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Original Article

Assessment of antibiogram and resistogram of pathogenic bacteria isolated from irrigated wheat field water

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Abstract
The present work concerned with the study of bacterial pathogens associated with irrigated wheat field water. Study was carried out to check the efficacy of heavy metals and antibiotics against five isolated bacterial pathogens. Different morphological and biochemical tests were performed to identify and characterize bacterial pathogens. Among isolated strains, two belong to genus *Staphylococcus* spp., other two were *Pseudomonas* spp. and one strain belonged to genus *Salmonella* spp. Effect of various parameters such as temperature and pH on growth of pathogens were also studied. Antibiogram and resistogram analysis were carried out through agar disc and agar well diffusion methods. Optimum temperature for growth of all bacterial strains was 37°C and pH 7 except *Salmonella* which had pH 6. Among all the antibiotics Oxacillin was less potent as three bacterial strains showed resistance against it while others antibiotics were more potent. Minimum inhibitory concentration of heavy metals for all bacterial isolates was different. It was concluded that irrigated pathogenic bacteria are highly resistant to heavy metals.
Key words: Bacterial pathogens, irrigated wheat field water, antibiotics, heavy metals, antibiogram, resistogram

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INTRODUCTION

Bacteria play important roles in cycling of carbon and many other important elements in the environment. Biological fixation of atmospheric nitrogen is also carried out by some bacteria. They also involved in methane production, reduction of sulfate, decomposition of vegetal and other product residues, and in biotransformation of metals (Haines *et al*., 2002). Fresh water from canals, rivers and streams are usually used for irrigation purpose which may contain hazardous microorganisms such as plant pathogens which are harmful for cultivation of many crops e.g. Cyanobacteria are pathogenic bacteria which can decrease the plant growth due to release of toxic substances (Kulik, 1995).

The most well-known potentially pathogenic bacteria which are present in water are *Salmonella, Shigella* and *Escherichia coli.* These enteric bacteria which are present in the intestinal tract of animals and humans are pathogenic in nature and present in large quantities in digestive tract. These pathogens are excreted with feces and contaminate the irrigated water used in fields. Coliform bacteria are naturally found in soil, decaying organic matter and digestive tract of warm blooded animals and they have capability to survive longer in the water (Welch *et al*., 2000). Some bacteria show growth promoting effect on plants such as *Pseudomonas* spp., (Van peer and Schipper, 1989) and *Scenedesmus* spp., (Mazur *et al*., 2001). But some species of *Pseudomonas* cause plant diseases like leaf spot, leaf stripe, wilt and similar diseases (Pelczar *et al*., 1993).

Heavy metals tolerance or resistance reflects the capability of bacteria to survive in an environment with high concentration of heavy metals. In other words heavy metals resistant bacteria are those which can accumulate high concentration of heavy metals in it without dying. The assay is called resistogram analysis. Heavy metal resistant bacteria have the plasmid containing the heavy metal resistant genes and presence of these bacteria in any environment is

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the indication of environmental pollution with heavy metals (Ehrlich, 1986). On the other hand, the ability of microorganisms to bear the effect of antibiotics is called the sensitivity of microbes (Presscott *et al*., 1999). Many antibiotics have been used in last several decades in medical, veterinary and agricultural practices to avoid contamination and cure the infectious diseases (Chelossi *et al*., 2003). Resistance against antibiotics or heavy metals can be natural or acquired or can be transmitted by vertically or horizontally (Alanis, 2005). Now a days, certain medicinal plants/traditional herbs are used to study antibacterial activity against infectious pathogens. The current study was focused on the isolation from irrigated water of wheat field, identification characterization the pathogenic bacteria and determination antibacterial activity of different medicinal plants. Furthermore, resistogram and sensitivity test on these pathogens was studied. This work was done because of the fact that these microorganisms are present everywhere in natural environment and are of economic importance as beneficial and pathogenic ones.

MATERIALS AND METHODS

Samples collection

Samples were collected from water standing in wheat fields of rural area, Faisalabad. These fields were irrigated with canal water. Sample was taken in the sterilized falcon tubes. After taking the water sample, the tubes were immediately closed to avoid the contamination.

Isolation and identification of bacteria

Nutrient agar broth (NAB) was used for the growth of bacterial colonies. The sterilized media was poured in the sterilized Petri dishes in the laminar air flow cabinet. On Petri dishes 50 µl of water sample was evenly spread with the help of sterilized spreader. Plates were incubated at 37°C for 24 h. After incubation single colony was isolated through streaking method. Different morphological tests such as Gram's staining, endospore and acid fast staining, and motility tests were performed. Various biochemical tests *viz.,* catalase test, urease, gelatin hydrolysis, carbohydrate fermentation, litmus milk, triple sugar iron, methyl red, Voges proskauer test, citrate test, indole, oxidase, hydrogen sulphide production, blood agar and selective media were performed for the identification and characterization of isolated bacteria (Perveen *et al*. 2011, Andleeb *et al*., 2014; Bashir *et al*., 2014; Mehmood *et al*., 2015).

Effect of temperature on bacterial growth

Luria-Bertani (LB) medium was prepared by dissolving 2.5 g yeast extract, 5 g trypton and 5 g NaCl in 500 ml of double distilled water (d_2H_2O). Then medium was autoclaved at 121^{o}C for 20 min and poured into three sets of sterilized test tubes, each set consists of five test tubes for each strain. Bacterial culture was inoculated on tubes under aseptic conditions. One set of test tubes was placed in incubator at 37^{o}C for 12 h, the second set was kept at 4^{o}C for 12 h and the third set of test tubes was placed at 25^{o}C for 12 h. After 12 h the optimal density was measured at 600 nm wave length with spectrophotometer.

Effect of pH on growth of bacteria

Luria-Bertani (LB) medium was prepared by dissolving 2.5 g yeast extract, 5 g trypton and 5 g NaCl in 500 ml of double distilled water (d_2H_2O). Then autoclaved medium was poured into five sterilized test tubes, and pH values 4, 5, 6, 7 and 8 were optimized. After sterilization of medium it was inoculated with isolated bacterial strains under aseptic conditions and incubated at 37^{o}C for 12 h. After incubation, optimal density was checked at 600 nm by using spectrophotometer.

Sensitivity test

Antibiotic resistance of isolated bacterial strains was studied by using agar disk diffusion method (Presscott *et al*., 1999; Kirby-Bauer, 1996). Seven different antibiotic discs *viz*., Ampicillin (10 ug), Carbenicillin (100 ug), Cloxacillin (5 ug), Methicillin (5 ug), Oxacillin (1 ug), Polymyxin B (300 ug) and Teicoplanin (30 ug) with different potency were used. The inhibitory effect was recorded by measuring the diameter of the zone of inhibition after 24-48 h in millimeter (mm; Seeley *et al*., 1990).The results of sensitivity tests were expressed as (0) for no sensitivity, (<1-6 mm) for low sensitivity, (>6-12 mm) for moderate sensitivity and (>12-33 mm) for high sensitivity.

Resistogram analysis

Minimum inhibitory concentration (MIC) of heavy metals for bacteria isolated from

irrigated water was determined through agar well diffusion method (Rios *et al*., 1988). Heavy metals such as zinc (Zn^{+2}), copper (Cu^{+2}), Ferrous (Fe^{+2}), mercury (Hg^{+2}), Nickel (Ni^{+2}), chromium (Cr^{+2}) and cobalt (Co^{+2}) are used. The isolated bacterial strains were poured with medium under aseptic conditions. The solutions of heavy metals ranging from 200 µg - 900 µg were added in wells on nutrient agar plates, and then theses plates were incubated at 37^{o}C for 24 h. The growth was observed on next day.

RESULTS

Identification of bacterial pathogens

It was observed that strains 1, 3 and 4 were Gram-negative bacteria (*Pseudomonas* sp. and *Salmonella* sp.) whereas strains 2 and 5 were Gram-positive bacteria (*Staphylococcus* sp.). Endospore and acid fast staining indicated negative results (Table I). Biochemical tests revealed that all isolated strains showed positive results in case of catalase activity, carbohydrate fermentation, litmus milk, and methyl red tests. Glucose, fructose and sucrose fermentation indicated by strains 2, 3 and 5. All isolated strains showed negative results against indole and Voges proskaure tests. These strains didn't show growth on various tested culture medium such as MacConkey agar, blood agar and Eosin methyl blue (EMB) agar (Table I). Gelatin hydrolysis was observed in case of all strains except strain 3.

Table I: Biochemical tests for isolation and identification of bacterial pathogens

Test	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
Gram's staining	-ve	+ve	-ve	-ve	+ve
Endospore staining	-ve	-ve	-ve	-ve	-ve
Acid Fast staining	-ve	-ve	-ve	-ve	-ve
Motility test	+ve	-ve	+ve	+ve	-ve
Catalase	+ve	+ve	+ve	+ve	+ve
Urease	+ve	-ve	-ve	+ve	-ve
Gelatin hydrolysis	+ve	+ve	-ve	+ve	+ve
Carbohydrate Fermentation	+ve	+ve	+ve	+ve	+ve
Litmus Milk	+ve	+ve	+ve	+ve	+ve
Glucose Fermentation	-ve	+ve	+ve	-ve	+ve
Fructose Fermentation	-ve	+ve	+ve	-ve	+ve
Sucrose Fermentation	-ve	+ve	+ve	-ve	+ve
Methyl red	+ve	+ve	+ve	+ve	+ve
Voges Proskauer	-ve	-ve	-ve	-ve	-ve
Citrate	-ve	-ve	+ve	-ve	-ve
Indole	-ve	-ve	-ve	-ve	-ve
Hydrogen Sulphide	-ve	-ve	+ve	-ve	-ve
MacConkey agar	-ve	-ve	-ve	-ve	-ve
Blood agar	-ve	-ve	-ve	-ve	-ve
EMB agar	-ve	-ve	-ve	-ve	-ve
Pseudomonas Selective medium	+ve	-ve	-ve	+ve	-ve
Staphylococcus Selective medium	-ve	+ve	-ve	-ve	+ve

(+) indicates presence and (-) absence

Effect of temperature and pH on bacterial growth

All isolated strains were grown at various temperatures and the maximum growth was observed at 37°C as compared to 25°C and 4°C (Figure 1). On the other hand, growth of *Pseudomonas* spp. was also observed at 25°C. Among the effect of pH on the bacterial growth, *Pseudomonas spp.* and *Staphylococcus spp.* showed maximum growth at pH 7 whereas *Salmonella spp*. reduced growth at same pH (Figure 2). .

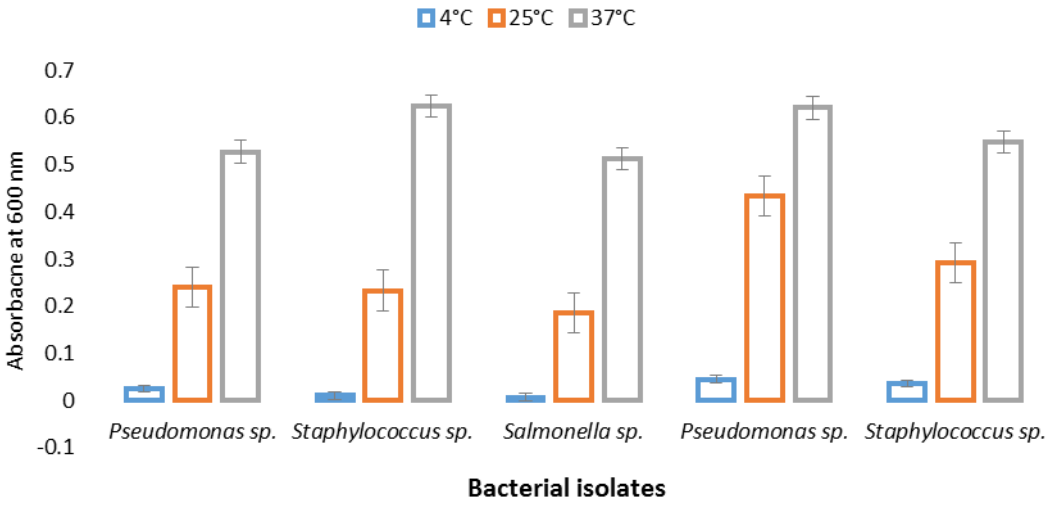

Figure 1: Effect of various temperatures on bacterial growth isolated from cotaminated wheat field water.

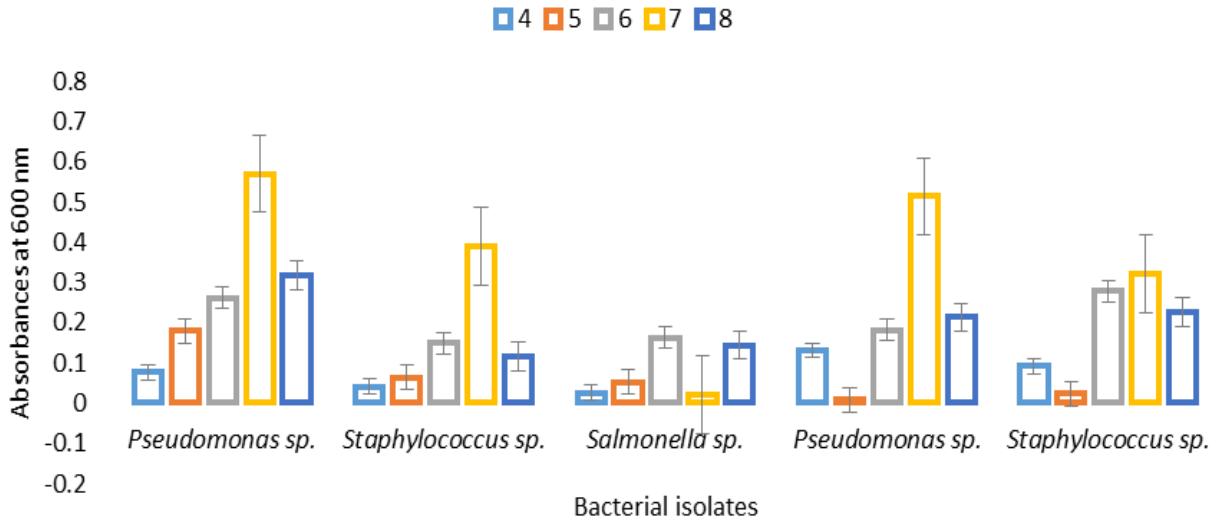

Figure 2: Effect of various pH on bacterial growth isolated from irrigated wheat field water.

Antibiogram analysis

The sensitivity of standard antibiotics against bacterial pathogens is analyzed. The results were observed through agar disc diffusion method (Figure 3). In case of antibiotic resistance strain 1 showed resistance against Oxacillin (1 µg) while showed maximum sensitivity (8.0±0.0 mm zone of inhibition) to Teicoplanin (30 µg). Strain 2 was sensitive to all antibiotics and showed maximum sensitivity of 11.7±0.0 mm of zone of inhibition to Carbencillin (100 µg). Strain 3 showed resistance against Carbenicillin (100 µg) and Oxacillin (1 µg) while it showed maximum sensitivity (6.5±0.0 mm

zone of inhibition) to Methicillin (5 µg). Strain 4 remained sensitive to all applied antibiotics and showed maximum sensitivity (14.0±0.0 mm zone of inhibition) to Cloxacillin (5 µg). Strain 5 had resistance against Oxacillin (1 µg) and showed maximum sensitivity (8.2±0.0 mm zone of inhibition) to methicillin (5 µg).

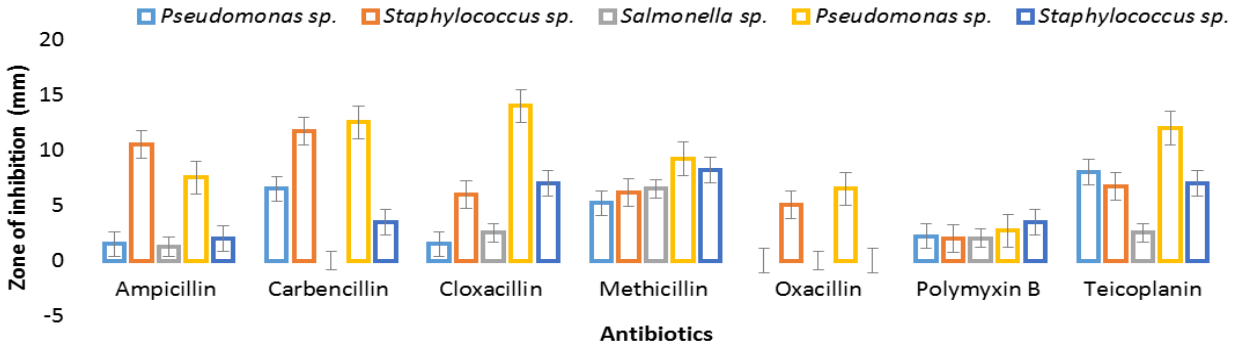

Figure 3: Antibiogram analysis against bacterial pathogens isolated from irrigated wheat field water.

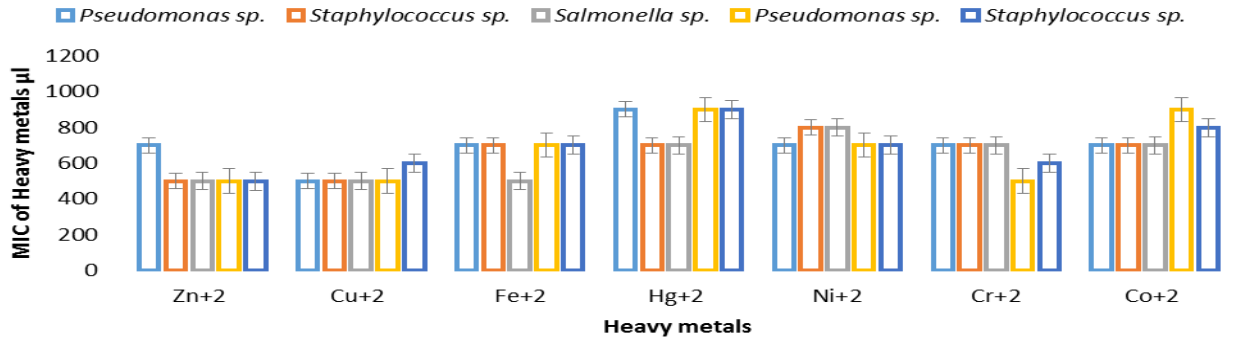

Figure 4: Resistogram analysis of various concentrations of heavy metals against bacterial pathogens isolated from irrigated wheat field water.

Resistogram analysis

The sensitivity with various concentrations (MICs) of heavy metals against pathogens is analyzed called resistogram analysis. Different concentration ranges (200-900 µg) of heavy metals were tested against isolated bacterial pathogens through agar well diffusion method. It was observed that all tested pathogens showed maximum resistance in the presence of all tested heavy metals. The maximum resistance of bacterial strains was observed at 700 and 800 µg concentrations (Figure 4).

DISCUSSION

Five bacterial strains were isolated in which two bacterial strains belonging to genus *Staphylococcus*, other two strains belonging to genus *Pseudomonas*, while one strain belonging to genus *Salmonella* were found. These observations indicated that bacterial strains belonging to genus *Staphylococcus* and *Pseudomonas* were present abundantly in the irrigated water while genus *Salmonella* was also present in the water.

All bacterial strains remained sensitive to all antibiotics used in this study. So these antibiotics were proved to be potent. In these antibiotics, Ampicillin (10 µg) is a semisynthetic Pencillin and act against wide spectrum of bacteria. It is a strong bactericidal. Polmyxin B (300 µg) was also applied. It is a polypeptide antibiotic which has ability to injure the cell membrane structure. It also affects normal permeability of the bacterial cell membrane (Pelczar *et al*., 1993). These studies of antibiotic resistance of bacterial strains showed Oxacillin (1 µg) as less potent antibiotic because three bacterial strains have resistance against it, while other antibiotics were proved as potent antibiotics. Antibiotic resistant bacteria present

in irrigated water can spread from animals to humans via food chain which can enter into the body and cause severe diseases. Antibiotic resistant bacteria have become a serious healthcare problem worldwide (Tenover, 2006). Minimum inhibitory concentration (MIC) of heavy metals (Zn^{+2}, Cu^{+2}, Fe^{+2}, Hg^{+2}, Ni^{+2}, Cr^{+2} and Co^{+2}) was also observed in this research work. Different concentrations of these heavy metals were applied. Minimum inhibitory concentration of heavy metals for each strain was different. Heavy metals are generally toxic and harmful for both Gram-negative and Gram-positive bacteria and some heavy metals such as mercury (Hg^{+2}) was considered to be very toxic for bacteria, humans and animals (Mahler *et al*., 1986).

Mechanism of heavy metal resistance in bacteria takes several forms which includes transformation of toxic material resulting in release of metal ions (Williams *et al.,* 1993). Nies (2000) explained that microbial detoxification of heavy metals by enzymatic action can occur by transfer of metal ions into less toxic or non-toxic by the process of enzymatic oxidation or reduction. Non-enzymatic detoxification of heavy metals can also done by precipitation of inorganic metabolic products such as sulfides, carbonates and phosphates (Macaskie *et al*., 1987; Roane and Kellogg, 1996).

CONCLUSION

Presence of heavy metal resistant bacteria in the irrigated water is a proof of heavy metal polluted environment and incidence of heavy metals resistant bacteria indicated the environmental contamination with these heavy metals.

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