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RESEARCH ARTICLE
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MICROBIAL ASSESSMENT OF INDUSTRIAL WATER COLLECTED FROM MAJOR INDUSTRIAL CITIES OF PAKISTAN

Zoha Zainab[1], Hassan Adil[2], Wajeeha Rehman[3], Ghania Syed[4], Muneeba Rasool[1], Tayyaba Qureshi[1], Malik Arsalan Ali[5], Muhammad Quddusе Ahmad Rao[6], Muhammad Arqam[1], Mohsin Gulzar Barq[1*],

[1]Department of Microbiology, University of Central Punjab, Lahore, 54000, Pakistan.
[2]Department of Biotechnology, University of Karachi, Pakistan.
[3]College of Earth and Environmental Sciences, University of the Punjab, Lahore, 53700, Pakistan
[4]Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, 53700, Pakistan
[5]Faculty of Sciences, Engineering and Technology, University of Adelaide, Adelaide, 5000, Australia.
[6] Institute of Molecular Biology and Biotechnology, University of Lahore, 54000, Lahore, Pakistan.

***Corresponding author:** Mohsin Gulzar Barq
*Tel.: +92-042 35880007 (171), Corresponding e-mail address: mohsin.barq@yahoo.com

Abstract:
The provision of clean and safe drinking water is a fundamental human right and critical to public health. Pakistan is currently grappling with water scarcity and contamination, so ensuring the hydro-environmental safety is a matter of supreme concern. This study examines the extent and nature of microbial contaminants in surface, ground, waste, and drinking water sources across five major industrial cities. 300 water samples retrieved from Lahore, Karachi, Faisalabad, Sialkot, and Gujranwala were subjected to microbiological characterization and quantification. The study performed between January 2022 and January 2023 was focused on total coliforms, fecal coliforms, *E. coli, Enterococcus faecalis, Pseudomonas aeruginosa,* and *Legionella pneumophila* count. Our findings reveal a pervasive presence of bacterial contaminants in water sources, with alarming prevalence rates observed in Karachi. The highest contamination levels were associated with wastewater samples (100%), highlighting the urgent need for effective wastewater treatment methods. Moreover, antibiotic resistance has been detected in specific bacterial strains. All results were evaluated against the international water quality standards established by the WHO, EPA, and NSDWQ. It is imperative to take immediate action to address the complex challenges associated with water contamination. These efforts need to be thorough and involve collaboration between government regulatory agencies and industry stakeholders.

Keywords: Water pollution, drinking water quality, Public health, industrial wastewater, antibiotic resistance, water resources, microbial contamination, Pakistan

Introduction:

Water envelopes 70% of Earth's surface, but only 0.5% is freshwater, suitable for human consumption. The United Nations' first water conference in 2023 pointed out that 2 billion people (26% of the population) across the globe don't have access to safe drinking water, and 3.6 billion

(46%) do not have access to proper hygiene and sanitation facilities (WHO 2008a). Poor administration, governance problems, population explosion, climate crisis, deforestation, urbanization, and industrialization have disturbed the circle of life in Pakistan (Afridi et al. 2019).

Pakistan is rapidly developing with a significant concentration of textile mills, manufacturing, processing factories, and mining sectors populating major urban vicinities (Akhtar et al. 2021). The wastewater industrial excrement is discharged into nearby water aquifers, streams, canals, sewer systems, or agricultural land (Kumar et al. 2023). The churned-out liquid vitiated by microbial contamination (bacteria, viruses, protozoa), toxic chemicals (organic and dissolved inorganic contaminants) and industrial wastes (metals, acids, salts, ions) pose grave threats to public health and water quality (Mahfooz et al. 2019). Pakistan ranks 137 in wastewater treatment and 144 in sanitation and drinking water amongst 180 countries according to the Environmental Performance Index (EPI) report of 2022 (Solomon 2023).

A single intake of dirty water is responsible for acute health concerns compared to long-term health issues due to chemical pollutants (Paul et al. 2015). Ground and surface water are the two main resources but are contaminated by animal or human feces in Pakistan. This polluted water consumption has caused 80% of waterborne diseases and even contributed to 33% of fatalities in Pakistan (Ahmed et al. 2017). The three most prevalent types of bacteria assessed in water quality testing are *Escherichia coli,* total coliforms, and fecal coliforms. Additionally, *Enterococcus*, in recreational water bodies, *Pseudomonas aeruginosa*, the ubiquitous opportunistic pathogen (Muddassir et al. 2022), and *Legionella pneumophila* in contaminated water aerosols compromise water purity standards (Bej et al. 1991).

In previous years, PCRWR (Pakistan Council of Research in Water Resources) has independently as well as under the government, conducted considerable studies for aquatic quality analysis to timely strategize a practical methodology against the prevailing water crisis. The “National Water Quality Monitoring Program” (NWQMP) 2002-2006 report presented 84% unsafe water sources, but the same regions re-assayed by WHO in 2015-2016 portrayed 69% unavailability still in the region (Rasheed et al. 2021).

In the previous reports, of the 27 water aliquots from Islamabad, 74% had bacterial infiltration, 41% of which was *E. coli* (Ahmed et al. 2017). Similarly, freshwater from 425 randomized schools in ten Sindh districts demonstrated 62% groundwater and 38% surface water sources, with 49% *E. coli,* followed by various bacterial intrusions recorded. Drinking water samples in several cities of Sindh had *Shigella, P. aeruginosa, Enterococcus, Salmonella* species, and different strains of *E. coli*. While in Lahore, among 100 samples from diverse areas, 59% (42% *E. coli* and 54% coliform tainted) were classified as unfit for drinking. In parallel, potable water unavailability is a dire issue in Baluchistan. Researchers have paid very little attention to a few reports published on the province's water quality evaluation outside of those by PCRWR. The data highlights 70-100% microbiological contamination attributed to poor facilities and resources in the dry arid region of Baluchistan (Rasheed et al. 2021).

Water purification plants in developed cities are limited and mostly non-functional, making instances of microbiological contamination difficult to address. A multitude of independent studies demonstrate elevated microbial concentration in drinking water across the country, but no comprehensive statistics have been compiled and analyzed till now. Little or no knowledge specifies *E. faecalis, P. aeruginosa, and Legionella* prevalence rates. Additionally, the lack of data hinders understanding and the underlying implications of the multidrug-resistant (MDR) microbial situation in Pakistan.

This research endeavors to shed light on microbial diversity found in industrial water sources across the five major cities of Pakistan. By evaluating the prevalence of total coliforms, fecal coliforms, *E. coli, P. aeruginosa, E. faecalis, and L. pneumophila*, we aim to provide a comprehensive understanding of water quality challenges faced by the nation. Furthermore, we explore the implications for public health, the environment, and industrial practices. Determination of drinking water quality primarily depends on the source, treatment in water treatment plants, distribution systems, storage tanks, household filters, and effluent water quality (WHO 2008b). These exact parameters were selected for our study to create a similar pattern in understanding the source and cause of the water fouling.

Materials and Methods:

- ***Site delineation and sample collection:***

A total of 300 industrial water samples were collected from the industrial areas of five major cities: Lahore (102), Sialkot (48), Faisalabad (56), Karachi (56), and Gujranwala (32). For sample area stratification, the water sources were divided into drinking water, groundwater, reverse osmosis (RO) treated water, processed and potable water, wastewater, and cooling tower or air conditioning drained water. The standard method of water sampling by the American Public Health Association was employed. The samples were transported at 2-8 ºC, temperature-controlled, light-proof, and disinfected, labeled containers with a field sample data sheet on the very same day of collection. The duration of the study was between January 2022 to January 2023.

- ***Bacterial identification and susceptibility testing:***

The collected water sample (1 mL) was spread on a Nutrient Agar plate and incubated overnight to proceed with initial identification via Gram staining. Germinated colonies were streaked on selective media as mentioned in Table 1 and kept for 24 to 48 hours at specific optimum temperatures. Additionally, 100 mL of the water sample was membrane filtered (0.45mm pore size), and membranes were incubated on specific media plates. The identified bacterial isolates were validated by performing biochemical confirmatory tests, i.e., Catalase test, Oxidase test, Lactose fermentation test, Indole test, Citrate utilization test, Methyl Red (MR), and Voges-Proskauer (VP) test.

Table 1: Selective media used to grow specific types of microorganisms.

Selective Media	Bacteria
M-Endo Agar	Total coliforms examination
Slantze and Bartley medium	Enterococci (*E. faecalis*)
MPA Agar	*P. aeruginosa*
mFC Agar	*E. coli*
Buffered Charcoal Yeast Extracted Agar	*L. pneumophila*

- ***Antibiotic Susceptibility Testing:***

A modified Kirby-Bauer disc diffusion method following the Clinical and Laboratory Standard Institute (CLSI) guidelines (Hudzicki 2012) was conducted with Amikacin, Norfloxacin, Chloramphenicol, and Penicillin antibiotic discs. Bacterial inoculum of 0.5 McFarland standard was spread and observed for the zone of inhibition on MH agar plates following overnight incubation.

- ***16S rRNA identification:***

Bacterial DNA was extracted using Thermo Scientific's GeneJET Genomic DNA Extraction kit. Each isolated bacterial conserved 16S rRNA regions were amplified for strain determination using universal primers 27F and 1492R (Barq et al. 2021) in a thermal cycler (Model FTC41H2D, Bibby Scientific Ltd., UK) and visualized using a 1% Agarose gel on a 254-nanometer UV Transilluminator (foto/UV 15, Model 33017, Fotodyne, USA).

- ***Statistical analysis:***

The statistical analysis was performed using SPSS software (IBM SPSS Statistics, Version 28.0). The difference among mean values was analyzed by Duncan’s Multiple Range Test (DMRT). Phylogenetic trees were made by aligning multiple sequences with the help of Mega XI (Liang et al. 2011).

Results:

The bacterial testing revealed total coliforms in 98 samples, *E. coli* (ZM-1) in 81 samples, *Enterococcus faecalis* (ZM-2) in 56 samples, *Pseudomonas aeruginosa* (ZM-3) in 59 samples, and *Legionella pneumophila* (ZM-4) in 6 samples. The prevalence of total and fecal coliform revealed concerning results. Notably, total coliforms (42%) and fecal coliforms (35%) were detected in the drinking water of Sialkot (n=14). Groundwater samples of Faisalabad showed 100% total and fecal coliform contamination. RO water of Lahore (50%) and Karachi (50%) exhibited greater microbial growth levels as compared to other cities. Process water from Gujranwala was extensively polluted (100%) with coliforms and fecal coliforms. All the samples (100%) of wastewater released from industries of Sialkot, Karachi, and Gujranwala were contaminated with coliforms. All of these water samples showed total and fecal contamination greater than the permissible values set by WHO, NSDWQ, and the U.S. EPA (0MPN/100mL) as seen in Figures 1 and 2.

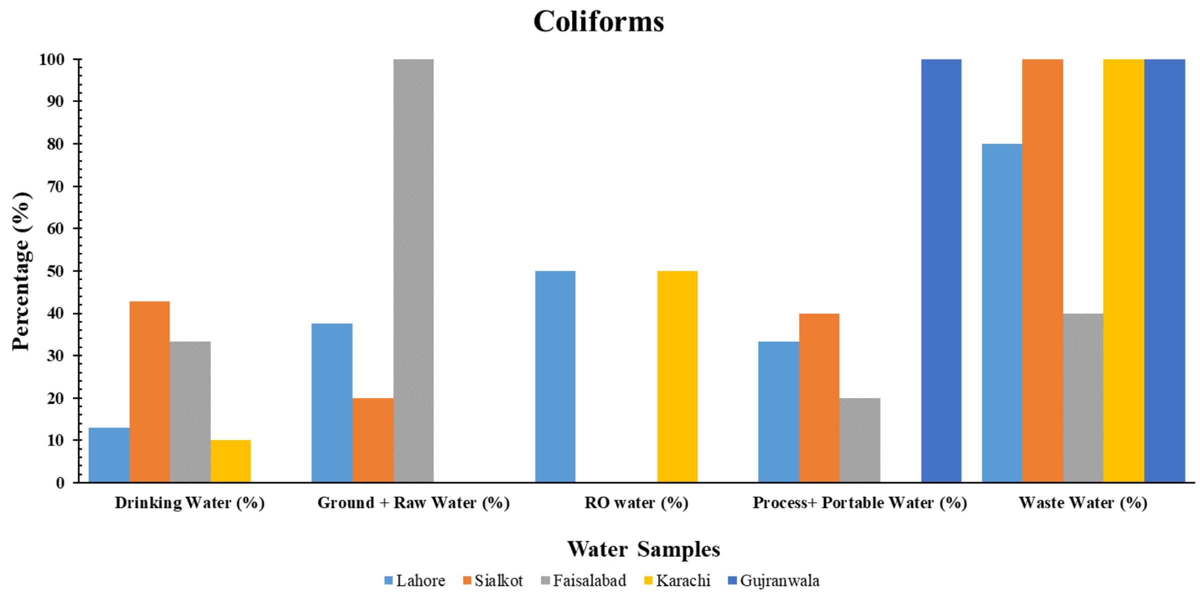

Figure 1: **The percentage of Coliforms detected in different water samples collected from Lahore, Sialkot, Faisalabad, Karachi, and Gujranwala. Values represent means (n=3) and error bars represent standard error. Different lower-case letters show a significant difference at P < 0.05 (Duncan's Multiple Range Test).**

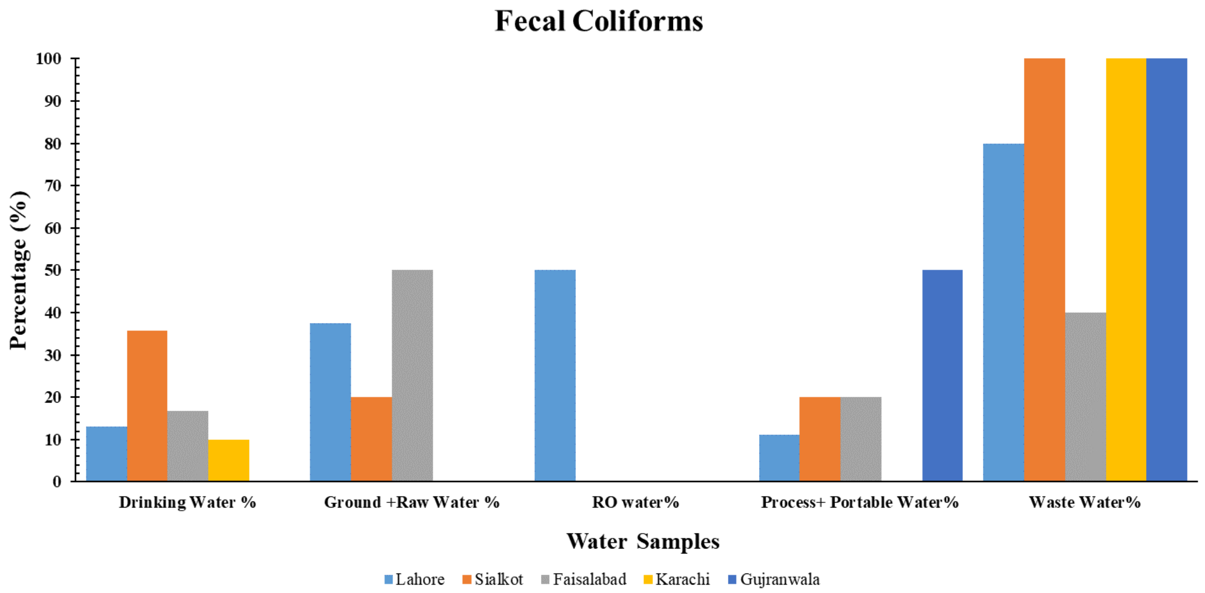

Figure 2: **The percentage of fecal coliforms detected in different water samples collected from Lahore, Sialkot, Faisalabad, Karachi, and Gujranwala. Values represent means (n=3) and error bars represent standard error. Different lower-case letters show a significant difference at P < 0.05 (Duncan's Multiple Range Test).**

The highest prevalence rates of *Enterococcus faecalis* were found in the drinking water (42%) of Sialkot. Groundwater and process water samples from Faisalabad were also significantly contaminated (50% and 20%, respectively). Enterococcus contamination was observed in all wastewater samples, with the highest in Karachi (100%). No sample of RO plant samples showed contamination, indicating the effective working of filtration treatment plants, as seen in Figure 3.

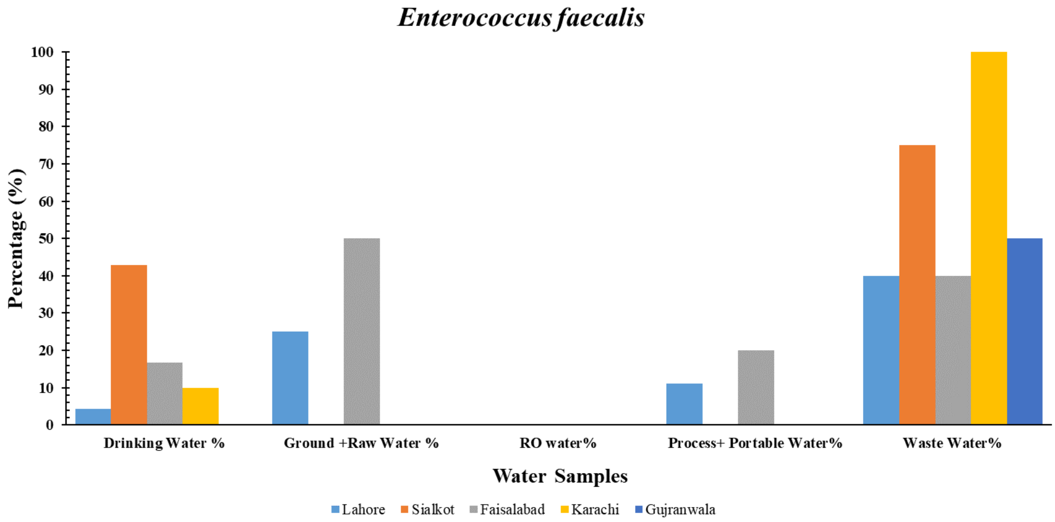

Figure 3: **The percentage of fecal coliforms detected in different water samples collected from Lahore, Sialkot, Faisalabad, Karachi, and Gujranwala. Values represent means (n=3) and error bars represent standard error. Different lower-case letters show a significant difference at P < 0.05 (Duncan's Multiple Range Test).**

The analyzed samples for *Pseudomonas aeruginosa* were pronounced highly unfit for intake in Faisalabad (17%) and Gujranwala (17%). Gujranwala's groundwater samples (50%) were comparatively more contaminated. Processed water in Faisalabad (20%) was more contaminated with *Pseudomonas.* All wastewater samples from Karachi (100%) were contaminated, while RO water samples had no microbial growth reported, depicting a safe water supply for user intake, as evident in Figure 4.

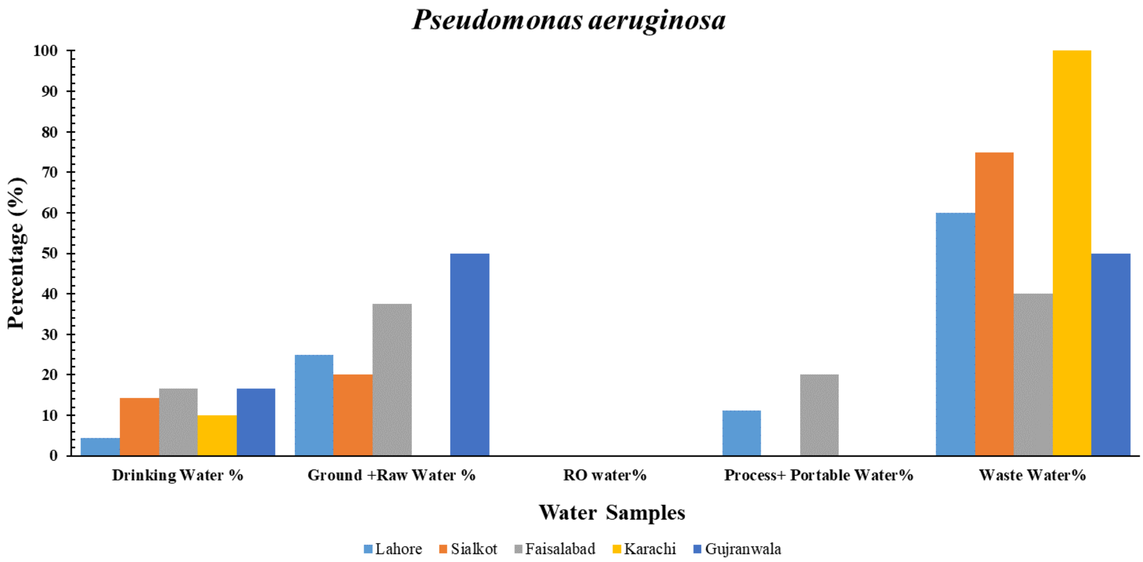

Figure 4: **The percentage of fecal coliforms detected in different water samples collected from Lahore, Sialkot, Faisalabad, Karachi, and Gujranwala. Values represent means (n=3) and error bars represent standard error. Different lower-case letters show a significant difference at P < 0.05 (Duncan's Multiple Range Test).**

Legionella pneumophila was detected in 25% of Lahore samples and 50% of Karachi samples, specifically in cooling towers, AC drains, and chillers' wastewater as shown in figure 5.

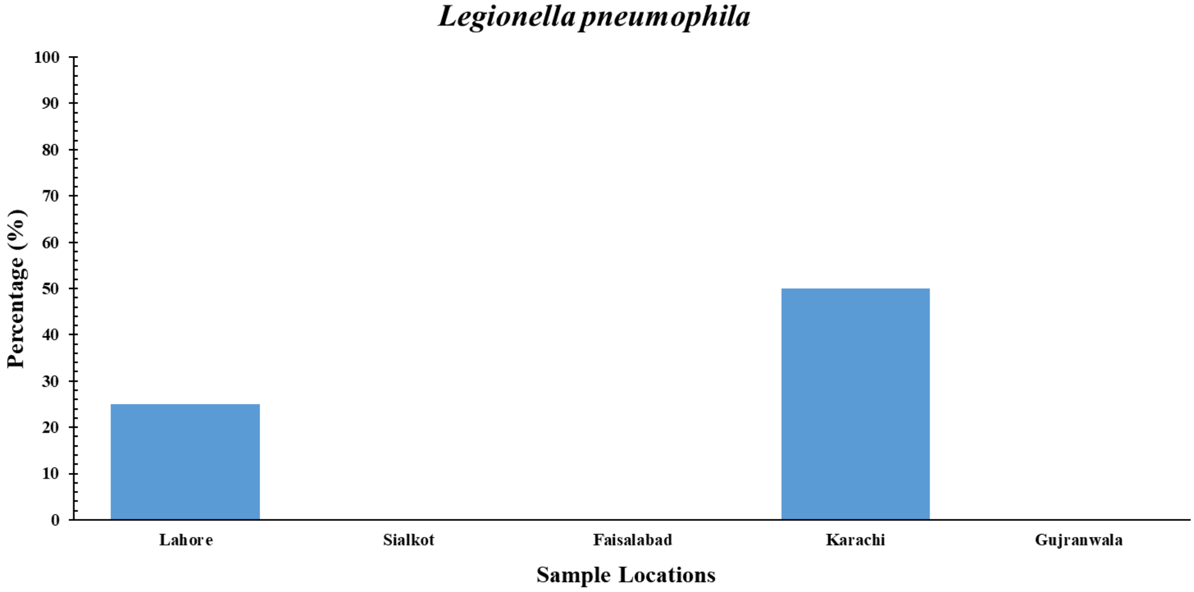

***Figure 5:* The percentage of fecal coliforms detected in different water samples collected from Lahore, Sialkot, Faisalabad, Karachi, and Gujranwala. Values represent means (n=3) and error bars represent standard error. Different lower-case letters show a significant difference at P < 0.05 (Duncan's Multiple Range Test).**

The Gram staining profiling showed only *Enterococcus faecalis* isolates being positive. The rest of the isolates (*E. coli, Pseudomonas aeruginosa, Legionella pneumophila*) appeared as pink Gram-negative bacteria. Further evaluation through biochemical tests is detailed in Table 2.

Table 2: Biochemical profiling of ZM-1, ZM-2, ZM-3, and ZM-4 isolated from different water samples of Lahore, Sialkot, Faisalabad, Karachi, and Gujranwala.

Sr.#	Tests	Description	Results			
			ZM-1	ZM-2	ZM-3	ZM-4
1.	Catalase	Bubble Formation	+	-	+	+
2.	Oxidase	Purple coloration	-	-	+	+
3.	Indole	Cherry red ring formation	+	--	-	--
4.	Methyl Red	Red tint after adding methyl red	+	--	-	--
5.	Voges-Proskauer	Reddish brown color	-	--	-	--
6.	Citrate	Green to blue color change	-	--	+	--
7.	Lactose fermentation	Gas production	+	--	-	--

16S rRNA sequencing of 04 morphologically different strains ZM1, ZM2, ZM3, and ZM4 confirmed with BLAST as *Escherichia coli* (Figure 7), *Enterococcus faecalis* (Figure 8), *Pseudomonas aeruginosa* (Figure 9), and *Legionella pneumophila* (Figure 10).

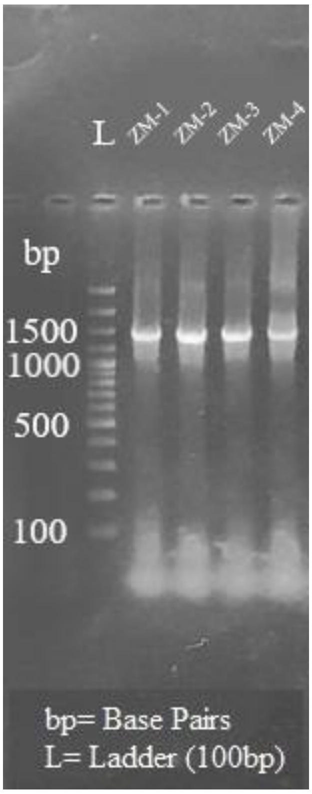

Figure 6: **Agarose gel (1.5%) electrophoresis of amplicons.3 µl of PCR product was loaded (Lane 1-4); L= 1kb-bp DNA molecular weight ladder. Lane 1= ZM-1, Lane 2= ZM-2, Lane 3= ZM-3, and Lane 4= ZM-4.**

Additionally, 16S rRNA gene amplification and sequencing were performed to construct phylogenetic trees, aiding in understanding genetic relationships among the bacteria.

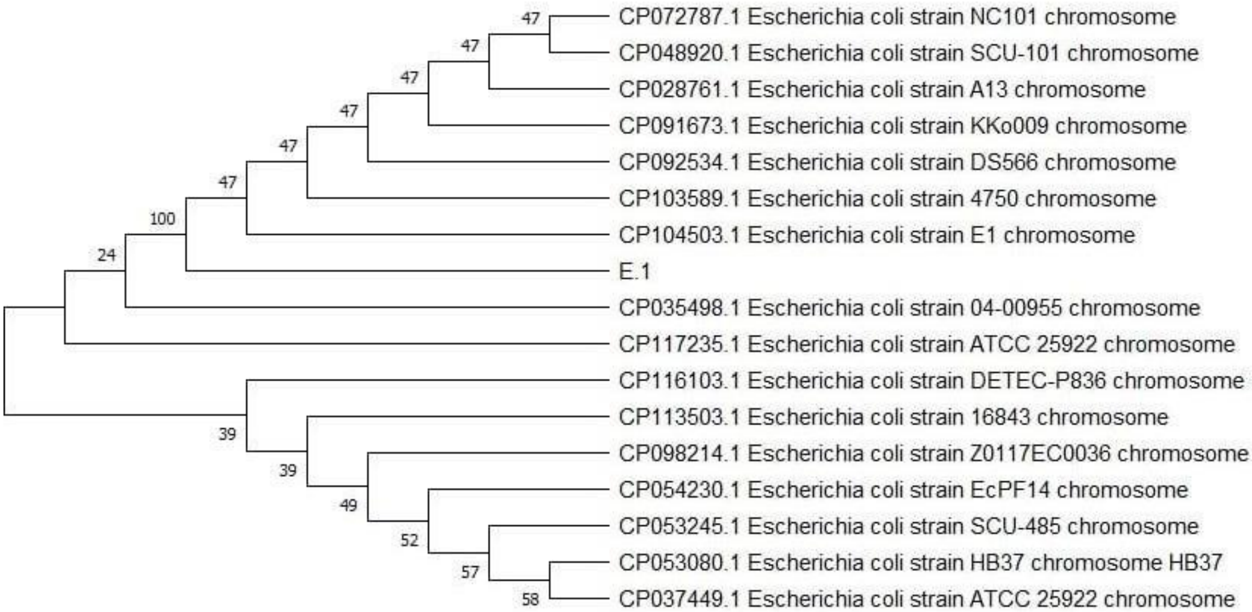

Figure 7: **Evolutionary relationship of ZM-1. A bar value of 0.05 indicates nucleotide substitutions per nucleotide position. Bootstrap values are shown as the percentage of 500 replicates displayed at the branch points.**

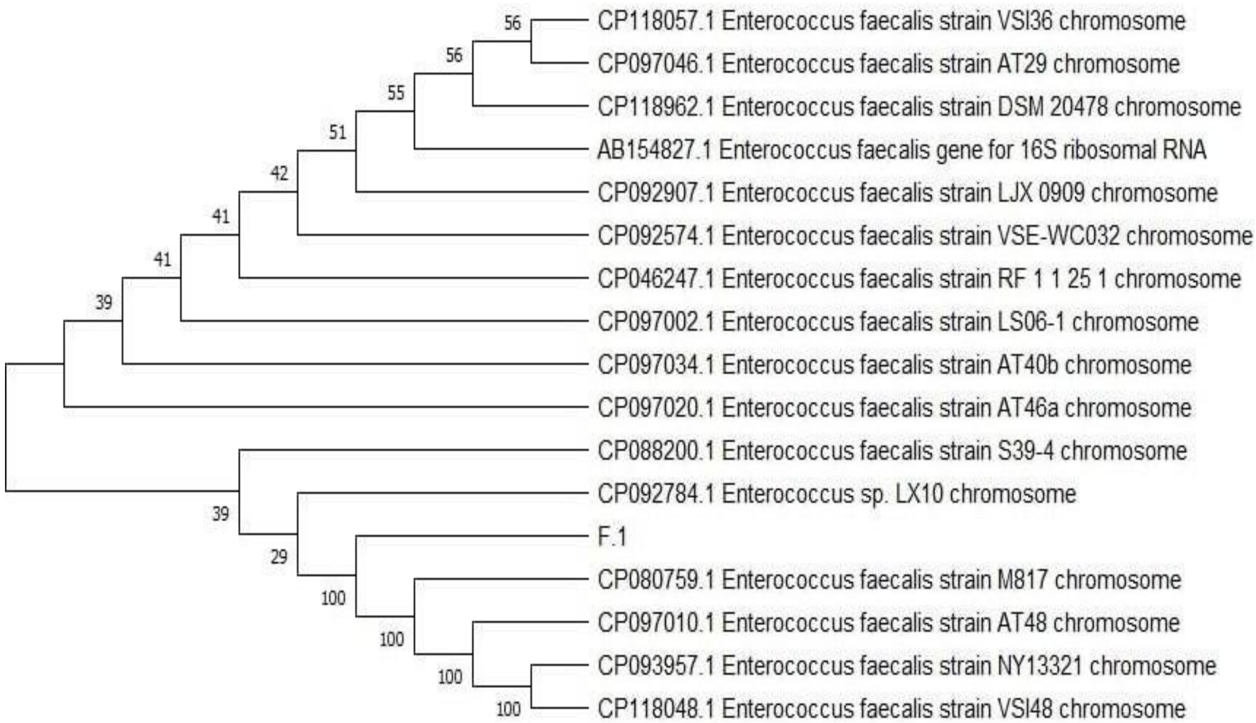

Figure 8: **Evolutionary relationship of ZM-2. A bar value of 0.05 indicates nucleotide substitutions per nucleotide position. Bootstrap values are shown as the percentage of 500 replicates displayed at the branch points.**

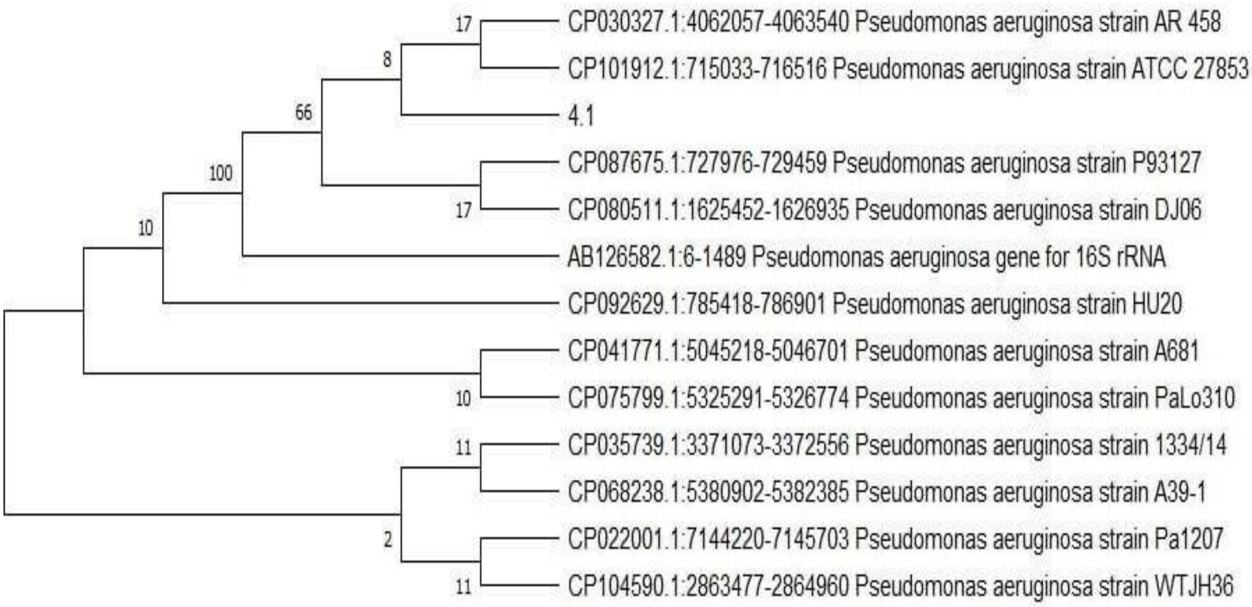

Figure 9: **Evolutionary relationship of ZM-2. A bar value of 0.05 indicates nucleotide substitutions per nucleotide position. Bootstrap values are shown as the percentage of 500 replicates displayed at the branch points.**

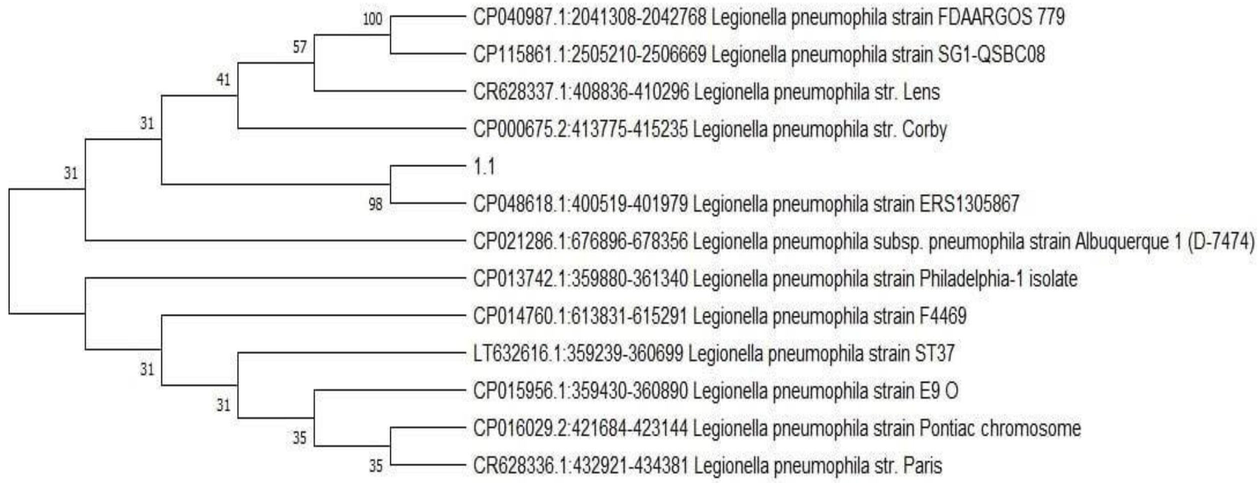

Figure 10: **Evolutionary relationship of ZM-2. A bar value of 0.05 indicates nucleotide substitutions per nucleotide position. Bootstrap values are shown as the percentage of 500 replicates displayed at the branch points.**

Antibiotic susceptibility testing exhibited wider zones than CLSI-Pakistan's standard, indicating resistance to Penicillin in *E. coli* and *P. aeruginosa* and Amikacin resistance in *Enterococcus faecalis*. Moreover, *Pseudomonas* isolates showed resistance to Chloramphenicol.

Discussion:

According to WHO, a 100ml sample of drinking water should have no coliform bacteria reported in it (Muddassir et al. 2022). With the aggravating climate issues, the bacteria observed in our study, namely *E. coli, Enterococcus, Legionella,* and *Pseudomonas,* are expected to be emerging pathogens spread by water (Fong 2020; Kuwajima et al. 2019).

There have been several separate investigations on microbial count in water samples in Pakistan. According to initial studies by the Pakistan Council for Research in Water Reservoirs (PCRWR), bacterial counts are often identified in water, with the readings compared to the National Standards for Drinking Water Quality (NSDWQ) (Mahfooz et al. 2019). PCRWR conducted a detailed water quality survey in 2020 that focused on monitoring the water quality of 29 major cities of Pakistan as well as many surveys in the previous years from 2002 to 2006, and 2015 to 2016. Despite various efforts, very little work has been done to obtain a complete set of standard for the nationwide picture of water quality in Pakistan (Khan and Mohsin 2023; Rasheed et al. 2021; Ummah 2019).

Understanding the link between a possible increase in antimicrobial resistance with the release of resistant microbial strains in wastewater was of particular concern. The study showed resistance of *E. coli* and *P. aeruginosa* to Penicillin. Their transmission to the environment can pose serious public health challenges. This was seen in 33 wastewater treatment plants and surface and groundwater (O'Flaherty and Cummins 2017). It emphasizes the importance of treating wastewater effectively, either by chlorination, chemical treatment, or filtration, before release. However, it is still unclear whether the presence of environmental antibiotic-resistant bacteria in untreated and treated drinking water has any substantial impact on human exposure to antibiotic-resistant pathogens (Sanganyado and Gwenzi 2019).

Karachi has long served as the central hub for businesses, marketing, and industrial corporations. With a population of 20.3 million, according to the National Digital Census 2023, the city hosts 60% of our industries (Solomon 2023; Yale University 2020). These hundreds of industries spill into rivers and streams. The findings raise certain questions on the industrial growth linkage to contaminated water intake. In the study conducted, the maximum amount of bacterial contamination was seen in wastewater samples. For Karachi, 100% prevalence rates for each bacterial category (total coliform, fecal coliform, *E. coli,* and *Enterococcus)* were observed. *Legionella* had the highest (50%) prevalence as compared to other cities, raising further concern.

The PCRWR report of 2021 showed 93% of the water in Karachi as unsafe. To the sad plight of the city, the supply and sewerage pipelines of the city have never been changed. Another study showed total coliforms in 48 out of 50 (96%) tested samples, which exceeded the permissible limit of WHO and PEPA (Aslam Ali et al. 2019). The safe water status in Karachi has drastically decreased from 25% in 2002 (Bernstein et al. 2007) to a disappointing 7% safe water recorded in 2020. These results are very close to our study for wastewater samples (100%). Contrarily, drinking water showed only 10% of bacterial organisms present. Groundwater, process water, and RO water showed no growth. This means pipelines might be the root cause of bacterial entry into the water supply chain. No growth means the water in the plant is properly treated and the system is working efficiently.
In the case of Lahore, the city gets water from the surface and underground through wells and tube wells around the city. Surface water contaminations are linked to urbanization, agriculture, and manufacturing discharge (Sasakova et al. 2018). Also, groundwater is contaminated due to physical changes in the landscape or untreated waste released from anthropogenic sources, be it agriculture, factories, or sewerage (Li et al. 2018). The capital of Punjab province, Lahore, had 100% of water samples as unfit for utilization by humans according to reports from 2003 to 2006. Over time, water quality has risen to about 70%. Despite an increase recorded in 2015, the report of 2020 showed results similar to those seen in 2010. They align closely with our present study in terms of microbial intrusion in almost all water sources.
Similar comparable research in 2021 revealed contaminated filtered drinking water supplies with 51% for total coliform and *E. coli*. Even higher contamination levels were found in an unfiltered water supply with total coliforms and *E. coli* reaching 84.5%. Our study showed a much lower level of contamination in drinking water (13%), implying the supply channel for drinking purposes has comparatively better quality but is still higher than the WHO and NSDWQ standards.
Faisalabad is home to various textile and agro-based (flour, cotton, sugar) industries in the country. Of the 59% categorized as unsafe samples, 14% were due to bacterial contamination. The city's water quality has increased from 20% safe water supply in 2010 to 40% recorded in 2020 (Mahfooz et al. 2019; Nasir et al. 2016). According to our study, all samples were seen as contaminated except for the RO water. The possible reasoning for this compromised water quality is waste dumped in landfills, contact with feces, faulty pipelines, and untreated release of wastewater into aquifers. In another research conducted in Faisalabad, water sources near the Samundari drain showed 90% of groundwater samples as unfit and exceeding WHO standards. Likewise, our results also showcased bacterial growth in the groundwater of industrial areas (Nasir et al. 2016).
Sialkot is the provider of sports supplies to the whole world. The city has many manufacturing, textile, bleaching, leather, metallurgy, and pharmaceutical industries. All industrial water samples collected from these areas presented contamination, except the RO water. This can be associated with production line microbes through seeping, contact, spillage, or leaking, as reported (Masood et al. 2020) regarding groundwater pollution. However, the PCRWR data contradicts this with a 90% safe water supply in 2010-2015 and a 100% safe water supply in 2020 (Rasheed et al. 2021).
Interestingly, Gujranwala showcased *Pseudomonas aeruginosa* prevalent in all sources, along with the least microbial invasion detected in samples, except process water and wastewater categories. Our results were analogous to Muddassir's work in depicting multi-drug resistance (MDR) behavior in *P. aeruginosa* (Muddassir et al. 2022). Within a span of 10 years from 2010-2020, the water quality in the city has worsened from 10% to 50%. The PCRWR 2021 report showed that 50% of polluted sources were rooted in unsafe domestic sewage disposal and inadequate disinfection practices.

Significant bacterial infiltration in water samples underscores the need for an urgent policy and improved management practices. Microbial growth in water supply and public health connection aligns with the criteria of the World Health Organization (WHO), Environmental Health Concern (Amin et al. 2024; Iqbal et al. 2014; Yale University 2020). The government of Pakistan is investing in water treatment infrastructure and regulation policies; however, more efforts are needed.

Conclusion:

Our comprehensive study served as an addition to the critical issue of microbial contamination in the drinking water supply. It extended beyond the mere identification of contaminants in industrial wastewater and rather delved into the intricate web of factors contributing to the contamination of water supplies. Among all the water samples tested, wastewater samples were the most contaminated, and *Legionella pneumophila* was detected in Karachi (50%) and Lahore (25%). The drinking water of Sialkot had severe total coliform, fecal coliforms and *Enterococci* growth in industrial samples. In Faisalabad and Gujranwala, *Pseudomonas aeruginosa* was most prevalent. Additionally, groundwater from Faisalabad displayed maximal incidence of coliforms, fecal coliforms and *Enterococci* as compared to other cities. RO water collected from Lahore and Karachi had elevated coliform levels. Similarly, processed water in Gujranwala had substantial coliforms and fecal coliform pollution.

Moreover, our research unveils a pressing concern, the rise of antibiotic-resistant bacteria in wastewater. Resistance observed in *E. coli and P. aeruginosa* against Penicillin entails potential consequences of untreated wastewater, introducing antibiotic-resistant pathogens into the water supply. Different ratios displayed in five major industrial cities reaffirm the widespread nature of the issue. It is imperative that government bodies, regulatory agencies, and industries, collaborate to implement effective policies and infrastructure improvements.

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