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Original Article

Source tracking of extensively drug resistant *Salmonella* Typhi in food and raw vegetables using molecular approaches

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Abstract

Introduction: Extensively drug resistant (XDR) strains of the *Salmonella* lineages have been reported to spread from Africa to South Asia. XDR strains are resistant to fluoroquinolones, chloramphenicol, co-trimoxazole, and ampicillin, resulting in treatment failure. The objectives of this study included the investigation of transmission of *S.* Typhi lineages and the identification of the potentially contaminated sources of the XDR typhoid outbreak from different urban areas by using molecular techniques.

Methodology: Environmental samples, including food samples, were collected from different towns and the susceptibility of each isolate to the antimicrobial agents was examined. Molecular identification of different *Salmonella* lineages including *S.* Typhi, *S.* Paratyphi A, H58, and XDR was carried out through multiplex PCR.

Results and Conclusions: A total of 328 environmental samples including raw vegetables, water, and bakery items were collected. More than half of the tested samples (64%) found harboring *Salmonella* spp. The *Salmonella* was confirmed through PCR amplification of species-specific markers that showed the presence of *S.* Typhi (40%), *S.* Paratyphi A (8%), H58 (7%), and XDR *S.* Typhi (6%). Raw vegetables had the highest number of *Salmonella* spp., indicating consumption of raw vegetables as a possible source of salmonellosis. XDR status was also affirmed through phenotypic antimicrobial susceptibility testing.

Key words: Antimicrobial susceptibility; drug resistance; food-borne infections; salmonellosis.

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Introduction

Typhoid is a systemic febrile disease that can be fatal, which necessitates early antibiotic treatment. It is estimated that there are 14.3 million cases of typhoid worldwide with 128,000 to 161,000 deaths annually [1,2]. The incidence rate of typhoid fever is highest in South Asia, followed by Southeast Asia, Western sub-Saharan Africa, Eastern Sub-Saharan Africa, and Oceania [3]. Pakistani citizens have the highest infection rate of *Salmonella enterica* serotype Typhi (*S.* Typhi) in Southeast Asia, with a reported rate of 493.5 cases per 100,000 people. People living in the Punjab and Sindh provinces of Pakistan are at the highest risk of contracting typhoid [4].

Antibiotic drug resistance in foodborne *Salmonella* is a major concern for public health safety. This organism spreads mostly in low-resource, socioeconomically underdeveloped places, via fecal-oral pathway. Lack of adequate sanitary facilities, tainted water, and disregard of food safety regulations are all frequent factors that create an ideal environment for the organism to flourish and transmit through various pathways, including direct animal contact, utilization of contaminated food and water, via fecal-oral route, and very infrequently through person-to-person contact [5]. In 94% of the total cases, Salmonellosis is thought to be spread by food, which may get contaminated through cross-contamination, environmental contamination, or by poor hygienic handling. The widespread presence of this pathogen in the human food chain has already been reported. In the United States, about 350 culture positive cases are reported annually to the Centers for Disease Control and Prevention (CDC). Most of these patients reported to have traveled abroad in the past 30 days [6,7].

Lately, the emergence of multidrug resistant (MDR) *S.* Typhi has caused a shift to the use of fluoroquinolones (e.g., ciprofloxacin) from conventionally consumed first-line antibiotic drugs such as, ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole [8]. For the last two decades, increasing resistance to fluoroquinolones (intermediate

or complete insensitivity to the ciprofloxacin drug) particularly in South Asia has led to the utilization of third-generation cephalosporins such as ceftriaxone as the recommended first line treatment [1-9]. Many multidrug resistant (MDR) *S.* Typhi strains have a variety of antibiotic resistance genes on self-transmissible incompatibility type (IncHI1) plasmids [1-9]. The predominant *S.* Typhi lineage in various regions of Eastern Africa and Asia is genotype 4.3.1, also known as haplotype 58 (H58), which is linked to increased levels of drug resistance and decreased susceptibility to fluoroquinolones [9]. Indeed, extensively drug resistant (XDR) *S.* Typhi is also an MDR H58 strain that had attained an IncY plasmid from an isolate of *E. coli* bearing the *bla*CTX-M-15 and *qnr*S resistance genes, which conferred resistance to ceftriaxone and third generation cephalosporins. As a result, azithromycin remained the only effective oral treatment for XDR S. Typhi. Yet, azithromycin-resistant strains of *S.* Typhi and *S.* Paratyphi A have also been reported recently from Bangladesh [10] and Pakistan [11]. It has raised serious concerns about the possibility that this mutation would eventually occur in XDR strains and will further limit its treatment options.

From the last decade, healthcare sectors in Pakistan have remained engaged in managing XDR Typhoid Fever. A typhoid outbreak due to the spread of *S.* Typhi, was described in February 2018. Between the period of November 2016 and September 2017, 339 cases of this emergent drug-resistant (XDR) strain of Typhi were recorded in Pakistan, predominantly in two cities, Hyderabad and Karachi. The most populous metropolis, Karachi, has witnessed an overwhelming increase recently. According to the Pakistan National Institute of Health, raising the total number of cases documented from January 1, 2017, till August 14, 2021, to 15,224. One case of travel-associated case was also proclaimed from the United Kingdom [10]. Even with the control measures by the local government, there was a marked increase in reported cases from 2017 to 2019 [11,12]. This continuous surge in drug resistance is alarming for the health care systems around the world, particularly for developing countries where antimicrobial resistance surveillance is not effective. World Health Organization also recommends continuous surveillance to identify the emergence of any new resistant strain to address the resistance issue promptly [13]. Therefore, this study was conducted to identify the potential sources responsible for the spread of XDR *S.* Typhi and to pave a path for the assessment of clonal transmission.

Methodology

Setting, Duration, and Study Type

A cross-sectional study was conducted in collaboration with the Pakistan Council of Scientific and Industrial Research (PCSIR) and Dow University of Health Sciences (DUHS), Karachi. The environmental samples were randomly collected from September 2020 to September 2021 from six different towns of Karachi namely Gulberg town, Gulshan town, Sadder town, Orangi town, Liaquatabad town, and Malir town. A total of 328 environmental samples were collected from various food stalls (raw vegetables, chickpea salad, *samosay*, cooked or uncooked food, etc.) shops and bakeries. The samples were collected in sterile containers (of 30 mL capacity) and were transported to the lab immediately for processing. All packaged or sealed food and bottled water were excluded from this study. The sample size was calculated through Open EPI with 7% precision and design effect (Deff) of 2 at a 95% confidence level (with an extra 10 % due to noncompliance).

Isolation of Salmonella

Pre-enrichment (non-selective enrichment)

The sample (25 g for solids and 10 mL for liquids) was added to 225 mL Lactose broth and Trypticase Soya broth (TSB) taken in a sterile, wide-mouth, screw cap bottle (500 mL) and incubated for 1 hour at room temperature. It was followed by mixing the contents through swirling and the pH was adjusted to 6.8, if necessary. After mixing, the sample was incubated at 35 ℃ for 24 hours [14].

Selective enrichment

An aliquot of 1 mL from the pre-enrichment medium was transferred to 10 mL Tetrathionate broth (TTB) while an aliquot of 0.1 mL from pre-enrichment was transferred into 10 mL Rappaport-Vassiliadis Soy Peptone (RVS) broth. Both the aliquots were incubated at 37 ℃ for 24 hours [14].

Selective plating method

After selective enrichment, a loopful of the culture from each aliquot was streaked on different selective media including Xylose Lysine Desoxycholate (XLD) agar, Hektoen enteric (HE) agar, & Bismuth sulfite (BS) agar which were then incubated at 37 ℃, for 24 hours [14].

Biochemical Characterization

According to the standard protocol outlined in the FDA's Bacteriological Analytical Manual, the

presumed colonies of *Salmonella* were then processed through a series of biochemical tests, including the Triple Sugar Iron (TSI), Lysine Iron Agar slant test (LIA), Urease and Citrate test [15].

Antimicrobial Susceptibility testing by Disc Diffusion method

Antimicrobial susceptibility pattern of the *Salmonella* spp. was determined by Kirby-Bauer's disk diffusion method on Mueller-Hinton agar (MHA) by using the commercially available antibiotic discs (OXOID) including Trimethoprim/Sulfamethoxazole (SXT), Ciprofloxacin (CIP), Chloramphenicol (C), Ampicillin (AMP), Meropenem (MEM), Ceftriaxone (CRO), Cefixim (CFM), Azithromycin (AZM). The results of disk diffusion were interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. *Salmonella* strains were classified as MDR if found resistant to trimethoprim/sulfamethoxazole, ampicillin, and chloramphenicol, whereas those MDR strains that was also resistant to ciprofloxacin and ceftriaxone were categorized as XDR [17–19].

DNA Extraction

DNA extraction was performed by using a commercially available Promega DNA extraction kit. At first, 1mL of freshly grown (overnight) bacterial culture was centrifuged for 2 minutes at 13,000–16,000 × *g* to pellet the cells, followed by the addition of 600 µL lysis solution. After gentle mixing, it was placed at 80 °C for 5 minutes and then kept at room temperature for cooling. After that RNase Solution (3 µL) was added and incubated for 15 to 60 minutes at 37 °C. After incubation, 200 µL acetone was added for protein precipitation at room temperature. After vortex and incubation for 5 minutes on ice, tubes were subjected to centrifugation for 3 minutes at 13,000–16,000 × *g*. The supernatant was shifted to a tube containing 600 µL isopropanol. The contents were mixed by inverting the tube until the development of a fine thread of DNA, which was separated by centrifugation for 3 minutes at 13,000–16,000 ×*g*. Then pellet was washed with 70% ethanol and air-dried at room temperature for 10-15 minutes. The DNA was solubilized in rehydration solution (100 µL) overnight at 4 °C or for 1 hour at 65 °C. Molecular identification was performed by using specific primers for *Salmonella*, *S.* Typhi, *S.* Paratyphi A, *S*. Typhi XDR, and *S*. Typhi H58. Primer sequences were taken from previous studies [20].

Molecular Confirmation of Salmonella species by invA gene

Salmonella was detected by PCR, targeting the *inv*A gene specific primer (Table 1) as previously described [21]. PCR reaction mixture contained nuclease-free water (5.5 µL), PCR master mix (12.5 µL) (Fischer Scientific, UK), reverse and forward primers (1 µL each), and DNA template (5 µL). The initial denaturation of the template DNA was carried out at 95 °C for 5 minutes, trailed by 29 denaturation cycles at 95 °C for 1 minute, annealing at 55 °C for 1 minute, elongation at 72 °C for 1 minute, and final extension at 72 °C for 10 minutes.

Multiplex PCR for detection of high-risk lineages of S. Typhi and S. Paratyphi A

The confirmed isolates for *Salmonella* spp. by *inv*A gene were further screened for *S.* Typhi, *S.* Paratyphi A, *S.* Typhi H58, and XDR *S.* Typhi by using multiplex PCR based on single nucleotide polymorphisms (SNPs) specified for these lineages of clinical importance. Primer sequences were taken from previous studies (Table 1) [22]. Each PCR reaction contained 5 µL of DNA, 12.5 µL of 2 × PCR Master Mix (Thermo Fischer Scientific, UK), 1 µL each of the reverse and forward primers, and 5.5 µL nuclease-free water to a final volume of 25 µL. All PCR reactions were performed on the thermal cycler (BioRad Laboratories Inc., USA) under the subsequent cycling conditions of 95 °C for 2 minutes, lined by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 45 seconds,

Table 1. Primers with amplicon sizes used in Multiplex PCR assay for each target sequence.

Primer Name	Target	Target gene	Primer sequences	Product Size
*Inv*A	*Salmonella*	*Inv*A	F 5' GTGAAATTATCGCCACGTTCGGGCA3' R 5' TCA TCG CAC CGT CAA AGG AAC C 3'	275
ST_227	*Salmonella typhi*	*STY0307*	F 5' GGC AGA TAT ACT TTC GCA GGC A 3' R 5' CCC AGA ACC AAA TTT GCT TAC A3'	227
SPA_305	*S.Paratyphi* A	*Intergenic region SSPA1732a-SSPA1724*	F 5' AGG GAT GAG AAT TTT CAG ACG T 3' R 5' ACC CCA GCT CTG AGA GAT TAT CT 3'	305
XDR_425	XDR *Salmonella typhi*	*STY0962*	F 5' TGA ATG GTT CTG GTC TGG CG 3' R 5' CTA AAC CAC GAC GGC TCA GT 3'	425
H58_509	*S. typhi* H58	*STY2513*	F 5' GGG CTT GAT GGC TTC ATT AGT 3' R 5' ACA GGT TGT ACG CCT TTC CA 3'	509

Table 2. Summary of environmental samples screened through Microbiological, biochemical and Molecular techniques.

Sampling subjects	No. of samples screened	No. of positive samples on Microbiological analysis	Biochemical Testing	*Inv*A PCR positive	*S. typhi* by Multiplex PCR	*S. paratyphi* A	H58	XDR S. *typhi*
Environmental samples	328	226	210	201	132	23	25	21

elongation at 68 °C for 1 minute, with the final extension at 68 °C for 10 minutes. The PCR amplicons were preserved at – 40 °C till future use.

Gel Electrophoresis and Visualization of PCR Products

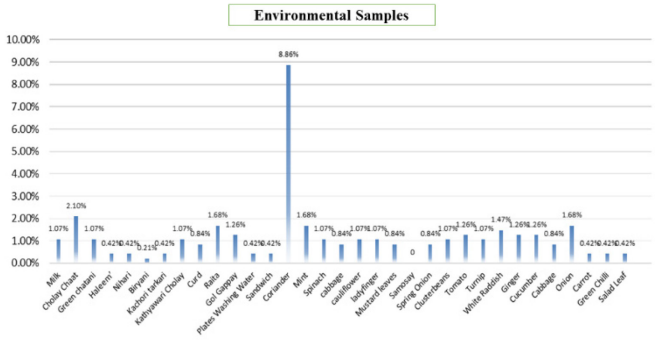
Agarose gel (1.5%) prepared in TBE buffer containing 2 µL ethidium bromide was used for the electrophoresis and visualization of PCR products. A 100-base pair ladder (New England biolabs) was used to evaluate the size of DNA products. A volume of 6 µL of amplified DNA product was loaded into the separated well. Gel electrophoresis was carried out for 45 minutes at 100 volts.

Results

Isolation of Salmonella by selective enrichment method

In this study, a total of 328 environmental samples were randomly collected from different areas of Karachi city from September 2020 to September 2021 which were microbiologically investigated through selective enrichment technique for the isolation of *Salmonella*. In all, 226 (68%) samples were contaminated with *Salmonella* as summarized in Table 2. The isolation rate of *Salmonella* spp. from the samples collected during the winter was 34%, which was significantly less as compared to the summer season, where the isolation rate was 66%. Additionally, it was observed during the collection of raw vegetables that more samples i.e., 39.5% were found contaminated with *Salmonella* sp. gathered in the evening in comparison to the samples i.e., 8% collected in the morning. The raw vegetables were also collected from the farmer's market in the morning and upon screening *Salmonella* spp. could not be isolated from any of those samples. It is correlated with the fact that there must have been a contamination source from the environment or unhygienic handling of the raw vegetables. Thus, the prevalence of *Salmonella* spp. in various samples was investigated and it was revealed that coriander had the highest occurrence, followed by chickpea salad, mint, onion, white radish and tomato while other environmental samples such as eggs with yolk and shell, curd, ice cream, sugarcane juice and other cooked food from stalls had no evidence of *Salmonella* spp. The details of environmental samples are summarized in the Figure 1.

Figure 1. Prevalence of *Salmonella* spp. in various environmental samples.



This graph exhibits the presence of *Salmonella* isolates in terms of percentages, revealing coriander to have the highest occurrence of Salmonella spp. i.e., 8.86%, followed by chickpea salad, mint, onion, white radish and tomato.

In this study, the positive *Salmonella* colonies chosen were based on the presence of pink colonies with black centers on XLD agar, grey-black colonies on BS agar, and blue-green or blue colonies with or without black centers. However, several of the analyzed samples showed yellow colonies on XLD plates, as a result of lactose fermentation, which is a defining trait used to distinguish *Salmonella* spp. from *E. coli*. Various studies reported other *S. enterica* serovars and *S. enterica* serovar Typhimurium grow as yellow colonies on XLD agar [23,24].

Biochemical Characterization

All the selected isolates were TSI positive, LIA positive, urease negative, and Citrate test positive. Thus, after microbiological and biochemical testing it can be deduced that out of a total of 328 screened samples, 210 (64%) samples were found to be contaminated with *Salmonella* (Table 2).

Antimicrobial Susceptibility testing by Disc Diffusion method

Salmonella Typhi showed the highest resistance to Ciprofloxacin (95%) and Ampicillin (80%) followed by Chloramphenicol (70%), Ceftriaxone (50%), Cifixim (40%), Trimethoprim/Sulfamethoxazole (15%), Azithromycin (2%) and no resistance shown to Meropenem. MDR cases were found as 28% (n = 58),

whereas 15% (n = 31) isolates appeared XDR. The sensitivity and resistance pattern to common antimicrobial agents is shown in Table 3.

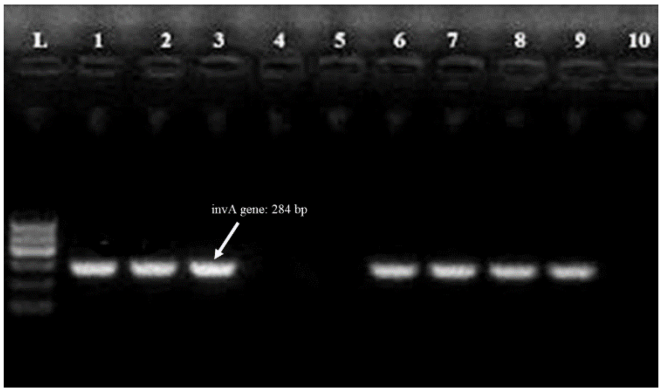
Molecular Confirmation of Salmonella spp. using PCR

All the isolates were screened and confirmed for *Salmonella* spp. by PCR using a specific pair of primers for the *inv*A gene. Out of 210 environmental samples, 201 were identified as *Salmonella* spp. through *inv*A gene primer as shown in Figure 2.

Multiplex PCR for detection of high-risk lineages of S. Typhi and S. Paratyphi A

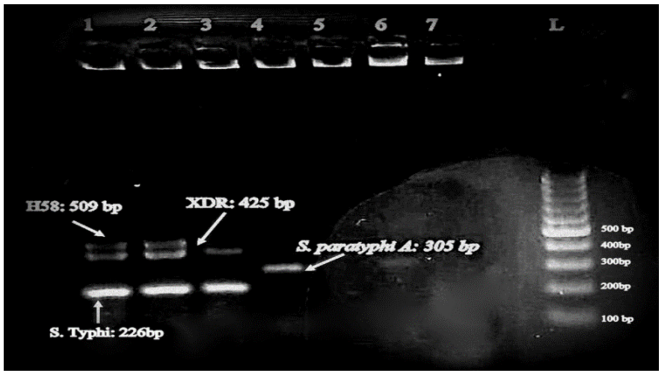
In brief, each of the positive isolates tested for the *inv*A gene went through multiplex PCR for *Salmonella*

Figure 2. DNA visualization on agarose gel showing invA gene encoding *Salmonella*.



Each lane from 1 to 9 show bands on 284 bp representing *Salmonella* spp. in environmental samples and Lane L represents DNA marker 100 bp ladder.

Figure 3. Agarose Gel image showing results of Multiplex PCR amplified products for lineage identification of *Salmonella* spp.



Results show different band depicting the presence of *Salmonella* lineages in environmental samples on the basis of respective band sizes in accordance with the ladder. Lane 1,2 and 3 are showing three bands representing *S.* Typhi at 227 bp, XDR S. Typhi at 425bp and H58 at 509 bp. Lane 4 shows a single band at 305 bp representing *S.* Paratyphi A and Lane L is showing 100bp DNA ladder.

Table 3. Antimicrobial Susceptibility Pattern of *Salmonella* isolates.

Antibiotics	**Resistance, n** (%)
Ampicillin	168 (80)
Azithromycin	4 (4)
Ceftriaxone	105 (50)
Trimethoprim	31 (15)
Ciprofloxacin	199 (95)
Chloramphenicol	147 (70)
Meropenem	0 (0)
Cifixim	84 (40)

lineage identification, which depicted that 65% (132/201) were *S.* Typhi, 11% (23/201) were *S.* Paratyphi A, 12% (25/201) were H58 and 10% (21/201) were XDR *S.* Typhi. The outcomes of *Salmonella* spp. isolation and subsequent lineage identification using multiplex PCR are shown in Table 2 and Figure 3.

Discussion

Salmonellosis is a zoonotic disease, and represents a serious medical problem, causing great concern over the consumption of contaminated food [21]. In this study, *Salmonella* spp. was isolated from different food samples (chickpea salad, *gol gappay*, *raita, kathyawari* chickpeas, etc.), and from raw vegetables (coriander, onion, mint, spinach, ladyfinger, and tomato) which in a majority of the countries including Pakistan are often consumed raw as a salad (Figure 1). In our study, coriander was the most contaminated by *Salmonella* spp. i.e., 8.86% of all collected environmental samples, which is concerning from a food safety point of view. Similar findings on the detection of *Salmonella* in raw or cooked food, beverages, and milk have also been reported previously [12,25–28]. Generally, these pathogenic bacteria are introduced in the aquatic environment primarily through untreated or treated wastewater discharge, soil leaching, and surface runoff, posing a significant risk of widespread disease outbreaks. Apart from this, *Salmonella* can adhere to plant tissues and its effective biofilm-forming ability allows it to survive under adverse conditions [29]. The possible reason for our finding could be due to the cross-contamination during the handling of raw vegetables or showering the contaminated water on vegetables to keep them fresh during selling, and due to the unhygienic surrounding environment. It is also observed that vegetable carts are usually placed near the garbage dumping sites or poultry shops which are the common vehicle of infection transmission for *Salmonella* outbreak [30,31]. It has been outlined that the growth of *Salmonella* Newport strain remained unstirred by the replacement of irrigation water,

suggesting that *Salmonella* adheres firmly to the sprout [32,33].

In this study, previously reported multiplex PCR primers were used that not only distinguished *S*. Typhi from *S*. Paratyphi A but also identified high and low-risk lineages of *S*. Typhi of clinical importance. These primers were designed from the highly conserved sequences across all strain-specific regions in their reference genomes like STY0307 genes for *S*. Typhi and an intergenic region between SSPA1723a and SSPA1724 for *S.* Paratyphi A. Many studies have examined thousands of *Salmonella* genomes that are accessible and have discovered SNPs that are unique to particular lineages [32,34-36]. In several South Asian nations, the predominant strain of *S*. Typhi is genotype 4.3.1 (H58), which is highly resistant to 27 antibiotics [36]. The used marker for the multidrug-resistant lineage 4.3.1 (H58) is based on a C-T synonymous mutation (T349T) in the STY2513 gene at position 2348902 in the *S.* Typhi CT18 reference genome. All H58 4.3.1 lineage and their sub-lineage isolates are covered by this mutation. For the detection of XDR lineages which is genotype 4.3.1.1.P1, the diagnostic marker is based on a GA (E13E) synonymous mutation in the STY0962 gene [37-38]. Furthermore, in the present study, we focused on the investigation of XDR S*almonella* Typhi since this species is of global public health concern and is the cause of Salmonellosis. The multiplex PCR assay enables the identification of the *S*. Typhi lineages such as MDR H58 and XDR *S.* Typhi. Our data have suggested that the environment remains an important vehicle for the transmission of *Salmonella* in humans, especially through the consumption of certain foods that are usually consumed raw. According to our study coriander was the agent that contained the most amount of *Salmonella* spp.

In conclusion, the present study suggests that raw vegetables specifically coriander are potential reservoirs or sources of *S.* Typhi and MDR and XDR strains in Karachi, Pakistan. Further, to the best of our knowledge, this study is the first to report the prevalence of *S*. Typhi, MDR H58 and XDR *S.* Typhi originating from environmental samples by using Multiplex PCR. Our study represents an important contribution to providing a large data set to identify *Salmonella* spp. in particular to characterize the level and dynamics of antimicrobial resistance in these pathogens present in leafy greens. Furthermore, this study can be helpful for government authorities enforcing monitoring of food safety and hygiene to prevent food-borne pathogen spread.

Ethical approval

Ethical approval was obtained with the code IBC KU-267/2022 from the University of Karachi

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Authors' Contributions

NUH, investigation, formal analysis and writing-initial draft; MS, conceptualization, data curation, funding, resources and writing-editing final draft; ZAM, conceptualization, resources, writing-initial draft; QAH, formal analysis; SK, conceptualization and resources. All the authors approved final draft and submission of the manuscript.

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