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MOLECULAR EVALUATION OF SUL1 AND CTX DRUG RESISTANCE AND VIRULANCE GENES IN VIBRIO CHOLERAE IN PESHAWAR, PAKISTAN

Original Research

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ABSTRACT

Background: *Vibrio cholerae* remains a major cause of cholera outbreaks in resource-limited countries, particularly in South Asia. The disease is transmitted through contaminated water and food, leading to acute watery diarrhea, dehydration, and potentially fatal outcomes if untreated. In Pakistan, recurring outbreaks and the emergence of multidrug-resistant strains of *V. cholerae* have raised significant public health concerns. Understanding the epidemiology, antimicrobial resistance, and virulence profile of circulating strains is critical to improving treatment strategies and outbreak management.

Objective: To determine the antimicrobial susceptibility pattern and molecular detection of *sul1* (resistance) and *ctx* (virulence) genes in *V. cholerae* isolates from Peshawar, Pakistan.

Methods: This cross-sectional study was conducted from January to December 2024 at Hayatabad Medical Complex, Peshawar. A total of 50 stool and rectal swab samples from patients with suspected cholera were cultured and confirmed as *V. cholerae* O1, serotype Ogawa. Phenotypic identification included Gram staining, biochemical tests, API-20E, and string test. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method. Molecular detection of *sul1* and *ctx* genes was carried out using PCR with specific primers.

Results: Out of 50 isolates, 28 (56%) were from females and 22 (44%) from males. Age distribution showed 29 (58%) cases in patients >20 years, 13 (26%) in 11–20 years, and 8 (16%) in 1–10 years. Geographically, Hayatabad reported the highest cases (6). Cholera cases peaked in August (9) and September (8). Resistance to meropenem and TMP-SMX was observed in 47 (94%) and 45 (90%) cases, respectively. The *ctx* gene was detected in 47 (94%) and *sul1* in 45 (90%) isolates.

Conclusion: The study highlights a significant prevalence of virulent, multidrug-resistant *V. cholerae* strains in Peshawar, particularly during the monsoon season. Targeted interventions, enhanced surveillance, and antimicrobial stewardship are urgently required.

Keywords: Antimicrobial resistance, Cholera, Khyber Pakhtunkhwa, *sul1* gene, *Vibrio cholerae*, Virulence genes, Waterborne diseases.

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INTRODUCTION

Cholera is a life-threatening, acute diarrheal illness that has persisted as a global health concern for centuries. It is classified as a notifiable disease by the World Health Organization (WHO), underscoring its potential to cause large-scale outbreaks if not rapidly identified and controlled (1). Caused by the ingestion of *Vibrio cholerae*—primarily through contaminated food or water—cholera is endemic in many low-resource settings where sanitation infrastructure is inadequate. Although modern medicine has enabled effective management through rehydration therapy and antibiotics, untreated cases can rapidly deteriorate, leading to severe dehydration and death. The historical roots of cholera trace back over a millennium in the Ganges Delta of India, from where it eventually spread across continents. Since the first pandemic in 1817, the disease has caused seven global outbreaks, resulting in millions of deaths worldwide, with the seventh pandemic beginning in 1961 and still ongoing in several regions (2). *V. cholerae* O1 and O139 serogroups are primarily responsible for epidemic cholera, with O139 emerging in 1992 and O1 variants evolving in 2002 to contain features of both the Classical and El Tor biotypes, which facilitated their rapid geographic spread across Asia and Africa (3). A striking example of the continued threat posed by cholera is the outbreak in Yemen, which recorded over 330,000 suspected cases and 1,759 deaths by 2017 alone (4).

Cholera's epidemiology is intricately linked to environmental, socioeconomic, and climatic factors. The primary reservoirs of *V. cholerae* include infected individuals and aquatic ecosystems, particularly brackish waters and estuaries (5). Transmission primarily occurs via ingestion of contaminated water or food, but human-to-human transmission, especially in healthcare settings, has also been observed during epidemics (6,7). The bacterium thrives on the chitinous surfaces of marine organisms, and the disease often spikes following natural disasters or flooding, which disrupt access to clean water. For instance, the 2010 floods in Pakistan precipitated a sharp rise in cholera incidence, with 164 laboratory-confirmed cases reported in areas previously considered low-risk (8). Similarly, Cox's Bazar has witnessed alarming spikes in acute watery diarrhea and confirmed cholera cases, reflecting the vulnerability of displaced and underserved populations (9). The pathogenicity of *V. cholerae* is largely attributed to its key virulence factors—cholera toxin (CTX) and the toxin co-regulated pilus (TCP)—which are expressed during intestinal colonization. TCP facilitates bacterial adherence and microcolony formation on the intestinal epithelium, while CTX triggers secretory diarrhea by disrupting ion transport mechanisms in host cells (10). These virulence factors are tightly regulated by the ToxR regulon, which responds to environmental cues such as bile acids or bicarbonate levels in the gastrointestinal tract (11). Once secreted through the type II secretion system, CTX binds to GM1 ganglioside receptors on intestinal epithelial cells, initiating endocytosis and intracellular signaling that ultimately leads to the characteristic profuse diarrhea of cholera (12). Additionally, the NanH enzyme enhances CTX uptake by modifying ganglioside structure, thereby facilitating toxin binding (13).

Despite the scientific understanding of its transmission and pathogenic mechanisms, cholera continues to pose a major public health challenge in resource-limited countries, especially in South Asia and sub-Saharan Africa. In Pakistan, inadequate sanitation, overcrowding, and the monsoon season contribute to annual surges in gastroenteritis and suspected cholera cases (14). Surveillance data from WHO further highlight that in 2020 alone, over 167,000 suspected cases were reported globally, with Pakistan, Yemen, and parts of Bangladesh among the most affected (15). Given the persistent threat of cholera, particularly in vulnerable regions, there is a critical need to enhance surveillance, improve water and sanitation infrastructure, and understand evolving epidemiological patterns and bacterial virulence. This study aims to investigate the recent trends, virulence dynamics, and epidemiological behavior of *V. cholerae* in affected regions, with a specific focus on contributing factors and implications for public health response.

METHODS

1. Study Setting and Sample Selection

This cross-sectional study was carried out in Peshawar, Khyber Pakhtunkhwa, from January to December 2024, targeting the detection and characterization of *Vibrio cholerae* among patients with acute watery diarrhea. Clinical samples were collected from patients presenting to Hayatabad Medical Complex, Peshawar, while microbiological and molecular analyses were conducted in the Microbiology Section of the Complex Medical Laboratory and Diagnostic Center, Peshawar. A total of 50 samples were collected using a purposive sampling approach, with the sample size calculated through OpenEpi software (https://www.openepi.com). Inclusion criteria

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encompassed patients of all ages exhibiting symptoms of watery diarrhea suspected to be of cholera origin. Exclusion criteria were not explicitly mentioned and should be incorporated for methodological transparency. Stool and rectal swab specimens were obtained from symptomatic individuals after obtaining informed verbal and written consent. Ethical approval for the study was secured from the Institutional Review Board (IRB) of the respective facility.

2. Phenotypic Identification

Initial isolation of *V. cholerae* was achieved by streaking stool and swab specimens onto selective media, including Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar, MacConkey agar, and Xylose Lysine Deoxycholate (XLD) agar, followed by Gram staining. After 16–20 hours of incubation at 37°C on TCBS agar, yellow, flat colonies approximately 2–3 mm in diameter were identified as presumptive *V. cholerae*. These colonies were sub-cultured on Luria-Bertani (LB) agar or nutrient agar for further processing. Biochemical profiling confirmed oxidase, catalase, ONPG, indole, sucrose, and mannitol positivity, while lactose fermentation remained negative—findings consistent with *V. cholerae*. Identification was further supported using the string test, performed by mixing bacterial colonies with 0.5% sodium deoxycholate on a glass slide; the formation of a mucoid "string" indicated a positive result (16). Additionally, API 20E test strips (bioMérieux) were inoculated with bacterial suspension and incubated at 37°C for 18–24 hours. Colorimetric reactions in the test wells were compared against a reference chart for *V. cholerae* confirmation (17).

3. Serotyping and Biotypic Identification

Isolated strains were serotyped using slide agglutination with polyvalent anti-O1 antibodies, followed by subtype differentiation with monovalent Ogawa and Inaba antisera (MAST ASSURE, UK). To distinguish between the classical and El Tor biotypes of *V. cholerae* O1, susceptibility to Polymyxin B and the Voges-Proskauer (VP) reaction were evaluated. Strains exhibiting resistance to Polymyxin B and a positive VP reaction were classified as El Tor biotype, while those sensitive to Polymyxin B with a negative VP test were identified as classical biotype strains.

4. Antimicrobial Susceptibility Testing

All confirmed isolates underwent antimicrobial susceptibility testing using the Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (18,19). Antibiotics tested included ampicillin (30 µg), meropenem (10 µg), ciprofloxacin (5 µg), erythromycin (15 µg), chloramphenicol (30 µg), amikacin (30 µg), azithromycin (15 µg), trimethoprim-sulfamethoxazole (23.5 µg/1.5 µg), tetracycline (30 µg), and ceftazidime (30 µg). Inhibition zones were measured and interpreted as susceptible, intermediate, or resistant based on standardized CLSI breakpoints.

5. DNA Extraction

Genomic DNA was extracted from isolates using the Archive Pure DNA Purification Kit (Hamburg, Germany), following the manufacturer's protocol. Extracted DNA was stored at 4°C for short-term use and at −20°C for long-term preservation.

6. Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed under sterile conditions to detect the presence of *ctx* and *sul1* genes associated with cholera toxin and sulfonamide resistance, respectively. PCR reactions were carried out in 25 µL volumes, starting with an initial denaturation at 95°C for 5 minutes, followed by 30 amplification cycles (95°C for 30 seconds, primer-specific annealing for 30 seconds, and 72°C for 30 seconds), and concluding with a final extension at 72°C for 10 minutes. The primers used included ctx forward (5′-TTTGTTAGGCACGATGATGGAT-3′), ctx reverse (5′-ACCAGACAATATAGTTTGACCCACTAAG-3′), sul1 forward (5′-CGGCGTGGGCTACCTGAACG-3′), and sul1 reverse (5′-GCAAGGCGGAAAGGAAGTGG-3′) (20,21). Amplified products were resolved via electrophoresis on 2% agarose gels (0.7% for genomic DNA) at 120 volts for 40 minutes. A 100 bp DNA ladder was used to assess amplicon sizes. Gels were visualized using the ABI gel documentation system.

Table1: Sequences of primers

S/No	Primers	Sequence	Reference
1	*ctx* Forward	5'- TTTGTTAGGCACGATGATGGAT-3'	(Jubyda *et al.,* 2023)
2	*ctx* Reverse	5'-ACCAGACAATATAGTTTGACCCACTAAG-3'	
5	*sul1* Forward	5'- CGGCGTGGGCTACCTGAACG-3'	(Thaotumpitak *et al.,* 2023)

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7. Statistical Analysis

Data analysis was performed using SPSS software version 22.0 and Microsoft Excel version 6.2.14. Descriptive statistics including frequencies, percentages, and ratios were computed to assess demographic distributions, seasonal trends, age groups, and vaccination status. Results were stratified according to relevant epidemiological and clinical variables to evaluate patterns of infection and antimicrobial resistance.

RESULTS

A total of 50 isolates of *Vibrio cholerae* were confirmed between January and December 2024. All isolates belonged to serogroup O1 and serotype Ogawa. The median age of the patients was 23 years, and females accounted for the majority of cases, with 28 (56%) infections, while 22 (44%) cases were identified in males. Adults over 20 years of age represented the largest affected group, with 29 cases (58%), followed by 13 cases (26%) in the 11–20-year age group and 8 cases (16%) among children aged 1–10 years. These findings indicate a notable concentration of cases in the adult population.

1. Area wise distribution of V. cholerae

The spatial distribution of cases across 13 localities in Peshawar showed that Hayatabad reported the highest number of cases, with 6 infections. Matra and Charsadda Road followed with 5 cases each. Several areas including Nasir Bagh, Tehkal, Noutia, Badaber, Defence Colony, Karkhano, and Hashtnagri each reported 4 cases. Gulbahar and Pishtakhara accounted for 3 cases, while Peshawar University recorded the lowest count with 2 cases. This pattern suggests heterogeneous geographic clustering, with certain urban areas exhibiting increased disease burden.

2. Age wise prevalence of V. cholerae

Among the 50 confirmed cases, the highest burden was observed in individuals aged above 20 years (29 cases, 58%), which may reflect occupational exposures, dietary habits, and access to healthcare. The 11–20-year age group accounted for 13 cases (26%), while only 8 cases (16%) occurred in children aged 1–10 years. These findings point toward a progressive increase in prevalence with age.

3. Gender-wise prevalence of V. cholerae

Females were disproportionately affected, comprising 56% (28) of the total cases, compared to males who represented 44% (22). This gender disparity could be attributed to social, environmental, or biological factors, though further exploration is needed to validate potential determinants.

4. Month-wise distribution of V. cholerae

The seasonal trend revealed fluctuations in case frequency throughout the year. A gradual increase was noted from January (2 cases) to May (7 cases), followed by a dip in June. The highest peaks were observed in August (9 cases) and September (8 cases), coinciding with the late summer and post-monsoon period. A sharp decline occurred in October, and cases remained low in November and December. These observations align with known seasonal cholera surges during humid and flood-prone months.

5. Antibiotic Resistance Pattern

Antibiotic susceptibility testing demonstrated widespread resistance among isolates. Meropenem showed the highest resistance, with 47 (94%) resistant strains. Ampicillin resistance was noted in 44 (88%) isolates, while ciprofloxacin and erythromycin resistance were observed in 30 (71%) and 43 (86%) isolates, respectively. TMP-SMX resistance was also prominent at 45 (90%). Resistance to azithromycin and amikacin was detected in 37 (74%) and 31 (62%) of isolates, respectively. Ceftazidime and chloramphenicol exhibited resistance in 35 (70%) and 20 (40%) of cases. Tetracycline showed a comparatively better susceptibility profile with 25 (50%) of strains susceptible, although 21 (42%) still exhibited resistance. Intermediate susceptibility responses were noted across all antibiotics, indicating partial efficacy in certain strains.

6. Molecular Identification of resistance and virulence gene

Molecular analysis revealed high prevalence of the ctx and sul1 genes. The ctx gene, responsible for encoding cholera toxin, was detected in 47 (94%) of the 50 isolates. Similarly, the sul1 gene, associated with sulfonamide resistance, was present in 45 (90%) isolates. Only

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3 samples (6%) tested negative for ctx, while 5 (10%) were negative for sul1. These findings confirm that the circulating strains in this population carry both virulence and resistance markers, underscoring the pathogenic and treatment-challenging nature of current isolates.

Table 2: Distribution of *V. cholerae* in different age groups

Variables		Number of cases, n (%)
Age group	1-10 Years	08 (16)
	11- 20 Years	13 (26)
	> 20 Years	29 (58)

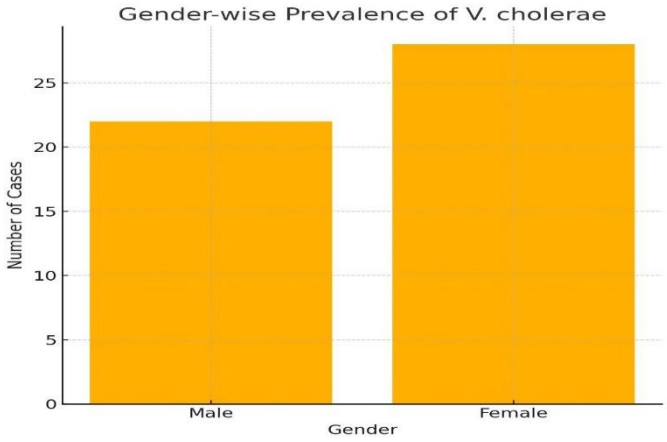


Figure 1 Gender-wise Prevalence of V. Cholerae

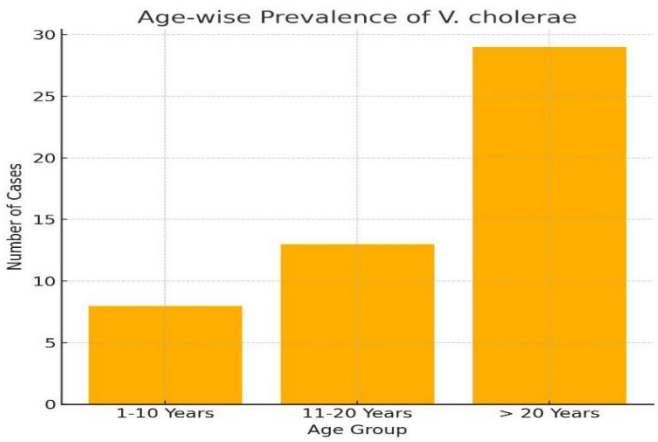
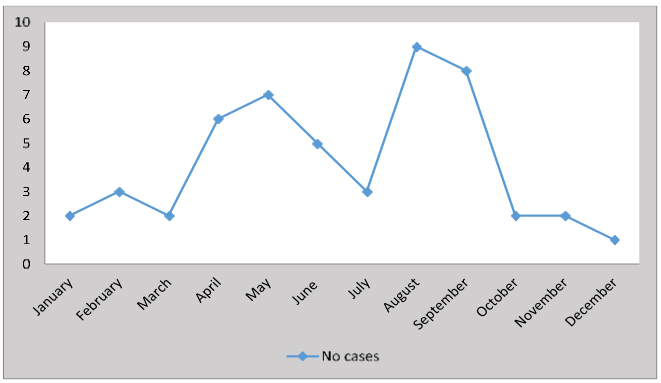
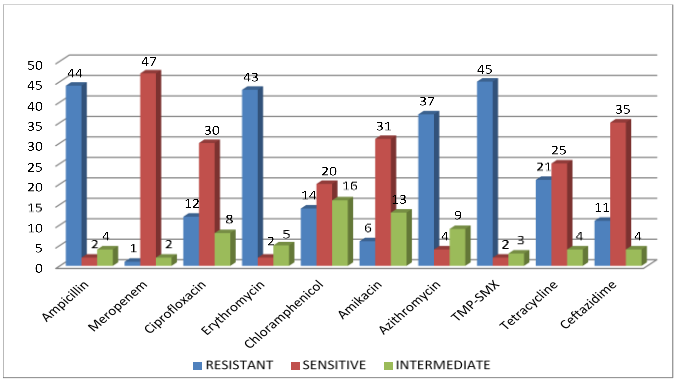


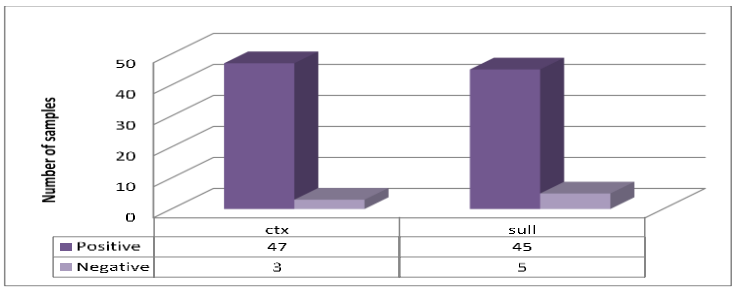
Figure 2 Age-wise Prevalence of V. Cholerae



Month wise distribution of *V. cholerae* in Peshawar



Antibiotic resistance pattern of *V. cholerae* in Peshawar



Ctx and sul1 gene prevalence

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DISCUSSION

The present study aimed to characterize *Vibrio cholerae* isolates obtained from various locations in Peshawar, Pakistan, with a focus on their prevalence, antibiotic resistance profiles, and molecular detection of virulence and resistance genes. The findings highlight a consistent predominance of *V. cholerae* O1, serotype Ogawa, and biotype El Tor among clinical isolates, aligning with prior reports from other endemic regions where similar biotypes remain dominant. The integration of both phenotypic and genotypic techniques allowed for a comprehensive assessment of strain characteristics and antimicrobial resistance, strengthening the diagnostic rigor of this investigation. The study confirmed a seasonal trend, with a concentration of cases between June and November, coinciding with monsoon-related environmental conditions that facilitate bacterial proliferation and water contamination. A similar seasonal clustering of *Vibrio* infections was previously reported in temperate climates, where increased water temperatures and flooding were found to enhance bacterial growth. In contrast to data from a German surveillance study which identified a higher infection rate in males, the current study observed a modest female predominance, with 56% of cases reported among women. This disparity may be influenced by local sociocultural roles, differing exposure to contaminated water sources, or sampling variation, and is reflective of how gender-based vulnerability may differ by region and context (22,23).

Antimicrobial resistance patterns observed in this study revealed concerning trends. A notably high level of resistance was found against meropenem (94%), ampicillin (88%), erythromycin (86%), and TMP-SMX (90%), while tetracycline remained the most effective agent with 50% susceptibility. These findings diverge from previous reports in which certain antimicrobials—such as ciprofloxacin, TMP-SMX, and tetracycline—exhibited near-complete susceptibility. For instance, studies conducted in East Africa demonstrated 100% sensitivity to ciprofloxacin and only moderate resistance to TMP-SMX and ampicillin (24,25). The significant discrepancies in antimicrobial efficacy underscore the influence of local antibiotic prescribing habits, environmental antibiotic residues, and the emergence of resistant clones driven by unregulated antimicrobial use. Furthermore, the detection of *ctx* and *sul1* genes in 94% and 90% of isolates respectively emphasizes the dual threat posed by toxigenic potential and drug resistance. The near-universal presence of *ctx* supports the notion that current strains retain full virulence capabilities, while the widespread detection of *sul1* reflects escalating resistance to sulfonamides. The molecular confirmation of both resistance and virulence determinants amplifies the clinical concern associated with these isolates, particularly in regions lacking robust treatment options.

Strengths of the study include the use of standardized microbiological and molecular protocols, as well as the geographic diversity of samples from multiple localities in Peshawar, which enhances the representativeness of the findings. However, several limitations must be acknowledged. The study relied on a relatively small sample size, which may not fully capture the heterogeneity of circulating strains. Moreover, exclusion criteria and patient clinical data such as dehydration status, hospitalization, or prior antibiotic use were not reported, limiting the ability to correlate microbial characteristics with clinical outcomes. Additionally, the absence of follow-up or environmental sampling from water sources restricts the ability to identify potential reservoirs or transmission pathways. To enhance future research, longitudinal surveillance with larger sample sizes and comprehensive clinical metadata should be prioritized. Inclusion of environmental isolates and whole-genome sequencing could provide greater insight into transmission dynamics, resistance mechanisms, and evolutionary trajectories. Regional stewardship programs should also be informed by real-time susceptibility data to preserve the efficacy of frontline antibiotics and mitigate the spread of resistant *V. cholerae* strains. In conclusion, the findings underscore the continued public health burden of cholera in under-resourced settings, exacerbated by the emergence of multidrug-resistant and toxigenic strains. The high prevalence of resistance to commonly used antimicrobials, along with the near-universal presence of virulence genes, calls for urgent measures in antibiotic regulation, clean water access, and sustained epidemiological monitoring.

CONCLUSION

This study highlights the ongoing public health challenge posed by cholera in Peshawar, particularly during the monsoon season when environmental factors amplify disease transmission. The identification of widespread antibiotic resistance and the presence of key virulence and resistance genes among clinical *V. cholerae* isolates emphasize the urgency of implementing robust antimicrobial stewardship and strengthening local surveillance systems. The observed gender distribution and resistance patterns, when compared with global findings, further underscore the role of regional disparities in shaping disease dynamics. These findings contribute valuable insights for guiding local public health strategies, improving diagnostic and treatment protocols, and preventing future outbreaks through evidence-based interventions.

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AUTHOR CONTRIBUTION

Author	Contribution
Maria Khan	Substantial Contribution to study design, analysis, acquisition of Data Manuscript Writing Has given Final Approval of the version to be published
Salman Ahmed	Substantial Contribution to study design, acquisition and interpretation of Data Critical Review and Manuscript Writing Has given Final Approval of the version to be published
Muhammad Rabnawaz	Substantial Contribution to acquisition and interpretation of Data Has given Final Approval of the version to be published
Farah Shireen	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Muhammad Mansoor Kamal	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Kabir Khan	Substantial Contribution to study design and Data Analysis Has given Final Approval of the version to be published
Muhammad Umair	Contributed to study concept and Data collection Has given Final Approval of the version to be published
Waqar Saeed	Writing - Review & Editing, Assistance with Data Curation
Muneeba*	Writing - Review & Editing, Assistance with Data Curation
Madiha Iqbal*	Writing - Review & Editing, Assistance with Data Curation

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