Research Article

Identification and Characterization of *Salmonella Enteritidis* and *Salmonella Typhimurium* in Table Eggs In Peshawar, Pakistan

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Abstract | In the present study, we investigated the prevalence of Salmonella in various parts of table eggs collected from various union councils of Peshawar, Khyber Pakhtunkhwa Pakistan. For this purpose, 200 egg samples were collected. The culturing of Salmonella from egg shell and their contents was carried out according to ISO guidelines for Salmonella isolation. A total of twenty-two (22) eggs were positive for Salmonella species. The antibiotic susceptibility testing revealed that Salmonella isolates were resistant to ampicillin, amoxicillin/clavulanic acid, cefotaxime, and kanamycin while isolates were susceptible to chloramphenicol, gentamicin, kanamycin and streptomycin. These positive samples were further investigated and confirmed through PCR by targeting serovars specific genes i.e rfbJ, fliC, fljB for *S. Typhimurium* and ST11, SPV, SefA for *S. Enteritidis*. The prevalence of Salmonella was found to be 22 (11%), out of which 17 (77.27%) isolates were *S. Enteritidis* and 5 (22.73%) isolates were found to be *S. Typhimurium* as confirmed through PCR. The presence of infectious Salmonella in table eggs in Peshawar presents a serious public health threat and should be monitored on routine basis for the presence of Salmonella.

Keywords: Table eggs, PCR, Public Health, Salmonella.

Received | May 07, 2021 **Accepted** | Deccember 28, 2021; **Published** | March 01, 2022
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Citation | Singh S, Gupta NR, Raza ST, Singh R, Srivastava S, Singh P (2022). Identification and characterization of *Salmonella Enteritidis* and *Salmonella Typhimurium* in table eggs in peshawar, pakistan. S. Asian J. Life Sci. 10(1): 7-13.
DOI | http://dx.doi.org/10.17582/journal.sajls/2022/10.1.7.13
ISSN | 2311–0589

INTRODUCTION

Eggs are source of variety of important nutrients such as vitamin D, vitamin B12, selenium and choline. Moreover, egg yolks contain antioxidants which may help in prevention of Age-Related Macular Degeneration (AMD), an eye disease in human (Chs, E, S 2010). However, raw eggs or inaccurately cooked eggs can lead to food borne infection (Savi et al., 2011). Salmonella is considered one of the most important foodborne pathogenic organisms. There are more than 2610 known serovars of Salmonella and many of these serovars are human pathogens (Guibourdenche et al., 2010). In many cases, S. enterica serovar Enteritidis and Salmonella enterica serovar Typhimurium contamination cause the salmonellosis in human (Asif et al., 2017). It is estimated that globally 93.8 million cases of gastroenteritis occur due to Salmonella species annually and about 80.3 million of these cases are food born and 155,000 deaths (Majowicz et al., 2010). In USA, there are estimates of 1.35 million infections, 26500 hospitalizations, and 420 deaths annually due to Salmonella (CDC, 2020). Eggs in form of shell eggs, liquid, frozen and its dried products are used as an economical food source (Downes and Ito, 2001). Due to widespread use of eggs as a food source, the safety of this product is important. Eggshells and egg contents can be contaminated by the bacteria in a variety of routs, such as during egg formation in the hen reproductive system or

the environmental conditions (Howard et al., 2012). Fresh eggs can be contaminated by Salmonella species due to two possible mechanisms, i) contamination on the outer shell surface and ii) internal egg content. Internal egg content contamination can be due to penetration through the eggshell or by direct contamination of egg contents before egg laying which is due to infection of the reproductive organs. This is considered to be the major route of egg contamination and it can be controlled by applying sanitary measures at the breeders level (hygiene practices, proper treatment and eventually vaccination (George et al., 2010). Contamination of eggs with Salmonella spp may occur by horizontal or vertical transmission at any stage of production. In vertical transmission egg yolk, albumin, membranes, or eggshells are contaminated in reproductive tract of the bird before eggs laying. While in horizontal transmission Salmonella spp is penetrated during or after egg laying via the egg shell from the gut or due to contaminated feces (Shinohara et al., 2008). Consumption of infected hen eggs with Salmonella spp is associated with many outbreaks of the disease in human population. Therefore, it is essential to determine the prevalence of the zoonotic Salmonella spp in table eggs for devising control measures for the disease. The present study was thus designed to investigate the prevalence and antimicrobial spectrum of *Salmonella Typhimurium* and *Salmonella Enteritidis* in poultry table eggs sold in general stores in Peshawar.

MATERIAL AND METHODS

The present study was carried out at Veterinary Research Institute (VRI), Khyber Pakhtunkhwa, Peshawar (34.0170° N, 71.5699° E). Identification of Salmonella isolates on conventional microbiological way was performed in Tuberculosis & Veterinary Public Health (VPH) section and Pathology and Bacteriology section of Center of Microbiology and Bacteriology (CMB); whereas molecular confirmation using PCR technique was done in Genomic laboratory, VRI, Khyber Pakhtunkhwa, Peshawar.

Sample size and collection

Twenty (20) Union Councils were randomly selected and then five general stores/marketing outlets per Union Council were randomly allocated. Similarly, 2 eggs per general store were collected in sterilized plastic bag. A total of 200 eggs were randomly collected from 100 marketing outlets located in 20 Union councils of Peshawar during the period from February to August 2019. The samples were immediately transported to CMB for further processing.

Isolation of Salmonella spp

2.3 Egg shell surface. A sterile cotton swab, soaked in sterilized normal saline was swabbed on egg surface and immersed in 10 ml normal saline solution followed by transmission to 90 ml of buffered peptone water and incubated at 37°C for 18 hours (Singh et al., 2010a).

Egg albumin and yolk

Eggs surface was sterilized by immersion in 70% alcohol for 2 min, air dried in biosafety cabinet Class-II/A2 for 10 minutes then cracked with a sterile knife. Samples of egg yolks and egg albumins were examined separately by adding 5 ml of each sample to 5 ml of normal saline solution. The solution was transferred to 90 ml of buffered peptone water and incubated at 37°C for 18 hours as described by (Singh et al., 2010b).

Selective enrichment of Salmonella

One ml pre-enriched sample was added in 10 ml of Tetrathionate Broth for all samples individually and incubated at 37°C for 24 hours and then streaking was done on Salmonella Shigella (SS) Agar and incubation at 37°C for 24 hours. Black centered colonies were considered positive for Salmonella and were streaked again on SS agar to get pure culture. After getting pure culture, some colonies were preserved in nutrient broth and glycerol for further molecular studies as described by (Ahmad et al., 2020).

Antibiotic Sensitivity Test of Salmonella isolates

Twelve (12) number of isolates were tested by Kirby Bauer disc diffusion technique on Mueller Hinton Agar (MHA) for antibiotic sensitivity test. Nine antimicrobial discs (Oxoid®) were used for antimicrobial susceptibility test including Ampicillin, Chloramphenicol, Erythromycin, Gentamicin, Kanamycin, Ciprofloxacin, Streptomycin, Amoxicillin- clavulanic acid and Cefotaxime-Clavulanic Acid. Antibiotic sensitivity profile was determined as described by ("Clinical & Laboratory Standards Institute: CLSI Guidelines" n.d.).

DNA extraction

DNA extraction was carried out through heat boiling method. Samples preserved in glycerol stock were cultured on SS Agar and then a single colony of Salmonella was inoculated in 1ml Nutrient broth and incubated properly. Next day, the Eppendorf tubes were centrifuged at 12000rpm. The supernatant was discarded without disturbing the pellet. To the pellet, 200µl sterile distilled water was added and vortexed properly. Now these tubes were kept in water bath containing boiling water for ten minutes at 100°C then centrifuged at 10,000 rpm for 10 minutes. Then extracted DNA was subjected to PCR reactions.

PCR screening using specific primers

The isolates of Salmonella were confirmed through PCR using gene specific primers. A highly conserved Type III secretion system gene invA was targeted for genus confir

Table 1: Prevalence of *Salmonella Enteritidis* and *Salmonella Typhimurium* in table eggs.

Sample Type	No of Samples	Total positive n (%)	Positive for Salmonella Enteritidis n (%)	Positive for Salmonella Typhimurium n (%)
Eggs	200	22 (11.00%)	17 (77.27%)	5 (22.73%)

Table 2: Prevalence of *Salmonella Enteritidis* and *Salmonella Typhimurium* in eggs' Shell, Albumin and Yolk.

Sample Type	No of Samples	Positive for S. Enteritidis-n(%)	Positive for S. Typhimurium –n(%)	Total positive n (%)
Shell	200	07 (3.5%)	02 (1%)	9 (4.5%)
Albumin	200	07 (3.5%)	03 (1.5%)	10 (5%)
Yolk	200	06 (3%)	0	6 (3%)

Table 3: Antibiotic Sensitivity Testing of *Salmonella Enteritidis* and *Salmonella Typhimurium*

S. #	Antimicrobial Agent	Antimicrobial concentration	Total No	Result		
				Sensitive n (%)	Intermediate n (%)	Resistant n (%)
1	Ampicillin	10 µg	12	0(0)	0(0)	12 (100)
2	Chloramphenicol	30 µg	12	(7) 58.33	1(8.33)	4 (33.33)
3	Erythromycin	15 µg	12	0 (0)	0 (0)	12 (100)
4	Gentamicin	10 µg	12	9 (75)	0(0)	3 (25)
5	Kanamycin	30 µg	12	5 (41.67)	3 (25)	4 (33.33)
6	Ciprofloxacin	05 µg	12	0 (0)	6 (50)	6 (50)
7	Streptomycin	10 µg	12	5 (41.67)	4 (33.33)	3 (25)
8	Amoxicillin- Clavulanic acid	20/10 µg	12	1 (8.33)	1 (8.33)	10 (83.33)
9	Cefotaxime- Clavulanic acid	30/10 µg	12	0 (0)	6 (50)	6 (50)

Table 4: List of primers for the detection of various Salmonella genes.

Gene	Oligonucleotide Sequence	AT	Size (bp)	Reference
InvA	GCTGGTTTTAGGTTTGGCGG CAAAGGTGACGCTATTGCCG	60°C	412	(Yasin et al.,2020)
rfbJ	CCAGCACCAGTTCCAACTTGATAC GGCTTCCGGCTTTATTGGTAAGCA	65°C	663	(Moosavy et al., 2015)
fliC	ATAGCCATCTTACCAGTTCCCCC GCTGCAACTGTTACAGGATATGCC	65°C	183	(Moosavy et al., 2015)
fljB	ACGAATGGTACGGCTTCTGTAACC TACCGTCGATAGTAACGACTTCGG	65°C	526	(Moosavy et al., 2015)
ST11	GCCAACCATTGCTAAATTGGCGCA GGTAGAAATTCCCAGCGGGTACTGG	65°C	429	(Moosavy et al., 2015)
SPV	GCCGTACACGAGCTTATAGA ACCTACAGGGGCACAATAAC	65°C	250	(Moosavy et al., 2015)
SefA	GCAGCGGTTACTATTGCAGC TGTGACAGGGACATTTAGCG	65°C	310	(Moosavy et al., 2015)

mation. *S. Typhimurium* and *S. Enteritidis* were screened individually in two separate PCR reactions by targeting serovars specific genes i.e rfbJ, fliC, fljB for *S. Typhimurium* and ST11, SPV, SefA for *S. Enteritidis*. The detail of primers is given in Table 4. The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with Ethidium Bromide and visualized under gel documentation system (Fas-Digi®).

RESULTS

A total of 200 egg samples were processed in which the number of positive samples for Salmonella through selective enrichment was 22 (11.00%) as shown in Table 1. Out of 22 positive samples 17 (77.27%) were found to be *Salmonella Enteritidis*, whereas, 5 (22.73%) of the isolates were found to be *Salmonella Typhimurium* as shown in Table 1.

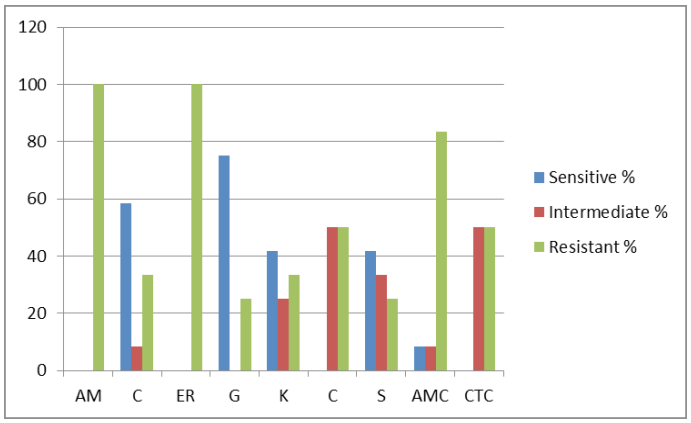

Figure 1: Antibiogram of Salmonella Isolates against various antibiotics. AM=Ampicillin, C=Chloramphenicol, ER=Erythromycin, G=Gentamicin, K=Kanamycin, C=Ciprofloxacin, S=Streptomycin, AMC=Amoxicillin-Clavulanic acid, CTC=Cefotaxime- Clavulanic acid.

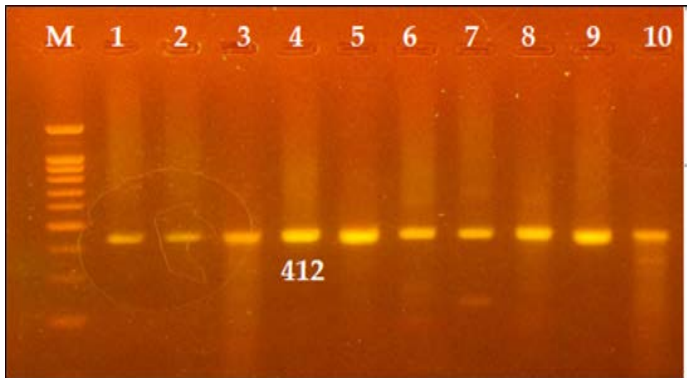

Figure 2: Gel electrophoresis image of invA gene. M represents 100bp Ladder while L4 represents 412 base pair product of invA gene.

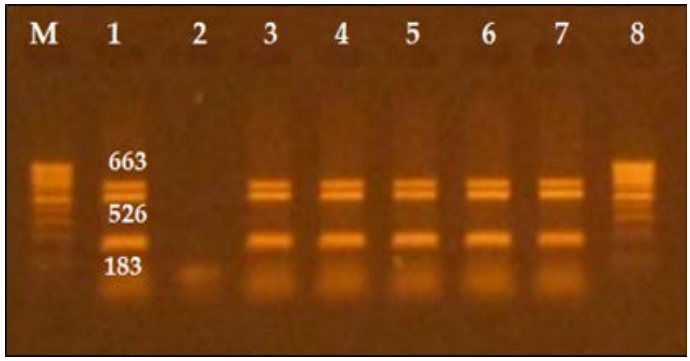

Figure 3: Gel electrophoresis image of rfbJ, fljB and fliC. M represents 100bp molecular ladder whiel L1 shows 663bp, 526bp and 183bp products of Salmonella Typhimurium specific gene i.e. rfbJ, fljB and fliC, respectively.

Total number of Salmonella isolates from different egg components was 25 (12.5%). Nine (9) (4.5%) isolates were detected in eggshell, 10 (5%) isolates were detected in albumin; whereas,7 (3.5%) isolates were detected in yolk (Table 2). The prevalence of Salmonella enterica Enteritidis was found to be 7 (3.5 %) from both eggshell and albumin contents and 6 (3%) from yolk content of eggs; whereas, the prevalence of *Salmonella Typhimurium* was found to be 2 (1%) from egg shell, 3 (1.5%) from albumin contents. The prevalence of *Salmonella Typhimurium* from egg yolk was 0 (Table 2). It is pertinent to mention here that Salmonella enterica Enteritidis was isolated from both albumin and yolk content of 03 number of eggs (Figure 5).

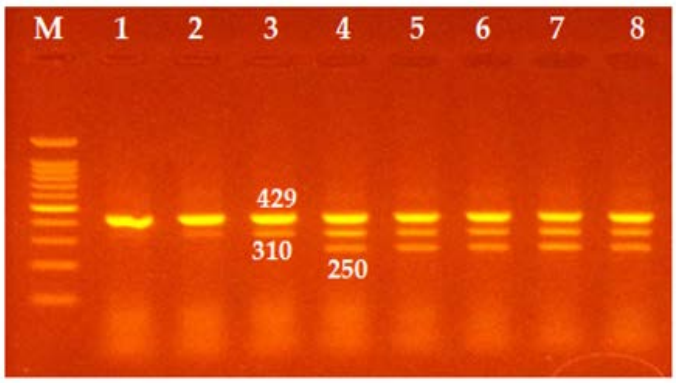

Figure 4: Gel electrophoresis image of SPV and ST11 and SefA. M represents 100bp molecular ladder whiel L1 shows 429bp, 310bp and 250bp products of Salmonella Enteritidis specific gene i.e. ST11, SefA and SPV, respectively.

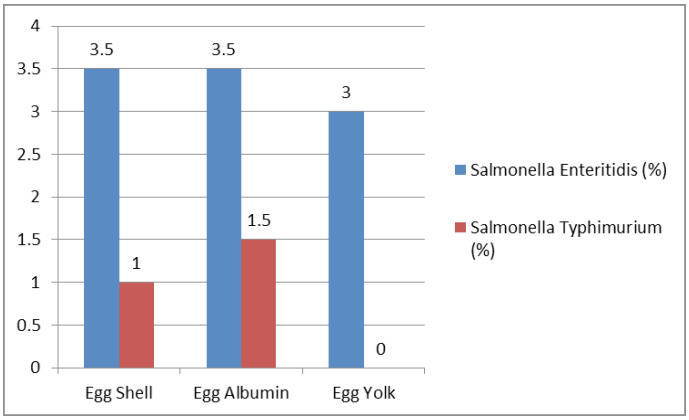

Figure 5: Percentage prevalence of *Salmonella Enteritidis* and *Salmonella Typhimurium* from egg shell and egg albumin.

The antimicrobial sensitivity pattern of 50% (n=12) isolates was done by Kirby-Bauer disk diffusion method. The Salmonella strains were interpreted as sensitive, intermediate and resistant based upon the formation of zone of inhibition using Clinical and Laboratory Standard institute (CLSI, 2019). The Salmonella Isolates were found to be highly resistant to the commonly used antibiotic in poultry industry. Highest resistance was noted against Ampicillin and Erythromycin (100%), followed by Amoxicillin-Clavulanic acid (83.33%), Ciprofloxacin and Cefotaxime-Clavulanic acid (50%), Chloramphenicol and Kanamycin (33.33%); whereas, least resistant (25%) was found against Gentamicin and Streptomycin. Gentamicin was

found highly effective against 75 % of Salmonella isolates followed by Chloramphenicol, which was effective against 58.33% of isolates; and Kanamycin and Streptomycin which were effective against 41.67 % isolates of Salmonella spp. Ampicillin, Ciprofloxacin and Cefotaxime-Clavulanic acid showed no efficacy (0%) against Salmonella isolates (Figure 1 and Table 3).

PCR screening of 25 biochemically confirmed Salmonella isolates revealed that (40%) isolates carried invA gene (Table 4). The isolates were then screened for *S. Typhimurium* and *S. Enteritidis* specific genes in two separate PCR reactions. Out of 25 isolates, 20 were identified to be *S. Enteritidis* while 5 were *S. Typhimurium*. The results of PCR are shown in Figure 2, Figure 3 and Figure 4.

DISCUSSION

The epidemiological studies depict that association between occurrence of Salmonellosis in human population and presence of Salmonella species in poultry products exist. Eggs contents can be contaminated with Salmonella species in the infected ovary of the hen. While contamination of egg shell can occur at any stage from laying to shifting to retail stores like contact with fecal material, feed, transportation, storage or during handling. In the present study, it was recorded that 11% eggs were contaminated with Salmonella. Our results were in agreement with similar studies conducted in Uruguay (9.35%) (Betancor et al., 2010) and India (7.5%) (Singh et al., 2010a).

In this study, contamination rate of Salmonella in eggs shell was found 4.5% while 8 % in eggs content (Albumin 5% and yolk 3 %). However, In similar study conducted In Pakistan, contamination rate in commercial eggs were found 40% in eggs shells while egg contents 8.33% (Akhtar et al., 2010) in which our results were in agreement with egg content contamination rate. Similarly, in another study, the prevalence of Salmonella species was recorded 34.12 % in eggs shell and 12.69% in egg content (Shahzad et al., 2012). In another study on prevalence of Salmonella species in South India, the contamination of egg shell with salmonella species was 6.1%, while in egg contents it was 1.8% (Suresh et al., 2006), the contamination value of egg shell is closed to the findings of the current study. Moreover, we recorded that in 22 contaminated eggs, 77.27% were *Salmonella Enteritidis* while 22.73% were *Salmonella Typhimurium*. These results are also in agreement with other study conducted in Pakistan in which Salmonella Enteritis was recorded as 75% while other serovars, *S. Typhimurium*, S. paratyphi B, S. pollorum and non-typable Salmonella e was less than 25% of the total isolates in poultry (Akhtar et al., 2010). *Salmonella Enteritidis* make colonies in ovaries and tissues surrounding ovaries of laying hens. There is a vertical transmission of infection from breeders to layers and which results in transmission to human through consumption of raw eggs (Akhtar et al., 2010). In a study in Europe few outbreaks occurred in human due to consumption of eggs in which, 3.5% were caused by *S. Typhimurium* and 77.2% by *S. Enteritidis* (Hazards (BIOHAZ) 2010). *Salmonella Enteritidis* is frequently isolated serovar from the egg contents and eggshells (Musgrove et al. 2005). Few studies in Iran have reported *S. Typhimurium* as a most frequent isolate (Jafari, et al., 2006). In another study, *Salmonella Typhimurium* was isolated from all of the egg shell samples (Jamshidi et al., 2010).

The use of antimicrobials in poultry industry for prevention of infections has been the source of antibiotic resistance in non-typhoidal Salmonella (Mehdi et al., 2018). The results of in vitro susceptibility test showed that all isolates were highly resistant to Ampicillin (100%), Erythromycin (100%) and Amoxicillin-Clavulanic acid (83.33%) while moderately resistant to Ciprofloxacin (50%), Cefotaxime-Clavulanic acid (50%) Kanamycin (33.333%) and Chloramphenicol (33.33%). Our results are in line with other findings who found 76.66% and 75 % of Salmonella isolates of poultry origin were resistant to Ampicillin (Rafiullah et al., 2018; Uddin et al., 2018). In similar study, 100 % of Salmonella isolates were found resistant to Erythromycin (Akhtar et al., 2010). Findings of this study are also in close agreement to another study wherein the authors reported 47.7% of Salmonella isolates were resistant to Ciprofloxacin and 41.1% to Chloramphenicol (Ahmad et al., 2020). In another study, 43% of Salmonella enterica isolates collected from poultry were found resistant to Ampicillin, Amoxicillin-Clavulanic acid, Ceftiofur, Cefoxitim and Ceftriaxone (Diarra and Malouin, 2014).

In conclusion, poultry table eggs are carrier of zoonotic strains of Salmonella in the Peshawar region that is potentially a high a threat to public health. Moreover, the dominant serovars like *S. Enteritidis* and *S. Typhimurium* are highly resistant and virulent and are of great public health concern. The results of the present study showed that both eggs shells and contents were contaminated which suggest that sources of contamination were most probably the poultry farms as well as at retail shops. Similarly, poor transportation, storage condition and handing could further enhance contamination of the eggs. Therefore, a proper surveillance system is urgently needed that could ensure a routine sampling pattern from different regions of Khyber Pakhtunkhwa to monitor trends in the burden of Zoonotic Salmonellosis due to resistant strains including the detection of epidemics. Furthermore, the use of antimicrobials in poultry industry needs to regularize to protect and improve human health. Moreover, the general public may be advised to properly disinfect the eggs before

storing and always fully cook the eggs.

ACKNOWLEDGMENT

The present study was carried out with the financial support of Director General (Research), Livestock & Dairy Development Department, Khyber Pakhtunkhwa, Peshawar; and Project Incharge, ADP Scheme: 36/170091, "Characterization of Cattle Genetic Resources of Khyber Pakhtunkhwa through Genetic Markers and Molecular Techniques", Peshawar.

CONFLICT OF INTEREST

The authors of the manuscript declare that there exists no conflict of interest among authors.

NOVELTY STATEMENT

The following statement may be written under the Novelty statement "The present study was conducted for the first time in Pakistan to determine molecular based detection of Salmonella Enteritidis and Salmonella Typhimurium from table eggs".

AUTHORS CONTRIBUTION

Conceptualization, Imtiaz Ali Shah, Khalid Khan and Muhammad Tariq Zeb; Data curation, Muhammad Hasnain Riaz and Muhammad Tariq Zeb; Formal analysis, Muhammad Hasnain Riaz and Yasin Ahmad; Funding acquisition, Faizul Hassan; Investigation, Imtiaz Ali Shah, Maleeha Anwar, Rafi Ullah, Inamullah Wazir and Khalid Khan; Methodology, Maleeha Anwar, Muhammad Hasnain Riaz, Yasin Ahmad and Muhammad Tariq Zeb; Project administration, Faizul Hassan; Resources, Rafi Ullah, Abdur Raziq, Muhammad Ijaz Ali and Faizul Hassan; Supervision, Abdur Raziq and Muhammad Ijaz Ali; Writing – original draft, Muhammad Tariq Zeb; Writing – review & editing, Irshad Ahmad, Muhammad Ibrahim Rashid, Imtiaz Ali Shah.

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