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Original Research

Physicochemical, Molecular and Cultural Identification of Microbial Pathogens in Wastewater Irrigated Crops

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Abstract

Water scarcity is a global issue and the application of untreated wastewater for irrigation is a general practice in developing countries. Major drawbacks of wastewater irrigation include the presence of heavy metals and disease-causing bacterial pathogens. The present study was conducted for identification of the bacterial strains in wastewater and wastewater irrigated vegetable and crops. XLT-4 cultural media was used for culture-based detection of different bacterial pathogens (red with black center for *Salmonella*, yellowish for *E. coli* and small pink colonies for *Shigella*). The presence of these disease-causing pathogens was indicated in almost all samples. For further confirmation, microscopic analysis was performed, which showed gram negative rod shaped colonies. Various biochemical identification tests (catalase, urease, oxidase and lactase) also confirmed the presence of pathogenic bacterial species in analyzed samples. PCR based diagnosis was carried out using species specific primers for Mdh gene of *E. coli*, IpaB gene of *Salmonella* and IpaH gene of *Shigella.* 16s rRNA was amplified in all observed samples as positive control. The Mdh gene amplification was observed in wastewater, pumpkin, sugarcane, maize, brinjal, lettuce, spinach, cabbage and radish samples, while IpaB gene was amplified

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in pumpkin, sugarcane, maize, brinjal, lettuce, spinach, cabbage and radish samples, and IpaH gene was amplified only in lettuce. These observations depicted that wastewater irrigated vegetables and crops were highly contaminated with pathogenic bacterial strains. Therefore, treated wastewater should be used for irrigation otherwise it may lead to health problems in humans.

Keywords: wastewater, vegetables irrigation, *Salmonell*, *Shigella*, *E. coli*

Introduction

Surface water scarcity is a global problem which gets more intense in the areas having extreme climate. The ever-increasing population has widened the demand-supply gap of freshwater availability. This situation has stimulated the invention and execution of innovative plans for freshwater management and wastewater recycling. Additionally, frequent occurrences of longer periods of drought, salinity and poor irrigation distribution system has made the use of untreated wastewater as a substitute for irrigation a common practice especially in developing countries [1]. Approximately 10% of the world's population consumes wastewater irrigated food products [2]. In Pakistan, untreated wastewater is extensively applied to crops and vegetables for irrigation due to surface water shortage. According to a survey, it has been found that about 32,500 hectares are being irrigated with untreated wastewater [3, 4].

The benefits and drawbacks of wastewater application to agricultural fields has been discussed in detail in previous literature [5-7]. For example, on one hand, the presence of essential nutrients (N, P and K) and organic matter in wastewater reduced the need for synthetic fertilizers, however, on the other hand, it served as an active source for polluting ground water, soil and food crops with heavy metals and pathogens. The presence of a number of foodborne pathogens has been reported in wastewater i.e., *Campylobacter* spp., Enterohemorrhagic *E. coli, Staphylococcus, Bacillus, Salmonella, Shigella*, adenoviruses, enteroviruses, noroviruses and rotaviruses [8-10].

Vegetables are rich in proteins, vitamins, fibers and carbohydrates. In Pakistan, about 26% of the consumed vegetables are being irrigated with untreated wastewater [3]. The edible parts of vegetables and fruits such as chili, cabbage, tomato, cantaloupes and sprouts grown under such conditions were found to be highly contaminated with harmful microbes like *Salmonella, Shigella, E. coli,* etc. Some of the main sources of pathogens in wastewater included human feces, eggs, poultry and seafood [11]. Scientists working on wastewater polluted areas confirmed the contamination of tomato fruit with *Salmonella enterica* responsible for foodborne illnesses, when the crop was irrigated using wastewater [12, 13].

Pathogenic microbes and helminthes present in wastewater have been reported to cause intestinal infection in which patients suffered from fever, diarrhea and cramps. Bacillary dysentery caused by Enterovasive *Escherichia coli* (EIEC) and *Shigella* have resulted in approximately 1.1 million deaths [14]. Previous reports have documented different areas which encountered disease outbreaks due to intake of fresh produce linked to the detection of *E. coli* (lettuce and spinach), *Salmonella typhimurium* and *S. Newportin* (tomato) [15]. Similarly, frequent outbreaks of waterborne diseases have been associated with increased consumption of infected raw leafy vegetables (salad) and fruits However, the type of crop, method of irrigation and harvesting practices were identified as important factors for determining the frequency of such infections [16, 17].

Waterborne epidemics are more likely to break in the areas where untreated wastewater is used in the cultivation of edible crops. Identification of such potential hazards is therefore imperative to mitigate the associated risks. The use of untreated wastewater for irrigation of vegetables and crops has been a common practice in Uchkara region of Faisalabad. However, evaluation of suitability and safety of such practices has not been evaluated. The current investigation was conducted to probe the occurrence of bacterial pathogens in wastewater and wastewater irrigated crops and vegetables. Hence, this research explored suitability of wastewater for irrigation purposes and its effects specially on urban communities where reduced surface water availability is a burning issue.

Materials and Methods

Culture-Based Detection of Bacterial Strains

Irrigated wastewater samples, obtained from healthcare units, domestic zones, commercial markets, industries and factories, were evaluated for the existence of *E. coli* (yellowish colony), *Salmonella* (red colony with black centre), and *Shigella* (small pink colonies). Wastewater samples were placed in antiseptic plastic bottles from three different sites of Uchkara, Faisalabad and XLT-4 agar was used for culturing bacteria. Wastewater samples were lined on XLT-4 solid media by decontaminated loop and incubated at 37ºC for 16-18 hours. Each distinctly visible colony was collected and transferred into XLT-4 liquid medium and kept at 37ºC for 24 hours [18]. After 24 hours, DNA was extracted from the bacteria growing at XLT-4 liquid medium.

Samples from eight wastewater irrigated vegetables (ladyfinger, brinjal, pumpkin, green chili, cabbage, spinach, lettuce and radish) and two crops (sugarcane and maize) were collected from different sites of Uchkara (Faisalabad). The samples were washed with distilled water, sterilized with 70% ethanol, and dried in fume hood. A homogenous mixture of vegetable samples was prepared by grinding and streaked on XLT-4 solid growth media. The streaked culture plates were aligned at 37ºC for 16-18 hours. Distinct and well defined colonies were collected, shifted to XLT-4 liquid medium and placed at 37ºC for 24 hours [18, 19]. This liquid cultures were used for DNA isolation.

Physicochemical Identification

Shape, motility and tests for gram staining, catalase, oxidase, urease, H_2S and lactose were performed according to the standard methods [20].

Genomic DNA Isolation

A volume of 1.5 mL bacterial culture (grown up to saturation) was taken into microcentrifuge tubes and centrifuged to get bacterial cell pellet. TE buffer (570 µL) was added, vortexed thoroughly to dissolve pellet and incubated at 37ºC for 1 hour. Further, 5 M NaCl (100 µL) and CTAB/NaCl (80 µL) solutions were added and incubated at 65ºC for 10 minutes followed by the addition of chloroform/isoamyl alcohol in the ratio of 24:1 (700 µl) and centrifuged for 5 minutes. Supernatant was taken (500 µL) and mixed with Phenol/Chloroform/Isoamyl alcohol (500 µL), again centrifuged for 5 minutes. Subsequently, the supernatant was taken and mixed with100% ethanol (600 µL), followed by incubation at -20ºC for 20 minutes. Later, the samples were centrifuged at 10,000 rpm for 15 minutes. Ethanol was discarded. Again, pellet was washed with 70% ethanol and air dried at 37ºC before dissolving in 50 µL of autoclaved dH_2O. DNA concentration was determined through spectrophotometer (UV visible spectrophotometer model S-22, Boeco, Germany) by measuring the absorbance at 260 nm.

PCR Based Detection of Bacterial Strains

For polymerase chain reaction (PCR) based detection of pathogen transmission in vegetables and crops, species-specific primers were used (Table 1). Primer specificity was determined by nucleotide BLAST available at https://blast.ncbi.nlm.nih.gov. PCR was executed using DNA (100 ng) along with forward and reverse primer each. PCR cycle was run as initial denaturation at 94ºC for 5 minutes followed by 33 cycles of 94ºC for 1 minute, 50ºC for 1 minute, 72ºC for 1 minute and final extension at 72ºC for 10 minutes. PCR products were electrophoresed on 1.5% agarose gel.

Results

A total of 11 sources (waste water, 8 vegetables and 2 crops) were checked for bacterial contamination. Wastewater samples showed the presence of pathogenic *E. coli* and *Salmonella* as indicated by colony morphology (Fig. 1A). All vegetables and crop samples showed various bacterial colonies on XLT-4 agar plates. XLT-4 provided with lactose and sodium thiosulfate helped in distinguishing various bacterial colonies. *Salmonella* and *Shigella* did not utilize lactose and thus colonies remained pink or red (Fig. 1B). The agar turned red in the presence of *Salmonella* type colonies. Most strains of *Salmonella* (*Salmonella typhi*) utilize sodium thiosulfate and produce H_2S gas, which resulted in the formation of red colonies with a black center. *E. coli* and *Klebsiella* utilize lactose instead of sodium thiosulfate and therefore, colonies appeared yellow, mucoid and agar medium turned yellow.

Two vegetables (green chili and ladyfinger) were found to be contaminated with lactose fermenting bacteria such as *E. coli* and *Klebsiella* as shown by yellow, mucoid colonies (Fig. 1B and 1G).

Table 1. Primer sequences used for PCR based detection of bacterial strains.

Primer	Sequence	Amplicon (size)	Annealing (temp.)	Reference
16S	5'AGAGTTTGGATCMTGGCTCAG	1400 bp	50ºC	Shine and Dalgarno (1974)
rRNA	CGGTTACCTTGTTACGACTT'3			
Mdh	5'GGTATGGATCGTTCCGACCT	304 bp	49.5ºC	Omar and Barnard (2014)
	GGCAGAATGGTAACACCAGAGT'3			
IpaB	5'GGACTTTTTAAAGCGGCGG	314 bp	52ºC	Kong et al. (2002)
	GCCTCTCCCAGAGCCGTCTGG'3			
IpaH	5'CCTTGACCGCCTTTCCGATA	606 bp	50ºC	WoseKinge and Mbewe (2010)
	CAGCCACCCTCTGAGGTACT'3			

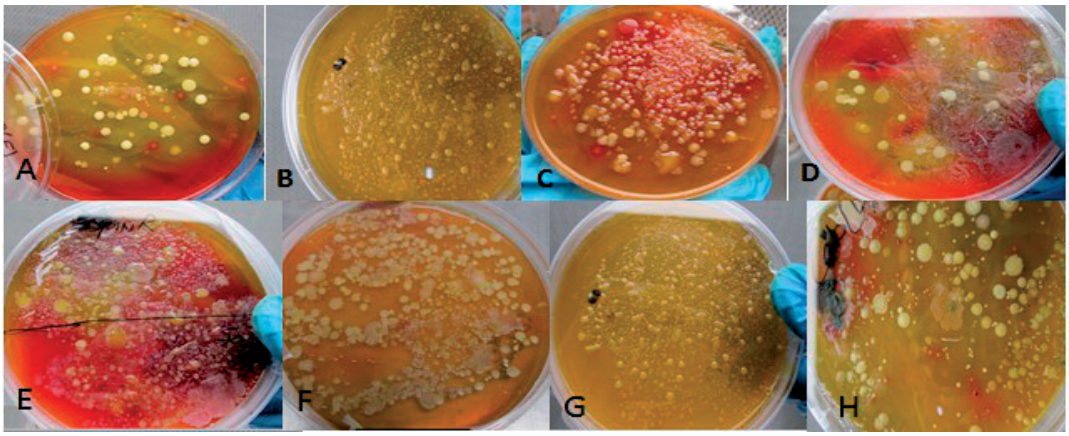

Fig. 1A. Biochemical Identification of Bacterial Strains. Bacterial Colonies on XLT-4 Agar Plates in (A) wastewater, (B) green chili, (C) cabbage (D), lettuce (E), spinach (F), pumpkin (G), ladyfinger (H), sugarcane and (I) Radish.

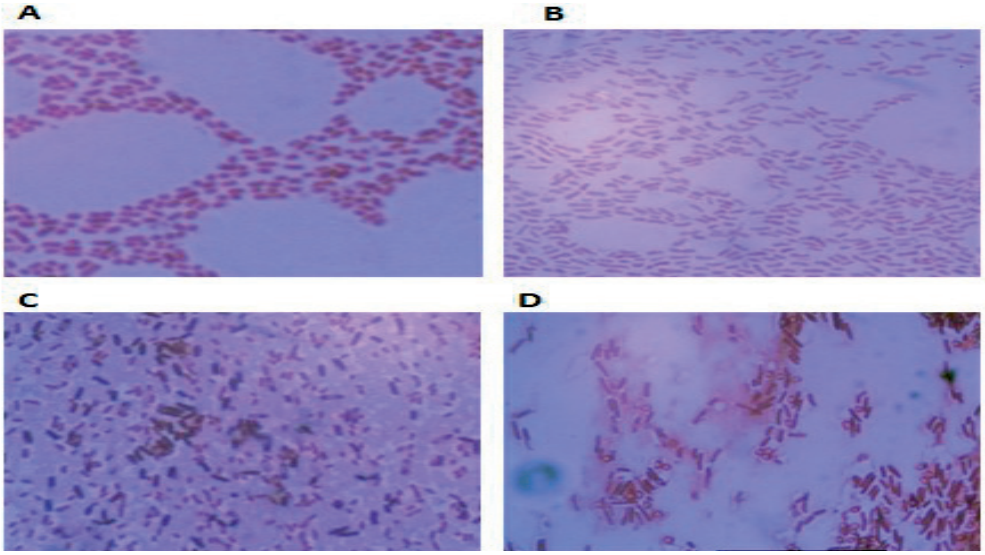

Fig. 1B. Gram staining of bacterial colonies show gram (-) rods of (A) *Salmonella*, (B) *Shigella*, (C) *E. coli* and (D) *Klebsiella.*

The remaining samples (wastewater, cabbage, lettuce, spinach, pumpkin, sugarcane and radish) were observed to be contaminated with both lactose fermenting and non-fermenting bacteria such as *E. coli* (yellow mucoid colonies), *Salmonella* (red colonies with a black center), and *Shigella* (small pink colonies) (Fig. 1A, 1C, 1D, 1E, 1F, 1H and II). These results showed that wastewater was indeed contaminated with bacterial pathogens thus exposing human health to higher risk.

All samples (wastewater and 10 wastewater irrigated vegetable and crops) exhibited positive results for bacterial contamination on XLT-4 agar culture media and liquid growth media confirming the presence of pathogenic bacteria in wastewater. Different bacterial colonies were examined through gram staining under the microscope that showed negative rods (Table 2). None of the examined bacterial colonies showed gram-positive rods (Fig. 1B). Various biochemical tests conducted in the study also confirmed the presence of pathogenic bacterial species in all samples.

All samples were additionally tested for PCR based confirmation. Good quality DNA was isolated by phenol:chloroform method and used as a template for PCR. The PCR findings displayed the amplification of 16S rRNA (used as positive control) in all the isolates (Table 3). PCR based identification was carried out using species-specific primers for Mdh gene of *E. coli*, IpaB gene of *Salmonella* and IpaH gene of *Shigella*. The PCR results showed the amplification of 16S rRNA, Mdh and IpaB genes in wastewater samples while IpaH gene was only detected in lettuce leaf samples (Fig. 2 and Fig. 3).

In spinach, 16S rRNA, Mdh and IpaB genes were successfully amplified in leaf, root and stem as compared to gene IpaH which did not amplify in this sample. Gene Mdh was detected in both leaf and fruit samples of ladyfinger. PCR detected Mdh gene in green chili leaf samples but not in fruit with any gene primer. In pumpkin leaf and fruit, both Mdh and IpaB genes were successfully amplified. While in brinjal,

Table 2. Physicochemical identification of bacterial colonies.

Characteristics	*Salmonella* spp.	*E. coli*	*Shigella* spp.
Shape	Rod	Rod	Rod
Gram stain	Negative (-)	Negative (-)	Negative (-)
Catalase	Positive (+)	Positive (+)	Positive (+)
Oxidase	Negative (-)	Negative (-)	Negative (-)
H2S	Positive (+)	Negative (-)	Positive (+)
Lactose	Positive (+)	Negative (-)	Negative (-)
Urease	Negative (-)	Negative (-)	Negative (-)

IpaB and Mdh genes were amplified in leaf and fruit respectively.

In maize crop, both leaf and grain samples showed amplification of Mdh gene while IpaB gene was amplified in leaf only. In leaf and root samples of sugarcane, both Mdh and IpaB genes were successfully amplified, while these genes were not identified in cane juice. In cabbage leaf and root, both Mdh and IpaB genes were amplified. IpaH gene was only amplified in lettuce leaf showing the contamination of lettuce with *Shigella* spp. while in radish leaf both Mdh and IpaB genes were amplified. Mdh gene was also amplified in radish root (Fig. 3). Culture and PCR based bacterial diagnosis in wastewater and wastewater irrigated crops and vegetables (Fig. 4), clearly highlighted presence of microbial pathogens.

Discussion

By 2030, United Nations is determined to provide clean water and sanitation to all, particularly the unprivileged sections of society. The accomplishment of this task seems to be difficult due to fast growing population, unplanned and random urbanization and industrial growth. All these factors are equally responsible for the deterioration of fresh water reserves thus making it almost unavailable for agriculture sector; the largest consumer of water in the world [21]. Therefore, irrigating agricultural fields with untreated waste water due to the shortage of clean surface water and high treatment cost has become a regular practice of farming community dwelling particularly in water scarce peri urban areas of the world [22].

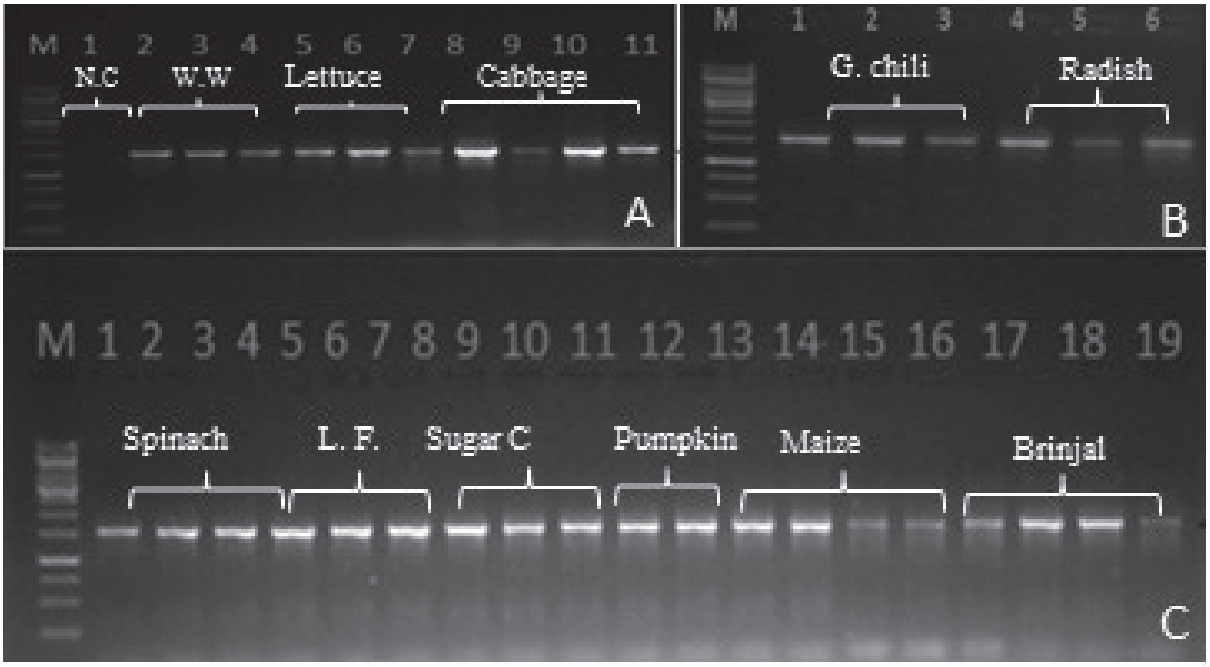

Fig. 2. Electrophoretic analysis of PCR-amplified target of 16S rRNA gene on 1% agarose. (A) Negative control (lane 1), amplified 16S rRNA gene in wastewater (lane 2-4), lettuce leaves (lane 5-7), cabbage stem, leaf and root (lane 8-11). M: 1Kb DNA ladder. (B) In green chili leaf (lane 1-3), radish (4-6). (C) In spinach leaf and stem (lane 1-3), lady finger leaf and fruit (lane 4-6), sugarcane leaf, root and juice (lane 7-9), pumpkin fruit and leaf (10-11), maize grain and leaf (lane 12-15), brinjal fruit and leaf (16-19). M: 1Kb DNA ladder (size marker).

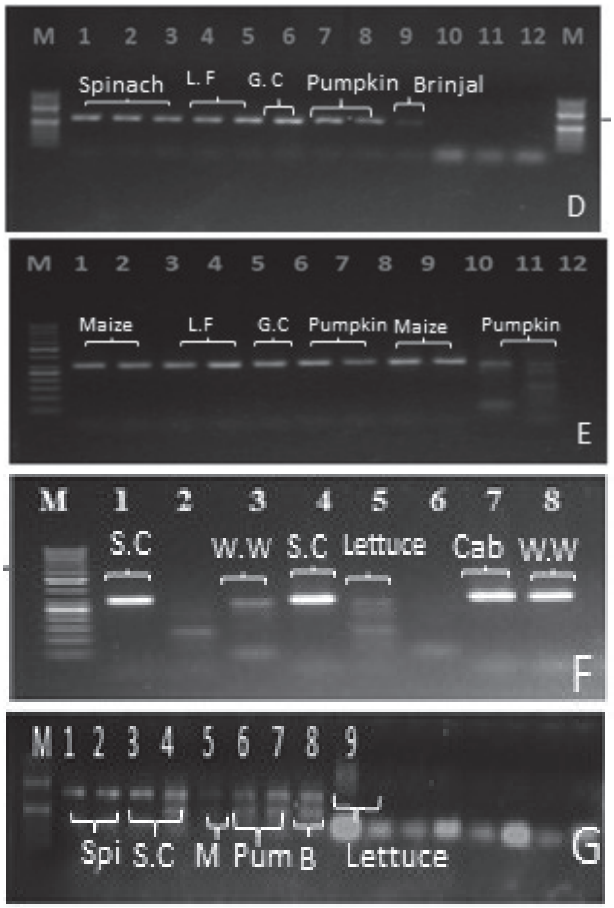

Fig. 3. 2% agarose gel picture of pathogen genes. (D) Amplification of Mdh gene in spinach leaf, stem and root (lane 1-3), ladyfinger leaf and fruit (lane 4, 5), green chili leaf (lane 6), pumpkin leaf and fruit (lane 7, 8), brinjal fruit (lane 9), lane 10-11 with no amplification. Lane M: 50 bp size marker. (E) Amplification of Mdh gene in maize leaf and grain (lane 1, 2) ladyfinger leaf and fruit (lane 3, 4), green chili leaf (lane 5), pumpkin leaf and fruit (lane 6, 7), maize leaf and grain (lane 8, 9), lane 10 and 11 amplified IpaB gene in pumpkin leaf and fruit. Lane M: 50 bp size marker. (F) Amplification of Mdh gene in sugarcane root and leaf (lane 1, 4), cabbage root (lane 7), wastewater (lane 8). Lane 3 and 5 amplified IpaB gene in wastewater and lettuce. Lane M: 50 bp size marker (G) Amplified IpaB gene in spinach root and leaf (lane 1, 2) sugarcane leaf and root (lane 3, 4), maize leaf (lane 5), pumpkin leaf and fruit (lane 6, 7), brinjal leaf (lane 8), lane 9: amplified IpaH gene in lettuce leaf, Lane M: 50 bp size marker.

Wastewater, being enriched with nutrients, is a preferred choice for irrigation due to its cheap and prompt availability. Untreated wastewater application is responsible for disseminating pollutants, heavy metals and pathogenic microbes, thus raising serious social and ecological concerns About 20 million hectares in 70 countries around the world are irrigated with wastewater [23]. Heavy metals like Pb, Zn, Ni, Se and Hg discharged from industries, get accumulated in soils and edible plant parts through irrigation practices [24-27]. Wastewater contaminated with microbial pathogens like bacteria and viruses pose health risk and result in a number of diseases [28]. Keeping these issues in mind, this study focused on identification of microbial species found in waste water irrigated edible vegetables and crops and could prove useful in suggesting remedies to revert environmental degradation.

In the present studies, 11 sample sources (one of wastewater and 10 from vegetables and other crops) suspected to be contaminated with various bacterial pathogens were analyzed. The culture and PCR based diagnosis showed that all the samples were contaminated (Table 3, Fig. 2 and Fig. 3). One of the pathogens detected in wastewater and many crop samples was *Salmonella.* This gram-negative bacterium formed red colonies with black center on XLT-4 media (Fig. 1A). In this study, most of the samples were contaminated with it including vegetable samples of spinach leaf and root, sugarcane leaf and root, brinjal fruit, and radish leaf and root. Earlier studies reported *Salmonella* in vegetables like tomato, cantaloupes and sprouts [16]. Moreover, it has been reported that contaminated soil and water are the chief reservoirs of *Salmonella* contamination in vegetables such as tomato [29].

Several cases of outbursts from fresh produce included incidence of *E. coli* O157: H7 (lettuce, spinach), *S. typhimurium* and *S. Newport* (tomato) and Hepatitis A (onion) [30]. Polluted water is the main source of contamination of crops and soil in which *E. coli* propagates and gets transmitted to inner tissues of lettuce where it persists for a long time [31, 32]. In this study, pathogenic *E. coli* strain was detected in all samples including wastewater, green chili, spinach, lettuce, sugarcane, maize and brinjal proving that the samples were highly contaminated with this pathogen.

Shigella spp. was only detected in lettuce showing that this pathogenic species got access to leaves of lettuce as this vegetable is usually grown on the soil surface and contamination may have occurred from wastewater irrigation [33]. Due to health and environmental risks associated with its use, the scientific approach demands treatment before irrigation with wastewater. However, many of the developing countries do not have sufficient resources for wastewater treatment and fresh water is also scarce; therefore, untreated wastewater is used for irrigation purposes [32, 34]. The data of this experiment illustrated that the consumption of vegetables and crops grown using wastewater can be hazardous.

Wastewater should be treated before use in irrigation to avoid contamination of edible commodities. Nevertheless, wastewater irrigation can be practiced to grow non-edible crops [35]. It can also be applied to fruits and vegetables that are preserved for a longer period as pathogens are killed due to heat and radiations used during preserving techniques [36]. The risks associated with wastewater can be greatly reduced by adopting some precautions e.g., sedimentation and flocculation. Various treatments such as oxidation ponds, filtration, activated sludge procedure, activated carbon treatment, chlorination and lime coagulation can

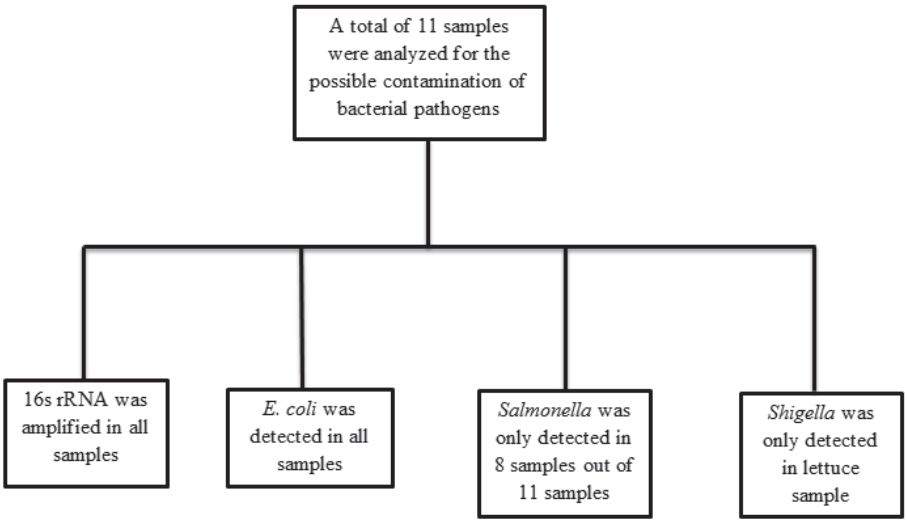

Fig. 4. Schematic layout of the experiment for the diagnosis of bacterial pathogens in wastewater and wastewater irrigated crops and vegetables.

Table 3. Identification of bacterial strains through PCR of 16S rRNA, Mdh, IpaB and IpaH genes.

Crop/ Vegetable	Part of the Plant Checked for bacterial contamination	16S rRNA	*Salmonella* (IpaB)	*E. coli* (Mdh)	*Shigella* (IpaH)
Spinach	Leaf	+	+	+	-
	Root	+	+	+	-
	Stem	+	+	+	-
Ladyfinger	Leaf	+	-	+	-
	Fruit	+	-	+	-
Green chili	Leaf	+	-	+	-
	Fruit	+	-	-	-
Pumpkin	Leaf	+	+	+	-
	Fruit	+	+	+	-
Brinjal	Leaf	+	+	-	-
	Fruit	+	-	+	-
Maize	Leaf	+	+	+	-
	Grain	+	-	+	-
Sugarcane	Leaf	+	+	+	-
	Root	+	+	+	-
	Juice	+	-	-	-
Cabbage	Leaf	+	+	+	-
	Root	+	+	+	-
Lettuce	Leaf	+	+	-	+
Radish	Leaf	+	+	+	-
	Root	+	-	+	-

remove viral and bacterial pollution in wastewater by up to 50% to 90% [37-41]. Therefore, the adoption of such treatment procedures before the use of wastewater for irrigation of edible crops and vegetables is crucial.

Conclusions

The use of wastewater for irrigation purposes is increasing day by day. Major concerns are associated with the consumption of heavily contaminating disease causing microbial species that come into contact with plants via water supply and cause various foodborne illnesses. In this study, the presence of *E. coli* in wastewater and wastewater irrigated pumpkin, sugarcane, maize, brinjal, lettuce, spinach, cabbage and radish samples was confirmed. Similarly, *Salmonella* was present in pumpkin, sugarcane, maize, brinjal, lettuce, spinach, cabbage and radish samples. Moreover, *Shigella* was identified in lettuce. The confirmation of pathogenic microbial species in mentioned plants indicated that the approach of wastewater treatment must be adopted before its application in agriculture. Otherwise, the bacterial pathogens entering the food chain through wastewater can cause various illnesses in humans.

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Conflict of Interest

All authors declare that there is no conflict of interest with any individual, commercial or financial institution other that mentioned in the acknowledgement section.

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