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UNVEILING MICROBIAL RISKS: ISOLATION AND CHARACTERIZATION OF BACTERIA FROM HOSPITAL WASTEWATER IN HEVELLIAN

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ABSTRACT

Hospital wastewater serves as a complex reservoir of microbial communities influenced by diverse sources including patient excretions, medical procedures, and cleaning practices within healthcare facilities. Upon collection, hospital wastewater samples undergo rigorous processing and analysis to isolate and identify bacterial species. The current study focus on isolation and identification of bacteria from hospital wastewater. Samples of hospital wastewater were collected from various public and private hospitals in Havelian, Abbottabad. Bacterial growth was identified by using different biochemical tests and Gram staining techniques. A total of 30 samples were analyzed using a combination of microscopy, Gram staining, and biochemical tests (oxidase, catalase, coagulase and other additional test). The findings indicated that most of the isolates (18/30) were Gram-positive rod-shaped bacteria, indicating a prevalence of this type of bacteria in hospital wastewater. The biochemical tests revealed a range of positive and negative results, indicating diversity among the isolates. The positive results for oxidase, catalase, and coagulase suggest the presence of enzymes that enable bacteria to survive and thrive in wastewater environments. The test results indicate the potential presence of enteric bacteria, which is a concern for public health. Findings of this study have implications for wastewater treatment and management in healthcare settings.

Key words: Hospital wastewater, Pathogen, Bacteria, Gram staining

INTRODUCTION

The vast invisible world that surrounds and inhabits us is made up of microscopic organism’s bacteria. Lactobacilli are rod-shaped, gram-positive bacteria that are found in milk, dairy products, fermented foods, and our oral, intestinal, and vaginal microbiota. Plantarum, *L. acidophilus*, *L. reuteri*, and others are among the most common species. A Gram-negative rod called *Helicobacter pylori* colonizes the stomach's mucosal lining. It is the leading cause for gastritis and peptic ulcers. It produces cytotoxins and ammonia which damage the stomach epithelium leading to abdominal pain, nausea, vomiting, and bloating. *H. pylori* is present in half of the world's population, however, most of them are asymptomatic while only a few develop gastritis and ulcers (Oppong *et al*., 2019).

Since different aspects of the environment act as carriers, the external environment is crucial in the spread of microbes to people (Ullah *et al*., 2025). A number of bacterial infections are primarily caused by hospital wastewater. Immunocompromised individuals are susceptible to opportunistic infections, which can arise from hospital wastewater. When the immune system is weak, bacteria and fungi are typically dangerous and cause illnesses (Saadabi *et al*., 2011).

Hospital wastewater (HWW) is hazardous and contagious, and it differs greatly from wastewater released from other sources. Operations (surgery) rooms, wards, laboratories, laundry, polyclinics, research units, radiology, and medicine and nutrient solutions used in microbiology labs are among the many micro- and macro pollutants that are released into the environment (Pradenas, *et al.,* 2020). Radioactive isotopes, pharmaceuticals, stock cultures, heavy metals, media, pathogens, medications, cotton particles, disinfectants, and chemical compounds are examples of both micro- and macro pollutants. Hospital wastewater also contains antibiotic residues and a number of resistant bacteria, which may prevent susceptible bacteria from growing and increase the number of resistant bacteria in the receiving water. When resistant bacteria are released into the environment, they either serve as reservoirs for antibiotic-resistance genes (ARGs) that could endanger public health or as vectors to carry a transmissible gene (Pradenas *et al.*, 2020).

Bacillus is the most common genus of pathogenic bacteria, accounting for 80% to 90% of the total, while Staphylococcus and Streptococcus range from 5% to 10%, according to research on HWW. The wastewater from hospitals and labs also contains a variety of bacteria, such as Pseudomonas, Enterococcus, and Acinetobacter species, that have the ability to withstand drugs. Among gram-positive bacteria, Staphylococcus aureus is the most prevalent pathogen with a high level of multidrug resistance (MDR). Studies show that the percentage of MDR bacteria varies based on the size and origin of HWW, ranging from 0.58% to 40% (Parida *et al*., 2022).

MATERIALS AND METHODS

Study area

This study was conducted in district Abbottabad, a small valley located in the outer most Himaliyas, between the Indus and Kashmir in the west and east, respectively. Abbottabad constitutes one of the districts within the North West Frontier Provinces (NWFP, now KPK) of Pakistan. The district encompasses an area of 1,969 km.

Sample Collection

Samples of hospital wastewater were collected from various public and private hospitals in Havelian, Abbottabad. The sampling was conducted using personal protective equipment, including gloves, to minimize contamination. Wastewater samples were collected directly from the drainage system using sterile syringes to ensure the integrity of the samples. Each sample was immediately transferred into sterile, sealed bottles labeled with unique identifiers and sealed with Scotch tape to prevent leakage and maintain sample integrity during transportation.

Serial Dilution

Serial dilution is performed to reduce the microbial load in samples and facilitate the isolation of individual bacterial colonies.

Procedure:

Label a number of sterile bottles or test tubes. One milliliter of the initial wastewater sample was extracted and transferred to the first tube, which held nine milliliters of sterile saline solution (1:10 dilution). Gently invert or vortex to mix well. Move 1 milliliter from the first tube to the second, which has 9 milliliters of sterile saline solution (1:100 dilution) in it. As needed, repeat the procedure for further dilutions (such as 1:1000 and 1:110,000). Blend each dilution well. For bacterial culture, plate each dilution onto the proper agar plates.

Identification of bacteria:

The resultant growth was identified by using different biochemical tests and Gram staining techniques as described by Moyes *et al.,* (2009) .

Gram staining

Gram staining is a widely used method for differentiating between gram positive and gram negative bacteria. Whereas Gram-positive bacteria are purple, Gram-negative bacteria are pink. To create a complex, iodine solution was added after cells had first been stained with crystal violet dye. The sample was finally stained red with safranin after a decolorizer, such as acetone or ethyl alcohol, was added. Because safranin was less intense than crystal violet, it did not interfere with the purple coloration of Gram-positive cells. However, a pink stain was present in the decolorized Gram-negative cells (Moyes *et al.,* 2009).

Biochemical tests

After Gram staining, different biochemical tests were performed for the conformation of different microorganisms.

Catalase Test

The enzyme catalase converts hydrogen peroxide into oxygen and water. The enzyme was present when hydrogen peroxide was mixed with a tiny inoculum of bacterial isolate, and oxygen bubbles quickly formed. Poor or absent bubble formation was a sign of catalase deficiency (Reiner, 2010).

Coagulase Test

Coagulase test was used for differentiating the species *S. aureus* positive from Coagulase Negative *S. aureus. S. aureus* produces coagulase, an enzyme that converts soluble fibrinogen found in plasma to impenetrable fibrin (Katz, 2010). On a sterile slide, a drop of regular saline was added to a few colonies of the cultured sample. Then drop of human plasma was mixed with the test suspension. Observed agglutination or clumping. Agglutination within 5-10 seconds was considered as positive. identified by tube coagulase test (Katz, 2010).

Oxidase Test

The result of this test is dependent on the existence of a cytochrome oxidase enzyme in bacteria, which catalyzes electron transfer between donors of electrons and the reactant dye. The concentration of tetramethyl-p-phenylene diamine dihydrochloride in the sample has dropped to a deep purple color. Pasteurella, Neisseria, Brucella, Vibrio, and pseudomonas were screened with this test, and the results were positive. Oxidase is not present in Enterobacteriaceae. In particular, the oxidase chemical is prepared as a 10g/l or 1% solution of tetramethyl-p-phenylene diamine dihydrochloride. Tetramethyl-p-phenylenediamine dihydrochloride was used as the substrate to soak filter paper. Smeared the colony in the filter paper after selecting it for testing using a wooden or platinum loop. looked for a color change to deep violet or blue within 10–30 seconds on the contaminated patch on paper (Shields and Cathcart, 2010).

RESULTS AND DISCUSSION

Thirty samples collected from Hospitals in Havelian, Civil Hospital Havelian, Hameed Azam Hospital Havelian, Zafran Hospital, Fatima General Hospital, Ayub Medical Complex, Inor Hospital, Shaheed Benazir Bhutto Hospital, Allied Hospital, Shaheena Jameela Hospital, Abbot Hospital were used in this study. Thirty samples were collected from various public and private hospitals. These samples were processed using different agar media including nutrient agar, MacConkey agar, and LB agar. Culturing techniques such as streaking, spreading, and serial dilution were employed to isolate bacterial colonies from the samples.

Morphological characterization:

Bacterial isolates were then characterized by morphology, by using different mediums that nutrient agar and MacConkey medium. On nutrient agar gram positive isolate produce yellow and white colonies that are present on the surface of the medium. MacConkey medium used as a differential medium for gram negative isolates. All gram-negative isolate produced pink colonies.

Gram staining

The isolated cultures were used for Gram staining which showed that the organisms are Gram negative and gram positive. 24 samples were gram-positive bacteria and 6 samples were gram-negative bacteria (Table 1). Bacterial isolates appear rod, cocci, singly and in pairs, and some in chains.

Catalase test

Catalase testing showed that more bacterial isolates were positive. Bubbles of oxygen were produced when a drop of hydrogen peroxide was added to its isolates (Table 1).

Coagulase test

The results of the coagulase test for 7 isolated bacteria were show positive to produce aggregates and for 23 bacteria show negative results, they were unable to form aggregates or clumps (Table 1).

Oxidase test

The all isolated bacteria were tested for the oxidase test. Out of 30, 24 were negative hey were unable to produce any color and 6 were positive show blue colour (Table 1).

Table 1. Gram Staining and different tests.

Total Samples	30
Gram Positive	24
Gram Negative	6
Catalase Positive	18
Catalase Negative	12

Coagulase Positive	6
Coagulase Negative	24
Oxidase Positive	6
Oxidase Negative	24

Biochemical characterization of bacterial isolate

After Gram staining, different biochemical tests were performed for the conformation of different microorganisms.

Table 2. Biochemical Characterization of each isolated bacteria.

SAMPLE	GRAM STAINING RESULTS	SHAPE	CATALASE	OXIDASE	COAGULASE
S 1	+	Rod	+	_	_
S 2	+	Rod Cluster	+	_	_
S 3	+	Rod Cluster	+	_	_
S 4	+	Rod	+	_	_
S 5	+	Rod	+	_	_
S 6	_	Rod	+	+	_
S 7	_	Diplo Bacilli	+	_	_
S 8	+	Rod	+	_	_
S 9	+	Rod Chin Cluster	+	_	_
S10	+	Rod	+	_	_
S11	+	Cocci Cluster	+	_	_
S12	_	Diplo Bacilli	+	_	_
S13	+	Rod	_	_	_
S14	+	Cocci Cluster	+	_	_
S15	+	Rod Pair	_	_	+
S16	_	Rod	+	+	_
S17	+	Rod	_	_	_
S18	+	Cocci Cluster	+	_	_
S19	+	Rod Pair	_	_	+
S20	+	Rod	_	_	_
S21	+	Cocci Cluster	+	_	_
S22	+	Rod Pair	_	_	+
S23	_	Rod	+	+	_
S24	+	Cocci Chain	_	+	_
S25	+	Rod Pair	_	_	+
S26	+	Cocci Chain	_	+	_
S27	_	Diplo Bacilli	+	_	_
S28	+	Cocci Chain	_	+	_
S29	+	Rod	_	_	+
S30	+	Rod Pair	_	_	+

The identification and characterization of these bacterial species from hospital wastewater highlight their diverse metabolic capabilities and adaptation to environmental conditions. Understanding these characteristics is crucial for assessing potential health risks associated with wastewater contamination and for developing effective management strategies. The present study aimed to isolate and identify bacteria from hospital wastewater samples collected from various private and public sources. A total of 30 samples were analyzed using a combination of microscopy, Gram staining, and biochemical tests (oxidase, catalase, coagulase and other additional test). The findings indicated that most of the isolates (18/30) were Gram-positive rod-shaped bacteria, indicating a prevalence of this type of bacteria in hospital wastewater. This finding is consistent with previous studies by Ghenghesh *et al*. (2001) that have reported Gram-positive bacteria as the dominant flora in wastewater.

The biochemical tests revealed a range of positive and negative results, indicating diversity among the isolates (Table 2). The positive results for oxidase, catalase, and coagulase suggest the presence of enzymes that enable bacteria to survive and thrive in wastewater environments. The test results indicate the potential presence of enteric bacteria, which is a concern for public health. Findings of this study have implications for wastewater treatment and management in healthcare settings. The presence of Gram-positive rod-shaped bacteria and enzyme-producing bacteria highlights the need for effective treatment strategies to remove pathogens and prevent the spread of infections Limitations of the study by (Kour *et al*. 2021) include the small sample size and the reliance on conventional biochemical tests, which may not have detected all possible bacterial species. Future studies could consider using molecular techniques, such as 16SrRNA sequencing, to provide a more comprehensive understanding of the bacterial community in hospital wastewater. The results of the bacterial identification highlight a diverse range of bacterial species present in the hospital environments. Gram-positive bacteria were predominant, which is consistent with typical findings in clinical settings. The prevalence of rod-shaped bacteria suggests environmental adaptations that favor survival and colonization in hospital settings.

Conclusion

This research contributes to our understanding of the bacterial diversity in hospital wastewater and emphasizes the need for effective wastewater management and public health strategies to prevent the spread of infections. The findings of this study can inform policy and practice in healthcare settings, ultimately improving public health and environmental safety. Healthcare facilities should prioritize effective wastewater treatment and management practices. Regular monitoring of wastewater quality should be conducted to detect potential pathogens and enzyme-producing bacteria. Further research should investigate the antimicrobial resistance profiles of bacteria in hospital wastewater. By addressing these recommendations, we can work towards reducing the risk of waterborne infections and improving environmental health.

REFERENCES

Ghenghesh, K, S., N. M. Saed, A. El-Ghodban, A. Rahouma and S. Abeid (2001). Dirty money: toxigenic and methicillin-resistant *Staphylococcus aureus* (MRSA) from used bank notes, *Jamahiriya Med. J.*, 1: 54-56.

Katz, D. S. (2010). *Coagulase test protocol. American Society for Microbiology Laboratory Protocols.* Available online*: https://www*. asmscience. org/content/education/protocol/protocol, 3220.

Kour, G., R. Kothari, H. M. Singh, D. Pathania and S. Dhar (2021). Microbial leaching for valuable metals harvesting: versatility for the bioeconomy. *Environmental Sustainability*, 4: 215-229.

Moyes, R. B., J. Reynolds and D. P. Breakwell (2009). Differential staining of bacteria: gram stain. *Current Protocols in Microbiology,* 15: A. 3C. 1-A. 3C. 8.

Oppong, T. B., H. Yang, C. Amponsem-Boateng and G. Duan (2019). Hand hygiene habits of Ghanaian youths in Accra. *International Journal of Environmental Research and Public Health*, 16: 1964.

Parida, V. K., D. Sikarwar, A. Majumder and A. K. Gupta (2022). An assessment of hospital wastewater and biomedical waste generation, existing legislations, risk assessment, treatment processes, and scenario during COVID-19. *Journal of environmental management*, 308: 114609.

Pradenas, L., M. Fuentes and V. Parada (2020). Optimizing waste storage areas in health care centers, *Annals of Operations Research*, 295: 503-16.

Reiner, K. (2010). Catalase test protocol. *American society for microbiology*, 1: 1-9.

Saadabi, A. M., L. F Ali, A. B. Omer, G. A. Ahmed and R. K Al Asa (2011). Isolation and Identification of Pathogenic Bacteria and Fungi from Some Sudanese Banknote Currency. *J. Applied Sciences Research*, 7: 129-33.

Shields, P. and L. Cathcart. (2010). Oxidase test protocol. *American society for microbiology*, 4: 1-9.

Ullah, F., R. ullah, W. Sajjad, J. Zahir, A. Waqar, Y. Aziz, S. Rehman, K. Ullah, Q. S. Haq and Z. Ullah (2025). Unseen threats in circulation: pathogenic bacteria and antibiotic resistance on Pakistani currency notes. *Indus Journal of Bioscience Research*, 3: 579-87.

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