Journal of Institute of Science and Technology, 25(2), 119-124 (2020)
ISSN: 2469-9062 (print), 2467-9240 (e)
Doi: https://doi.org/10.3126/jist.v25i2.33748

Research Article

REVISITING THE REVISED CLSI BREAKPOINT FOR DETECTING FLUOROQUINOLONES RESISTANCE IN SALMONELLA

Suchitra Thapa[1*], **Basudha Shrestha**[2], **Sarita Manandhar**[3]

[1]*Department of Microbiology, Amrit Campus, Tribhuvan University, Kathmandu, Nepal*
[2]*Department of Microbiology, Kathmandu Model Hospital, Kathmandu, Nepal*
[3]*Department of Microbiology, Tri-Chandra Multiple Campus, Tribhuvan University, Kathmandu, Nepal*
[*]*Corresponding author: suchitrathapa69@gmail.com, suchitra.thapa@ac.tu.edu.np*

*(**Received**: July 31, 2020; **Revised**: October 08, 2020; **Accepted**: October 15, 2020)*

ABSTRACT

Resistance towards fluoroquinolones and treatment failure is a matter of concern in enteric fever. The present study was undertaken to analyze the susceptibility pattern of *Salmonella* towards fluoroquinolones using 2006 and 2013 Clinical Laboratory Standards Institute (CLSI) breakpoint for interpretation and revisit the efficacy of Nalidixic acid resistance (NAR) as a phenotypic marker. A retrospective analysis of the zone of inhibition (ZOI) diameter data and minimum inhibitory concentration (MIC) data of 105 *Salmonella* was conducted. The ZOI diameter analysis showed that all isolates were susceptible to Ofloxacin and Ciprofloxacin using the previous 2006 ZOI breakpoint. However, with the revised 2013 breakpoint of Ciprofloxacin, the susceptibility percent dropped significantly and for Ofloxacin the breakpoint was not revised, so the percentage remained unchanged. The MIC analysis showed that all isolates were susceptible towards Ofloxacin and 97.14 % of isolates were susceptible to Ciprofloxacin using the previous 2006 MIC breakpoint, while the susceptibility decreased for both antibiotics with the revised 2013 MIC breakpoint. Statistically, the ZOI diameter of Nalidixic acid and MIC values of Ciprofloxacin and Ofloxacin was negatively correlated. To conclude, the revision of breakpoints addresses the problem of screening fluoroquinolones resistance but the emerging fluoroquinolones resistance situation is still a matter of concern in healthcare facilities of Nepal. Thus a reliable screening method is need of the hour as NAR cannot be considered a reliable marker to screen fluoroquinolones resistance.

Keywords: Fluoroquinolones, Ciprofloxacin, Nalidixic acid resistance, *Salmonella*, CLSI

INTRODUCTION

Enteric fever is a systemic infection caused by *Salmonella enterica* serotype Typhi (*S. Typhi*) and *S. enterica* serotype Paratyphi (*S. Paratyphi*) A, B, and C. In both serotypes, antibiotic resistance is occurring with increased frequency throughout Asia (Crump *et al.,* 2003). This emergence of resistance and decreasing levels of susceptibility of *Salmonella enterica* to a wide spectrum of antimicrobial is a matter of concern as it limits the availability of antimicrobials for therapeutic usage in the future. WHO has ranked fluoroquinolones resistant *Salmonella* as a high priority pathogen in 2017 (Tacconelli *et al.,* 2018). Due to the rise in antibiotic resistance and the inability of Clinical Laboratory Standards Institute (CLSI) breakpoint to efficiently detect the decreased susceptibility of fluoroquinolones, several studies from various countries (Sjolund-Karlsson *et al.,* 2014; Crump *et al.,* 2003) including Nepal (Acharya *et al.,* 2012) suggested to re-evaluate the CLSI (CLSI, 2006) interpretative criteria of fluoroquinolones. Thus in the year 2011, CLSI recommended NAR as a surrogate marker for the screening of fluoroquinolones resistant strains (CLSI, 2011).

But due to the presence of fluoroquinolone resistance among Nalidixic acid susceptible (NAS) isolates, this screening method was not considered a reliable marker (Humphries *et al.,* 2012). To overcome this problem, CLSI breakpoint was revised in 2012 for Ciprofloxacin (CLSI, 2012) and in 2013 for Ofloxacin (CLSI, 2013) for *Salmonella* isolates. Since the recommendation of CLSI has considerable influence on many countries, the guidelines were revised timely to accommodate the changing trend of antibiotic susceptibility of pathogens (Humphries *et al.,* 2019; Crump *et al.,* 2003).

In Nepal, antibiotic resistance among Gram negative bacteria is increasing (Thapa *et al.,* 2006) and screening of such resistance is routinely done using the disc diffusion (DD) method following CLSI standard guidelines. But this DD method has been claimed inadequate to determine to reduce susceptibility of fluoroquinolones by many studies (Gupta *et al.,* 2020; Bhetwal *et al.,* 2017; Khanal *et al.,*2017) so NAR was used as a surrogate marker in those cases. Unfortunately, the fluoroquinolones resistance among *Salmonella* continued to increase in Nepal (Gupta *et al.,* 2020; Pokharel *et al.,* 2016; Karki *et al.,* 2013) and to implement an appropriate treatment for enteric fever patients, an efficient screening method along with a standard CLSI guideline is utmost necessary (Bhetwal *et al.,* 2017; Khanal *et al.,*2017). These guidelines should be continuously monitored for their efficacy and suggest for re-evaluation if necessary. Thus this study aims to analyze the data of ZOI diameter and MIC value of *Salmonella* against fluoroquinolones using

the previous (2006) and revised (2013) CLSI breakpoints and to re-examine the efficacy of NAR marker as screening test for fluoroquinolones susceptibility.

MATERIALS AND METHODS

A total of 105 *Salmonella* isolates were acquired from Kathmandu Model Hospital and Kirtipur Hospital in 2010. The isolates were identified by conventional biochemical methods and serotyped by agglutination with specific antisera (Denka Seiken Co. Ltd. Tokyo, Japan). These bacterial isolates were subjected to antibiotic susceptibility testing (AST) by Kirby-Bauer DD method following the guidelines of CLSI (CLSI, 2006) for quinolones and fluoroquinolones (Nalidixic acid-30µg, Ofloxacin-5µg, Ciprofloxacin-5µg). The MIC values of Ciprofloxacin, Ofloxacin, and Nalidixic acid were determined using the broth dilution method (Andrews, 2001) following the CLSI guidelines. For comparative analysis, breakpoints of fluoroquinolones for *Salmonella* from previous 2006 CLSI (CLSI, 2006) and revised 2013 CLSI standard guidelines (CLSI, 2012, 2013) were used. All the data were analyzed using commercially available software programs (SPSS 20.0 for Windows, SPSS Inc. and WHONET2019, WHO).

RESULTS

Of the 105 blood culture positive isolates, 69 (65.71 %) were *Salmonella* Paratyphi A and 36 (34.29 %) were *Salmonella* Typhi.

Zone of inhibition diameter of fluoroquinolones

Based on the interpretation ZOI diameter, the susceptibility percentage of Ciprofloxacin was significantly reduced by 91.43 % as compared to the breakpoints of 2006 CLSI and 2013 CLSI guideline (Table 1). While the ZOI breakpoint for Ofloxacin was not revised so the susceptibility percentage remained 100 % (105).

Minimum inhibitory concentration of fluoroquinolones

Based on MIC interpretation the susceptibility percentage of both Ciprofloxacin and Ofloxacin was significantly reduced by 62.38 % and 73.33 % respectively as compared with the breakpoints of 2006 CLSI and 2013 CLSI guideline (Table 2).

Table 1. Susceptibility percentage of Ciprofloxacin (CIP) (n=105)

Antibiotic Code	Zone of inhibition diameter (mm) Mean ± S.D.	2006 CLSI interpretative criteria		Revised CLSI interpretative criteria	
		Susceptible isolates (Percentage)	Breakpoint (mm)	Susceptible isolates (Percentage)	Breakpoint (mm)
CIP	22.03 ± 3.44	105 (100 %)	S≥21; R≤15	9 (8.57 %)	S≥31; R≤20

Table 2. Susceptibility percentage of Ciprofloxacin (CIP) and Ofloxacin (OF) (n=105)

Antibiotic Code	MIC Range (µg/mL)	MIC_{50}	MIC_{90}	2006 CLSI guidelines		Revised CLSI guidelines	
				Susceptible isolates (Percentage)	Breakpoint (µg/ml)	Susceptible isolates (Percentage)	Breakpoint (µg/ml)
CIP	0.003-2	0.125	0.5	102 (97.14%)	S≤1; R≥4	26 (24.76 %)	S≤0.064; R≥1
OF	0.01-2	0.5	1	105 (100 %)	S≤2; R≥8	28 (26.67 %)	S≤0.125; R≥2

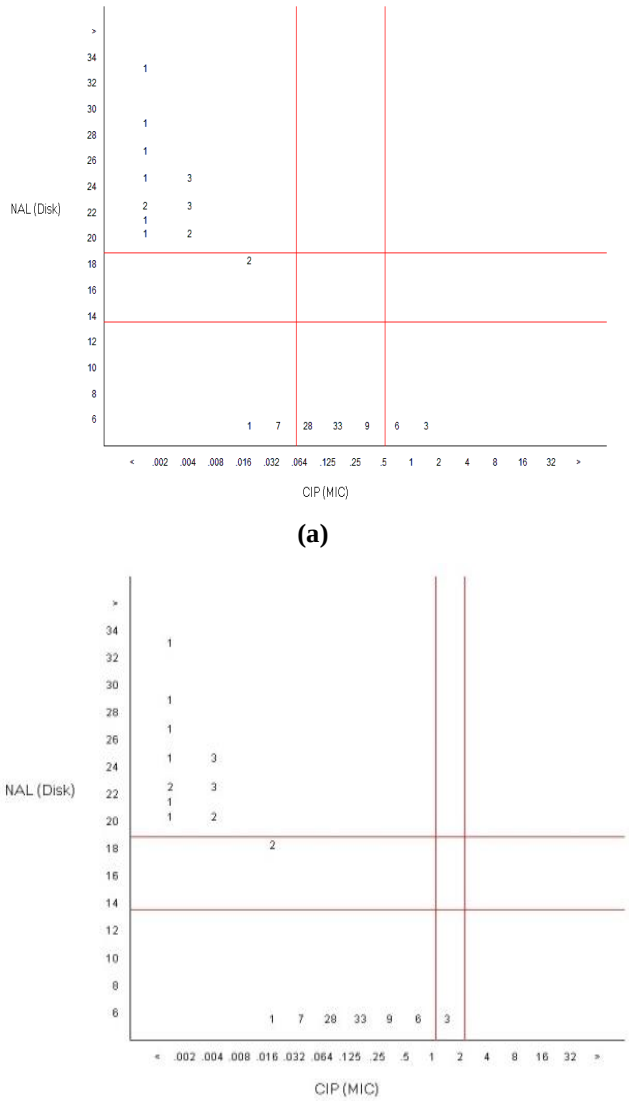
Screening of fluoroquinolones resistance using nalidixic acid as a marker

The distribution of MIC of fluoroquinolones among the NAR and NAS was tested for Ciprofloxacin and Ofloxacin each using the Mann Whitney test and was found to be highly significant ($p < 0.01$). Also, Pearson's correlation was computed to assess the relationship between the ZOI diameter of Nalidixic acid with MIC of Ciprofloxacin (($r = -0.316$, $p<0.05$) and Ofloxacin ($r = -0.407$, $p<0.05$) each separately. There was a low negative correlation between ZOI diameter of Nalidixic acid and MIC of Ciprofloxacin and Ofloxacin which is distinctively visible in the scatterplot, as shown in Figs. 1 and 2. Regression analysis was done and the coefficient of determination was computed which showed a poor relationship of Nalidixic acid ZOI diameter with MIC of ciprofloxacin ($R^2= 0.10$, $p<0.05$) and MIC of Ofloxacin ($R^2= 0.166$, $p<0.05$).

DISCUSSION

In this study, a notable decrease in susceptibility percentage of *Salmonella* towards Ciprofloxacin was observed following the revised 2013 ZOI breakpoint (Table 1) and 2013 MIC breakpoint (Table 2). This decrease in Ciprofloxacin susceptibility is a worrisome situation (especially from the ZOI interpretation) since the

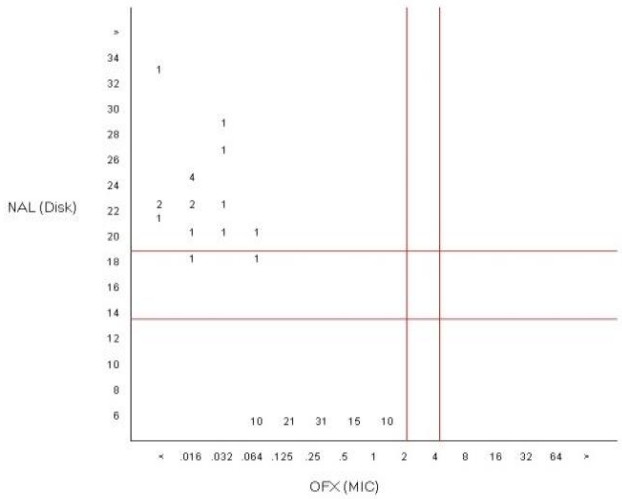
susceptibility dropped to 8.5 % which is a question for its usage in the treatment regime. However, the susceptibility percentage dropping to as low as 3 % has been reported in some studies from India with the revised Ciprofloxacin breakpoints (Saksena *et al.*, 2016; Balaji *et al.*, 2014). Several reports have questioned the efficacy of Ciprofloxacin as a drug of choice (Gupta *et al.*, 2020; Pokharel *et al.*, 2016; Saksena *et al.*, 2016; Balaji *et al.*, 2014) and a randomized trial in Nepal has strictly suggested not to use fluoroquinolones in enteric fever treatment not even the fourth generation Gatifloxacin (Arjyal *et al.*, 2016).



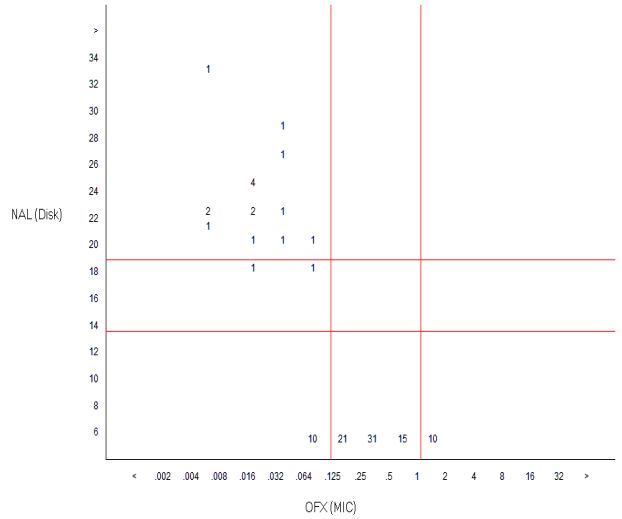
(a)

(b)

Fig. 1. Scatterplot showing correlation of MICs of Ciprofloxacin and ZOI diameter of Nalidixic acid of *Salmonella* isolates (n = 105) using breakpoints as recommended by (a) 2006 CLSI and (b) 2012 CLSI guidelines. Horizontal and vertical solid lines represent their respective breakpoints



(a)



(b)

Fig. 2. Scatterplot showing correlation of MICs of Ofloxacin and ZOI diameter of Nalidixic acid of *Salmonella* isolates (n = 105) using breakpoints as recommended by (a) 2006 CLSI and (b) 2013 CLSI guidelines. Horizontal and vertical solid lines represent their respective breakpoints

Therefore, due to the changing trend of antibiotic susceptibility in *Salmonella*, diagnostic laboratory should not depend on the revised breakpoints and must routinely check the efficacy of the current breakpoint in conjunction with treatment response and recommend CLSI for revisions if necessary.

The scenario of Ofloxacin is even worse; since the detection of reduced susceptible isolates was not successfully achieved in this study from the 2013 breakpoints as the ZOI breakpoint was not revised in the case of Ofloxacin. But the scatterplot of MIC of Ofloxacin clearly showed a shift in the cluster of susceptible isolates

into intermediate zone in the two plots (Figure 2). These intermediately susceptible isolates are recorded as susceptible isolates in ZOI interpretation, due to lack of revised ZOI breakpoint. This situation may lead to a treatment failure if the laboratories solely depend on ZOI interpretation.

In particular, a randomized trial of the treatment response in enteric fever in Nepal with such a situation was well documented (Thompson *et al.*, 2017). Therefore a revision of the current Ofloxacin ZOI breakpoint is necessary (Das *et al.*, 2016; Sjolund-Karlsson *et al.*, 2014). Earlier in 2011, CLSI recommended Nalidixic acid as a phenotypic marker for screening isolates with decreased fluoroquinolones susceptibility (Guzmán-Martín *et al.*, 2018) and was endorsed for very long period (Hakanen *et al.*, 1999) but now with the evolution in resistant mechanism in *Salmonella*, the efficacy of the test has been frequently questioned. In this study, the efficacy was validated using a correlation and regression analysis. Though the NAS and NAR populations clustered at a specific MIC range of Ciprofloxacin and Ofloxacin, the outliers were distinctly observed in both the scatterplots of Ciprofloxacin and Ofloxacin (Figs. 1 and 2), which signifies that the Nalidixic acid screening test has some limitations, and was confirmed statistically. The distribution of MIC of Ciprofloxacin and Ofloxacin was highly significant among NAS and NAR groups when tested separately indicating relation between them.

A similar finding has been reported by numerous studies worldwide (Das *et al.*, 2016; Acharya *et al.*, 2012; Hakanen *et al.*, 1999). Subsequently, a negative correlation between Nalidixic acid ZOI diameter and MIC of each fluoroquinolones indicated that the higher the ZOI diameter of Nalidixic acid (higher susceptibility), less will be the MIC of fluoroquinolones (higher susceptibility). In other words higher the NAR, the higher will be the resistance against fluoroquinolones and vice-versa but the correlation coefficient was very low to be taken into serious consideration which should be duly noted. A similar correlation in *Salmonella* has been reported by many researchers (Das *et al.*, 2016; Acharya *et al.*, 2012; Hakanen *et al.*, 1999).

Furthermore, the regression analysis revealed a very low coefficient of determination for MIC of Ciprofloxacin and Ofloxacin with ZOI diameter of Nalidixic acid. A similar result was also reported by Acharya *et al*. (2012). The coefficient of determination points out that only 10 % of variance in Ciprofloxacin MIC can be predicted by Nalidixic acid ZOI diameter, while the rest of the variance in MIC could be due to other factors. Similarly, 16.6 % of variance in MIC of Ofloxacin can be predicted by Nalidixic acid ZOI diameter while the rest of the variance in MIC could be due to other factors. Thus the NAR cannot be solely used as a phenotypic marker for detecting resistance of Ciprofloxacin and Ofloxacin (Das *et al.*, 2016). Additionally, it also clarified that NAR does not confirm the presence of resistance towards Ciprofloxacin. This inefficiency of screening fluoroquinolones susceptibility clears out the usefulness of NAR as a marker in such isolates. Screening of such isolates and other resistant isolates is essential for the proper treatment of any infection and for this an efficient and reliable screening technique with an updated breakpoint is need. Resistance towards the newer fluoroquinolones (Levofloxacin and Gatifloxacin) has been reported by others too (Arjyal *et al.*, 2016; Das *et al.*, 2016; Sjolund-Karlsson *et al.*, 2014). Studies have shown that this revised 2013 CLSI breakpoint (MIC and ZOI) of fluoroquinolones considerably trace fluoroquinolones resistant isolates of *Salmonella* (Saksena *et al.* 2016; Balaji *et al.* 2014; Humphries *et al.*, 2012).

CONCLUSION

The revised 2013 CLSI guideline was able to reliably detect decreased susceptibility to fluoroquinolones in *Salmonella* but this should not overshadow the fact that the fluoroquinolones susceptibility is continuously emerging even among the new fluoroquinolones like Gatifloxacin. And while revising the breakpoints, CLSI should consider both MIC and ZOI breakpoints for revision since many laboratories solely depend on the disc diffusion method for screening antibiotic susceptibility. Also, as fluoroquinolones are still the drug of choice in many healthcare facilities of Nepal, a suitable detection method is required to reliably screen the fluoroquinolones resistant isolates in the routine laboratory.

ACKNOWLEDGEMENTS

The authors are grateful to the laboratory staff of Kathmandu Model Hospital and Kirtipur Hospital for bench facilities. We also acknowledge the patients who provided clinical samples.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Suchitra Thapa designed the research proposal, performed the laboratory works, analyzed the data, and wrote the manuscript. Basudha Shrestha and Sarita Manandhar contributed to conceptualizing and supervising the research work.

REFERENCES

Acharya, D., Malla, S., Bhatta, D. R., Adhikari, N., & Dumre, S. P. (2012). Current fluoroquinolones susceptibility criteria for Salmonella need re-evaluation. *Kathmandu University Medical Journal*, *10*(1), 66-71.

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, *48* (suppl.), 5-16.

Arjyal, A., Basnyat, B., Nhan, H. T., Koirala, S., Giri, A., Joshi, N., ... & Adhikari, N. (2016). Gatifloxacin versus ceftriaxone for uncomplicated enteric fever in Nepal: an open-label, two-center, randomized controlled trial. *The Lancet Infectious Diseases*, *16*(5), 535-545.

Balaji, V., Sharma, A., Ranjan, P., & Kapil, A. (2014). Revised ciprofloxacin breakpoints for Salmonella Typhi: its implications in India. *Indian Journal of Medical Microbiology*, *32*(2), 161-163.

Bhetwal, A., Maharjan, A., Khanal, P. R., & Paraguli, N. P. (2017). Enteric fever caused by *Salmonella enterica* serovars with reduced susceptibility of fluoroquinolones at a community based teaching hospital of Nepal. *International Journal of Microbiology*, *2017*, 2869458. https://doi.org/10.1155/2017/2869458

CLSI (2006) *CLSI Performance standards for antimicrobial susceptibility testing*. Approved standards 9th edition, CLSI M2-A9, CLSI, Wayne, PA, USA: Clinical and Laboratory Standards Institute.

CLSI (2011) *CLSI Performance standards for antimicrobial susceptibility testing*. 16th informational supplement. CLSI M100-S16. CLSI, Wayne, PA, USA: Clinical and Laboratory Standards Institute.

CLSI (2012) *CLSI Performance standards for antimicrobial susceptibility testing*. 22nd informational supplement. CLSI M100-S22. CLSI, Wayne, PA, USA: Clinical and Laboratory Standards Institute.

CLSI (2013) *CLSI performance standards for antimicrobial susceptibility testing*. 23rd informational supplement.CLSI M100-S23. CLSI, Wayne, PA, USA: Clinical and Laboratory Standards Institute.

Crump, J. A., Barrett, T. J., Nelson, J. T., & Angulo, F. J. (2003). Re-evaluating fluoroquinolone breakpoints for *Salmonella enterica* serotype Typhi and non-Typhi salmonellae. *Clinical Infectious Diseases*, *37*(1), 75-81.

Das, S., Ray, U., & Dutta, S. (2016). Revisit of fluoroquinolone and azithromycin susceptibility breakpoints for *Salmonella enterica* serovar Typhi. *Journal of Medical Microbiology*, *65*(7), 632-640.

Gupta, S. K., Panday, R. C., Sah, J. P., Dhakal, G., Mahaseth, D., Shah, K., & Sharma, B. (2020). Prevalence of *Salmonella* species with nalidixic acid resistance in the enteric fever patients of Nepal. *British Journal of Medical & Health Sciences*, *2*(7), 314-321.

Guzmán-Martín, J. L., Navarro-Marí, J. M., Expósito-Ruiz, M., & Gutiérrez-Fernández, J. (2018). Nalidixic acid surrogate test for susceptibility to ciprofloxacin in Salmonella. Revisiting the question. *Journal of Medical Microbiology*, *67*(7), 965-967.

Hakanen, A., Kotilainen, P., Jalava, J., Siitonen, A., & Huovinen, P. (1999). Detection of decreased fluoroquinolone susceptibility in salmonellas and validation of nalidixic acid screening test. *Journal of Clinical Microbiology*, *37*(11), 3572-3577.

Humphries, R. M., Abbott, A. N., & Hindler, J. A. (2019). Understanding and addressing CLSI breakpoint revisions: a primer for clinical laboratories. *Journal of Clinical Microbiology*, *57*(6), e00203-19. https://doi.org/10.1128/JCM.00203-19

Humphries, R. M., Fang, F. C., Aarestrup, F. M., & Hindler, J. A. (2012). In vitro susceptibility testing of fluoroquinolone activity against Salmonella: recent changes to CLSI standards. *Clinical Infectious Diseases*, *55*(8), 1107-1113.

Karki, S., Shakya, P., Cheng, A.C., Dumre, S. P., & Leder, K. (2013). Trends of etiology and drug resistance in enteric fever in the last two decades in Nepal: A systematic review and meta-analysis. *Clinical Infectious Diseases*, *57*(10), e167-e176.

Khanal, P. R., Satyal, D., Bhetwal, A., Maharjan, A., Shakya, S., Tandukar, S., & Parajuli, N. P. (2017). Renaissance of conventional first-line antibiotics in *Salmonella enterica* clinical isolates: Assessment of MICs for therapeutic antimicrobials in enteric fever cases from Nepal. *BioMed Research International*, *2017*, Article ID 2868143. https://doi.org/10.1155/2017/2868143

Pokharel, P., Lekhak, B., Amatya, R., Bhattarai, S., & Pokharel, P. (2016). Enteric fever caused by *Salmonella enterica* serovar paratyphi A: An emerging health concern in Nepal. *African Journal of Microbiology Research*, *10*(42), 1784-1791.

Saksena, R., Nayyar, C., & Manchanda, V. (2016). Six-year susceptibility trends and effect of revised clinical laboratory standards institute breakpoints on ciprofloxacin susceptibility reporting in typhoidal Salmonellae in a tertiary care pediatric hospital in Northern India. *Indian Journal of Medical Microbiology*, *34*(4), 520-525.

Sjölund-Karlsson, M., Howie, R. L., Crump, J. A., & Whichard, J. M. (2014). Fluoroquinolone susceptibility testing of *Salmonella enterica*: detection of acquired resistance and selection of zone diameter breakpoints for levofloxacin and ofloxacin. *Journal of Clinical Microbiology*, *52*(3), 877-884.

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., ... & Ouellette, M. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet Infectious Diseases*, *18*(3), 318-327.

Thapa, E., Thapa, R., Singh, A., Pokharel, B., & Devkota, U. (2016). Antibiotic resistance pattern of bacterial isolates from post-tracheostomised patients attending tertiary care hospital in Nepal. *Journal of Institute of Science and Technology*, *21*(1), 129-132.

Thompson, C. N., Karkey, A., Dongol, S., Arjyal, A., Wolbers, M., Darton, T., ... & Baker, S. (2017). Treatment response in enteric fever in an era of increasing antimicrobial resistance: an individual patient data analysis of 2092 participants enrolled into 4 randomized, controlled trials in Nepal. *Clinical Infectious Diseases*, *64*(11), 1522-1531.