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RESEARCH ARTICLE

Molecular Epidemiology of Zoonotic *Salmonella Enteritidis* Isolated from Poultry and Human Sources by Multi Locus Sequence Typing

Shakera Sadiq[1*], Mansur-ud-Din Ahmad[1], Mamoona Chaudhry[1], Haroon Akbar[2], Muhammad Hassan Mushtaq[1], Faisal Shehzad[1], Saima Hassan[1] and Muhammad Umar Zafar Khan[3]

[1]Department of Epidemiology and Public Health, University of Veterinary and Animal Sciences, Lahore, Pakistan
[2]Department of Parasitology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[3]Lanzhou Veterinary Research Institute Chinese Academy of Agricultural Sciences Beijing China
*Corresponding author: shakera.sadiq@uvas.edu.pk

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A B S T R A C T

Salmonella enterica, *sub specie* enterica, serovar *Enteritidis* is still one of the major zoonotic serotypes responsible for infection in poultry and humans worldwide. For the tracking of bacterial sources and to determine the distribution of *Salmonella* from infected people, typing technologies are highly essential. Multilocus sequence typing is one of the subtyping techniques based on sequencing of three to seven well conserved housekeeping genes within the bacterial genome. It is a valuable tool for exploring the genetic diversity of known serovars and the identification of genetic clusters of *Salmonella*. The present study was conducted in Lahore from January 2019 to June 2019. A total of 320 samples were collected from poultry (n=250) and human sources (n=70). *Salmonella* was phenotypically confirmed by biochemical tests and molecularly by targeting the *ompc* gene for genus and *sdf1* gene for serovar *Enteritidis*. MLST was performed on three *Salmonella Enteritidis*, and three untyped isolates. All the *S. Enteritidis* isolates fall in ST (11) with eBG4. MLST identified the untyped isolates as *Salmonella Infantis* (ST 32), *Salmonella Agona* (ST13), and *Salmonella Brederny* (ST505). The results of MLST showed the clonal relationship between *S. Enteritidis* isolated from poultry and human sources. MLST scheme resulted in total of 4 ST's with all *S. Enteritidis* fall in (ST 11). Phylogenetic analysis revealed the same genetic lineage between all *S. Enteritidis* and a different lineage for other isolated serovars. In conclusion the results of present study suggested that MLST can be used as a subtyping technique to find out clonal relationship between seovars and can also be used as alternative method for serotyping of *Salmonella*.

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INTRODUCTION

Salmonella enterica *sub spp.* enterica serovar *Enteritidis* is zoonotic serotype responsible for infection in poultry and humans worldwide (Bakeri *et al.*, 2003; Mammina *et al.*, 2012; Toro *et al.*, 2016; Guard *et al.*, 2018). It is also considered as one of the emerging foodborne zoonosis and evidence depicts a clear epidemiological association between human *Salmonella* outbreaks and consumption of commercial poultry products (mainly undercooked meat and eggs) (Deng *et al.*, 2014; Borges *et al.*, 2017).

For the tracking of bacterial sources and to determine the distribution of *Salmonella* from infected people typing technologies are highly essential (Foley *et al.*, 2009). Subtyping of *Salmonella Enteritidis* is difficult as it is more genetically homogenous and its clonal circulation is less as compared to other serotypes (Mammina *et al.*, 2012; Deng *et al.*, 2014). Traditional methods of phenotypic typing such as serotyping, bio-typing, phage typing and antimicrobial susceptibility testing are insufficient for epidemiological purposes (Lukinmaa *et al.*, 2004; Hyeon *et al.*, 2013) Molecular subtyping methods have made revolutions in epidemiological identification of microbial strains. These methods have

been developed based on of three main mechanisms of discrimination between microbial strains: (1) restriction analysis of bacterial DNA, (2) amplification of particular genes, polymerase chain reaction (PCR), and (3) DNA sequence polymorphism, identification at specific loci in the genome (Foley *et al.*, 2009). However, many of these have not been standardized internationally.

MLST was first described and developed in 1998 (Maiden *et al.*, 1998) and has now become the gold standard for population genetic analyses and long term epidemiology of pathogenic microbes (Achtman *et al.*, 2012). MLST is an important tool for exploring the genetic diversity of known serovars and the identification of genetic clusters. This technique is highly suitable for long term global epidemiology of pathogens. It is now used to characterize several pathogenic microbes and has rapidly been gaining recognition as one of the best molecular sub-typing techniques (Kotetishvili *et al.*, 2002; Zakaria *et al.*, 2020). This technique is based on the sequencing of the number of different housekeeping genes (Maiden *et al.*, 1998). The MLST approach only uses a small fraction of the whole genome (that is usually between 3 and 7 housekeeping genes of approximately (450-500 base pairs), which is considered to be a representative of entire genome diversity (Didelot and Maiden, 2010). MLST is fully portable, can be used as a powerful tool for global epidemiology as data is stored in a single MLST database and can be interrogated via the internet electronically (Urwin and Maiden, 2003). This scheme is highly objective and allows the comparison of isolates between laboratories and the evolutionary relationships between isolates from diverse sources can be inferred (Hughes *et al.*, 2010). Multilocus sequence typing (MLST), has also been used to subtype *Salmonella* strains for epidemiological evaluation and genetic relatedness (Kotetishvili *et al.*, 2002; Wang *et al.*, 2020).

According to author's knowledge, no study has been conducted in Pakistan on Multilocus sequence typing of *Salmonella Enteritidis*. So the present study was conducted for molecular epidemiology of *Salmonella Enteritidis* through a Multi-locus sequence typing technique.

MATERIALS AND METHODS

Sample collection: A cross sectional study was conducted in Lahore from January 2019 to June 2019. A total of 320 samples were collected from commercial broiler farms (n=150), poultry meat retail shops (n=100), hand swabs of poultry farm workers (n=50) and stool samples of diarrheal patients (n=20). All the samples were properly labelled and transferred to laboratory of Epidemiology and Public Health for further microbiological processing.

Isolation and biochemical confirmation of *Salmonella:* *Salmonella* was isolated according to ISO protocol: 6579:2002. All the samples were pre-enriched in tetrathionate broth (CM0029, Oxide). For culturing and isolation of *Salmonella*, Xylose Lactose Tergitol™ 4 (XLT4, CM1061, Oxide) media was used. Presumptive well isolated, pink with black centered colonies (Fig. 1) were further confirmed by biochemical tests (triple sugar iron test (TSI) and urease test).

Molecular confirmation of *Salmonella Enteritidis* strains: All the phenotypic confirmed isolates were further molecularly confirmed by PCR. The PCR was optimized for the molecular confirmation of genus *Salmonella* and serovar *Enteritidis*. PCR amplification of the *ompc* gene (204 bp) encoding biosynthesis of outer membrane protein C, was used for the preliminary identification of genus *Salmonella* (Alvarez *et al*., 2004). *Sdf1* (304 bp) gene which is reported to be highly unique and specific for serovar *Enteritidis* was used for the identification of *Salmonella Enteritidis* (Agron *et al.*, 2001; Alvarez *et al.*, 2004; Clavijo *et al.*, 2006).

Selection of *Salmonella* isolates for MLST: Due to financial constrains a total of six isolates (*Salmonella Enteritidis*, n=3 untyped *Salmonella* genus, n=3) selected randomly, were used for Multi Locus Sequence typing. Among the selected *S. Enteritidis* strains two were selected from poultry source and, one from human source (stool sample). Similarly, two untyped *Salmonella* were selected from poultry source and one from human source.

DNA extraction: For MLST analysis all isolates were refreshed in Luria-Bertani (LB)) broth with an overnight incubation at 37ºC. Genomic DNA was extracted from purified colonies by using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer protocol.

Optimization of Multi locus Sequence Typing technique: Multilocus sequence typing technique was optimized for seven housekeeping genes following the protocol of (Kidgell *et al.*, 2002) in the Department of Epidemiology and Public Health, University of Veterinary and Animal Sciences (UVAS), Lahore. The seven genes used for MLST were *aroC* (chorismate synthase), *dnaN* (DNA polymerase III beta subunit), *hemD* (uroporphyrinogen III cosynthase), *hisD* (histidinol-dehydrogenase), *purE* (phosphoribosylaminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase) and *thrA* (aspartokinase+homoserine dehydrogenase). The following primers were used for PCR amplification (Table 1).

Preparation of reaction mixture: For the amplification of the genes, 50 µl reaction mixtures were prepared in seven individual PCR tubes. The reaction mixture consisted of 4ul template DNA, 2ul forward primer, 2ul reverse primer, 25ul master mix (Lucigen, USA), and remaining 17ul DNase RNase free water to make the total volume 50ul. Negative control was also prepared with all components except template DNA. All the seven PCR tubes were placed in a thermal cycler (Bio-Rad).

Cycling conditions: The cycling conditions comprises of 30 cycles with an initial denaturation temperature of 98ºC for 10 seconds, an annealing temperature of 55 ºC for 10 seconds and, a final extension temperature of 72ºC for 1 minute (Noda *et al.*, 2011). Reactions were carried out in thermal cycler (Bio-Rad).

All the amplified PCR products were observed as positive bands by running on (1.5%) agarose gel stained with ethidium bromide using gel electrophoresis system (Bio Rad). Bands were visualized in Gel Doc system (Syngene).

Sequencing of PCR products: All the positive PCR products were purified and sequenced commercially from Macrogen Company, Korea. The following sequencing primers were used. (Table 2) (Kidgell *et al.*, 2002).

RESULTS

The results revealed 16% (40/250) prevalence of *Salmonella* in poultry samples while 7 % (5/70) in human samples. Among the positive poultry samples 5(12%) isolates were confirmed as *Salmonella Enteritidis* and among the positive human samples 1(20%) isolates was detected as *Salmonella Enteritidis* (Fig. 2). A total of six isolates (*S. Enteridis* n=3, untypes *Salmonella* n=3) were selected randomly for MLST analysis.

Analysis of sequences: The PubMed MLST scheme assigns allele numbers to isolates and according to these allele numbers specific sequence types (ST's) are assigned. Genetically close sequence types (ST's) are then combined in e Brust Groups (eBG). All the sequences (forward and reverse) of samples and references were combined and trimmed using the BioEdit software and submitted to PubMed MLST scheme for assigning either existing or novel allele numbers against seven housekeeping genes *(aroC, dnaN, hemD, hisD, purE, succA, thrA*). The seven genes in all *S.Enteritidis* represents the same allele numbers *(aroC=5, dnaN=2, hemD=3, hisD=7, purE=6, succA=6, thrA=11)*. On the basis of these allelic profiles all *S.Enteritidis in* the present study belonged to ST 11, and eBG 4. The untyped serovars were identified as *Salmonella* serovar *Bredernay*, *(aroC=157, dnaN=142, hemD=49, hisD=16, purE=40, succA=35, thrA= 3)* (ST=505; eBG=119), *Salmonella* serovar *Agona*, *(aroC=3, dnaN=3, hemD=7, hisD=4, purE=3, succA=3, thrA=7)* (ST=13; eBG=54), and *Salmonella Infantis*, *(aroC=17, dnaN=18, hemD=22, hisD=17, purE=5, succA=21, thrA=19)* (ST=32; eBG=31) (Table 3).

Phylogenetic Analysis: All the allelic sequences of each isolate were concatenated into the sequence of *aroC, dnaN, hemD, hisD, purE, succA, thrA* with a composite sequence length of 3,336 bp. The sequences were then aligned by using multiple sequence alignment (MUSCLE) in MEGA X software. Evolutionary analysis was performed by constructing a Neighbor-joining tree with Kimura 2 algorithm. *Salmonella* serovar *Bredernay*, *Agona*, and *Infantis* were selected as outgroups. Mean pairwise and overall distances among samples were calculated after jukes-Canter correction. Mean overall distance among samples was 0.03. Mean pairwise distance between three pairs of samples excluding MLST reference is given in Table 4.

Phylogenetic analysis: According to phylogenetic analysis *S. Enteritidis* isolates from poultry and human sources follow the same genetic lineage of ST (11). In MLST all concatenated sequences of ST (11) belong to the clades of ST (136) and ST (183). The (Fig. 3) represents the same genetic lineage of ST (11) with ST (11)/MLST reference, ST (11) (Kidgell, 2002) and clades of ST (136)/MLST scheme, ST (183)/MLST scheme. The other three *Salmonella Infantis*, *Agona* and *Bredernay* follow the different lineage with ST (32), ST (13) and ST (505).

DISCUSSION

Salmonella enterica serovar *Enteritidis*, is one of the most common non-typhoidal zoonotic bacteria responsible for the highest incidence of foodborne salmonellosis in humans worldwide (Retamal *et al.*, 2015). Multilocus sequence typing of *Salmonella Enteritidis* was performed for the first time in Pakistan. We have successfully used this technique for molecular epidemiology of *Salmonella Enteritidis* isolated from human and poultry samples. As the serotyping facility of *Salmonella* is not commercially available in Pakistan so we also used this technique as an alternative method of serotyping as suggested by Achtman *et al.* (2012). MLST was performed on three *Salmonella Enteritidis and* three untyped *Salmonella* isolated from poultry and human samples.

Table 1: Selection of specific primers for the Multilocus sequence typing of *Salmonella Enteritidis* (Achtman *et al.*, 2012)

Primer	Sequence 5′–3′
aroCforward	5'-CCTGGCACCTCGCGCTATAC-3'
aroCreverse	5'-CCACACACGGATCGTGGCG-3'
dnaNforward	5'-ATGAAATTTACCGTTGAACGTGA-3'
dnaNreverse	5'-AATTTCTCATTCGAGAGGATTGC-3'
hemDforward	5'-GAAGCGTTAGTGAGCCGTCTGCG-3'
hemDreverse	5'-ATCAGCGACCTTAATATCTTGCCA-3'
hisDforward	5'-GAAACGTTCCATTCCGCGCAGAC-3'
hisDreverse	5'-CTGAACGGTCATCCGTTTCTG-3'
purEforward	5'-ATGTCTTCCCGCAATAATCC-3'
purEreverse	5'-TCATAGCGTCCCCCGCGGATC-3'
sucAforward	5'-AGCACCGAAGAGAAACGCTG-3'
sucAreverse	5'-GGTTGTTGATAACGATACGTAC-3'
thrAforward	5'-GTCACGGTGATCGATCCGGT-3'
thrAreverse	5'-CACGATATTGATATTAGCCCG-3'

Table 2: Primers for the sequencing reactions: (Achtman *et al.*, 2012)

Primer	Sequence 5′–3′
aroC forward(s)*	5'-GGC ACC AGT ATT GGC CTG CT-3'
aroC reverse (s)	5'-CAT ATG CGC CAC AAT GTG TTG-3'
dnaN forward (s)	5'-CCG ATT CTC GGT AAC CTG CT-3'
dnaN reverse (s)	5'-CCA TCC ACC AGC TTC GAG GT-3'
hemD forward (s)	5'-GTG GCC TGG AGT TTT CCA CT-3'
hemD reverse (s)	5'-GAC CAA TAG CCG ACA GCG TAG-3'
hisD forward (s)	5'-GTC GGT CTG TAT ATT CCC GG-3'
hisD reverse (s)	5'-GGT AAT CGC ATC CAC CAA ATC-3'
purE forward (s)	5'-CGC ATT ATT CCG GCG CGT GT-3'
purE reverse (s)	5'-CGC GGA TCG GGA TTT TCC AG-3'
sucA forward (s)	5'-AGC ACC GAA GAG AAA CGC TG-3'
sucA reverse (s)	5'-GGT TGT TGA TAA CGA TAC GTA C-3'
thrA forward (s)	5'-ATC CCG GCC GAT CAC ATG AT-3'
thrA reverse (s)	5'-CTC CAG CAG CCC CTC TTT CAG-3'

s* primers for sequencing.

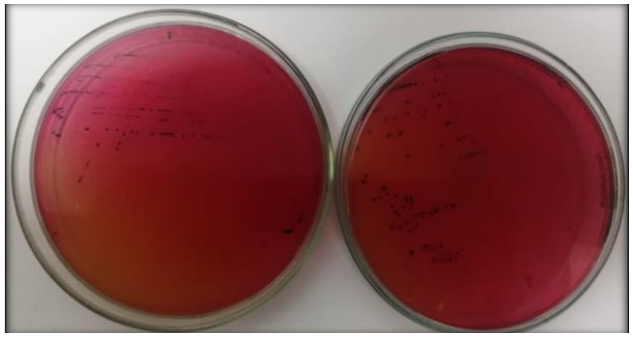

Fig. 1: Phenotypic confirmation of *Salmonella* on XLT 4 media (pink with black centered colonies).

Table 3: Allelic profiles and designation of sequence types through MLST scheme

Sample ID	Source	Country	aroC	dnaN	hemD	hisD	purE	sucA	thrA	ST	eBG	Serovar
CH19	Poultry	Pakistan	5	2	3	7	6	6	11	11	4	*Enteritidis*
CH15	Poultry	Pakistan	5	2	3	7	6	6	11	11	4	*Enteritidis*
Human Sample	Stool sample	Pakistan	5	2	3	7	6	6	11	11	4	*Enteritidis*
LF	Poultry farm	Pakistan	157	142	49	16	40	35	3	505	11	*Bredernay*
H2TM	Human hand swab	Pakistan	3	3	7	4	3	3	7	13	54	*Agona*
AF4	Poultry farm	Pakistan	17	18	22	17	5	21	19	32	31	*Infantis*

Table 4: Mean pairwise distances by Jukes-Cantor model

Salmonella_Enteritidis _Reference_from_MLST_Scheme_(ST11)									
Salmonella_Enteritidis /ST(11)/(Kidgel2002)	0.011								
ST_136/MLST_Scheme	0.001	0.012							
ST_183/MLST_Scheme	0.000	0.012	0.001						
Chicken_Sample_number_15/ *Salmonella_Enteritidis* /Pakistan/2019/ST(11)	0.000	0.011	0.001	0.000					
Chicken_sample_number_19/*Salmonella_Enteritidis* /Pakistan/2019/ST(11)	0.000	0.011	0.001	0.000	0.000				
Human_sample/*Salmonella_Enteritidis* /Pakistan/2019/ST(11)	0.000	0.011	0.001	0.000	0.000	0.000			
Chicken_sample/*Salmonella_Agona*/Pakistan/2019/ST(13)	0.057	0.060	0.058	0.057	0.057	0.057	0.057		
Human_sample/*Salmonella_Bredeney*/Pakistan/2019/ST(505)	0.059	0.060	0.060	0.059	0.059	0.059	0.059	0.015	
Chicken_sample/*Salmonella_Infantis*/Pakistan/2019/ST(32)	0.050	0.061	0.051	0.050	0.050	0.050	0.050	0.014	0.014

The numbers of base substitutions per site between sequences are shown. Analyses were conducted using the Jukes-Cantor model [1]. This analysis involved 9 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 3337 positions in the final dataset.

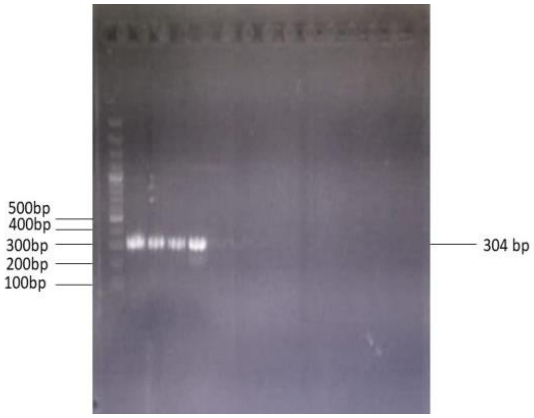

Fig. 2: Gel image showing amplification bands of *Salmonella Enteritidis* targeting *sdf I* gene (304 bp).

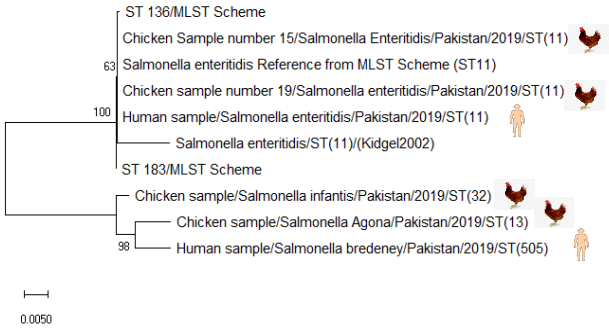

Fig. 3: Phylogenetic analysis of human and poultry isolates: Evolutionary analysis by Maximum Likelihood method: The phylogenetic analysis was inferred by using the Maximum Likelihood method and Kimura 2-parameter model [1]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 10 nucleotide sequences. There were a total of 3291 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2].

According to our first-time reported results, all the *Salmonella Enteritidis* strains were characterized as ST (11). MLST scheme did not detect any difference in nucleotides on seven housekeeping genes. All the *S. Enteritidis* strains were assigned the same allelic numbers according to Pubmed MLST scheme *(aroC=5, dnaN=2, hemD=3, hisD=7, purE=6, succA=6, thrA=11)*. The results of our study are inconsistent as reported from Japan where all 30 *S.Enteritidis* strains belong to ST (11) (Noda *et al.*, 2011), reported from Iran where all 76 *S. Enteritidis* strains belong to ST(11) (Ghaderi *et al.*, 2015), from Brazil where among 46 *S. Enteritidis* strains, 44 belong to (ST 11) (Campioni *et al.*, 2015) and in Korea where all 20 *S. Enteritidis* belong to ST(11) (Hyeon *et al.*, 2013). Many *Salmonella* STs are clustered in one group called as eBGs (e Brust groups) which comprises of genetically related isolates. ST (11) is the most common ST in eBG4 that predominately comprises of *Salmonella Enteritidis* (Achtman *et al.*, 2012).

The findings of our study in comparison to other studies reported showed no allelic differences in seven housekeeping genes with the same ST (11). The absence of mutations in housekeeping genes suggested the clonal ST lineage remained survived from the last three decades without any genetic changes being produced as a result of mutations (Baumberg, 1995; Noda *et al.*, 2011).

The concatenated sequence analysis showed, ST (11) belongs to clade ST (136) and ST (183) (Fig. 5.1). *Salmonella* Agona (ST13), *Bredernay* (ST32), and *Infantis* (ST505) were used as outgroup belongs to a different lineage from ST11.In our study despite the fewer number of samples, all belong to ST (11) which justify that as reported from all the above-mentioned studies, a highly clonal population of *S. Enteritidis* is found in humans and poultry food samples of Pakistan also. According to the study in Japan from 1973 to 2004, Noda and his colleagues further supported the speculation of Rabsch that from 1980s ST (11) lineage of *S. Enteritidis* acquired a niche in reproductive tissues of chicken which then remain persist in the population (Rabsch *et al.*, 2000; Noda *et al.*, 2011) and the same speculation can be applied to our country where *S. Enteritidis* in poultry and human samples follow the same lineage.

On the other side only ST (11) consists of human *S. Enteritidis* strains according to the global MLST scheme. MLST can be used as a replacement for serotyping for the designation of serovars to isolates as it is less technically demanding. In this study due to financial constraints, we could perform MLST on only three untyped *Salmonella* isolates which were identified as *S. Agona*, *S. Infantis* and *S. Bredernay*. Not even a single study in Pakistan has

reported the prevalence of *S. Agona* and *S. Bredernay* before and only one study was conducted on *S. Infantis* in Faisalabad. To implement control strategies for *Salmonella* from poultry food chain and to prevent its zoonotic impact it is crucial to have knowledge about serotypes prevailing in our poultry food chain. The application of MLST for *S. Enterica* serotypes in our laboratories will be highly beneficial for researchers and ultimately for stakeholders of the poultry sector in Pakistan. However, this technique is sometimes less useful for outbreak investigation and local epidemiology due to low discriminatory power (Torpdahl *et al.*, 2005; Achtman *et al.*, 2012).

Conclusions: MLST technique can also be used for the identification of genetic clusters of *Salmonella* enterica as well as for surveillance at national and international levels.

Ethical permission: This study was approved by Ethical Review Committee for Animals and Humans, University of Veterinary and Animal Sciences, Lahore.

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Authors contribution: SS and MDA designed the study. MC and HA supervised the study. SH and FS help in collection of the samples and SS performed laboratory work, MHM and UZK help in analyses the data. All authors approved the manuscript draft.

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