Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2019.080

RESEARCH ARTICLE

Antibiotic Susceptibility Pattern of Salmonellae Isolated from Poultry from Different Districts of Punjab, Pakistan

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ARTICLE HISTORY (19-153)

Received: April 08, 2019
Revised: May 05, 2019
Accepted: May 06, 2019
Published online: June 10, 2019

Key words:
Antibiotic resistance
PCR
Poultry
S. enteritidis
S. gallinarum

A B S T R A C T

Salmonella is one of the most common bacterial pathogens of poultry which not only affect poultry; its transmission to human food chain is a threat to public safety. The irrational use of antibiotics in poultry industry has resulted in antibiotic resistant strains. Aim of the present study was to isolate, characterize and determine antibiotic susceptibility pattern of salmonellae. A total of 150 samples including droppings, liver and intestinal content of poultry were collected from different Districts of Punjab. Out of 150 samples, 44.66% (n=67) were positive for salmonellae. Salmonellae were identified using genus specific and serovar specific polymerase chain reactions. Out of 67 salmonellae, there were 34(52.3%) *Salmonella gallinarum*, 21(31.34%) *Salmonella enteritidis* and 12 (17.91%) unidentified salmonellae. Antibiotic susceptibility pattern of all the isolates was determined by Kirby-Bauer disc diffusion method. Overall, salmonellae (n=67) showed higher level (≥75%) of resistance to nalidixic acid (98.5%), ampicillin (98%) and amoxicillin (95.5%), intermediate level (>40%- <75%) of resistance to gentamicin (61.2%), chloramphenicol (61.2%), tetracycline (59.7%), ciprofloxacin (67.2%) and ceftazidime (52.3%) and low level (≤40%) of resistance to cefotaxime (31.4%), ceftriaxone (26.9%), sulfamethoxazole (26.9%) and cefixime (20.9%). Occurrence of antibiotic resistant salmonellae in poultry insinuate for its continuous monitoring and exploration of alternatives of antibiotics for its control in poultry and further transmission to human beings.

To Cite This Article: Yasmin S, Nawaz M, Anjum AA, Ashraf K, Ullah N, Mustafa A, Ali MA and Mehmood A, 2020. Antibiotic susceptibility pattern of salmonellae isolated from poultry from different districts of Punjab, Pakistan. Pak Vet J, 40(1): 98-102. http://dx.doi.org/10.29261/pakvetj/2019.080

INTRODUCTION

Poultry sector is one of the fastest growing sectors in agriculture industry, which accounts for 32.7% of total meat production in Pakistan (Anonymous, 2018). In recent years, poultry enterprises have developed rapidly in Pakistan. However, different infectious diseases pose a serious threat to the survival of poultry industry (Abbas *et al*., 2008, 2017a, 2017b, 2019; Ashraf *et al*., 2017; Idris *et al*., 2017; Mahmood *et al*., 2017; Naqvi *et al*., 2017). These diseases inflict heavy economic losses to the poultry industry. Major bacterial diseases of poultry include fowl typhoid, enteritis, fowl cholera, colibacillosis and salmonellosis (Mustafa and Ali, 2005).

Salmonella (Gram negative, rod shaped, motile and facultative anaerobe) belongs to family Enterobacteriaceae. It causes economic losses of worth billions, every year (Wales and Davies, 2011). On the basis of 46 lipopolysaccharides and 114 flagellar antigens, more than 2610 serovars of salmonellae have been identified (Xiong *et al.*, 2018). *Salmonella* genus contains host specific serovars including *Salmonella pullorum* and *Salmonella gallinarum* which cause bacillary white diarrhea and fowl typhoid, respectively in birds. *Salmonella typhimurium* and *Salmonella enteritidis* (non-host specific salmonellae) are transmitted from contaminated poultry products to human food chain and pose a major threat to public safety (Girmay *et al.*, 2015). Prevalence of salmonellae from poultry products has been reported worldwide (Adeyanju and Ishola, 2014).

Antibiotic resistance is one of the major problems of public health concern. Antibiotics are frequently used for

the treatment and control (prophylactic use) of *Salmonella* in poultry industry. *Salmonella spp.* show resistance to quinolones, nalidixic acid and their derivatives such as flouroquinolones (Su *et al.*, 2004). Worldwide irrational use of antibiotics in food animals has led to the emergence of drug resistant *Salmonella* which can be transferred to humans through consumption of contaminated food (Sandvang *et al.*, 1998). In developing countries like Pakistan, there is no regulation or control for the mitigation of *Salmonella* from poultry and accurate diagnosis and targeted antibiotics are still not practiced (Wajid *et al.*, 2018). European Union restricted the use of antibiotics as growth promoters since January, 2006 due to the emergence of resistant bacterial pathogens. In 2017, U.S. banned the use of medically important antibiotics for growth promotion. In accordance with order of Supreme court of Pakistan vide order suo motto human right case no. 7230-P, Pakistan has also banned the medically important antibiotics in livestock and poultry. Plant extracts, probiotics, nano-particles and bacteriophages can be used as alternatives of antibiotics in poultry (Ahmed *et al.*, 2016). Keeping in mind the importance of antibiotic resistance, current study was designed to isolate and determine the antibiogram of salmonellae from commercial poultry of different districts of Punjab, Pakistan.

MATERIALS AND METHODS

Sample collection: A total of 150 samples were collected from commercial poultry birds. Poultry droppings (n=50), liver (n=50) and intestine (n=50) samples were collected from five districts of Punjab, Pakistan, including Lahore, Narowal, Sialkot, Sheikhupura and Gujranwala region. Samples were transported to Department of Microbiology, University of Veterinary and Animal Sciences, Lahore and stored at 4°C until further analysis.

Isolation of salmonellae: Samples were enriched in selenite broth. After 24 hours enrichment at 37°C, 100-200µl sample was plated on Salmonella Shigella agar, followed by incubation at 37°C for 24-48 hours. Black centered colonies were sub-cultured for purification (Orji *et al.*, 2005). Pure colonies were stored in nutrient broth supplemented with 15-20% glycerol as well as in cryobeads at -20°C.

Identification of salmonellae: All isolates were identified by microscopic analysis (Gram's staining) and conventional biochemical profiling using Indole production, Methyl red, Voges Proskauer (VP), citrate utilization, sugar fermentation, H_2S production using Triple sugar Iron (TSI) medium and Urease production tests following Bergey's manual of Determinative Bacteriology (Holt *et al.*, 1994). DNAs were isolated from biochemically identified isolates of *Salmonella* using commercially available DNA extraction kit (GeneAll). Molecular identification was achieved by polymerase chain reactions (PCR) using genus and serovar specific primers as described in Table 1**.** PCR mixture (25 µl) was prepared using nuclease free water (7.5µl), nTaq Master mix (Wizbio solutions) (12.5 µl), forward and reverse primers (1.5µl each) and DNA template (2µl). Prepared reaction mixtures were then placed in T100™ thermal cycler (Bio-Rad) and programming was performed according to specific conditions as described in Table 1. Amplicons were electrophoresed on 1.5% agarose gel at 80 volts for 50 minutes. Gel was visualized using gel documentation system (Cleaver Scientific, UK).

Antibiotic resistance profiling: Susceptibility testing was performed using Kirby-Bauer disc diffusion method with minor modifications (Bauer *et al.*, 1966). Briefly, a lawn of test organism (0.5 McFarland) was prepared by swabbing on Mueller Hinton agar. Antibiotic discs (Oxoid) were placed on agar surface at appropriate distance. After 16-24 hours incubation at 37°C, diameter of zones of inhibition was recorded (mm). Isolates were marked as resistant, intermediate or sensitive following the standards provided by Clinical laboratory standards institute.

RESULTS

Out of 150 samples collected, 44.6% (67/150) were positive for salmonellae. Salmonellae (n=67) were isolated from Lahore (14/67, 20.9%), Gujranwala (14/67, 20.9%), Sheikhupura (15/67, 22.3%), Sialkot (12/67, 17.9%) and Narowal (12/67, 17.9%) districts. On the basis of polymerase chain reaction salmonellae were identified as *S. gallinarum* (34), *S. enteritidis* (21) and other salmonellae (12) as shown in Table 2. Representative PCR amplicons of *S. enteritidis* and *S. gallinarum* resolved on agarose gel are shown in Fig. 1. Overall, salmonellae (n=67) showed higher level (≥75%) of resistance to nalidixic acid (98.5%), ampicillin (98%) and amoxicillin (95.5%), intermediate level (>40% - <75%) of resistance to gentamicin (61.2%), chloramphenicol (61.2%), tetracycline (59.7%), ciprofloxacin (67.2%) and ceftazidime (52.3%), and low level (≤40%) of resistance to cefotaxime (31%), ceftriaxone (26.9%), sulfamethoxazole (26.9%) and cefixime (20.9%). *Salmonella enteritidis* showed higher level of resistance to nalidixic acid (100%), ampicillin (95.2%), amoxicillin (95.2%) and ciprofloxacin (76.2%) followed by intermediate level of resistance to tetracycline (61.9%), ceftazidime (52.4%), gentamicin (52.4%) and chloramphenicol (42.9%), and low level of resistance to cefotaxime (38.1%), ceftriaxone (33.3%), sulfamethoxazole (28.6%) and cefixime (14%). *Salmonella gallinarum* showed higher level of resistance to ampicillin (100%), nalidixic acid (97%) and amoxicillin (94.1%), intermediate level of resistance to ceftazidime (64.7%), chloramphenicol (64.7%), gentamycin (61.8%), tetracycline (58.8%) and ciprofloxacin (58.8%) and low level of resistance to ceftriaxone (23.5%), cefotaxime (23.5%), cefixime (23.5%) and sulfamethoxazole (20.6%). Other salmonellae (n=12) had high level of resistance to ampicillin (100%), amoxicillin (100%), chloramphenicol (83.4%), gentamicin (75%), ciprofloxacin (75%), nalidixic acid (100%), intermediate level of resistance to tetracycline (58.3%), sulfamethoxazole (41.7%) and cefotaxime (41.7%), and low level of resistance to ceftriaxone (25%), cefixime (25%) and ceftazidime (16.7%) as described in Table 3. District wise antibiotic resistance pattern of salmonellae is given in Table 4. District wise antibiotic resistance pattern was also similar to the overall antibiotic resistance pattern of salmonellae with slight variations.

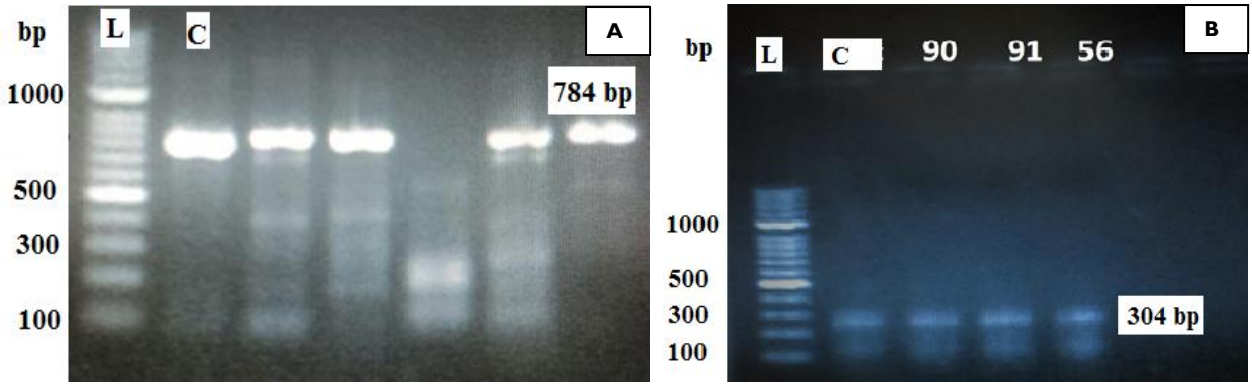

Fig. 1: Representative PCR based identification of (A) *Salmonella* genus and (B) *Salmonella enteritidis*. L: 100 base pair (bp) ladder; C: Positive control.

Table 1: Primers used in current study

Target Organism	Primers (5'----------3')	Target gene	Amplicon (bp)	Tm	Reference
Salmonella genus	F:GGAACGTTATTTGCGCCTGCTGAGGTAG R:GCATGG ATTTTGCC GGCG AGATTGTG	hilA	784 bp	51°C	(Ohud *et al.*, 2012)
S. enteritidis	F:TGTGTTTTATCTGATGCAA GAGG-3' R: TGAACTACGTTCGT TCTTC TGG-3'	ompc	304 bp	58°C	(Modarressi and Thong, 2010)
S. gallinarum	F:GATCTGCTGCCAGCTCAA R:GCGCCCTTTTCAAAACATA	*glg*C	300 bp	55°C	(Kang *et al.*, 2011)

Table 2: Distribution of different salmonellae isolated from different sample types collected from different districts

Sample	Distribution of isolates salmonellae in different districts															Total no. of positive samples		
	Lahore (n=14) n(%)			Gujranwala (n=15) n (%)			Sheikhupura (n=14) n(%)			Sialkot (n=12) n(%)			Narowal (n=12) n(%)					
	S.G	S.E	U.S	S.G	S.E	U.S	S.G	S.E	U.S	S.G	S.E	U.S	S.G	S.E	U.S	S.G	S.E	U.S
Liver	2(67)	0(0)	1(33)	2(50)	2(50)	0(0)	2(40)	1(20)	2(40)	2(100)	0(0)	0(0)	2(67)	1(33)	0 (0)	10(59)	4(24)	3(17)
Intestine	3(50)	2(33)	1(17)	2(50)	1(25)	1(25)	3(50)	1(17)	2(33)	2(40)	2(40)	1(20)	2(50)	1(25)	1(25)	12(48)	7(28)	6(24)
Droppings	3(60)	2(40)	0(0)	3(50)	2(33)	1(17)	2(50)	2(50)	0(0)	2(40)	2(40)	1(20)	2(40)	2(40)	1(20)	12(48)	10(40)	3(12)
Total	8(57)	4(29)	2(14)	7(50)	5(36)	2(14)	7(47)	4(27)	4(27)	6(50)	4(33)	2(17)	6(50)	4(33)	2(17)	34(51)	21(31)	12(18)

n: Number of isolates; S.G: *Salmonella gallinarum*; S.E: *S. enteritidis*; U.S: Unidentified *Salmonella* serovar.

Table 3: Antibiotic Susceptibility patterns of *Salmonellae*

Antibiotic	Disc (µg)	Antibiotic resistance profile											
		S. enteritidis (n=21)			*S. gallinarum* (n=34)			*Salmonella* spp* (n=12)			Total (n=67)		
		S n(%)	I n (%)	R n (%)	S n (%)	I n (%)	R n (%)	S n(%)	I n (%)	R n (%)	S n (%)	I n (%)	R n (%)
AMP	10	0(0)	1(4.8)	20(95.2)	0(0)	0(0)	34(100)	0(0)	0(0)	12(100)	0(0)	1(2)	66(98)
AMX	30	1(4.8)	0(0)	20(95.2)	0(0)	2(5.9)	32(94.1)	0(0)	0(0)	12(100)	1(1.5)	2(3)	64(95.5)
CFM	5	16(76.1)	2(9.5)	3(14)	23(67.6)	03(8.9)	08(23.5)	8(66.6)	1(8.3)	03(25)	51(76.1)	2(3)	14 (20.9)
CRO	30	12(57.2)	2(9.5)	7(33.3)	17(50)	9(26.5)	8(23.5)	6(50)	3(25)	3(25)	35(52.2)	14(20.9)	18(26.9)
CTX	30	11(52.4)	2(9.5)	8(38.1)	25(73.5)	1(3)	8(23.5)	7(58.3)	0(0)	5(41.7)	43(64.1)	3(4.5)	21(31.4)
CAZ	30	0(0)	10(47.6)	11(52.4)	0(0)	12(35.3)	22(64.7)	0(0)	10(83.3)	2(16.7)	0(0)	32(47.7)	35(52.3)
CN	30	7(33.3)	3(14.3)	11(52.4)	13(38.2)	0(0)	21(61.8)	2(16.7)	1(8.3)	9(75)	22(32.9)	4(5.9)	41(61.2)
TE	30	7(33.3)	1(4.8)	13(61.9)	12(35.3)	2(5.9)	20(58.8)	5(41.7)	0(0)	7(58.3)	24(35.8)	3(4.5)	40(59.7)
CHL	30	8(38.1)	4(19)	9(42.9)	10(29.4)	2(5.9)	22(64.7)	1(8.3)	1(8.3)	10(83.4)	19(28.4)	7(10.4)	41(61.2)
NAL	30	0(0)	0(0)	21(100)	0(0)	1(3)	33(97)	0(0)	0(0)	12(100)	0(0)	1(1.5)	66(98.5)
CIP	5	2(9.5)	3(14.3)	16(76.2)	9(26.5)	5(14.7)	20(58.8)	3(25)	0(0)	9(75)	14(20.9)	8(11.9)	45(67.2)
SXT	25	10(47.6)	5(23.8)	6(28.6)	17(50)	10(29.4)	7(20.6)	7(58.3)	0(0)	5(41.7)	34(50.7)	15(22.4)	18(26.9)

*unidentified Salmonella serovar; n: number of isolates; AMP: ampicillin; AMX: amoxicillin; CFM: cefixime; CRO: ceftriaxone; CTX: cefotaxime; CAZ: ceftazidime; CN: gentamicin; TE: tetracycline; CHL: chloramphenicol; NAL: nalidixic acid; CIP: ciprofloxacin; SXT: sulfamethoxazole.

Table 4: District wise Antibiotic resistance pattern of salmonellae isolated from poultry

Antibiotic	District wise Antibiotic resistance pattern of salmonellae														
	Lahore (n=14)			Sheikhupura (n=15)			Gujranwala (n=14)			Sialkot (n=12)			Narowal (n=12)		
	S n(%)	I n (%)	R n (%)	S n (%)	I n (%)	R n (%)	S n(%)	I n (%)	R n (%)	S n (%)	I n (%)	R n (%)	S n (%)	I n (%)	R n (%)
AMP	0(0)	0(0)	14(100)	0(0)	1(6.7)	14(93.3)	0(0)	0(0)	14(100)	O(0)	0(0)	12(100)	0(0)	O(0)	12(100)
AMX	0(0)	0(0)	14(100)	0(0)	2(13.3)	13(86.7)	1(7.1)	0(0)	13(92.9)	0(0)	0(0)	12(100)	0(0)	0(0)	12(100)
CFM	10(71.5)	1(7.1)	3(21.4)	11(73.3)	1(6.7)	3(20)	11(78.6)	0(0)	3(21.4)	10(83.3)	0(0)	2(16.7)	9(75)	0(0)	3(25)
CRO	8(57.1)	3(21.4)	3(21.4)	9(60)	3(20)	3(20)	7(50)	3(21.4)	4(28.6)	7(58.4)	1(8.3)	4(33.3)	4(33.4)	4(33.3)	4(33.3)
CTX	8(57.1)	01(7.1)	5(35.7)	12(80)	1(6.7)	2(13.3)	7(50)	1(7.1)	6(42.9)	8(66.7)	0(0)	4(33.3)	8(66.7)	0(0)	4(33.3)
CAZ	0(0)	8(57.1)	6(42.9)	0(0)	9(60)	6(40)	0(0)	6(42.9)	8(57.1)	0(0)	5(41.6)	7(58.4)	0(0)	4(33.3)	8(66.7)
CN	6(42.9)	1(7.1)	7(50)	5(33.3)	1(6.7)	9(60)	4(28.5)	0(0)	10(71.5)	3(25)	1(8.3)	8(66.7)	4(33.3)	1(8.3)	7(58.4)
TE	5(35.7)	0(0)	9(64.3)	7(46.7)	0(0)	8(53.3)	4(28.6)	2(14.3)	8(57.1)	5(41.6)	0(0)	7(58.4)	3(25)	1(8.3)	8(66.7)
CHL	1(7.1)	3(21.4)	10(71.5)	3(20)	0(0)	12(80)	7(50)	2(14.3)	5(35.7)	1(8.3)	2(16.7)	9(75)	7(58.4)	0(0)	5(41.6)
NAL	0(0)	0(0)	14(100)	0(0)	0(0)	15(100)	0(0)	1(7.1)	13(92.9)	0(0)	0(0)	12(100)	0(0)	0(0)	12(100)
CIP	2(14.3)	3(21.4)	9(64.3)	4(26.7)	1(6.7)	10(66.6)	3(21.4)	2(14.3)	9(64.3)	3(25)	1(8.3)	8(66.7)	2(16.7)	1(8.3)	9(75)
SXT	10(71.5)	03(21.4)	01(7.1)	11(73.3)	1(6.7)	3(20)	5(35.7)	4(28.6)	5(35.7)	4(33.3)	4(33.4)	4(33.3)	4(33.3)	3(25)	05(41.7)

DISCUSSION

Salmonella cause major health problems in humans and poultry birds. *Salmonella gallinarum* cause fowl typhoid in poultry (Paiva *et al.,* 2009). Fowl typhoid is controlled by vaccination but it is still present in poultry industry worldwide (Penha Filho *et al.*, 2016). *Salmonella enteritidis* cause ovarian infection in layer and food poisoning in humans. Food poisoning caused by *Salmonella* is one of the leading causes of food borne infections (Majowicz *et al.,* 2010). Antibiotics are commonly used for the control and treatment of salmonellae and other bacterial infections in poultry which result in emergence of antibiotic resistance. Emergence and transmission of antibiotic resistant salmonellae from poultry to human food chain is one of major threats to public safety (Yoon *et al.,* 2017). Present study reports the antibiotic susceptibility pattern of *S. enteritidis*, *S. gallinarum* and other salmonellae isolated from poultry. Present study employed *Salmonella* genus specific, *S. gallinarum* specific and *S. enteritidis* specific PCR for rapid detection. PCR based identification of *S. enteritidis* and *S. gallinarum* have been preferred in previous studies as well (Yoshida *et al.*, 2016). Out of 67 salmonellae isolated in present study, 34 were *S. gallinarum* and 21 were *S. enteritidis*, whereas 12 isolates remained unidentified. Results of present study are in accordance with previous reports, which also described that *S. gallinarum* and *S. enteritidis* are highly prevalent in poultry (Lye *et al.*, 2010).

Antibiotic resistance in bacteria of poultry origin including *Salmonella* has increased with time (Álvarez-Fernández *et al.,* 2012). There are many reports of high prevalence of multiple drug resistant and extensively drug resistant *Salmonella* in poultry throughout the world, including Pakistan (Akhtar *et al.,* 2010; Asif *et al.,* 2017; Yoon *et al.,* 2017). Current study reported high level of resistance to penicillin group of antibiotics (ampicillin, amoxicillin) which is in accordance with previous studies (de Oliveira *et al.,* 2005; Akhtar *et al.,* 2010; Álvarez-Fernández *et al.,* 2012; Asif *et al.,* 2017). Resistance to nalidixic acid (98.5%) and ciprofloxacin (67.2%) is also in accordance with previous studies where a high level of resistance to quinolones has been reported in *Salmonella* (de Oliveira *et al.,* 2005; Yoon *et al*., 2017; Nhung *et al.,* 2017). Parvej *et al.* (2016) reported slightly lower level of ciprofloxacin resistance (46.4%) in *Salmonella enterica* as compared to present study. Resistance to third generation cephalosporins in *Salmonella* of poultry origin as reported in current study is alarming and use of these antibiotics should be monitored carefully. A low level of resistance to ceftriaxone (14.42%), ceftazidime (22.85%) and cefotaxime (20%) has also been reported in *Salmonella* of poultry origin from Kohat, Pakistan (Ramadhan *et al.,* 2017). In present study, resistance to sulfamethoxazole (26.9%), gentamicin and tetracycline was on the lower side as compared to some of the previous studies. Asif *et al.* (2017) reported 80% resistance to tetracycline while Taddele *et al.* (2012) reported 100% resistance to gentamicin.

Conclusions: Occurrence of salmonellae showing resistance to commonly used antibiotics (ampicillin, amoxicillin, nalidixic acid, ciprofloxacin, chloramphenicol and tetracycline especially to third generation cephalosporins) in poultry insinuates for continuous monitoring and regulation of antibiotic use in poultry sector. It also insinuates for exploration of alternatives to antibiotics including medicinal plants, probiotics and bacteriophages for control and treatment of salmonellae in poultry and to prevent its subsequent transmission to human food chain.

Acknowledgements: This research work was partially funded by Higher Education Commission/NRPU project # 7069/Punjab/NRPU/R&D/HEC/2017.

Authors contribution: SY, MN, KA and AAA designed the research. SY, MN and MAA collected samples. SY, NU and AM performed experiments. SY, MN, AAA and KA analyzed the data. SY, MAA, AM and MN prepared the manuscript. All authors contributed in manuscript revision and approved the final version for submission.

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