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## Molecular identification and antibiogram profile of *Vibrio cholerae* isolates from diarrheal patients in Northwestern Pakistan

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### Abstract

*Vibrio cholerae*, the causative agent of cholera, remains a significant public health concern in Khyber Pakhtunkhwa, Pakistan. This study investigated the prevalence and antimicrobial susceptibility patterns of *V. cholerae* in stool samples collected from diarrheal patients from July 2022 to August 2023. A total of 361 stool samples were collected and cultured on Thiosulphate Citrate Bile Sucrose Agar (TCBS) media. Ninety-one (25.20%) samples were identified as *V. cholerae* through biochemical tests followed by PCR amplification of the 16S rRNA gene. The highest infection rate was observed in the age group of  $\leq 18$  years (59.34%), with males (60.40%) being more affected than females (39.60%). The summer season accounted for the highest proportion of *V. cholerae* cases (68.13%). Chi-square tests revealed significant correlations between age groups, gender, and seasonal variation with *V. cholerae* positivity ( $p < 0.001$ ,  $p = 0.026$ , and  $p < 0.001$ , respectively). Geographically, Peshawar had the highest number of cases (37.36%), followed by Dir and Swat (24.17% each), and Mardan (14.30%). Antibiotic susceptibility testing showed that fluoroquinolones, particularly ciprofloxacin (71.93% susceptibility) and levofloxacin (66.67% susceptibility), were the most effective antibiotics. However, high resistance levels were observed against sulfamethoxazole-trimethoprim (71.42%), azithromycin (64.92%), tetracycline (77.50%), ampicillin (81.25%), and erythromycin (94%). Multidrug-resistant and extensively drug-resistant isolates constituted 19.78% and 17.58% of the total, respectively. District-wise analysis revealed varying susceptibility patterns, suggesting differing antibiotic selection pressures and possible circulation of region-specific strains. These findings highlight the importance of targeted preventive measures during high-risk periods and among vulnerable demographic groups, as well as the need for continued surveillance of antibiotic resistance in *V. cholerae*.

### 1. Introduction

*Vibrio cholerae*, the etiological agent of cholera, continues to pose a major public health threat in low- and middle-income countries, particularly in regions where inadequate sanitation and limited access to safe drinking water persist. Cholera, an acute diarrheal infection, is contracted through the ingestion of food or water contaminated with specific serogroups of *V. cholerae*, namely O1 or O139 (Kaper et al., 2014). The disease is characterized by its rapid onset, profuse watery diarrhea, and vomiting, leading to severe dehydration and potentially death if left untreated (Kumar & Sharma, 2014). Globally, it is estimated that cholera affects between 1.3 to 4.0 million people, resulting in 21,000 to 143,000 deaths annually (Asumah et al., 2023) with South Asia and Sub-Saharan Africa experiencing the highest disease burden (Ali et al., 2015).

In recent years, Pakistan has been experiencing an occurrence of cholera epidemics, with a significant increase between 2022 and 2024. The epidemics coincided with unparalleled monsoon flooding that significantly impaired water supply systems and sanitary facilities, establishing the optimal conditions for the

transmission of *V. cholerae*. The World Health Organization (WHO, 2023) and the Global Task Force on Cholera Control (GTFCC, 2024) reported that several provinces, including Sindh, Punjab, and Khyber Pakhtunkhwa (KP), have recorded persistent clusters of confirmed *V. cholerae* O1 cases. Karachi and Lahore continued to be chronic endemic hotspots, while additional foci were identified in Peshawar, Swat, and other flood-affected districts (National Institute of Health Pakistan, 2023). Post-flood evaluations indicated significant cross-contamination between sewage and drinking water pipelines, particularly in informal settlements and peri-urban areas (UNICEF Pakistan, 2023). Consistent with findings in other South Asian contexts (Asantewaa et al., 2024; Debes et al., 2016), the convergence of extreme weather events, urban overcrowding, and decreasing public health infrastructure have increased cholera risk in Pakistan, highlighting the critical necessity for integrated surveillance and water safety measures. The bacterium *V. cholerae* is transmitted via the fecal-oral route, emphasizing the importance of hygiene and sanitation in preventing its spread (Kanjiyal et al., 2010). Outbreaks are frequently linked to contaminated water sources, inadequate

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sewage disposal, and poor food handling practices. The ability of *V. cholerae* to persist in aquatic environments, coupled with its capacity to form biofilms, contributes to its environmental resilience and poses ongoing risks for transmission. The World Health Organization emphasizes that improving access to safe drinking water, sanitation, and hygiene interventions is crucial for effective cholera control and prevention (Taylor et al., 2015). Despite advancements in medical treatment, cholera continues to pose a threat, particularly in areas afflicted by poverty, conflict, and natural disasters.

Despite the fact that several studies have investigated the prevalence and molecular epidemiology of the disease in Pakistan, there is still a significant gap concerning the emergence of *V. cholerae* and the patterns of antimicrobial resistance (AMR) in KP province. Previous surveillance reports have mainly focused on coastal or flood-affected areas of Sindh and Punjab particularly Karachi and Lahore where *V. cholerae* O1 has been primarily isolated (Hussain et al., 2024). In contrast studies in KP provide limited data even though there have been growing reports of water born epidemics related to inadequate sanitation and unsafe drinking water in the region. The current study shows a shift towards more resistant and possibly hyper virulent genotypes propagating in northern regions, in contrast to previous findings from southern Pakistan that mainly focused on the genetic variety of O1 and non-O1 strains isolated (Hussain et al., 2024). Furthermore, this study highlights an alarming evolution in resistance profiles as the high resistance to frontline antibiotics, such as tetracycline, erythromycin, and ampicillin, contrasts with previous national trends where these antibiotics remained effective. This emphasizes the need for region-specific research to guide empirical strategies for treatment and educate local public health initiatives. Targeted surveillance and molecular characterization of *V. cholerae* strains in KP are essential for managing future outbreaks and reducing the risks of AMR because of the region's geographic, socioeconomic, and climatic variety.

This study aimed to investigate the prevalence of *V. cholerae* in stool samples collected from diarrheal patients across several districts of KP, Pakistan, including Peshawar, Mardan, Swat, and Dir. The study also sought to determine the antimicrobial susceptibility patterns of the isolated *V. cholerae*, providing valuable insights into the current resistance landscape in the region. The data collected during this period could be useful for analyzing the general trends in cholera cases in the region. By identifying the circulating *V. cholerae* strains and their

susceptibility profiles, the findings of this study can inform public health interventions and guide appropriate treatment strategies to combat cholera effectively.

## 2 Materials and Methods

### 2.1 Research Design

The study was conducted from July 2022 to August 2023 at IBGE, University of Agriculture Peshawar, to investigate the prevalence of *V. cholerae* in stool samples and identified the susceptibility pattern in four KP districts; Peshawar, Mardan, Swat and Dir. The healthcare facilities from which samples were collected are illustrated in Figure 1.

### 2.2 Inclusion and Exclusion criteria for sample collection

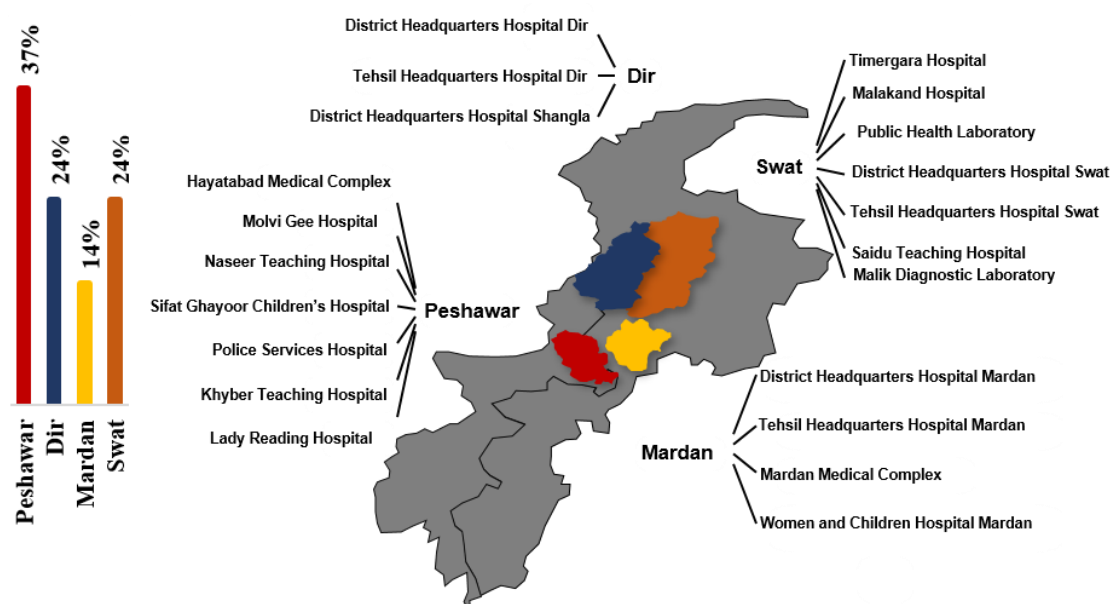
Stool samples from all participants who reported diarrhea for up to three months were collected particularly patients who passed out watery or soft stools. Samples have been collected from patients of all ages, including both male and female. The study excluded participants who were already on antibiotics. Moreover, Participants who reported constipation, were excluded from the study in order avoid wrong isolation.

### 2.3 Sample Collection

Three hundred and sixty-one (361) stool samples in total had been collected from diarrheal patients. Each participant was provided with clean, dry, wide-mouth screw-capped universal container to be used for collecting samples. Each sample was given a unique laboratory number on the labels, which were applied accurately and appropriately. The samples were taken to the laboratory in 1-3 hours after collection for processing.

### 2.4 Bacterial isolation and Identification

Samples were inoculated into an alkaline peptone water broth for enrichment, and after three hours of incubation, they were further cultured on the Thiosulphate Citrate Bile Sucrose Agar (TCBS) media. Following a 24-hour incubation period at 37 °C, the culture plates were examined for the growth of *V. cholerae*. Gram staining and biochemical tests including indole, Methyl Red and Voges-Proskauer were performed (Zereen et al., 2019).



**Figure 1** Distribution of *Vibrio cholerae* cases in four districts of Khyber Pakhtunkhwa, Pakistan from July 2022-August 2023.

## 2.5 Antimicrobial Susceptibility Testing

Using the Kirby-Bauer disc Diffusion method, the antimicrobial susceptibility had been evaluated. The culture was taken up from the plate using a sterile loop; it was suspended in alkaline peptone water (APW) and incubated for 6-8 hours at 37 °C. As per the McFarland Standard ( $1.5 \times 10^8$  CFU/mL), the microbial suspension required for AST was prepared. It was then spread throughout the Mueller-Hinton agar (MHA) plate using a sterile lawn swab. Using guidelines from the Clinical Laboratory Standards Institute (CLSI, 2018), antibiotic-incorporated plates were incubated at 37 °C and zone sizes were interpreted within 18-24 hours of incubation (Raveendran et al., 2008). The antibiotics used were; Ceftriaxone; CRO, Ampicillin; AMP, Tetracycline; TET, Azithromycin; AZM, Chloramphenicol; C, Ciprofloxacin; CIP, Trimethoprim/sulfamethoxazole; SXT, Erythromycin; ERY, Levofloxacin; LVX, Cefixime; CFM; Amikacin; AMK, Ceftazidime; CTZ, Colistin; CST. Bacterial isolates have been classified according to their resistance profiles following the standardized criteria proposed by Magiorakos et al., (2012) and Ahmad et al., (2025). Isolates showing resistance to at least one antibiotic across three or more antimicrobial categories were classified as multidrug-resistant (MDR). Isolates exhibiting resistance to all but one or two types of antimicrobials were classified as extensively drug-resistant (XDR). This classification allowed a uniform evaluation of resistance severity and facilitated comparison with global research on antimicrobial resistance trends. To ensure alignment with globally accepted breakpoint criteria, these definitions were used in conjunction with antimicrobial susceptibility values that were interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI M45, 2018) guidelines.

## 2.6 Sequencing of 16s rRNA gene

Genomic DNA was extracted with the TIAN amp Bacteria DNA Kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China) and subsequently analyzed. Universal primers 27F (5'-GGTTGGCCAATCTACTCCAGG-3') and 1492R (5'-TGGTCTCCTTAAACCTGTCTT-3') were used for the 16S rRNA gene amplification (Kai et al., 2019). Each PCR reaction contained 5 µl of template DNA, 2.5 µl of each forward and reverse primer, 25 µl of master mix, and 14 µl of PCR-grade water, resulting in a total reaction volume of 49 µl. The PCR cycling parameters included an initial denaturation at 94 °C for 1 minute, succeeded by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 54 °C for 1 minute, and extension at 72 °C for 3 minutes. A final extension was performed at 72 °C for 1 minute. The PCR product was subjected to sequencing. Sequence data followed the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) to discover homologous sequences. A multiple sequence alignment was conducted with Clustal W in MEGA version 11, and a phylogenetic tree was generated using the maximum likelihood method to evaluate the genetic relationships among the *V. cholerae* isolates (Dhillon et al., 2003; Sun et al., 2024).

## 2.7 Statistical Analysis

IBM SPSS Statistics (version 26.0 Corp., Armonk, NY, USA) was used for all statistical analyses. To evaluate correlations between *V. cholerae* positive results and categorical characteristics such as age group, gender, and season, chi-square ( $\chi^2$ ) tests were used. Statistical significance was considered as a p-value of less than 0.05.

## 3 Results

### 3.1 Occurrence of *V. cholerae* in Khyber Pakhtunkhwa

Out of 361 stool culture samples, 123 samples showed growth where 91 (25.20%) were reported as *V. cholerae* during the study. PCR amplification confirmed these isolates as *V. cholerae* (Figure 2). The study evaluated the prevalence of infection in the population according to age and gender. The age group of  $\leq 18$  years had the highest infection rate in KP, at 54 (59.34%). The age group of 19-40 years had 22(24.17%) positive cases, and the age group of over 40 years had 15 (16.49%) positive cases. Moreover, frequency of male patients was higher 55 (60.40%) than female 36 (39.60%). Seasonal analysis indicated that summer season represented highest ratio 68.13% of *V. cholerae* cases. The percentage was calculated in the total of 91 positive *V. cholerae* cases.

Chi-square tests were performed to statistically support the observed relationships between seasonal and demographic parameters and *V. cholerae* positivity. Age groups and cholera positivity were found to be significantly correlated ( $\chi^2 = 46.23$ ,  $df = 2$ ,  $p < 0.001$ ), with the majority of cases (59.34%) occurring in the  $\leq 18$  year group. The results are consistent with earlier findings that demonstrate children are more susceptible because of weakened immunity and increased exposure to the environment. Males (60.40%) were more affected than females (39.60%), according to the gender-wise distribution, which also revealed a statistically significant correlation ( $\chi^2 = 4.94$ ,  $df = 1$ ,  $p = 0.026$ ). This could be due to variations in occupational exposure or behavior. Seasonal variation in cholera cases was also significant ( $\chi^2 = 61.78$ ,  $df = 3$ ,  $p < 0.001$ ), with the summer season reporting the highest number of cases (68.13%). The significance of focused preventative measures at high-risk periods and among vulnerable demographic groups is highlighted by these statistical results, which also support the accuracy of the trends that have been observed.

### 3.2 Distribution of *V. cholerae* by Age group

Out of 91 positive cases of *V. cholerae*, the data showed that thirty-four (37.36%) cases were in the Peshawar population, which was considered at risk. Furthermore, thirteen (14.30%) positive cases were noted in Mardan, twenty-two (24.17%) positive cases in both Dir and Swat (Figure 1). Twenty-two (24.17%) patients in Peshawar were of  $\leq 18$  year age group, six (6.60%) patients in the age group of 19-40 years, while six (6.60%) patients in the age group of above 40 years. Eight (8.79%) patients in Dir were of  $\leq 18$  years age group, ten (10.98%) patients were in the age group of 19-40 years and four (4.40%) patients in the age group of above 40 years. In Mardan, eleven patients (12.10%) belonged to the age groups of  $\leq 18$  years, one patient (1.10%) both in the age group of 19-40 years and above 40 years respectively. Thirteen (14.27%) patients in the  $\leq 18$  year age group, five (5.50%) in the 19-40 year age group, and four (4.40%) in the above 40 year age group were observed in Swat (Figure 3).

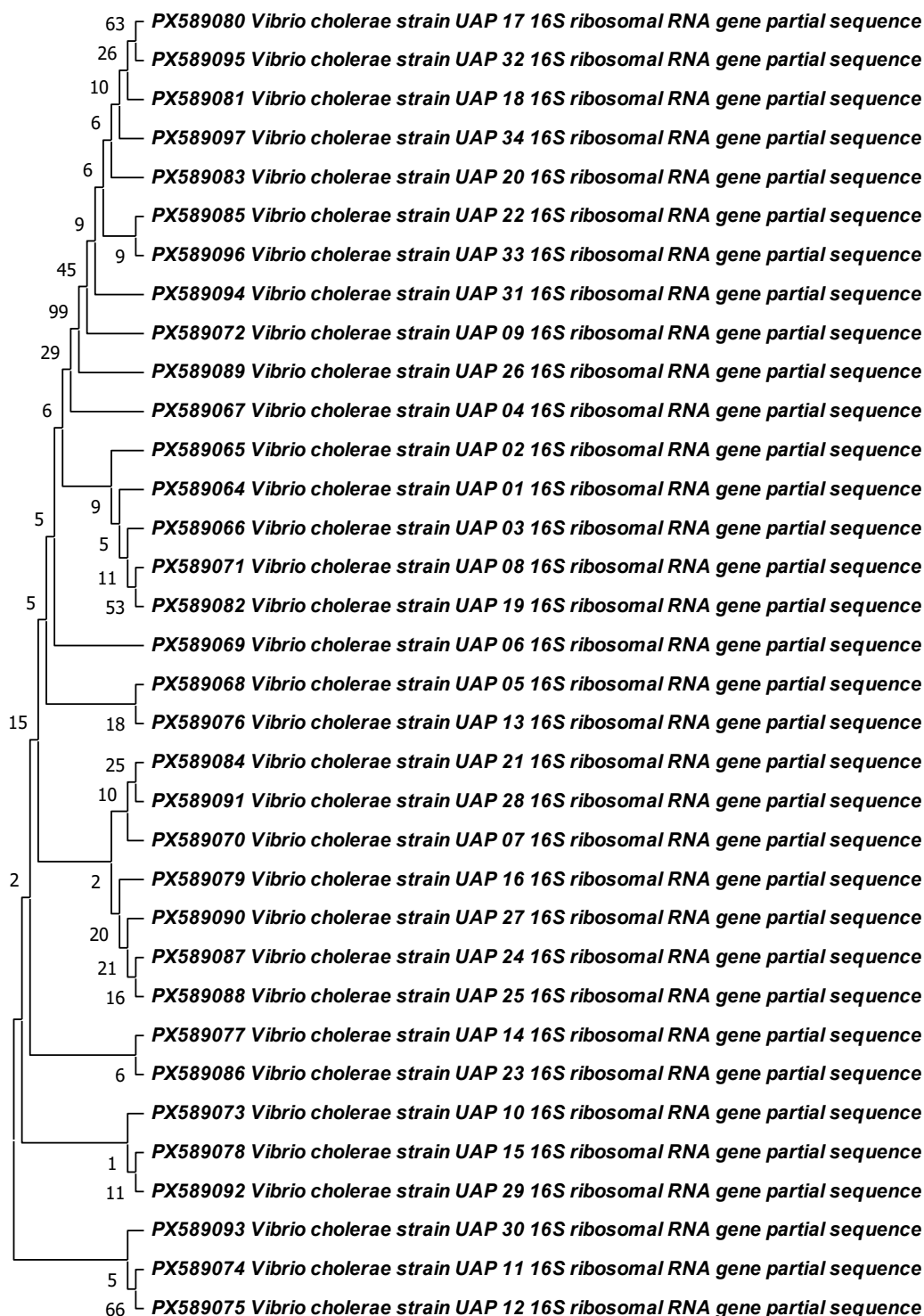
### 3.3 Distribution of *V. cholerae* by Gender

In Peshawar and Dir, there were more male positive cases than female positive cases i.e., twenty-seven (79.41%) male and seven (20.59%) female positive cases in Peshawar while fourteen (63.63%) male and eight (36.37%) female positive cases in Dir. In contrast, Swat and Mardan showed lower ratio of male cases as compared to female cases i.e., there were eight (36.37%) male and fourteen (63.63%) female positive cases in Swat while six (46.16%) male and seven (53.84%) female positive cases in Mardan had been observed (Figure 4).

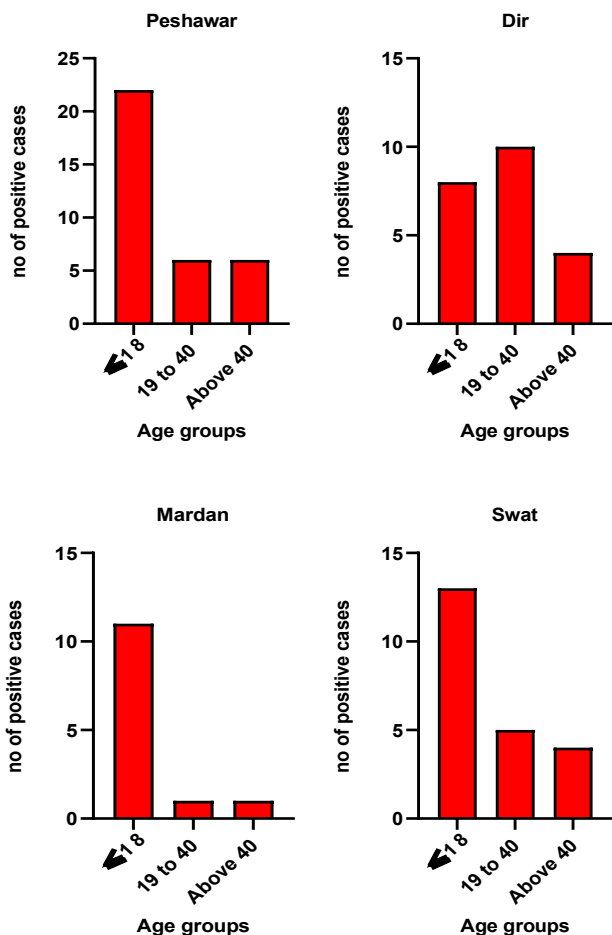
### 3.4 Distribution of *V. cholerae* by Season

In Peshawar, the prevalence of cholera was lowest in spring and autumn season, i.e., four (11.76%), while increased to twenty-six (76.48%) in summer season. In the winter season no cases of *V. cholerae* were detected. In Dir, the prevalence of *V. cholerae* was same in summer and autumn season, i.e. eleven (50%) cases were recorded in both the seasons, but no cases were reported in the winter and spring seasons. According to the Mardan prevalence, there were no cases in the spring season and only one (7.70%) positive case was found in the winter.

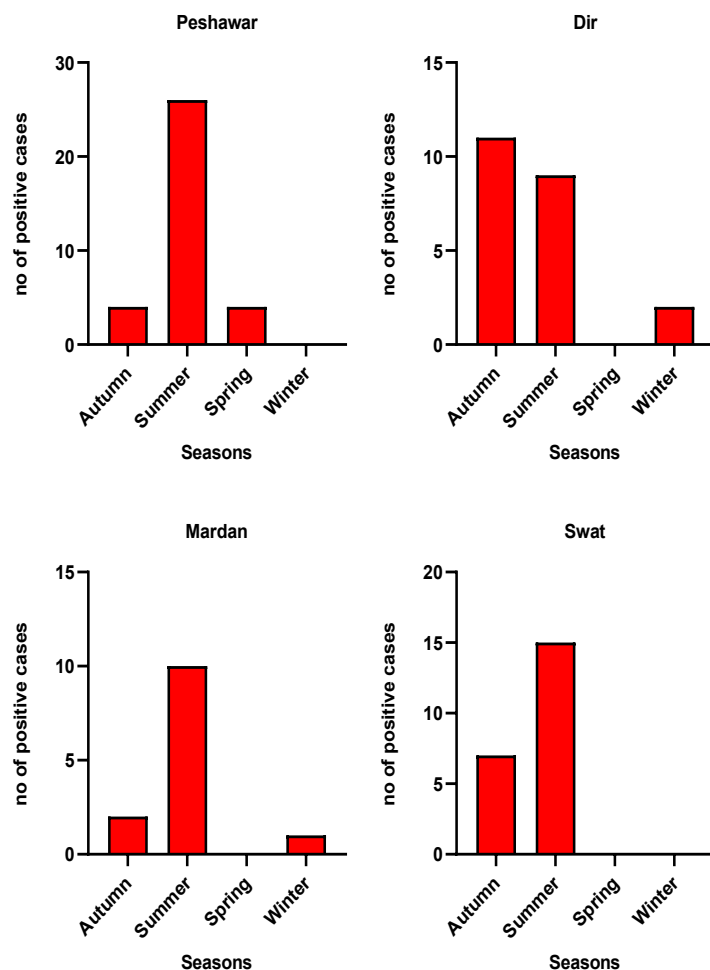
Ten (76.9%) positive cases of *V. cholerae* were reported during the summer, whereas two (15.40%) positive cases were identified during autumn season. In Swat, during the summer season, fifteen (68.18%) positive cases were observed. Conversely, there were seven (31.82%) cases reported in the autumn. Spring and winter seasons did not show any cases. According to the results, there were more cases of *V. cholerae* in Peshawar during the summer season than the other seasons i.e., twenty-six, followed by nine positive cases in Dir, ten positive cases in Mardan while fifteen cases of *V. cholerae* were reported in Swat (Figure 5).



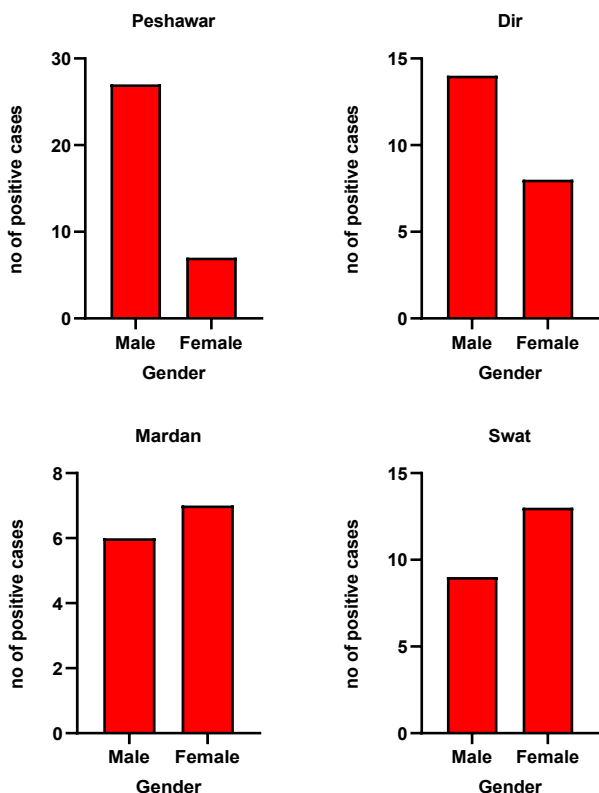
**Figure 2** Phylogenetic analysis based on 16S rRNA gene sequences of *Vibrio cholerae* isolates. The tree was constructed using the Maximum Likelihood method in MEGA 11 software. Each isolate was labeled with its corresponding identifier (UAP; University of Agriculture Peshawar).



**Figure 2** Age wise distribution of *Vibrio cholerae* positive cases in Khyber Pakhtunkhwa, Pakistan.



**Figure 4** Seasonal distributions of *Vibrio cholerae* positive cases in Khyber Pakhtunkhwa, Pakistan.



**Figure 3** Gender wise occurrence of *V. cholerae* in Khyber Pakhtunkhwa, Pakistan.

### 3.5 Overall Antibiotic Susceptibility Profile

Ninety-one *V. cholerae* isolates tested for antibiotic susceptibility testing. Fluoroquinolones constituted the most effective antibiotic class, with 66.67% of isolates exhibiting susceptibility to levofloxacin (LVX) and 71.93% to ciprofloxacin (CIP). Conversely, elevated resistance levels have been observed against various frequently utilized drugs, including sulfamethoxazole-trimethoprim (SXT; 71.42% resistant), azithromycin (AZM; 64.92% resistant), tetracycline (TET; 77.50% resistant), and ampicillin (AMP; 81.25% resistant). *V. cholerae* exhibited significant resistance to erythromycin (ERY; 94%), aligning with the emerging patterns of macrolide resistance documented in South Asia. According to these profiles, 19.78% (18/91) of isolates were designated as multidrug-resistant (MDR) and 17.58% (16/91) were classified as extensively drug-resistant (XDR).

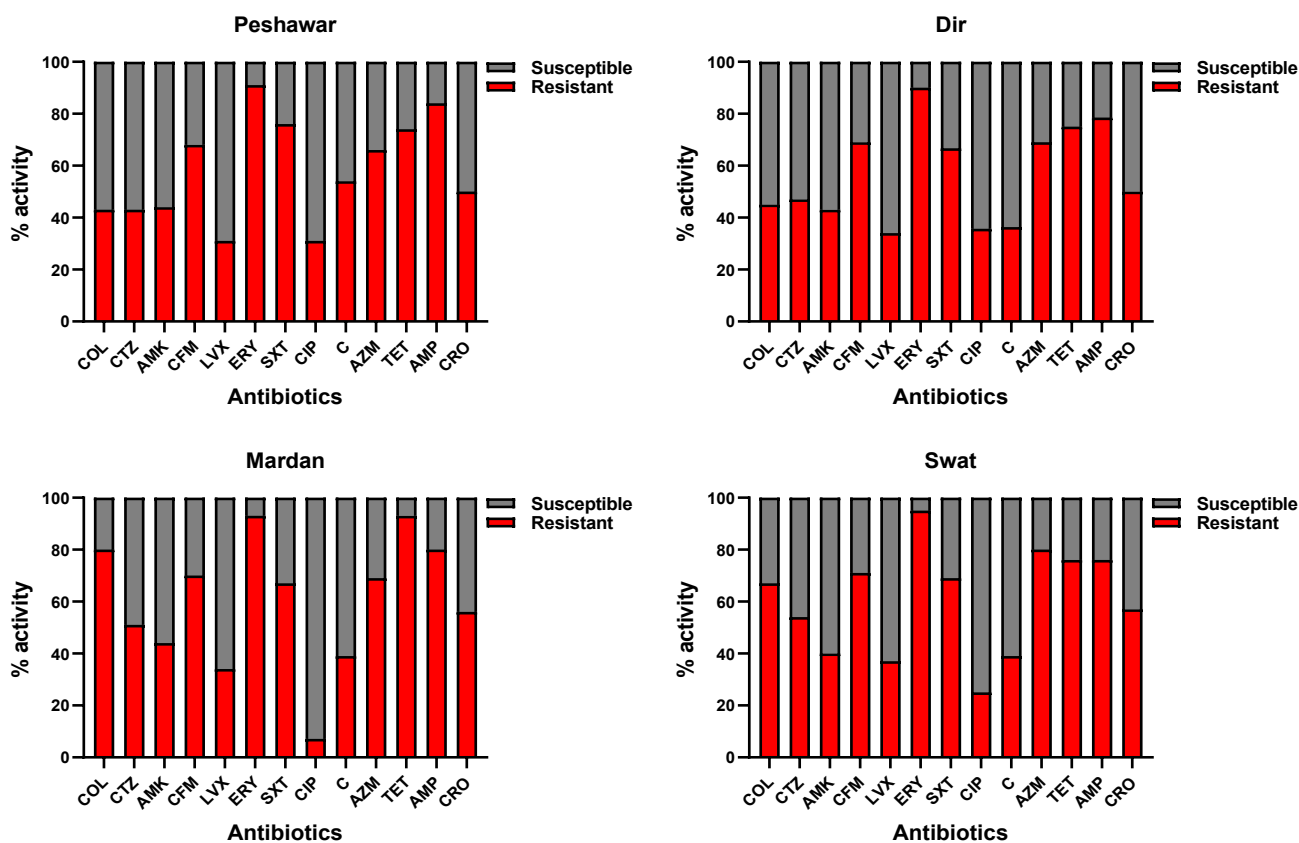
### 3.6 Most Effective and Ineffective Antibiotics

Among all isolates, the antibiotics with the lowest effectiveness were AMP (81.25% resistance), AZM (64.92% resistance), ERY (94% resistance), and colistin (90% resistance), indicating established intrinsic and acquired resistance mechanisms. In contrast, ciprofloxacin (71.93% susceptibility) and levofloxacin (66.67% susceptibility) exhibited the high levels of effectiveness against *V. cholerae*, although susceptibility was moderate rather than high.

### 3.7 District-Wise Antibiotic Resistance Patterns

Distinct geographical susceptibility patterns were observed across the four sampled districts. In Peshawar, fluoroquinolones exhibited moderate efficacy (69% susceptibility to CIP and LVX), while significant resistance remained against AMP (84%), ERY (91%), SXT (76%), and TET (74%). In Dir, the most highly effective agents were chloramphenicol (63.64% susceptibility) and CIP (64.28% susceptibility), whereas resistance to AMP (78.57%), TET (75.00%), and SXT (66.67%) was significant.

Mardan exhibited the highest fluoroquinolone efficacy, demonstrating 92.86% susceptibility to ciprofloxacin; however, resistance to tetracycline and erythromycin persisted at 93%, with moderate resistance observed for colistin and ampicillin at 80%. In Swat, CIP (75%) and amikacin (60%) showed the highest activity, while ERY (95%), AZM (80%), and TET (76%) showed poor effectiveness. The variability across districts suggests differing antibiotic selection pressures and possible circulation of region-specific *V. cholerae* strains (Figure 6).



**Figure 5** Antimicrobial resistance profile of *Vibrio cholerae* isolates against 13 antibiotics in four districts of Khyber Pakhtunkhwa, Pakistan. Red bars indicate resistance; Black bars show susceptibility, while grey bars show intermediate activity.

### 4 Discussion

The present study investigated the occurrence of *V. cholerae* in KP, Pakistan, revealing a significant prevalence of the pathogen within the region. A high ratio of tested samples of *V. cholerae* were being positive indicating a notable presence of the bacteria in the studied population. The findings highlighted the importance of addressing the public health concerns associated with *V. cholerae* in KP. It is crucial to continuously monitor and mitigate the factors contributing to the spread of this infectious disease.

The age distribution of the infected individuals revealed that the highest infection rate was observed in the age group of  $\leq 18$  years, suggesting that younger individuals are disproportionately affected by *V. cholerae* infection in KP. This pattern could be linked to higher exposure in informal or densely populated urban environments (Penrose et al., 2010). Furthermore, the age group of 19-40 and over 40 years indicated a decreasing trend of infection with increasing age. This age-related disparity in infection rates may be attributed to various factors, including differences in hygiene practices, immune status, and exposure levels among different age groups. Moreover, the study found a higher frequency of male patients,

compared to female patients, suggesting a potential gender-related difference in susceptibility or exposure to *V. cholerae* infection.

The seasonal analysis indicated that the summer season had the highest proportion of *V. cholerae* cases, highlighting the seasonal variability of *V. cholerae* infection in KP. This seasonal pattern may be associated with increased water contamination, changes in hygiene practices, and other environmental factors that favor the growth and transmission of the bacteria during the summer months (Kumar & Sharma, 2014). The geographical distribution analysis revealed that Peshawar had the highest burden of *V. cholerae*, indicating that the population in this region is at a higher risk of infection. In contrast, low cases at Mardan, Dir and Swat reflect spatial variability in cholera prevalence across KP. These variations are likely influenced by factors such as population density, sanitation infrastructure, water quality, and access to healthcare services.

These gender-specific differences may be related to variations in occupational exposure, hygiene practices, and healthcare-seeking behaviors between males and females in different regions. Understanding the reasons behind regional variations is crucial to target intervention effectively. This is particularly true given that conflict, climate change, and unplanned urbanization

are major factors in the spread of cholera (**Kaper et al., 1995**). The observed prevalence of *V. cholerae* in KP is consistent with the fact that cholera remains a significant public health challenge in many parts of the world, particularly in developing countries with inadequate water and sanitation infrastructure. In fact, one study reported that Sub-Saharan Africa and South-East Asia accounted for a substantial proportion of the global cholera burden between 2008 and 2012 (**Ali et al., 2015**). Also, cholera outbreaks continue to pose a threat to public health, especially in regions with limited resources for diagnosis, prevention, and control (**Ebob, 2020**).

Significant correlations between seasonal and demographic factors and *V. cholerae* positivity were validated by chi-square analysis. The majority of cases were observed in the 18-year-old group, and there was a significant correlation between age and infection ( $\chi^2 = 46.23$ ,  $df = 2$ ,  $p = 0.001$ ). This aligns with earlier reports that highlighted children's increased vulnerability due to lower immunity and greater environmental exposure (**Alam et al., 2006**). There was also a significant gender-based difference ( $\chi^2 = 4.94$ ,  $df = 1$ ,  $p = 0.026$ ), with males significantly more likely to be affected, which may be due to professional or behavioral risk factors. With a peak incidence in the summer, seasonal variation was highly significant ( $\chi^2 = 61.78$ ,  $df = 3$ ,  $p < 0.001$ ), which is consistent with previous studies that link increased cholera transmission to higher ambient temperatures and water scarcity (**Hussain et al., 2024**). These findings highlight the necessity of focused interventions among vulnerable demographic groups and during high-risk times.

The correlation between age group and cholera positivity was statistically significant ( $\chi^2 = 46.23$ ,  $df = 2$ ,  $p < 0.001$ ), despite the fact that the distribution of *V. cholerae* cases appeared to be higher among individuals under the age of 18. This could be partially explained by sample size limitations within specific age groups, especially the  $\geq 40$  age group, which included fewer cases and could have provided less statistical ability to identify an actual association. Furthermore, the observed distribution might have been affected by possible confounding variables such as diverse healthcare-seeking behaviors, exposure to contaminated water sources, nutritional status, and varying immune levels among age groups. According to previous studies, children and adolescents are frequently more susceptible to cholera because of more exposure and a lower level of acquired immunity (**Alam et al., 2006**). Nevertheless, clinical datasets that only include symptomatic or hospitalized patients may not consistently reflect these patterns. To further understand the role of age in cholera susceptibility and transmission dynamics, larger and more balanced age-group representation is essential in future community-based studies.

Our findings showed that *V. cholerae* isolates had different levels of antibiotic resistance in different districts. For instance, Peshawar had 100% levofloxacin resistance, Mardan had the highest ciprofloxacin susceptibility (92.86%), Swat had 100% erythromycin and levofloxacin resistance, and Dir had a more balanced resistance profile. These differences are probably due to a mix of different strains that are circulating, how antibiotics are used in the area, and pressures to choose certain strains at the facility level. Similar geographic variability has been observed in environmental and food-associated *V. cholerae* populations, highlighting the impact of regional antimicrobial practices and sanitation conditions.

The 100% erythromycin resistance reported in *V. cholerae* isolates is much higher than the global average of 20-60%. Therefore, all the results were checked again using CLSI M45 (2018) breakpoints (resistant  $\leq 13$  mm), and all isolates stayed resistant, which shows that this finding is not due to a mistake in the method. Quality control utilizing *E. coli* ATCC 25922 and adherence to appropriate disc storage protocols eliminates technical artifacts. Recent regional studies show that macrolide

resistance is on the rise, even though it is not common around the world. **Sharif et al., (2023)** found that 82-95% of isolates in Bangladesh were resistant. Molecular screening of our isolates identified *erm* and *mef* genes, corroborating the phenotypic findings and indicating the presence of resistant clones. These trends may be caused by the wrong use of antibiotics in aquaculture and pollution of the environment, which encourages clonal expansion.

In light of these findings, it is imperative to implement comprehensive strategies to control the spread of *V. cholerae* in KP, focusing on improving water quality, sanitation, and hygiene practices. The potential of oral cholera vaccines to prevent and control cholera outbreaks must be considered, as they are demonstrated to be safe and effective (**Zaman et al., 2020**). Prioritizing efforts to enhance access to clean water and sanitation facilities in vulnerable communities, especially in areas with high population density and poor infrastructure, is crucial. Given the high infection rates among younger individuals, health education programs targeting children and adolescents may be particularly effective in promoting safe hygiene practices and preventing the transmission of *V. cholerae*. Because climate change is a factor in the rise of diseases, climate-resilient strategies are needed to reduce the risk of future outbreaks (**Rana et al., 2023**).

The current study has its limitations and it is essential to acknowledge the various limitations of this study. First, the cross-sectional design limits the capacity to determine causal or temporal associations between *V. cholerae* infection and clinical, environmental, or demographic parameters. It is virtually impossible to tell if the observed relationships are the result of accidental correlations or causative risk factors because the data were gathered all at once. Additionally, selection bias might have been introduced by excluding patients who had recently taken antibiotics, which could have resulted in an underestimation of the actual prevalence of infection or antibiotic resistance. The findings' relevance to the larger patient population, especially those with partially treated illnesses, may also be impacted by this restriction. To overcome these limitations and provide a more thorough overview of cholera epidemiology and resistance trends, future community-based and longitudinal research with more expansive inclusion criteria are required.

The fact that it only processed clinical samples collected from hospital patients, which could not accurately represent the diversity or prevalence of *V. cholerae* in the general population. The results' generality may be limited by this sampling strategy's potential to ignore mild cases and asymptomatic carriers who prefer not to seek medical attention. Furthermore, it is more difficult to gain insight into environmental reservoirs and transmission routes when environmental sampling had not collected, such as from food, water, or sanitary sources. Prior research has emphasized how crucial it is to include clinical data with environmental surveillance in order to better understand cholera dynamics and determine infection sources. For a more thorough and representative examination of *V. cholerae* epidemiology and transmission pathways, future studies should include community-based and environmental sampling techniques.

Data collection was restricted to a single calendar year, which might not sufficiently represent inter-annual variations in *V. cholerae* transmission patterns. This is another major limitation of this study. Climate and environmental factors including temperature, rainfall, and water availability are known to affect the occurrence of cholera, and they may vary greatly from year to year. Because of this, the seasonal patterns that have been noted, including the summertime peak in cases, could not be entirely transferable to other years or more general climate circumstances. To confirm these results and gain a better understanding of the determinants and constancy of seasonal

cholera dynamics in the area, longitudinal studies involving several seasons and years are required.

Furthermore, 16S rRNA sequencing alone is insufficient to distinguish between toxigenic and non-toxigenic *V. cholerae*, and toxin gene analysis (e.g., *ctxA*, *rtxA*) was not conducted. Multivariate statistical adjustments for potential confounders were not utilized, which may affect the observed resistance patterns. Finally, quality control reference strains (e.g., *E. coli* ATCC 25922) were utilized during susceptibility testing, although this was not initially documented. Subsequent research that includes toxin gene analysis, extensive environmental sampling, longitudinal methodologies, and thorough statistical modeling will enhance comprehension of *V. cholerae* prevalence and antimicrobial resistance.

## 5 Conclusion

The study conducted from July 2022 to August 2023 at IBGE, The University of Agriculture Peshawar, examined the prevalence of *V. cholerae* in stool samples and identified the susceptibility pattern in several KP districts. The findings highlighted the need for improved sanitation and hygiene practices in the affected districts to reduce the prevalence of *V. cholerae*. The high infection rate among younger individuals suggested a need for targeted interventions and education programs for this age group. The observed antibiotic resistance patterns emphasize the importance of judicious antibiotic use and the development of alternative treatment strategies to combat MDR and XDR strains of *V. cholerae*.

## Statements and Declarations

### Human Ethics and Consent to Participate declarations:

This study was carried out in accordance with the Declaration of Helsinki (2013) and the ethical standards set out by the National Bioethics Committee (NBC) of Pakistan. Formal ethical approval had been obtained from the Institutional Research and Ethical Board (IREB) at Hayatabad Medical Complex, Peshawar, Pakistan. Informed consent, either written or verbal, was obtained from all participants or their legal guardians before sample collection, where appropriate. All data were completely anonymized, and no identifiable patient information was documented or utilized at any phase of the investigation.

### Availability of data and materials:

The datasets generated and/or examined in this study are available at National Center for Biotechnology Information (NCBI) with accession numbers from PX589064-97.

**Competing interests:** The authors have declared no conflict of interest.

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**Clinical trial number:** Not applicable.

### Authors' contributions

Conceived of or designed the study: Jawad Ahmad

Performed research: Aisha Irfan, Zala Khan and Jawad Ahmad

Analyzed data: Aisha Irfan and Zala Khan

Contributed new methods or models: All authors contributed to refining experimental procedures and optimizing laboratory workflows used in the study.

Wrote the paper: Aisha Irfan and Jawad Ahmad drafted the initial manuscript. Jawad Ahmad contributed to the final editing and approval of the manuscript.

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