

Antimicrobial Susceptibility Profiles and *In Vitro* Antibacterial and Anti-Virulence Effects of Aqueous Pomegranate Peel Extract on Clinical *Vibrio Cholerae* Isolates from Erbil, Iraq

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Abstract This study investigated the antimicrobial susceptibility patterns of clinical *Vibrio cholerae* isolates from Erbil, Iraq and evaluated the in vitro antibacterial and anti-virulence activity of aqueous pomegranate (*Punica granatum*) peel extract. Fifteen confirmed non-duplicate clinical *V. cholerae* isolates obtained from 257 diarrheal stool specimens (2024-2025) were identified by conventional microbiological methods, O1/O139 serotyping, VITEK 2 and confirmed by 16S rRNA gene amplification. Antimicrobial susceptibility testing was performed across ten antimicrobial agents according to CLSI guidelines. Antibacterial efficacy of the aqueous peel extract was evaluated by minimum inhibitory concentration (MIC), agar well diffusion and growth kinetic assays. Transcriptional responses of virulence-associated genes (*flaA*, *vpsT* and *luxO*) following exposure to 10 and 20 mg/mL of extract were quantified by RT-qPCR using the $2^{-\Delta\Delta C_T}$ method. All isolates (100%) were resistant to amoxicillin-clavulanic acid, whereas high susceptibility was retained for chloramphenicol (93.3%), imipenem (86.7%), amikacin (80.0%), tetracycline (80.0%) and doxycycline (73.3%), with intermediate responses in erythromycin (60.0%) and ciprofloxacin (53.3%). The aqueous peel extract demonstrated dose-dependent antibacterial activity, with MIC values ranging from 3 to 50 mg/mL MIC₅₀ = 25 mg/mL (; MIC₉₀ = 50 mg/mL) and inhibition zones of 19-24 mm at 50 mg/mL. Significant downregulation of *flaA* and *vpsT* was observed following 10 and 20 mg/mL treatment ($p < 0.001$), whereas *luxO* expression showed variable, moderate reduction. These findings provide updated regional baseline data on antimicrobial resistance in clinical *V. cholerae* and demonstrate that aqueous pomegranate peel extract exhibits direct *in vitro* growth-inhibitory and transcriptional modulatory activity. These findings remain strictly limited to *in vitro* laboratory observations and do not establish therapeutic efficacy; further chemical profiling of the aqueous phase, phenotypic motility/biofilm assays and *in vivo* functional validation are required.

Key Words *Vibrio cholerae*, Cholera Surveillance, Antimicrobial Susceptibility, Pomegranate Peel Extract, Virulence Gene Expression

INTRODUCTION

Cholera is an acute diarrheal illness caused by a gram-negative bacterium, *V. cholerae* and is still an ongoing challenge to the general health in the urban population due to constant strain on water, sanitation and hygiene systems [1]. Although effective preventive and curative measures exist, sporadic outbreaks and seasonal outbreaks still remain and thus, continuing laboratory monitoring and careful microbial characterization are needed [2].

In the city of Erbil, regular monitoring of the epidemiology of diarrheal diseases is carried out through the Erbil Central Laboratory where suspected cases of cholera are

confirmed by standard microbiological methods. However, the available data are mainly limited to case identification and reporting and there is little information on antimicrobial susceptibility trends and the molecular characteristics of circulating *V. cholerae* isolates. As a result, the ability to study the dynamics of local resistance and explain bacterial behavior extends beyond the determination of its phenotype [3].

Antibiotics are commonly used as a supplement to rehydration therapy to reduce disease severity and bacterial secretion. The development of antimicrobial resistance in *V. cholerae* has enhanced the need to develop localized data on susceptibility to support evidence-based therapy [2,4].

In Erbil, there is a shortage of systematically recorded antimicrobial resistance information on *V. cholerae*, which explains the necessity of new laboratory-based assessments of the problem [5].

In addition to more traditional antimicrobial approaches, there has been a growing interest in anti-virulence approaches to reduce bacterial pathogenicity without necessarily reducing bacterial viability. The antimicrobial and anti-virulence properties of the plant-derived constituents have been studied in vast detail. Historically, plant-derived bioactive compounds have treated many diseases and microbiological infections. Over the previous several decades, preventive and therapeutic herbs have become more popular [6], Pomegranate (*Punica granatum* L.) is a popular fruit from the Punicaceae family. It is native to northern India to Iran and it has been grown in the Mediterranean since ancient times. Pomegranate has a long history of medicinal use, Pomegranate phytochemicals are antibacterial according to studies high polyphenol concentration in pomegranate juice makes it antioxidant-rich, Pomegranate peel and its bioactive components have been tested against bacterial infections, including the tooth decay-causing *Streptococcus mutans* strain and *Rothia dentocariosa* clinical isolate [7]. However limited evidence exists on their effect on clinical *Vibrio cholerae* isolates. Pomegranate peel extract's effect on clinical *V. cholerae* isolates' virulence-related gene expression is unclear. This gap in knowledge led to the use of pomegranate peel extract in the present study to investigate its in-vitro antibacterial activity and potential effects on virulence-related gene expression and pomegranate peel extract has proven its ability to inhibit several of the enteric pathogens [8,9]. However, the available information on its effects on the virulence-related gene expression in clinical isolates of *V. cholerae* in Erbil is scarce.

Although cholera cases are still being detected in Erbil, integrative research combining epidemiological studies, antimicrobial susceptibility testing and molecular analysis of virulence control in *V. cholerae* remains deficient. Remarkably, transcriptional responses of vital virulence-related genes following exposure to plant-produced compounds have not been well characterized in locally circulating clinical isolates.

The current study aimed at defining clinical isolates of *V. cholerae* in Erbil using an integrated methodology that included epidemiological surveillance, antimicrobial susceptibility testing and molecular analysis of the expression of virulence-related genes. The following specific objectives were addressed: (1) evaluating the temporal distribution of suspected cholera cases from regional reference laboratory records during 2024-2025, (2) determining the antimicrobial susceptibility profiles of confirmed clinical isolates, (3) evaluating the *in vitro* antibacterial efficacy and growth kinetics of aqueous *Punica granatum* peel extract and (4) quantifying the transcriptional response of key virulence-associated genes (*flaA*, *vpsT* and *luxO*) upon extract exposure.

METHODS

Study Design, Cohort Denominators and Sample Collection

This cross-sectional laboratory study was conducted at the Erbil Central Laboratory, Erbil, Iraq, between October 2024 and December 2025. A regional surveillance dataset of 49,290 diarrheal stool records was analyzed for macro-epidemiological patterns, while a targeted screening cohort of 257 fresh stool specimens from acute gastroenteritis cases was processed in Cary-Blair transport medium (Oxoid, UK) for selective culture. From these, 15 non-duplicate clinical *V. cholerae* isolates were confirmed and used for all downstream assays.

Isolation and Identification of *Vibrio cholerae*

Stool samples were first inoculated in Alkaline Peptone Water (APW) (Oxoid, UK) and incubated at 35-37 °C For 5-8 hours. After enrichment, cultures were subcultured on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar, MacConkey agar and blood agar (Oxoid, UK). The plates were incubated at 35-37 °C and observed at 18-24 hours as per the normal laboratory identification procedures. *V. cholerae* colonies were presumptively identified by colony morphology on TCBS agar, Gram staining and traditional biochemical tests that included oxidase assay (Scharlau, Spain) and Triple Sugar Iron (TSI) agar reaction (HIMEDIA, India). Typical biochemical profiles (Gram-negative comma rods, oxidase positive, TSI acid butt/alkaline slant without H₂S) were used to identify presumptive colonies. All 15 confirmed non-duplicate isolates were serotyped by slide agglutination using polyvalent and monovalent *V. cholerae* O1 (Ogawa/Inaba) and O139 antisera (Denka Seiken, Japan) and their identification was independently confirmed using the VITEK 2 Compact automated system with GN ID cards (bioMérieux, France) with ≥98% confidence (Figure 1) [10].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of all 15 clinical isolates was performed using the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar (Scharlau, Spain) in strict compliance with Clinical and Laboratory Standards Institute (CLSI M100, 34th ed.) guidelines. Bacterial suspensions were adjusted to a 0.5 McFarland standard (1.5×10⁸CFU/mL). The 10-agent antibiotic panel comprised: amikacin (AK, 30 µg), Amoxicillin-Clavulanic Acid (AMC, 20/10 µg), Ciprofloxacin (CIP, 5 µg), Doxycycline (DO, 30 µg), Vancomycin (VA, 30 µg), (Clindamycin (CM, 2 µg), Erythromycin (E, 15 µg), Imipenem (IMP, 10 µg), Tetracycline (TE, 30 µg) and Chloramphenicol (CM, 30 µg). Non-standard agents such as clindamycin and amikacin were included to profile broader multidrug phenotypic co-resistance patterns in local clinical strains. Plates were incubated at 35±2°C for 18-24 h. Zone diameters were measured to the nearest millimeter and classified as Susceptible, Intermediate, or Resistant based on CLSI criteria.

Molecular Confirmation of *Vibrio cholerae*

DNA Extraction: Genomic DNA was extracted from overnight cultures grown on TCBS agar using the AddPrep Bacterial Genomic DNA Extraction Kit (AddBio, Korea) according to the manufacturer's instructions.

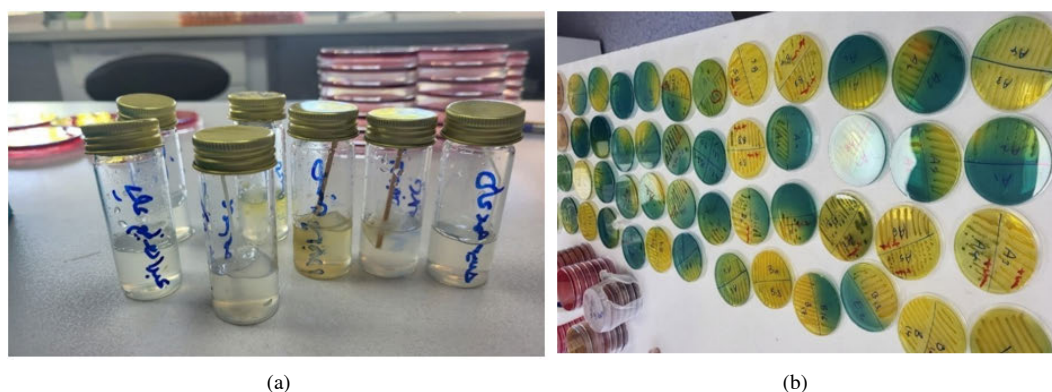


Figure 1: Isolation and Primary Laboratory Identification of Clinical *Vibrio cholerae* Isolates (a) Stool Specimens in Cary-Blair Transport Medium (b) Representative Yellow Colonies on TCBS Agar

Table 1: Molecular identification and GenBank Sequence Accession Mapping of Clinical *Vibrio cholerae* Isolates

Isolate Identifier	Isolate Accession	Query Coverage (%)	Sequence Identity (%)	GenBank Reference Accession	Organism Confirmation
Sample 1	PX927510	100%	100%	CP189101	<i>Vibrio cholerae</i>
Sample 2	PX927511	100%	100%	CP189138	<i>Vibrio cholerae</i>
Sample 3	PX927512	100%	100%	CP189059	<i>Vibrio cholerae</i>
Sample 4	PX927513	100%	100%	CP189240	<i>Vibrio cholerae</i>
Sample 5	PX927514	100%	100%	CP189053	<i>Vibrio cholerae</i>
Sample 6	PX927515	100%	100%	CP189170	<i>Vibrio cholerae</i>
Sample 7	PX927516	100%	100%	CP189228	<i>Vibrio cholerae</i>
Sample 8	PX927517	100%	100%	CP189334	<i>Vibrio cholerae</i>
Sample 9	PX927518	100%	100%	CP189128	<i>Vibrio cholerae</i>
Sample 10	PX927519	100%	100%	CP189232	<i>Vibrio cholerae</i>
Sample 11	PX927520	100%	100%	CP189139	<i>Vibrio cholerae</i>
Sample 12	PX927521	100%	100%	CP189210	<i>Vibrio cholerae</i>
Sample 13	PX927522	100%	100%	CP189101	<i>Vibrio cholerae</i>
Sample 14	PX927523	100%	100%	CP189138	<i>Vibrio cholerae</i>
Sample 15	PX927524	100%	100%	CP189059	<i>Vibrio cholerae</i>

PCR Amplification of the 16S rRNA Gene

Species-level confirmation of *V. cholerae* was performed by PCR amplification of the 16S rRNA gene using specific primers (forward: 5'-GGAAACGATGGCTAATACCG-3'; reverse: 5'-GCCCTTACCTCACCAACTAG-3'). PCR reactions were carried out in a total volume of 25 μ L containing genomic DNA template, primers and 2 $\times$ PCR master mix (AMPLIQON, Denmark). Thermal cycling conditions consisted of initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes, with a final extension at 72°C for 7 minutes. PCR products were separated on 1.5% agarose gel electrophoresis at 120 V for 30 minutes and visualized under ultraviolet illumination. The expected amplicon size was 372 bp. The results from the BLAST indicated that the highest query sequence was 100% identity Table 1.

Growth Curve Analysis of *Vibrio cholerae* Under Extract Treatment

To evaluate the effect of aqueous pomegranate peel extract on the growth dynamics of *Vibrio cholerae*, a growth curve assay was performed. Overnight bacterial cultures were adjusted to approximately 1×10^6 CFU/mL and inoculated into fresh Luria-Bertani (LB) broth containing different sub-inhibitory concentrations of the extract (50,25,12,6 and

3 mg/mL). Untreated cultures without extract served as growth controls. Cultures were incubated at 35-37°C with shaking and bacterial growth was monitored by measuring optical density at 600 nm (OD600) at regular time intervals every 60 mins over a 24 hour period. Growth inhibition was determined by comparing OD600 values of treated cultures with untreated control. The results were plotted as growth curves to demonstrate the dose-dependent inhibitory effect of the extract (Figure 8).

Phylogenetic Analysis

Partial 16S rRNA gene sequences obtained from representative clinical *Vibrio cholerae* isolates were subjected to phylogenetic analysis using IQ tree tool. Sequence alignment was performed against closely related reference sequences retrieved from the GenBank database. A maximum likelihood by using bootstrap branch replicates 1000 was constructed to confirm the taxonomic positioning of the clinical isolates within the *V. cholerae* clade. The analyzed isolates clustered closely with established *V. cholerae* reference strains, demonstrating high sequence similarity and supporting the molecular identification results. The phylogenetic tree further confirmed that the investigated isolates belonged to the same species group, with no major divergence detected among the clinical strains (Figure 2).

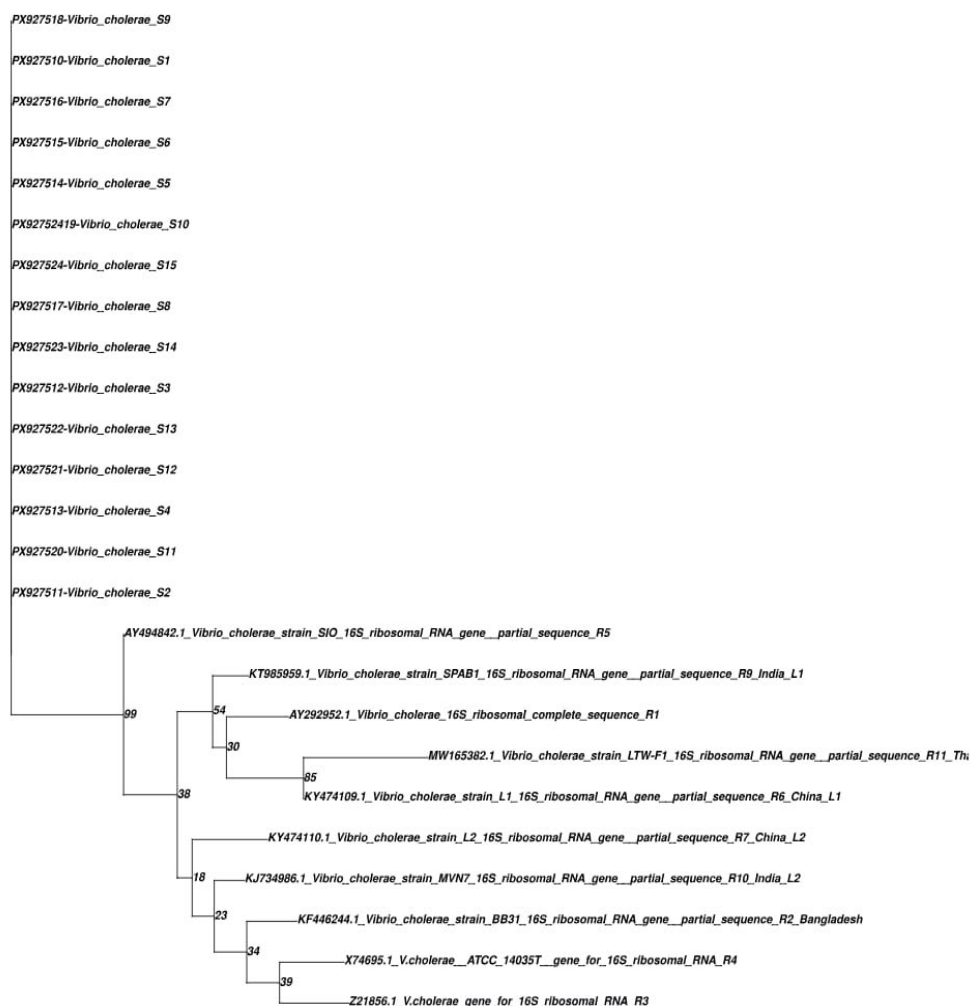


Figure 2: Maximum Likelihood Phylogenetic Tree based on Partial 16S rRNA Gene Sequences of Clinical *Vibrio cholerae* Isolates (*) and GenBank Reference Sequences, S = means sample, R = means references

Preparation of Aqueous Pomegranate

Peel Extract Pomegranate (*Punica granatum* L.) fruits were purchased from local markets in Erbil. Peels were excised, thoroughly rinsed with sterile distilled water, air-dried at ambient room temperature 22–25°C in the shade to constant weight and ground into fine powder using a laboratory mill. For aqueous extraction, 100 g of peel powder was macerated in 100 mL of sterile distilled water (1:1 w/v) at room temperature for 6 days with daily intermittent agitation. The mixture was filtered through Whatman No. 1 filter paper followed by a 0.45 µm cellulose acetate membrane filter and concentrated to dryness under reduced pressure using a rotary evaporator. The dried extract residue was reconstituted in sterile 0.25% (w/v) Carboxymethyl Cellulose (CMC) vehicle to generate a standardized stock solution and stored at 4°C. All biological assays (MIC, well diffusion, growth curves and RT-qPCR) were performed exclusively using this single batch of aqueous extract.

Gas Chromatography Mass Spectrometry (GCxMS) Analysis

For chemical profiling purposes, dried pomegranate (*Punica granatum*) peel powder (10 g) was extracted with deionized

distilled water using maceration at room temperature for 72 h with intermittent shaking. The extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator. The dried residue was stored at 4°C until analysis. GC-MS analysis was performed using an Agilent 7890A gas chromatograph coupled to a 5975C mass selective detector equipped with an HP-5MS capillary column. Helium was used as the carrier gas at a constant flow rate. The injector temperature was maintained at 250°C. The oven temperature was programmed from 60°C to 280°C at a controlled ramp rate. Mass spectra were recorded in Electron Impact (EI) mode at 70 eV over an appropriate mass scan range. Compounds were tentatively identified by comparing the obtained mass spectra with those available in the NIST mass spectral library. It is important to note that GC-MS profiling was conducted on aqueous extract solely to obtain a broad chemical fingerprint of peel constituents. All antibacterial and gene expression assays described in this study were performed using an aqueous extract prepared separately as detailed above.

Minimum Inhibitory Concentration (MIC) Determination

Pomegranate peel extract was formulated into Minimum Inhibitory Concentrations (MICs) of the isolates of *Vibrio cholerae* by the broth microdilution procedure. The suspension of bacteria was set up at a rough density of 1×10^6 CFU/mL. Two-fold dilutions of the extract (0.39 to 50 mg/ml) in Luria-Bertani broth for obtaining final concentrations ranging from 50 to 0.39 mg/mL (50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 mg/mL) the prepared extract dilutions were mixed and incubated with the bacterial inoculum at 35°C within 24 hours, a growth control of bacterial inoculum in LB broth without pomegranate peel extract was included to ensure bacterial growth under the test condition A vehicle control containing sterile distilled/deionized water without extract was also included to ensure that the extraction vehicle itself did not affect bacterial growth Following incubation, bacterial growth was determined in 600nm optical density and lowest concentration in which no bacterial growth was visible was remembered as MIC.

Agar Well Diffusion Assay

Agar well diffusion method was also used to assess the antibacterial activity. The standardized bacterial suspensions were used to inoculate Mueller Hinton agar plates which are equivalent to 0.5 McFarland standard. Wells (6MM diameter) were filled with different amounts of pomegranate peel extract (50,25,12,6 and 3m. mL⁻¹). The plates were then incubated at 37°C after a period of 24 hours and then the diameter of the inhibition zones was measured in millimeters.

RNA Extraction and Gene Expression Analysis

Aqueous pomegranate peel extracts were added to cultures of *Vibrio cholerae* at various chosen concentrations and incubated at 35°C over the duration of eight hours. Commercial RNA extraction kit (AddBio, Korea) was used to extract total RNA according to the instructions provided by the manufacturer. The NanoDrop spectrophotometer (thermo fisher scientific, USA) was then used to determine RNA concentration and purity. Total RNA (1,000 ng) was reverse-transcribed to cDNA using the AddScript cDNA Synthesis Kit (AddBio, Korea) following on-column DNase I digestion. Quantitative real-time PCR was performed using SYBR Green chemistry on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The 16S rRNA gene served as the validated internal reference gene. Relative expression fold changes were calculated using the comparative $2^{-\Delta\Delta C_T}$ method, utilizing untreated isolates as baseline calibrators. Samples showing no quantifiable cycle threshold within 40 cycles were designated as Not Detected (ND, indicating complete transcriptional suppression below the assay detection limit). Statistical comparisons were conducted on ΔC_T values prior to exponential transformation (Table 2).

Statistical Analysis

The statistical analysis was based on one-way Analysis of Variance (ANOVA), followed by Tukey's multiple-comparison test where appropriate where the difference was considered significant at $p < 0.05$. All experimental procedures were

triplicated, with independent biological replicates considered the experimental units and technical replicates used for repeated measurements the data obtained were reported as mean + SD. For the qRT-PCR analysis, relative gene expression was calculated using the comparative $2^{-\Delta\Delta C_T}$ method, with the untreated bacterial culture serving as the calibrator/control group. Technical replicates were averaged and were not treated as independent observations and qRT-PCR data were summarized at the biological replicate level and expressed as mean±SD. Differences in relative gene expression among the experimental groups were analyzed using one-way ANOVA followed by Tukey's multiple-comparison test, with $p < 0.05$ considered statistically significant.

RESULTS

Isolation and Molecular Confirmation of *Vibrio cholerae*

A total of 257 stool samples collected during the study period (October 2024- December 2025) were received at the Erbil Central Laboratory and processed with acute diarrheal disease patients. Presumptive *V. cholerae* isolates were identified by selective culture, microscopic study and biochemical characterization. Among them, 15 non-duplicate isolates met the phenotypic specifications of *V. cholerae* and were molecularly confirmed and analyzed. All 15 isolates were subjected to PCR amplification targeting the *V. cholerae* -specific 16S rRNA gene. All isolates produced the anticipated amplicon of 372 bp, which fulfilled species level identification. Negative controls did not give any amplification. Figure 3 shows a sample agarose gel electrophoresis of the 16S rRNA gene amplification. Besides its use in species identification, the 16S rRNA gene served as the reference gene for normalizing relative gene expression in the qRT-PCR study, owing to its recognized role as a housekeeping/reference gene in bacterial gene expression research. Nonetheless, the lack of independent validation for the stability of 16S rRNA expression after pomegranate extract therapy is a drawback of the current investigation. Subsequent research needs to assess many potential reference genes to validate the reliability of the chosen reference gene inside the particular experimental parameters.

Epidemiological Distribution of Suspected Cholera Cases in Erbil City

The Erbil Central Laboratory had records of surveillance of diarrheal stool samples and they reported that a total of 49,290 sample were captured in the months of the study (2024-2025); 17,264 cases were captured within the months of July through November of both years. The monthly analysis of suspected cases of cholera showed that there was temporal variation in the course of the study. In the year 2024, there were greater instances of suspected cases in the spring months and then again in the late summer month which was followed by a decline in the fall and winter months. In 2025, there were higher cases of suspected cases in the spring period and highest monthly cases were registered between April and May. The increased reported cases were seen during spring of 2025 as compared to the same months in 2024. Figure 4 shows the distribution of the suspected cases of cholera monthly in both years.

Table 2: Primer Sequences used for Quantitative Real-Time PCR Analysis of Virulence-Associated Genes (*flaA*, *vpsT*, *luxO*) and the Reference Gene (16S rRNA)

Primer Name	Forward Primers (5'-3')	Reverse Primers (5'-3')	Source
16S rRNA	GGAAACGATGGCTAATACCG	GCCCTTACCTCACCAACTAG	Pederson <i>et al.</i> [20]
<i>flaA</i>	GGATTAAAGATACGGATTTTG	CGAGATTGCAGAGTTTG	
<i>vpsT</i>	GTCCGCAGGATATTGAGCAT	GCCTTTGATCAGGGTATCCA	
<i>luxO</i>	GCGAAAGTGGTACAGGTAAG	CCCTTTGACGTGACCAAAC	

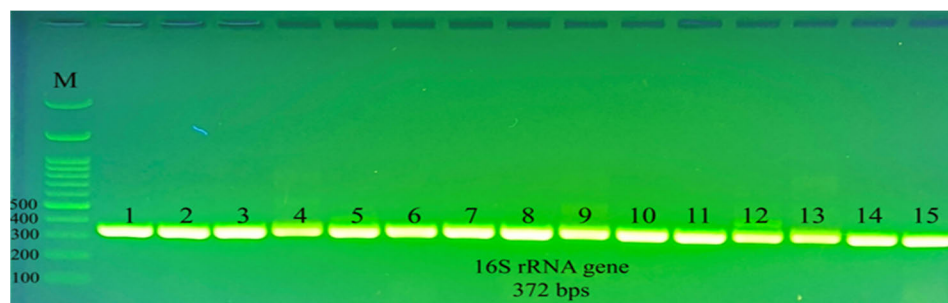
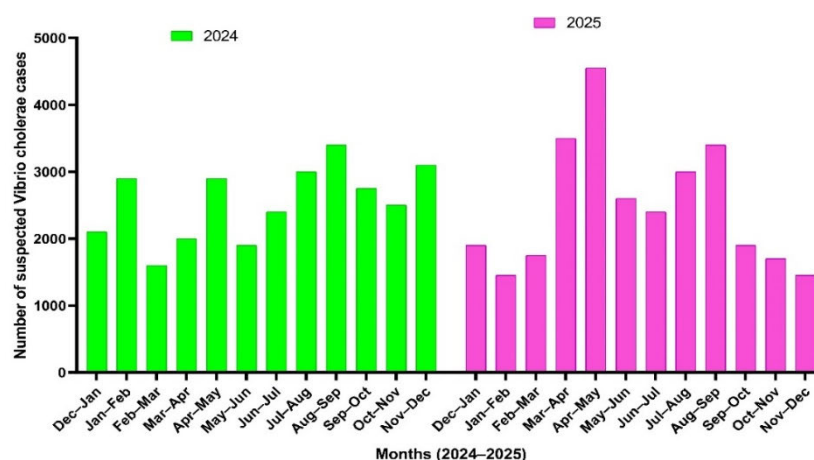
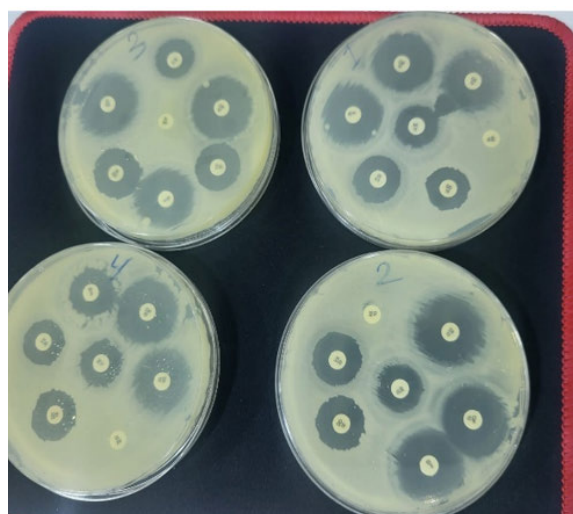

Figure 3: Agarose Gel Electrophoresis of Partial 16S rRNA gene Amplicons (372 bp) from Clinical *Vibrio cholerae* Isolates (M: DNA Ladder; Lanes 1-15: Clinical Isolates)


Figure 4: Monthly Distribution of Suspected Cholera Cases in Erbil City during 2024-2025

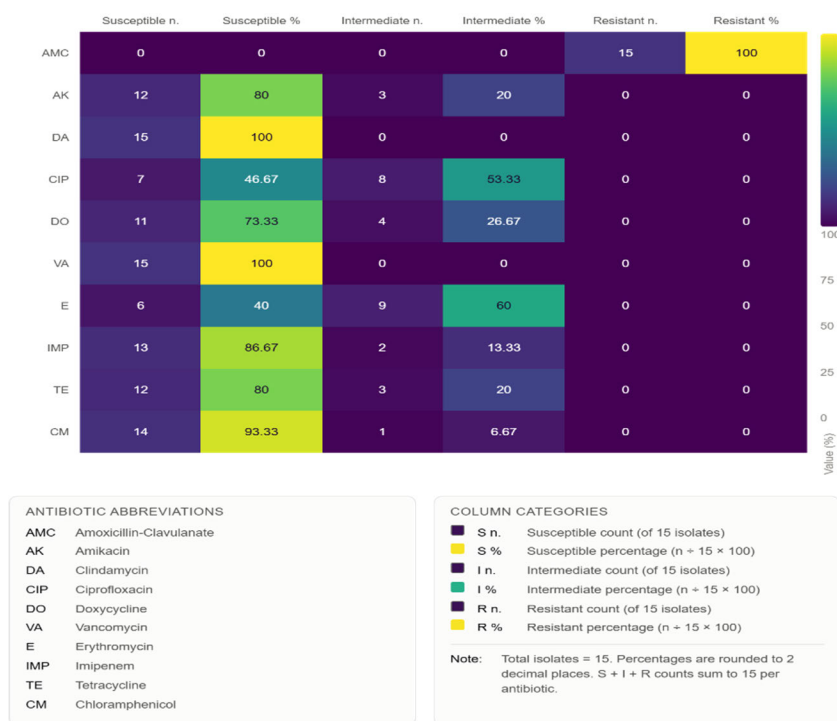

Figure 5: Representative Kirby-Bauer disk Diffusion Assay showing Antimicrobial Susceptibility Patterns of Clinical *Vibrio cholerae* isolates on Mueller-Hinton Agar

Antimicrobial Susceptibility Patterns of *V. cholerae* Isolates

Antimicrobial susceptibility testing of the fifteen confirmed *V. cholerae* was conducted using the Kirby-Bauer disk diffusion technique (Figure 5). The resulting susceptibility profiles displayed similar patterns in the test antibiotic panel. All isolates (100%) were resistant to amoxicillin-clavulanic acid. On the other hand, susceptibility to ciprofloxacin and imipenem was found to be high but Intermediate susceptibility was observed in 60.0% of isolates (9/15) against erythromycin and in 53.3% of isolates (8/15) against ciprofloxacin. The tested panel did not identify any isolate resistant to more than two major classes of antibiotics. The complete numerical distribution of susceptibility categories across all ten tested antimicrobials is presented in Table 3 and visualized via the isolate-level heatmap in Figure 6. The heatmap illustrates the relative susceptibility profiles of each isolate against the tested antimicrobial agents, with distinct color intensities corresponding to resistant, intermediate, or susceptible interpretations according to CLSI breakpoints.

Table 3: Antimicrobial Susceptibility Profiles of Confirmed Clinical *Vibrio cholerae* Isolates (n = 15)

Antimicrobial Agent	Disk Content	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Amikacin (AK)	30 µg	12 (80.00%)	3 (20.00%)	0 (0.00)
Amoxicillin-Clavulanic Acid (AMC)	20/10 µg	0 (0.00%)	0 (0.00%)	15 (100)
Ciprofloxacin (CIP)	5 µg	7 (46.67%)	8 (53.33%)	0 (0.00)
Doxycycline (DO)	30 µg	11 (73.33%)	4 (26.67%)	0 (0.00)
Clindamycin (DA)	2 µg	15 (100.00%)	0 (0.00%)	0 (0.00)
Erythromycin (E)	15 µg	6 (40.00%)	9 (60.00%)	0 (0.00)
Imipenem (IMP)	10 µg	13 (86.67%)	2 (13.33%)	0 (0.00)
Tetracycline (TE)	30 µg	12 (80.00%)	3 (20.00%)	0 (0.00)
Chloramphenicol (CM)	30 µg	14 (93.33%)	1 (6.67%)	0 (0.00)
Vancomycin (VA)	30 µg	15 (100.00%)	0 (0.00%)	0 (0.00%)


Figure 6: Heatmap Representation of Antimicrobial Susceptibility Patterns of 15 Clinical *Vibrio cholerae* Isolates

This approach highlights the uniform resistance observed against amoxicillin-clavulanic acid, while demonstrating variable responses among isolates to other antibiotic classes. The heatmap provides an integrated overview of resistance distribution and supports comparative interpretation of antimicrobial patterns across the isolates.

Chemical Composition of Pomegranate Peel Extract

The GC-MS chromatogram of the aqueous pomegranate peel extract demonstrated multiple peaks distributed across a broad retention time range, indicating the presence of diverse phytochemical constituents. Tentative identification using NIST library matching suggested the presence of various compound classes commonly reported in plant-derived extracts, including phenolic-related compounds, organic acids, esters and other secondary metabolites. The chromatographic profile reflects the complex chemical composition of pomegranate peel and supports its potential as a source of bioactive compounds. The

representative GC-MS chromatogram is presented in Figure 7 and Table 4. Because biological experiments in the present study were conducted using an aqueous extract, the GC-MS data are presented as indicative chemical fingerprinting rather than direct compositional characterization of the tested extract.

Antibacterial Activity of Pomegranate Peel Extract

Minimum Inhibitory Concentration (MIC): The Minimum Inhibitory Concentrations (MICs) of aqueous pomegranate peel extract in comparison with fifteen isolates of *V. cholerae* were found within a range of 3 to 50 mg/mL. Five of the isolates (Nos. 4, 5, 8, 14 and 15) had the lowest MIC values of 3 mg/mL⁻¹ which shows an increased susceptibility to the extract. Two of the isolates had a MIC value of 6 mg/mL⁻¹ and six isolates had a MIC value of 25 mg/mL⁻¹. The other three isolates required more concentration (50 mg mL⁻¹) to give them total effect of preventing growth. Table 5 shows the MIC values of the individual isolates.

Table 4: Tentatively Identified Compounds Detected by GC-MS Analysis in Pomegranate Peel Extract, Retention Times and Relative Peak Area

Tentatively identified compound	Retention time (min)	Relative peak area (%)
2,4,6-Trihydroxybenzoic acid	4.667	0.01
1-Naphthalenol	5.284	0
Dodecane	5.603	2.05
2-Carene	5.774	0.73
1,3,5-Benzenetriol	6.2	28.7
Trehalose	6.347	3.89
1,3,5-Benzenetriol	6.472	2.05
Trehalose	6.696	0.1
2-Naphthalenol	6.968	0.07
Tetradecane	7.427	1.9
1,3,5-Benzenetriol	7.696	6.27
Morpholine, 4-methyl-	7.743	9.76
2,4-Di-tert-butylphenol	8.541	5.51
Diethyl phthalate	9.201	38.92
Trehalose	9.56	0.05

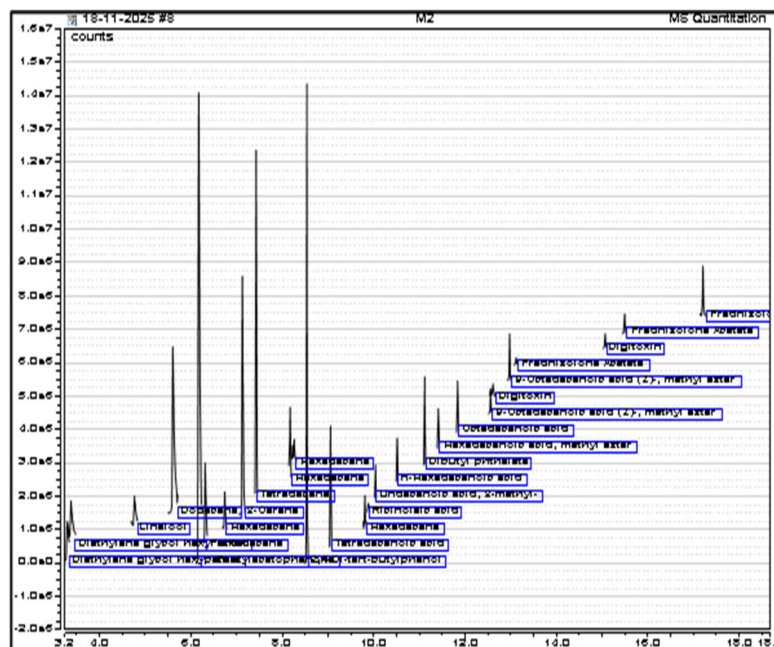
Relative peak area (%) was calculated from the integrated peak area for each reported peak relative to the sum of the integrated areas of the reported tentatively identified peaks. Compounds were tentatively identified by comparison of their mass spectra with the NIST mass spectral library

Table 5: Minimum Inhibitory Concentration (MIC) Values of Pomegranate Peel Extract against *Vibrio cholerae* Isolates

Pomegranate peel extract	<i>V. cholerae</i> isolates														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	50	25	25	3	3	25	50	3	25	6	25	6	50	3	25

Table 6: Inhibition Zone Diameters (mm; mean±SD of Triplicate Assays) of Aqueous Pomegranate Peel Extract against Clinical *V. cholerae* Isolates

Isolate No.	50 mg/mL	25 mg/mL	12 mg/mL	6 mg/mL	3 mg/mL	Vehicle control (0.25% CMC)
1	22.0±0.8	17.0±0.5	20.0±0.8	15.0±0.6	10.0±0.4	0.0±0.0
2	24.0±1.0	15.0±0.6	14.0±0.5	11.0±0.5	9.0±0.3	0.0±0.0
3	21.0±0.9	14.0±0.6	11.0±0.4	9.0±0.4	8.0±0.3	0.0±0.0
4	22.0±0.7	16.0±0.7	13.0±0.5	10.0±0.4	9.0±0.4	0.0±0.0
5	20.0±0.8	17.0±0.8	15.0±0.6	12.0±0.5	8.0±0.3	0.0±0.0
6	21.0±0.8	19.0±0.7	17.0±0.7	13.0±0.6	9.0±0.4	0.0±0.0
7	23.0±1.1	18.0±0.8	15.0±0.6	11.0±0.5	10.0±0.5	0.0±0.0
8	19.0±0.8	14.0±0.5	13.0±0.5	10.0±0.4	7.0±0.3	0.0±0.0
9	21.0±0.9	18.0±0.7	16.0±0.6	14.0±0.6	9.0±0.4	0.0±0.0
10	22.0±0.8	17.0±0.6	13.0±0.5	10.0±0.4	7.0±0.3	0.0±0.0
11	20.0±0.7	15.0±0.6	12.0±0.5	9.0±0.4	7.0±0.3	0.0±0.0
12	23.0±0.9	18.0±0.8	16.0±0.7	10.0±0.5	8.0±0.4	0.0±0.0
13	21.0±0.8	17.0±0.7	13.0±0.5	9.0±0.3	8.0±0.3	0.0±0.0
14	22.0±0.9	17.0±0.7	12.0±0.5	10.0±0.4	8.0±0.3	0.0±0.0
15	20.0±0.7	16.0±0.6	15.0±0.6	11.0±0.5	8.0 ± 0.3	0.0±0.0


Figure 7: GC-MS Total Ion Chromatogram of a Separately Prepared Aqueous Pomegranate (*Punica Granatum*) Peel Extract used for Exploratory Chemical Fingerprinting

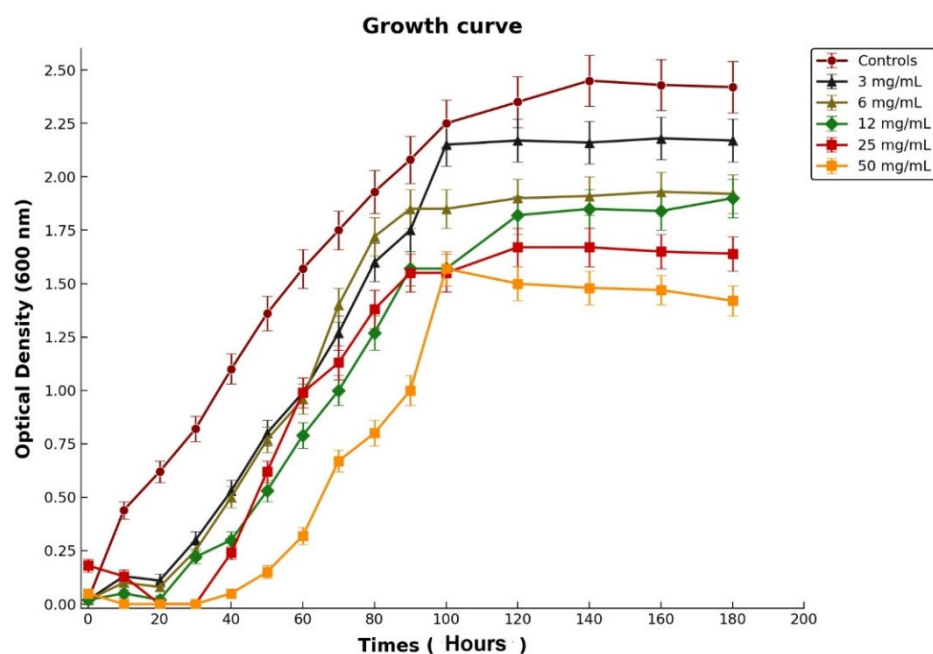


Figure 8: Growth Curve Analysis of *Vibrio cholerae* Isolates Treated with Different Concentrations of Aqueous Pomegranate Peel Extract

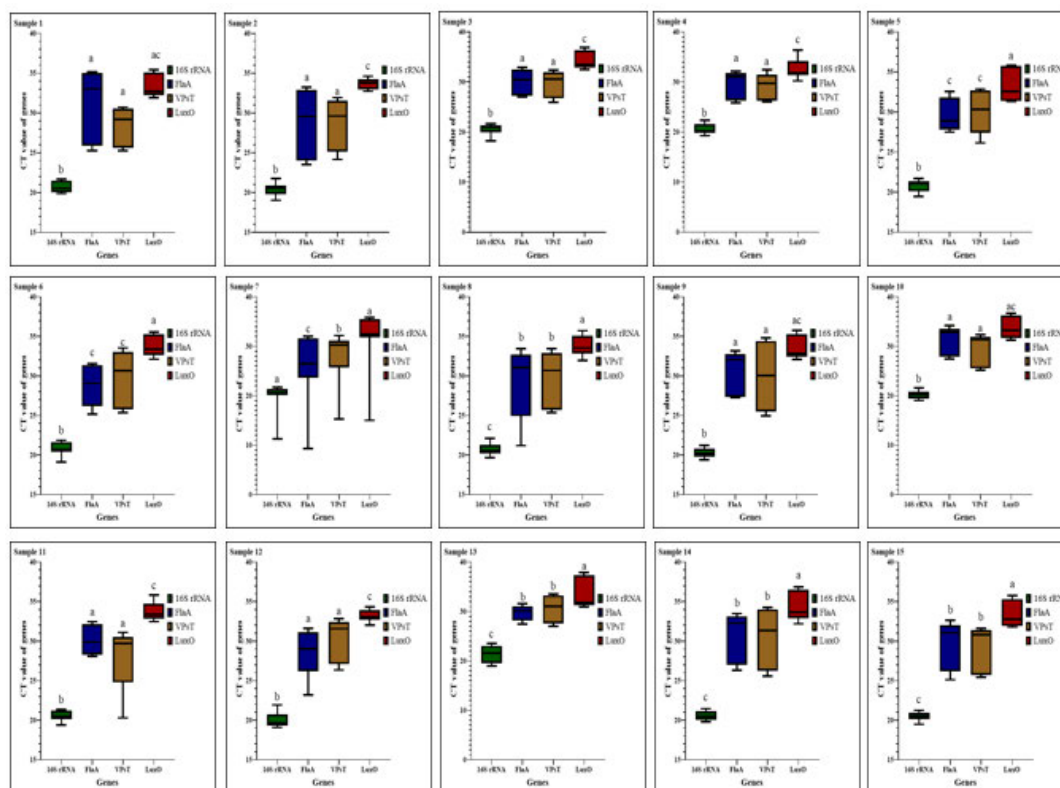


Figure 9: Relative Transcript Expression of Virulence-Associated Genes (*flaA*, *vpsT*, and *luxO*) in *Vibrio cholerae* Following Extract Exposure

Agar Well Diffusion Assay

Agar-well diffusion agar clearly showed that pomegranate peel extract had a dose-dependent antibacterial activity against all *V. cholerae* isolates studied. Inhibition zones of

between 19-24 mm were obtained at the highest concentration (50 mg mL⁻¹). Inhibition zones of 25 mg mL⁻¹ consisted of 14 mm to 19mm and those of lower concentrations (12, 6 and 3mg mL⁻¹) resulted in smaller inhibition zone.

Table 7: Relative transcript fold change ($2^{-\Delta\Delta C_T}$) of Virulence-Associated Genes (*flaA*, *vpsT*, *luxO*) Normalized to 16S rRNA in Clinical *V. cholerae* Isolates.

(ND: Not Detected, amplification $C_T > 40$, Reflecting Complete Transcript Suppression below Detection Limits)

Sample	flaA			vpsT			luxO		
	Control	10 mg/mL	20 mg/mL	Control	10 mg/mL	20 mg/mL	Control	10 mg/mL	20 mg/mL
1	1.35	ND	0.02	0.9	0.06	0.02	1.42	0.87	0.08
2	1.89	0.01	0.12	0.38	0.06	0.02	1.14	0.48	0.53
3	1.27	0.01	0.12	0.95	0.03	0.01	0.75	0.31	0.4
4	1.87	0.02	0.02	1.18	0.02	0.03	6.2	0.76	0.19
5	0.83	0.06	0.01	1.09	0.02	0.01	0.81	0.49	0.13
6	1.48	0.01	0.14	1.3	ND	0.04	1.4	0.69	0.11
7	1.23	0.05	0.02	1.29	0.02	0.03	0.72	0.7	0.32
8	1.82	0.02	0.02	0.95	0.01	0.05	0.67	0.52	0.34
9	0.34	0.01	0.02	0.59	ND	0.04	0.87	0.65	0.06
10	0.31	ND	0.02	1.39	0.01	0.01	0.28	0.48	0.38
11	0.25	0.02	0.06	1.64	0.04	0.05	1.27	0.63	0.21
12	0.73	0.08	0.02	0.33	0.01	0.01	1.08	0.49	0.13
13	2.69	0.01	0.02	1.67	ND	0.01	1.43	0.87	0.25
14	1.08	0.01	0.01	1.66	ND	0.01	0.43	0.81	0.28
15	1.1	0.02	0.01	1.22	0.02	0.01	1.14	0.55	0.19

Although there was variation in isolates, quantifiable inhibition zones were observed in all investigated concentrations of all isolates. For the concentrations, Table 6 tabulates the specific inhibition zone diameters of each isolate.

Transcriptional Responses of *Vibrio cholerae* to Pomegranate Peel Extract

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to identify the expression levels of *flaA*, *vpsT* and *luxO* genes of virulence in response to the treatment with pomegranate peel extract. The abundance of transcripts was adjusted to the 16S rRNA reference gene and was measured by the comparative $2^{-\Delta\Delta C_T}$ method. Pomegranate peel extract treatment resulted in down-regulation of *flaA* and *vpsT* compared to untreated controls and the strength of the repression was greater with higher extract concentrations. In comparison, the expression of *luxO* showed only a small drop when exposed. The results of all the genes in the control and treated conditions relative to each other in terms of expression are presented in Table 7 and the comparison of the change in gene expression is shown in Figure 8 and 9.

DISCUSSION

This study provided a detailed laboratory-based study of *V. cholerae* isolates collected in the city of Erbil, in which an epidemiological surveillance is combined with antimicrobial susceptibility analysis and the expression of virulence-related genes measured by molecular analysis. The study provided localized data on the dynamics of cholera and bacterial nature that are relevant to daily diagnostic and surveillance efforts by focusing on one metropolitan area.

The epidemiological examination revealed a strong seasonal trend in suspected cases of cholera with peaks in the number of cases during the spring months especially in 2025. Urban areas have broadly recorded seasonal cholera patterns and the patterns may coincide in variations in environmental and infrastructural factors of water supply and temperature changes. Even though the present experiment was not directly aimed at exploring causative reasons, therefore no

causal relationships can be established, the observed temporal patterns revealed indicate that the circulations of the *V. Cholerae* are persistent in the investigated region and that laboratory surveillance should be maintained [11].

The evaluation of antimicrobial susceptibility showed that all the known isolates of *V. cholerae* could resist amoxicillin and clavulanic acid but retained sensitivity to ciprofloxacin and imipenem. All these findings highlight the urgency of continuous surveillance of the dynamics of antimicrobial resistance in the local area [10]. Although the rehydration therapy is always the fundamental importance in treating cholera, antibiotics are also commonly used to shorten the illness period and shedding of bacteria. New susceptibility information regarding the city of Erbil would help to provide informed antimicrobial stewardship in the clinical environment [12,13].

In addition to traditional antimicrobial tests, the study determined the effectiveness of pomegranate peel extract on clinical isolates of *V. cholerae* in antibacterial activity. The extract was dose-effectively inhibitory as indicated by the minimum inhibitory concentrations and agar well diffusion tests. This research confirms prior findings that pomegranate peel contains a diverse variety of phytochemicals that vary by cultivar, geographical origin, extraction solvent and analytical circumstances. In previous GC-MS investigations of pomegranate peel, phenolic compounds, fatty acids, esters and other volatile or semi-volatile substances were found. In pomegranate peel extracts, 2,4-di-tert-butylphenol and hexadecanoic acid derivatives have been found, confirming the presence of similar chemical ingredients in this investigation. Previous research have shown that pomegranate peel extracts have antibacterial activity and that their biological effects vary depending on the extraction method and bacterial species studied. The complex mixture of phytochemicals in pomegranate peel has been linked to its antimicrobial activity and studies of its phenolic compounds have shown that chemical composition can affect antimicrobial activity. Thus, compounds like 2,4-di-tert-butylphenol, 2-carene, linalool and fatty acid derivatives in the present GC-MS profile may be promising candidates for

further study, The ranges of concentrations required to induce growth suppression were relatively high; however, the consistent antibacterial effect on all isolates incriminates the presence of bioactive compounds that can regulate bacterial growth *in vitro*, but library matching alone cannot determine their contribution to the antibacterial activity observed in this study. The current profile may vary from prior publications due to pomegranate variety, geographical origin, extraction circumstances and analytical approach [14,15].

Most importantly, transcriptomic analysis revealed that pomegranate peel extract treatment caused marked down-regulation of the *flaA* and *vpsT* genes that encode part of the motility and biofilm architecture, respectively. These observations indicate that the extract can put the virulence-related pathways of *V. cholerae* on the other hand, the expression of *luxO* was not much altered, which is a symptom of different expression sensitivities. Internal-generally-specific modulations of such kind have been reported in studies of anti-virulence interventions, highlighting the complexity of regulatory networks that control bacterial pathogenicity [16,17].

The transcriptional change was observed, which supports the hypothesis that phytochemicals can act not only by direct antibacterial action but also by altering the expression of virulence-related genes. This strategy may provide synergies of benefit by reducing pathogenic potential and causing a minimal selection pressure to resistance [18,19]. However, the existing findings are limited to *in vitro* observations and are thus to be taken cautiously.

There are a number of limitations that should be mentioned. A comparatively small group of verified *V. cholerae* isolates was used as a sample and the study was limited to one urban area. In addition, no functional tests were performed to assess the phenotypic characteristics like motility and biofilm formation. Nevertheless, the research provides useful background information on the city of Erbil and creates a platform in which future studies, including bigger sample sizes and further molecular studies, can be conducted based on it. Moreover, chemical profiling of the aqueous extract that was used in the biological assays was not conducted and this should be filled in future researches. GC-MS profiling was performed on a separately prepared aqueous extract rather than on the same batch used in the biological assays. Therefore, the detected compounds represent an exploratory chemical fingerprint and cannot be directly attributed to the antibacterial and gene-expression effects observed in this study. Future investigations should chemically characterize the same aqueous-extract batch used in the biological assays using appropriate LC-based analytical techniques to establish a direct relationship between chemical composition and biological activity.

CONCLUSION

The present work is a detailed laboratory evaluation of *V. cholerae* strains, obtained in the city of Erbil that combines epidemiological monitoring, antimicrobial resistance analysis and molecular evaluation of the expression of

virulence-related genes. The results showed evidence of seasonal fluctuation in suspected cases of cholera and also indicate that resistance to amoxicillin-clavulanic acid is constant with no evidence of resistance to various widely used antibiotics. The bacterial growth and phenotypic responses of the bacteria under the influence of pomegranate peel extract demonstrated through the antibacterial activity against all the tested isolates and down-regulation of important virulence-associated genes (*flaA* and *vpsT*), indicate that the pomegranate peel extract may have the capacity to regulate bacterial growth and the expression of select virulence-associated genes in the *in vitro* environment at the molecular level. The relatively intermediate impact on the *luxO* expression indicates that virulence pathways are differentially regulated in response to extract exposure. Despite its small sample size of confirmed isolates and its one-city design, the study contributes to baseline data on Erbil city and a more comprehensive body of knowledge on local *V. cholerae* features.

The obtained results based on *in-vitro* findings suggest the further development of antimicrobial monitoring along with the molecular methods of cholera studies however further *in-vivo* and functional validation is required to confirm their biological effects and safety before any therapeutic applicability can be considered.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Consent to Participate

Written informed consent was obtained from all patients (or parent/legal guardian for pediatric cases) prior to specimen collection in accordance with institutional clinical surveillance protocols approved by the ethics committee.

Availability of Data Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

Iman M. Rasul: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Visualization, Writing Original Draft. Salah Tofik J. Balaky: Supervision, Conceptualization, Review and Editing.

Ethical Statement

This study was reviewed and approved by the Medical Ethics Committee of Hawler Medical University, Erbil, Iraq (Paper code: 21, Meeting Code: 3). All procedures were performed in accordance with the ethical standards of the Declaration of Helsinki.

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