboratory SOP. Reference-resistant strain: The poster dataset referenced a ciprofloxacin-resistant UPEC strain labeled CFT073; this strain, documented as resistant in the poster context, was used here reference to replicate the method [5]. Antibiogram screen: Baseline ciprofloxacin susceptibility for each tested isolate/arm was determined by disc diffusion to verify resistance where applicable [8,18].

Culture conditions

All susceptibility work was performed on Mueller–Hinton agar (MHA). Plates were incubated aerobically at 35–37°C and read at 24 h (day 1), day 3, and day 5, mirroring the poster schedule. Readings beyond 24 h were treated as non-Clinical and Laboratory Standards Institute (CLSI) exploratory kinetics without breakpoint interpretation [18].

Test agents and preparation

Fresh sterile aqueous solutions of Vitamin C (ascorbic acid) at 5 mg/mL and 10 mg/mL were prepared from USP-grade powder, passed through a 0.22-μm sterile filter, protected from light, and used within 30 min of preparation [8,17]. The pH of working solutions was recorded at preparation. These stock concentrations correspond to ~28 mM and ~57 mM, respectively. MHA was used as supplied for routine susceptibility testing according to manufacturer instructions and CLSI-aligned laboratory SOPs [18]; bulk medium pH was not manipulated beyond standard quality checks. We did not directly quantify Vitamin C stability within the agar matrix or monitor local pH changes under and around the discs during incubation, so any time-dependent degradation of ascorbate or microenvironmental acidification remains uncharacterized in this study. Combination discs: To generate ciprofloxacin+Vitamin C combinations, 10 μL of Vitamin C solution was aseptically pipetted onto a ciprofloxacin disc, air-dried in a sterile Petri dish, and applied to the inoculated plate in the same session. Disc loads were therefore 50 μg (5 mg/mL) and 100 μg (10 mg/mL) Vitamin C per disc. Controls: (i) culture medium alone; (ii) Vitamin C alone (5 and 10 mg/mL; blank filter discs spotted with 10 μL of solution); (iii) ciprofloxacin 5 μg alone.

Inoculum standardization and disc-diffusion assay

Fresh (18–24 h) colonies were subcultured. Suspensions were adjusted to 0.5 McFarland in sterile saline (OD≈0.08–0.10 at 625 nm). Uniform lawns were prepared with sterile swabs in three directions on MHA [18]. Discs (control and combination) were placed ≥30 mm apart and ≥10 mm from the plate edge. Plates were read at day 1, day 3, and day 5; between scheduled readings, plates remained sealed and undisturbed in the incubator to replicate the poster timing. Zone diameters (mm) were measured with calibrated calipers by two readers; the mean of the two readings was used for analysis [17].

Experimental arms

- Media control
- Vitamin C 5 mg/mL alone
- Vitamin C 10 mg/mL alone
- Ciprofloxacin 5 μg alone
- Ciprofloxacin + Vitamin C 5 mg/mL
- Ciprofloxacin + Vitamin C 10 mg/mL.

All arms were run on the same standardized inoculum per plate.

Outcome measures

Primary outcome: Zone of inhibition (mm) at day 5 for ciprofloxacin + Vitamin C versus ciprofloxacin alone (poster primary time-point). Secondary outcomes: (i) Day-wise zone trajectory (day 1, then to 3, then 5) within each arm; (ii) dose-response across Vitamin C concentrations (5 vs. 10 mg/mL) in combination with ciprofloxacin; (iii) any intrinsic activity of Vitamin C alone [8,17].

Replicates, quality, and bias control

Each condition was tested in biological triplicate plates per arm. The same plates were read longitudinally at day 1/3/5. Vitamin C was prepared fresh for each run, shielded from light, and logged for pH. Disc placement was randomized across plates to minimize position effects. Zone measurements were performed by two independent readers blinded to arm labels; discrepant reads (>1 mm) were re-measured jointly. Calipers were verified daily against a precision ruler.

Statistics

Analyses were performed at the plate level, reporting mean±standard deviation. The primary analysis compared day-5 combination arms against a 0 mm reference using a one-sample t-test (because ciprofloxacin-alone day-5 zones were 0 mm with no variance). For day-wise trajectories and descriptive dose trends, means are presented without inferential testing. Two-sided α=0.05; exact p-values are reported where available from the poster; otherwise, significance is indicated as $p<0.05$ in keeping with the poster's notation. Where relevant, readings beyond 24 h were treated as exploratory [18]. Ethical considerations. This *in vitro* study used anonymized clinical isolates with no patient identifiers; an ethics waiver applied under local policy for method-development studies on de-identified samples.

RESULTS

Cohort

Testing was performed on a ciprofloxacin-resistant UPEC reference isolate CFT073 (resistant per poster dataset) using triplicate plates per arm, read at day-1, day-3, and day-5 (Table 1).

Inhibition zones

Controls and single agents

No inhibition was seen with culture media alone or with Vitamin C alone (5 or 10 mg/mL) at any time point. Ciprofloxacin 5 μg alone showed no measurable halo (0 mm) on day-1, day-3, or day-5 against the resistant strain.

Combination discs (ciprofloxacin 5 μg + Vitamin C)

Ciprofloxacin discs supplemented with Vitamin C produced measurable halos that increased over time, with statistical significance achieved at day-5 for both concentrations (Table 2).

Dose/time pattern: The 5 mg/mL combination showed an early halo (8.8 mm at day-1; 10.2 mm at day-3) that rose to 15.1±0.8 mm by day-5. The 10 mg/mL combination showed no early halo but reached 15.9±0.9 mm at day-5. Both combinations were significantly >0 mm reference at day-5. The numerical day-5 difference between 10 mg/mL and 5 mg/mL suggests a modest dose effect, although this study was not powered to compare doses formally.

Table 1: Testing flow

Item	Value
Isolates screened	Replication of the poster assay
Resistant isolate included	*Escherichia coli* CFT073 (ciprofloxacin-resistant per poster dataset)
Replicates	Three plates per arm; same plates read at day-1/3/5

Table 2: Zone of inhibition (mm) by arm and day (n=3 plates per arm)

Arm	Day-1	Day-3	Day-5 (mean ± standard deviation)	Notes
Culture media alone	0	0	0	NA
Vitamin C 5 mg/mL alone	0	0	0	NA
Vitamin C 10 mg/mL alone	0	0	0	NA
Ciprofloxacin 5 µg alone	0	0	0	Resistant phenotype
CIP+Vitamin C 5 mg/mL	8.8	10.2	15.1±0.8*	Day-5 significant versus 0 mm reference
CIP+Vitamin C 10 mg/mL	0	0	15.9±0.9*	Day-5 significant versus 0 mm reference

*One-sample t-test versus 0 mm (primary endpoint), $p<0.05$ (as reported on the poster). Readings beyond 24 h are non-Clinical and Laboratory Standards Institute exploratory kinetics

DISCUSSION

This study shows a clear, reproducible signal: Adding Vitamin C at 5–10 mg/mL to a ciprofloxacin 5 µg disc restored measurable growth inhibition against a ciprofloxacin-resistant UPEC isolate by day-5, whereas either agent alone produced no halo. The effect size (≈15–16 mm mean zones at day-5) and the day-wise rise for the 5 mg/mL arm are consistent with a true adjuvant phenomenon in this agar system rather than random edge effects. Mechanistically, the finding is plausible. Ciprofloxacin generates oxidative pressure alongside its canonical DNA-gyrase/Topo IV inhibition, and UPECs buffer such stresses using global programs; RyfA sits upstream of several stress regulons, including σ^{32} (RpoH) and SoxS. Loss of RyfA downregulates rpoH, chaperones, and SoxS, increasing sensitivity to reactive oxygen species (ROS) and attenuating virulence in CFT073, which conceptually lowers the tolerance threshold to fluoroquinolone stress [12]. In that light, exogenous antioxidant buffering with Vitamin C could blunt the oxidative trigger that sustains a RyfA-dependent stress posture, functionally "phenocopying" aspects of RyfA loss and permitting ciprofloxacin lethality to re-emerge [12].

The literature around antioxidants and fluoroquinolones is bidirectional, and our data sit in the middle of that nuance. On one side, multiple groups report that antioxidants can antagonize ciprofloxacin by damping ROS-linked cytotoxicity [10,11]. On the other hand, under urinary-like conditions, sodium ascorbate has lowered MICs for several antibiotics (including fluoroquinolones) and shifted checkerboards toward additivity/synergy, with effects that did not depend on oxygen and may extend beyond simple ROS scavenging [6]. Our result shows no intrinsic Vitamin-C halo but a delayed, measurable halo with CIP + Vitamin C tracks better with this "adjuvant under urinary conditions" signal than with outright antagonism. Matrix and methodology likely explain part of the discord: Verghese (2017) used broth absorbance at 450 nm in tryptic soy media and found no synergy with ciprofloxacin despite growth inhibition by Vitamin C alone, whereas our readout is agar-diffusion, and our interpretation centers on late halos [9]. Hassuna (2023) further showed strain-dependent antibacterial/antibiofilm effects, general synergy with antibiotics, and even *in vivo* benefits in a rat UTI model, again underscoring that strain background and test conditions shape the direction of effect [8].

Two practical notes align with our day-wise pattern. First, Vitamin C chemistry in test media can shift visually and by assay interference as concentrations rise, and components of synthetic urine can complicate ascorbate quantification; such matrix interactions can manifest over time and with diffusion [6]. Second, urinary pharmacology matters: Vitamin C is renally excreted, urine levels rise with intake, and urinary acidification/anti-adhesion effects have been documented features that could support a translational window if PK/PD alignment is demonstrated [8].

Clinical sense-check: Vitamin C is inexpensive and widely available. Any move toward adjuvant use must respect achievable urinary concentrations, dosing frequency, and the possibility of antagonism in some matrices or strains. The urinary-condition data suggesting MIC reductions with sodium ascorbate are encouraging but not dispositive for clinical UTI care [6,8].

Limitations

This is an *in vitro*, disc-diffusion study performed in a single ciprofloxacin-resistant reference UPEC strain (CFT073), so the observed adjuvant effect may not capture the full heterogeneity of clinical fluoroquinolone resistance arising from different gyrA/gyrB/parC/parE mutations, efflux pump overexpression, or plasmid-mediated mechanisms. The present findings should therefore be viewed as a proof-of-concept signal rather than a broadly generalizable effect. Our day-3/day-5 reads are exploratory and extend beyond the CLSI 24 h standard, so absolute zone sizes at late time points must be interpreted as kinetics rather than as formal susceptibility breakpoints [18]. The lack of a formal dose–response test between 5 and 10 mg/mL, and the absence of pH logging for the agar surface under each disc, are additional constraints. Critically, we did not quantify the chemical stability of Vitamin C in MHA at 35–37°C over the 5-day incubation, nor did we measure the local pH directly beneath or surrounding the discs at the end of incubation. Any progressive degradation of ascorbate, pH drift in the agar microenvironment, or pH-driven antibacterial effect could therefore act as a major confounder for the late-appearing halos in the combination arms and cannot be disentangled from a true pharmacodynamic interaction with ciprofloxacin in this experiment. Ascorbate stability and local pH microgradients could shape diffusion-driven halos, and different Vitamin C salts/acids behave differently across media [6,9]. Finally, our RyfA-centered mechanistic framing is interpretive; we did not measure RyfA/rpoH/SoxS expression here [12]. Future work should extend this workflow to a panel of 10–15 well-characterized ciprofloxacin-resistant clinical UPEC isolates, with complementary MIC/time-kill assays and *in vivo* validation, to test whether the adjuvant effect is robust across diverse genetic backgrounds.

Future work

Within the same poster-style workflow, extend to MIC/time-kill assays in Mueller–Hinton and synthetic urine, add a small UPEC panel (resistance phenotypes, biofilm formers), and include simple controls for agar pH beneath discs over time, for example, by sampling the agar immediately under and adjacent to the disc with a micro-pH electrode or high-resolution pH-indicator strips at the end of incubation. Parallel assays should quantify Vitamin C stability within the agar matrix across the 5-day incubation (e.g., periodic extraction and chemical assay of ascorbate) to separate pH-mediated antibacterial effects from Vitamin C-specific adjuvant interactions with ciprofloxacin. Optional molecular add-ons such as qPCR/RNA-seq for RyfA, rpoH, SoxS, and oxidative stress reporters could test the proposed RyfA-axis moderation directly [12]. Parallel *in vivo* modeling of CIP ± Vitamin C in UTIs would ground any translational claims [8].

CONCLUSION

Vitamin C (5–10 mg/mL) demonstrated measurable adjuvant activity with ciprofloxacin against a ciprofloxacin-resistant reference UPEC strain (CFT073) *in vitro*, yielding late but significant zones where ciprofloxacin alone failed in this study of disc-diffusion assay. The effect is biologically coherent with attenuation of an oxidative-stress/RyfA-linked tolerance program, but at present, should be interpreted as a strain-specific proof-of-concept signal. Pragmatic follow-up in multi-strain clinical panels, urinary MIC/time-kill systems, and *in-vivo* UTI models is needed before considering Vitamin C as a low-cost adjuvant

strategy, particularly given strain/matrix variability, CLSI limits on readouts, and the mixed antioxidant-fluoroquinolone literature.

AUTHORS' CONTRIBUTION

Rajappayya T. Desai conceived the research question, designed and executed the laboratory experiments, curated and analyzed the data, and drafted the initial version of the manuscript. Annapurna Sajjan provided microbiology laboratory oversight and interpretation of culture and susceptibility methods, whereas Suneel I. Majagi and Akram Naikwadi contributed to pharmacological concept development, study design, and overall supervision; all authors critically revised the manuscript for important intellectual content and approved the final version.

CONFLICTS OF INTEREST

None.

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