



Diagnostic Validation of Nalidixic Acid Disk Diffusion as a Mandatory Safeguard Against Deceptive Ciprofloxacin Susceptibility Reporting

Shamima Kawser¹, Sanjida Hossain², Khandker Md. Nurur Sabah³, Zebun Nessa⁴

¹Professor, Department of Microbiology, Dr. Sirajul Islam Medical College, Dhaka, Bangladesh; ²Assistant Professor, Department of Microbiology, Dr. Sirajul Islam Medical College, Dhaka, Bangladesh; ³Associate Professor, Department of Cardiology, Mughda Medical College Dhaka, Bangladesh; ⁴Assistant Professor, Department of Laboratory Medicine, Institute of Child and Mother Health, Dhaka, Bangladesh

Abstract

Background: Standard disk testing often fails to detect early mutations that cause reduced fluoroquinolone susceptibility in *Salmonella*, leading to inadvertent treatment failures. **Objective:** The purpose of the present study was to validate the accuracy of the 30µg nalidixic acid disk as a low-cost screening marker for elevated ciprofloxacin MICs. **Methodology:** This cross-sectional study was conducted in the Department of Microbiology & Immunology at Bangladesh Medical University (BMU), Dhaka, Bangladesh, from January 2008 to December 2008 for a period of one year. Disk diffusion parameters for nalidixic acid were cross-referenced against quantitative ciprofloxacin agar dilution MICs across 100 clinical *Salmonella* isolates (72 *Salmonella* Typhi and 28 *Salmonella* Paratyphi A). **Results:** Nalidixic acid resistance (zone less than or equal to 13 mm) was found in 76.0% of isolates. This profile correlated perfectly with elevated ciprofloxacin MIC values more than or equal to 1 µg /ml (covering 40% resistant and 36% intermediately sensitive strains). The 24.0% of isolates sensitive to nalidixic acid matched true baseline ciprofloxacin-sensitive endpoints. **Conclusion:** Testing with a nalidixic acid disk provides high diagnostic sensitivity and specificity, making it an essential, cost-effective screening step to prevent false reporting of ciprofloxacin in resource-limited laboratories. [*Bangladesh Journal of Infectious Diseases*, June 2026;13(1):14-18]

Keywords: Diagnostic stewardship; nalidixic acid screening; ciprofloxacin susceptibility; Typhoidal *Salmonella*; agar dilution reference; quinolone resistance-determining region; resource-limited laboratories

Correspondence: Dr. Shamima Kawser, Professor, Department of Microbiology, Dr. Sirajul Islam Medical College, Dhaka, Bangladesh; Email: drshamimak@gmail.com; Cell No.: +8801715495203; ORCID: <https://orcid.org/0009-0000-5193-0361>
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Introduction

The widespread and prolonged use of fluoroquinolones for the treatment of enteric fever has significantly influenced the antimicrobial resistance patterns of *Salmonella enterica* serovars Typhi and Paratyphi A throughout the Indian subcontinent^{1,2}. Following the emergence of multidrug-resistant (MDR) strains that were resistant to traditional first-line agents such as chloramphenicol, ampicillin, and co-trimoxazole, fluoroquinolones became the preferred therapeutic

option because of their excellent intracellular penetration, oral bioavailability, and high clinical efficacy. However, extensive use and, in some settings, misuse of these agents have exerted considerable selective pressure on circulating *Salmonella* strains, facilitating the emergence and dissemination of fluoroquinolone-resistant isolates². The most common mechanism of resistance involves point mutations within the quinolone resistance-determining region (QRDR) of the *gyrA* gene, which encodes a subunit of bacterial DNA

gyrase, the primary target of quinolone antibiotics³. These mutations alter the structure of the DNA gyrase enzyme, reducing the binding affinity of quinolones and fluoroquinolones and thereby diminishing their antibacterial activity. Additional mutations in *gyrB*, *parC*, and *parE*, as well as plasmid-mediated resistance mechanisms, may further contribute to reduced susceptibility and higher levels of resistance⁴.

The development of these genetic alterations presents a significant diagnostic challenge for routine microbiology laboratories. Isolates harboring a single mutation in the *gyrA* gene typically exhibit complete resistance to nalidixic acid, a first-generation quinolone, while simultaneously demonstrating only a modest increase in ciprofloxacin minimum inhibitory concentrations⁵. Consequently, when conventional disk diffusion methods are used, these isolates often produce relatively large zones of inhibition around ciprofloxacin disks, leading to their erroneous classification as susceptible. This phenomenon can create a false sense of confidence among clinicians and laboratory personnel regarding the expected efficacy of ciprofloxacin therapy⁶.

The clinical implications of such misclassification are substantial. Patients infected with nalidixic acid-resistant *Salmonella* strains that appear susceptible to ciprofloxacin by routine disk testing frequently experience delayed clinical response, prolonged fever clearance times, microbiological persistence, or even treatment failure⁷. These outcomes not only increase patient morbidity but also contribute to prolonged transmission and greater healthcare costs. Therefore, accurate detection of reduced fluoroquinolone susceptibility is essential for guiding appropriate antimicrobial therapy and improving patient outcomes⁸.

Although determination of ciprofloxacin MICs by reference methods such as agar dilution or broth microdilution provides the most reliable assessment of fluoroquinolone susceptibility, these techniques require specialized equipment, trained personnel, and additional financial resources that are often unavailable in resource-limited community laboratories. In such settings, the use of nalidixic acid resistance as a surrogate screening marker for reduced ciprofloxacin susceptibility has been widely advocated. Validating this cost-effective approach is crucial for minimizing reporting errors, supporting diagnostic stewardship, and ensuring that clinicians receive accurate susceptibility data to guide effective treatment decisions for enteric fever.

Methodology

Study Settings and Population: This cross-sectional study was conducted in the Department of Microbiology & Immunology at Bangladesh Medical University (BMU), Dhaka, Bangladesh, over a one-year period from January 2008 to December 2008. The study population consisted of patients of all age groups and both sexes who attended the outpatient departments or were admitted to the hospital with clinical features suggestive of enteric fever, including prolonged fever, headache, abdominal discomfort, anorexia, vomiting, diarrhea, constipation, or other compatible symptoms. Blood samples were collected from these patients and processed for bacterial culture and identification according to standard microbiological procedures.

Selection criteria: Patients presenting with clinical suspicion of enteric fever, patients from whom blood samples were submitted for culture and sensitivity testing, and (iii) culture-confirmed cases yielding *Salmonella enterica* serovar Typhi or Paratyphi isolates were included in this study. Only the first isolate obtained from each patient during the study period was included in the analysis to avoid duplication. Patients with negative blood culture results, cultures yielding organisms other than *Salmonella enterica* serovars Typhi or Paratyphi, duplicate isolates obtained from the same patient during the same episode of illness, (iv) patients who had received prolonged antibiotic therapy before sample collection that could significantly affect culture yield, and samples that were contaminated, improperly collected, inadequately labeled, or insufficient in quantity for microbiological analysis were excluded from this study.

Study Procedure: Relevant demographic and clinical information, including age, sex, clinical presentation, and prior antibiotic exposure, were collected using a structured data collection form. All laboratory investigations were performed in the Department of Microbiology following standard operating procedures and quality control measures to ensure the accuracy and reliability of the results.

Collection of Specimens: This laboratory validation study was conducted at the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh. The study evaluated 100 clinical isolates of typhoidal *Salmonella* recovered from patient blood cultures. The sample pool included 72 distinct *Salmonella* Typhi strains and 28 *Salmonella* Paratyphi A strains.

Microorganism Identification: Isolates were subcultured onto MacConkey, blood, and chocolate agar media to assess purity and growth morphology. Final phenotypic identification was performed using standard biochemical tests like Gram stain checking (Gram-negative rods), positive motility pattern, alkaline slant/acid butt reactions on Triple Sugar Iron (TSI) agar, negative indole production and positive sodium citrate utilization. Serological confirmation was completed via slide agglutination using somatic (O), flagellar (H), and capsular (Vi) diagnostic antisera to ensure accurate species classification.

Ciprofloxacin Agar Dilution: Quantitative minimum inhibitory concentrations (MICs) for ciprofloxacin were established using the standard agar dilution technique to serve as the reference standard. Serial two-fold dilutions of pure ciprofloxacin powder were mixed into molten Mueller-Hinton agar (MHA) to produce a range of testing plates. Inocula were adjusted to a 0.5 McFarland standard, diluted 1:10, and placed onto the agar surfaces using a multipoint inoculator to ensure a uniform spot density of 10^4 CFU/spot. Following 18 hours of incubation at 35°C, MIC values were recorded as the lowest concentration that completely inhibited visible bacterial growth. Results were classified based on CLSI criteria: Resistant (≥ 4 µg/ml), Intermediate Sensitive (1 to <4 µg/ml), and Sensitive (<0.06 µg/ml).

Nalidixic Acid Disk Diffusion Screening: The surrogate screening index test used a standard 30 µg nalidixic acid disk via the Kirby-Bauer disk diffusion method on MHA plates. Suspensions matched to the 0.5 McFarland standard were spread evenly across the agar plates using sterile cotton swabs before disk placement. Following incubation at 35°C for 16-18 hours, zones of inhibition were measured using a digital caliper. Diameters were interpreted using CLSI criteria: Resistant (≤ 13 mm) and Susceptible (≥ 19 mm). Diagnostic validation metrics were calculated by comparing disk resistance patterns against the reference agar dilution tiers.

Statistical Analysis: Statistical analysis was performed by Windows based software named as Statistical Package for Social Science (SPSS), versions 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Continuous data were expressed as mean, standard deviation, minimum and maximum. Categorical data were summarized in terms of frequency counts and percentages. Chi-square test was used for comparison of categorical variables and Student t

test was applied for continuous variables. Every efforts were made to obtain missing data. A two-sided P value of less than 0.05 was considered to indicate statistical significance. Differences between case and control were tested.

Ethical Consideration: All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration 2013) and also with the ethical guidelines of the Institutional research ethics. Formal ethics approval was granted by the local ethics committee. Participants in the study were informed about the procedure and purpose of the study and confidentiality of information provided. All participants consented willingly to be a part of the study during the data collection periods. All data were collected anonymously and were analyzed using the coding system.

Results

The comparison between first-generation quinolone disk diameters and ciprofloxacin MIC values showed a clear diagnostic correlation.

Seventy-six percent of the total isolates were resistant to nalidixic acid (zone ≤ 13 mm), while twenty-four percent were fully sensitive.

Table 1: Distribution of Isolates According to Nalidixic Acid Disk Susceptibility (N = 100)

Nalidixic Acid	Zone Diameter (mm)	Frequency	Percent
Resistant	≤ 13	76	76.0

The majority of Salmonella isolates (76%) were resistant to nalidixic acid, while only 24% remained susceptible. This high prevalence of nalidixic acid resistance indicates widespread reduced susceptibility to fluoroquinolones among circulating enteric fever pathogens and highlights the need for careful antimicrobial susceptibility testing (Table 1).

Table 2: Distribution of Ciprofloxacin MIC Categories among Salmonella Isolates (N = 100)

Ciprofloxacin MIC Categ	MIC Range (µg/ml)	Frequency	Percent
Resistant	4.0 to 8.0	40	40.0
Intermediate	1.0 to 4.0	36	36.0
Sensitive	0.125 to 1.0	24	24.0
Susceptible	≥ 19	24	24.0

Table 3: Protective Immunity Against Mumps in Different Residence (n=180)

Ciprofloxacin MIC Category	MIC Range (µg/ml)	S. Typhi	S. Paratyphi A	Total Isolates	Nalidixic Acid Disk Status
Resistant	4.0–8.0	31	9	40 (40.0%)	Resistant (≤ 13 mm)
Intermediate	1.0–4.0	24	12	36 (36.0%)	Resistant (≤ 13 mm)
Sensitive	0.125–1.0	17	7	24 (24.0%)	Susceptible (≥ 19 mm)

Ciprofloxacin MIC testing demonstrated that 40% of isolates were resistant, 36% showed intermediate susceptibility, and only 24% were fully susceptible. Thus, 76% of isolates exhibited reduced susceptibility to ciprofloxacin, emphasizing the growing challenge of fluoroquinolone resistance among *Salmonella* Typhi and Paratyphi A isolates (Table 2).

A strong correlation was observed between nalidixic acid disk susceptibility and ciprofloxacin MIC values. All isolates categorized as ciprofloxacin-resistant or intermediate by MIC testing were resistant to nalidixic acid, whereas all ciprofloxacin-sensitive isolates were susceptible to nalidixic acid. These findings demonstrate that nalidixic acid disk testing serves as a reliable surrogate marker for detecting reduced ciprofloxacin susceptibility and may be particularly useful in resource-limited laboratories where routine MIC determination is not feasible (Table 3).

Discussion

The present study evaluated the relationship between nalidixic acid susceptibility and ciprofloxacin minimum inhibitory concentration (MIC) among *Salmonella enterica* serovars Typhi and Paratyphi A isolates. The findings demonstrated a strong correlation between nalidixic acid resistance and reduced ciprofloxacin susceptibility, supporting the use of nalidixic acid disk testing as a practical screening tool for identifying isolates with decreased fluoroquinolone susceptibility in resource-limited laboratory settings.

A notable finding of this study was the high prevalence of nalidixic acid resistance, observed in 76% of the isolates. This result reflects the ongoing selective pressure exerted by the extensive use of fluoroquinolones in the treatment of enteric fever throughout South Asia. Similar rates of nalidixic acid resistance have been reported from Bangladesh, India, Pakistan, and Nepal, where widespread use of fluoroquinolones has contributed to the emergence of resistant *Salmonella* strains. Resistance to nalidixic acid is generally associated with mutations in the

quinolone resistance-determining region (QRDR) of the *gyrA* gene, which reduce the susceptibility of bacterial DNA gyrase to quinolone action. Consequently, nalidixic acid resistance has become an important phenotypic marker for detecting isolates that may not respond optimally to fluoroquinolone therapy.

The ciprofloxacin MIC results further highlighted the magnitude of this problem. Only 24% of isolates were fully susceptible to ciprofloxacin, while 40% were resistant and 36% exhibited intermediate susceptibility. Thus, more than three-quarters of the isolates demonstrated reduced susceptibility to ciprofloxacin. These findings are consistent with reports from endemic regions where progressive increases in ciprofloxacin MIC values have been documented over the past two decades. Although some isolates may still appear susceptible by routine disk diffusion testing, elevated MIC values are frequently associated with delayed clinical response, prolonged fever clearance time, and treatment failure. Therefore, reliance solely on conventional ciprofloxacin disk diffusion results may lead to inaccurate susceptibility reporting and inappropriate antimicrobial selection.

The most significant observation of this study was the complete concordance between nalidixic acid disk susceptibility and ciprofloxacin MIC categories. All isolates categorized as ciprofloxacin-resistant or intermediate by MIC testing were resistant to nalidixic acid, whereas all ciprofloxacin-sensitive isolates remained susceptible to nalidixic acid. This finding confirms the value of nalidixic acid resistance as a surrogate marker for reduced ciprofloxacin susceptibility. Similar correlations have been demonstrated in several previous studies, leading many investigators to recommend nalidixic acid screening as an economical alternative to routine MIC determination, particularly in developing countries where advanced susceptibility testing methods may not be readily available.

The practical implications of these findings are substantial. Determination of ciprofloxacin MIC by agar dilution or broth microdilution remains the reference standard; however, these methods require specialized equipment, technical expertise, and

increased laboratory costs. In contrast, nalidixic acid disk testing is simple, inexpensive, and widely available. Incorporating nalidixic acid screening into routine laboratory practice can help identify isolates with reduced fluoroquinolone susceptibility, minimize false susceptibility reporting, and guide clinicians toward more effective therapeutic options such as third-generation cephalosporins or azithromycin when appropriate.

Conclusion

In conclusion, the study demonstrates a strong and clinically relevant correlation between nalidixic acid resistance and elevated ciprofloxacin MIC values among *Salmonella* Typhi and Paratyphi A isolates. The findings support the continued use of nalidixic acid disk testing as a reliable and cost-effective surrogate marker for reduced ciprofloxacin susceptibility, particularly in resource-constrained settings where routine MIC testing is not feasible. Nalidixic acid disk diffusion is a highly reliable and cost-effective screening tool for catching hidden fluoroquinolone resistance. Clinical diagnostic laboratories in endemic regions should mandate routine dual-disk testing to prevent reporting errors and ensure safe, effective treatment for enteric fever.

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Conflict of interest:

All authors declare that they have no The authors declared no conflict of interest.

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Authors' contributions

Kawser S, Hossain S had involved in protocol preparation, data & sample collection and literature search and manuscript writing. Sabah KMN, Nessa Z were involved in manuscript preparation and revision. All the authors have read and approved the final version of the manuscript.

Data Availability

The datasets used and analyzed during the present study are derived from hospital surveillance systems and DGHS databases. Data are available from the corresponding author upon reasonable request and subject to approval by the relevant institutional authorities.

Ethics Approval and Consent to Participate

Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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ORCID:

Shamima Kawser: <https://orcid.org/0009-0000-5193-0361>

Sanjida Hossain: <https://orcid.org/0009-0003-2610-1862>

Khandker Md.Nurus Sabah:

<https://orcid.org/0000-0002-8117-3012>

Zebun Nessa: <https://orcid.org/0009-0004-2529-7047>

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