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Presence and Antibiotic Resistance of MDR *Salmonella* Isolates Recovered from *Zea mays* L. Farms Located near the Poultry Farms in Faisalabad-Pakistan

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Abstract

Background: *Salmonella* is the major food-borne pathogen associated with food products and causative agent of salmonellosis. Discharge of untreated wastes and leakage of poultry drainage in irrigation water might be the significant source of contamination in fields. The aim of this study was to investigate the presence of *Salmonella* in the rhizosphere and phyllo sphere of *Zea mays* L farm, following irrigation with ditch water contaminated with poultry drainage.

Methods: Total 6 maize farms in and around Faisalabad (Pakistan) were selected nearby the poultry farm area. Irrigated water, rhizosphere and leaves were analyzed for presence of *Salmonella*. A total of 160 samples were collected from different farms. Samples were cultivated on SS agar media and incubated at 37°C.

Results: Out of 160 samples, 39 showed positive growth for bacterial contamination. 18 samples were confirmed as *Salmonella* by morphological and biochemical characteristics. Our results indicated the presence of *Salmonella* isolates from irrigated water (n=10), from rhizosphere (n=5), from phyllo sphere (n=1) and from roots (n=2). Antibiotics susceptibility pattern of *Salmonella* isolates against routinely used antibiotics had indicated that 71% isolates were resistant to Tetracycline and Amikacin, and 65% resistance to Chloramphenicol. All the isolates were sensitive to Levofloxacin, Tobramycin, Cefepime, Gentamycin, Cefoxitin and Sulfamethoxale. All isolates were intermediate resistant to cefuroxime and ampicillin.

Conclusion: From obtained result it is confirmed that *Salmonella* spp. have been found in irrigation water mixed with poultry drainage and could be a source of *Salmonella* contamination to the crops located near the poultry farms.

Introduction

Salmonella is the major food-borne pathogen associated with food products and causative agent of salmonellosis. *Salmonella* is a natural inhabitant in the GIT (gastrointestinal tract) of birds, and humans [1]. Many of non-typhoidal *Salmonella* species known as NTS serotypes, which have a wide host range including human. It causes various complexities including diarrheal disease and certain intestinal complexities. The main source for salmonellosis has established been specific to animal origin including poultry products and drainage [2]. It has been found to be prevalent in soil and on water surface and persist there for a prolong periods of time [3].

Water has been shown as a carrier for pathogen transmission and a source of microbial contamination of crops and a vehicle for pathogen transmission [4,5]. Discharge of untreated wastes and leakage poultry drainage in the environment can leads to worsen the quality of water sources [6]. In different countries surveys are performed to check the *Salmonella* existence in certain leafy vegetables and different type of crops, their result identified *Salmonella* in up to 28% of the samples [7]. Raise in number of food-borne disease cases mainly associated to fresh fruits and vegetables [8]. It can also be associated to more consumption of ready-to-eat foods and food products [9,10]. In addition, on the basis of evidence obtained by plant pathologists and food microbiologists it is assumed that many of pathogens involved in enteric diseases have adapted to persist in or on plants [11,12]. These pathogens exist as part of plant's natural life cycle between infecting host.

Fields when irrigated with contaminated water or leakage of poultry drainage, pathogens may attach to different surfaces of plant including, roots, fruits, stems and other edible parts [13]. When adhere on fresh fruits and vegetables, pathogens may persist and multiply along the farm-to-fork continuum [14]. Multiple research studies have indicated that *Salmonella* and many other foodborne pathogens can be passed in soil by the drainage of wastes and also be transferred to plants in field by application of manure [15]. Recently study suggested that water is a reservoir to spread the *Salmonella* to plants [16].

Moreover, in this study other factor involve in *Salmonella* reachability to plants were investigated. The aim of this study was to investigate the persistence of *Salmonella* in phyllo sphere and rhizosphere and roots of maize fields, following irrigation with ditch water near the poultry farm areas in Faisalabad, Punjab-Pakistan. Moreover, *Salmonella* cause food-borne illnesses in humans, their sensitivity to various antibiotics is evaluated. This test demonstrated that the bacterium is multidrug resistance MDR. This multidrug resistance bacterium is a major contributor to the growing problem of antibiotic resistance in humans. When antibiotics are misused in poultry, then microbe become resistant to them.

Methods

Sample Collection

Different sample from irrigation ditch water, rhizosphere, roots and from phyllo sphere were collected from different region of Faisalabad. Three sample from each of farm was randomly collected. For collection of roots and rhizosphere, a sharp sterilized blade in 70% Ethanol was used. With the help of blade, roots from different plants were cut and collected in sterilized falcon tube. Approximately 2 to 3 roots per plant and each length of each root was around 9 - 11 cm was collected. Falcon tube was shaken for 3 to 4 min until the release of soil from roots. Then using the sterilized forceps remove the roots from the falcon tube and put into another 50ml falcon tube labelled by root sample, and previous tube was properly labelled as rhizosphere sample. Time and date were also mentioned on falcon tube. Samples were transported at 4°C and processed within 24 hours. For collection of water sample from different source sterilized falcon tube was used. Using gloved hands 30ml water sample was taken in 50ml of falcon tube from irrigation ditch. Properly labelled the falcon tube and transported the water sample to lab for further processing. Then leaves sample were collected using sterilized scissor in 70% of ethanol. Approximately 2 to 4 leaves per plant were collected and length of each leave was around 8 to 12 cm. Leaves were placed in properly labelled 50ml falcon tube. All the samples were safely transported to laboratory and screened for *Salmonella*.

Isolation of *Salmonella* by culturing Water and soil samples

Isolation of *Salmonella* was carried out by filtering the water by using syringe filter. Water samples were diluted 10-folds and then pre-enriched by mixing the 10 mL of water sample in 100 mL of nutrient broth. Similarly, soil samples were also diluted by making 10-fold dilution then pre-enriched by putting 10g of sample in 100ml of nutrient broth. Both the samples were incubated at 37°C for 24 hours. Then *Salmonella Shigella* (SS) agar was used for further isolation and purification. 1ml aliquot of each pre-enriched suspension was transferred to *Salmonella shigella* (SS) agar plate, incubated at 37°C for 24 to 36 hours. Colorless with black centers *Salmonella* colonies appeared, that were further confirmed as Gram negative by Gram staining.

Leaves and Root Samples

Collected leaves and root sample were dipped in 30 ml sterile phosphate buffered saline, vortexed for 2-3 min,

and then 2 ml aliquot of each suspension was transferred to 18 ml of nutrient broth, incubated at 37°C for 24 hours. Then 1ml of aliquot of each suspension plated onto the *Salmonella shigella* agar plate and incubated at 37°C for 24 to 36 hours. Colorless with black centers *Salmonella* colonies appeared, that were picked and confirmed as Gram negative by Gram staining.

Biochemical Characterization

Isolated organisms which showed *Salmonella* growth characteristics on SS media, were further confirmed by performing the following biochemical tests named as, Catalase, Oxidase, Triple Sugar Iron Test (TSI), Methyl Red/Voges-Proskauer (MR-VP) and Indole Test.

Testing of susceptibility of the isolates to antimicrobial agents

Susceptibility of pure isolates were checked to eleven (11) commonly used antimicrobial agents with the following disk contents; sulfamethoxazole /trimethoprim (25 µg), ampicillin (10 µg), levofloxacin (5 µg), Tobramycin (10 µg), Amikacin (30 µg), Tetracycline (10 µg) and Chloramphenicol (30 µg), gentamycin (30 µg), cefoxitin (30 µg), Cefepime (30 µg), Cefuroxime (30 µg) by the disk diffusion method [17] and based on recommendations of Clinical and Laboratory Standards Institute guidelines (CLSI, 2010). Mueller–Hinton agar (Oxoid) was made and poured into plates, spread the culture onto the agar by using the sterile wire loop and allow to dry for five minutes. Antimicrobial discs were placed on the agar plates with antibiotic disc dispenser. Plates were incubated at 37 °C for 24 hours.

Results

A total of 160 samples were collected from different farms. Out of 160 samples, 39 showed positive growth for bacterial contamination. 18 samples were confirmed as *Salmonella* by morphological and biochemical characteristics.

Biochemical Test	Result
Catalase	Positive
Oxidase	Negative
Methyl red	Positive
Vogues Prausker	Negative
Indole	Negative

Table 1: Biochemical test

Our results indicated that the presence of *Salmonella* isolates was detected in 10 samples of irrigation water, 5 samples of rhizosphere, 1 sample of phyllo sphere (leaves) and 2 samples of roots. As shown below in table 2, 5 samples out of 40 rhizospheric samples were shown the *Salmonella* positive results which means that the only 12.5% samples showed the *Salmonella* positive results. On the other hand, 10 water samples showed *Salmonella* positive results after morphological and biochemical characterization, it means 25% showed *Salmonella* positive results from water samples. Only 2 out of 40 roots samples and 1 out of 40 leaves samples confirmed the *Salmonella* isolates. It means that 5% and 2.5% *Salmonella* positive results showed by roots and leaves respectively. These results indicated that maximum *Salmonella* sp. was identified in water samples, then in rhizospheric soil and least amount *Salmonella* sp. was identified in roots and leaves. After processing total of 160 samples, we come to know that only 11.25% samples were shown *Salmonella* positive results.

Antimicrobial Susceptibility Testing

Antibiotics susceptibility pattern of *Salmonella* isolates against routinely used antibiotics had indicated that 71% isolates were resistant to Tetracycline and Amikacin, 65% showed resistance to Chloramphenicol. All the isolates were sensitive to Levofloxacin, Tobramycin, Cefepime, Gentamycin, Cefoxitin and Sulfamethoxale. All isolates were intermediate resistant to Cefuroxime, Ampicillin. Only 2 isolates were MDR. As all the result shown in table 3.

Sample Source	Sample processed	*Salmonella* +ve	*Salmonella* -ve	Prevalence of *Salmonella* (%)
Rhizosphere	40	5	35	12.5
Roots	40	2	38	7.40
Phyllosphere (leaves)	40	1	39	2.5
Water	40	10	30	25
Total	160	18	142	11.25

Table 2: Percentage of *Salmonella* after morphology study and biochemical characterization

Antimicrobial agents	Conc.	Standards (mm)			Results (mm)
		Sensitive	Intermediate	Resistance	
Cefepime	30 µg	>25	Nil	<18	25
Cefuroxime	30 µg	>23	15-22	<14	16
Ampicillin	10 µg	>17	14-16	<13	14
Tobramycin	10 µg	>15	13-14	<12	16
Amikacin	30 µg	>17	15-16	<14	14
Tetracycline	30 µg	>15	12-14	<11	0
Sulfamethoxazole	25 µg	>16	11-15	<10	25
Levofloxacin	5 µg	>17	14-16	<13	23
Chloramphenicol	30 µg	>18	13-17	<12	0
Gentamycin	30 µg	>15	13-14	<12	16
Cefoxitin	30 µg	>10	15-17	<14	21

Table 3: Antimicrobial susceptibility testing

Discussion

Contamination of *Salmonella* spp. in maize comprises negative effects on consumer and also has devastating financial impact on the maize industry. Thus, to improve public health, there is much need of research to check from where and how the contamination is being occur in crops including *Zea mays* L. This study was conducted to evaluate the presence of *Salmonella* in the *Zea mays* L farms located near the poultry farms, and to check the antimicrobial susceptibility of recovered isolates. Our study showed that 11.25% of sample were positive to *Salmonella* spp. from different sources. From irrigation

water source 25% sample were positive for *Salmonella* spp. it means water was contaminated and could serve as a carrier of *Salmonella* sp. to fields. Our study co related with study that, Irrigation water used in the fields contain lower than the 40% isolation rate [18]. It was reported worldwide that occurrence of *Salmonella* in various water bodies is ranging from 3 to 100% with frequency of positive samples [19].

The major source of human pathogens, such as *Salmonella*, is the poultry drainage or chicken litter, as poultry waste has the potential to pollute surface or ground water. In this study water showed the major reservoir of *Salmonella* with high density, it means poultry farm near the *Zea mays* L farms can impact on the *Salmonella* contamination of irrigated water. Furthermore, samples were collected from the *Zea mays* L farms located near the poultry farms area, so we presume that *Salmonella* contamination in ditch water was due to poultry drainage. It is anticipated that Irrigated water serve as reservoir to transmitted *Salmonella* to rhizosphere, roots, phyllo sphere of maize in field. As in this study, crop was irrigated with ditch water, not by any other method like spray irrigation, so after high *Salmonella* contamination in water, rhizosphere have 12.5% of *Salmonella* contamination found in this study. It is also reported in several studies that *Salmonella* can persist in farm environment for long period of time [20]. Several studies reported that there is improper waste management in Pakistan [21]. It is also indicated that country producing more organic waste, then the risk is also increased because the *S. typhimurium* acts as a potentially farm pathogen.

It is known that *Salmonella* is able to colonize fruit and vegetables and also leaf surfaces and it has been shown fruit contamination is due to early leaf colonization. S. Typhimurium have ability to survive in the rhizosphere, in the phyllo sphere and in the soil for at least 26- 28 days after the irrigation. In this study the isolation rate of *Salmonella* from phyllo sphere (leaves) is 2.50% and a previous study reported 1.5 % of confirmed *Salmonella* spp. from leaves [22]. Unexpectedly very low percentage of *Salmonella* confirmed on leaves surfaces. Many of factors can change the prevalence of pathogen on leaves surfaces. It was hypothesized that survival of *Salmonella* on plant surfaces could influenced by variations in environmental conditions (such as temperature, sunlight etc.) [22]. Level of pathogen could higher on plant surface when applied spray irrigation of water [22] but in this study we select those farms where water is applied through ditch water. From root sample more isolation of *Salmonella* 7.40% is confirmed then phyllo sphere because we hypothesized that irrigated water directly contacts with rhizosphere and root surface in contrast with phyllo sphere.

Several studies revealed that *Salmonella* shows multi drug resistance pattern [23]. Only 2 isolates in this study shows multidrug resistance. Multi drug resistance shown by the *Salmonella* in this study, suggests that there could be improper antibiotic improper usage or misused in that environment from where these isolates originated [24], or it is also possible that resistance to multiple antibiotics is due to isolates may have acquired the genes. Hence, from vegetables or fruit multidrug resistance strains of *Salmonella* conformation is a major concern for public health and food quality. One isolate from rhizosphere and one from irrigated water was particularly observed to be resistant to multidrug resistance. This shows a great public health concern as certain cases of salmonellosis require antibiotic therapy for treatment. In many studies it is reports that MDR *Salmonella* isolates have been suggested to be more virulent than non MDR *Salmonella* isolates [25]. This study requires great attention after detection of these multiple drug resistant *Salmonella* strains. Our findings indicate that these isolates recovered in this study have the potential to develop resistance for commonly used antimicrobial drugs and shows considerable health hazards to consumers, hence control measures should be adopted otherwise it can be life threatening condition. From our result it is confirmed that *Salmonella* was present in rhizosphere, root, and plant surface and able to colonize on the plant surface for prolong period of time after irrigation with contaminated water.

From all of above findings it is conclude that poultry waste mixed with the irrigation water through irrigations ditches and reach to the crops. As no any other significant source for the *Salmonella* reported. In this study we found *Salmonella* as the MDR pathogen so intake of food with such contamination is very lethal for human being. There must be a check in the disposal of poultry and dairy waste. It must be dumped to away from the agriculture farms to avoid contamination with irrigation water. In Faisalabad there is no such mechanism of poultry and dairy waste disposal, improper disposal leads to farm to fork contamination as reported in this study. So this study is very significant for the awareness among common man and for higher authorities to take significant action to avoid in future circumstances.

Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contribution

Conceived and designed the experiments: Arslan Ali, Saima Muzammil, Nimra Amjad.

Performed the experiments: Fatima javed, Arslan Ali, Nimra Amjad, Muhammad Zeeshan Ahmad, Muhammad Umar
Analyzed the data: Zain Ul Abbas, Fatima javed
Contributed materials/analysis/tools: Saima Muzammil, Sadaf Oranab, Maira Sajid
Wrote the paper: Arslan Ali.

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