INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
14–381/2015/17–6–1255–1259
DOI: 10.17957/IJAB/14.0035
http://www.fspublishers.org

Full Length Article

Tracing Transmission of *Salmonella enterica* subsp. *enterica* in Tomato Fruits

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Abstract

A major drawback of waste water irrigation to vegetables is the presence of lethal disease causing bacteria and the risk of their transmission to the tomato fruit. The presence of *S. enterica* subspecies *enterica* in fruiting bodies of tomato plant was traced, irrigated with contaminated waste water. Molecular and cultural diagnosis of *S. enterica* subsp. e*nterica* was carried out in tomato fruits after contaminated waste water irrigation to determine its transmission from water to tomato fruits. Sixteen genotypes were irrigated with waste water from germination to maturity. Tomato fruits were sampled and their flesh was used as inoculums for culturing the bacteria. No bacterial infection was detected in two genotypes while remaining fourteen accessions were found positive for bacterial growth in the culture. PCR amplification of bacterial 16srRNA was observed in all 14 genotypes depicting the bacterial infection in these fruits. But the diagnostic primers of *S. enterica* were amplified only in the fruits of two genotypes out of total 16. These Results showed that 87.5% genotypes were free of *S. enterica* subsp. e*nterica* contamination, depicting that most of the time pathogen was physiologically blocked from gaining access to the tomato fruit. It further confirmed the presence of variability for the potential of bacterial transmission from root to fruit in the tomato genotypes. The advantage of this variability can be harnessed in the form of breeding such genotypes, which present strong physiological barrier and defence system for the bacterial transmission to the tomato fruit.

Keywords: Peri-urban agriculture; Vegetables; Waste water irrigation; Pathogenic infection

Introduction

The global water scarcity issue led many countries to reuse the waste water for irrigation purpose. The recommended practice of irrigating crops requires the processing of waste water by filtration. But the heavy cost of filtration prevailed the irrigation by untreated waste water in the under developed and developing countries. Pakistan is one of these countries where untreated waste water is directly used for watering crops and vegetables in the periurban areas. This waste water usually consists of hospital, house hold and industrial sewage and effluents, which is a rich source of all types of human pathogens. The pathogens of orofecal route of transmission are real threat when this waste water is directly applied for agricultural purpose (Feenstra *et al*, 2000). Three main types of pathogens including viruses (Picornaviruses, Adenoviruses and Rotaviruses), protozoan /Helminth (*Entamoeba, Giardia, Trichomonas*), and bacteria (*Salmonella, Shigella, Mycobacterium, Klebsiella, Clostridium*) were found in waste water (National Research Council, 1996). These pathogens can survive for days to months in soil and on crops depending upon a number of factors such as soil pH, moisture content, moisture-holding capacity, organic matter, soil temperature and sunlight (Shuval *et al*., 1990). These pathogens result in disease transmission when pathogen-contaminated waste water is used for irrigation (Mapanda *et al.*, 2007). Usually the dense network of cellulosic fibres in plant cell wall and certain molecular immune system don't allow the entry of microorganisms into plant cells (Cooley *et al.*, 2003). However, if the cell wall is ruptured, or pathogens surpass the host innate immune system, the plant protoplast can be infected leading to the pathogen internalization (Warriner *et al.*, 2003). Different factors are responsible for the transmission of pathogens from fields to crops and crop type is the most important one determining pathogenic severity. Different sources of these pathogens are animal and human faeces, eggs, poultry, sea foods, juices, beef, milk, cheese, fruits, and vegetables irrigated with waste water (Zhao *et al*., 2008). Vegetables cultivated using waste water can be a source of infection for various lethal diseases for consumers (WHO, 2006). Among these, bacterial pathogens are those that are most commonly present in waste water and causing diseases. These bacterial pathogens cause a number of diseases like diarrhea, cholera, typhoid, and dysentery. A bacterial species commonly present in waste water is *S. enterica*, which can cause food poisoning, diarrhoea, typhoid fever and pneumonia. *S. enterica* consists of six

To cite this paper: Raja, S., A.A. Khan and H.M.N. Cheema, 2015. Tracing transmission of *Salmonella enterica* subsp. *enterica* in tomato fruits. *Int. J. Agric. Biol.*, 17: 1255–1259

subspecies: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *Indica* (Popoff, *et al.,* 2001, Popoff, *et al.,* 2004). Contamination of a crop by *S. enterica* can occur before or after harvesting but its transmission from soil to different plant parts depends on the crop. For crops, having edible part above the soil surface (such as tomato) are less contaminated than low growing crops like lettuce and parsley (Melloul *et al*., 2001). It is noteworthy that the food contaminated by *S. enterica* is more dangerous to human health when consumed raw.

The objective of this study was to test the hypothesis that waste water irrigation promotes salmonella internalization in tomato fruit. Secondly the variability among tomato genotypes for transmission of bacteria from water to tomato fruit was also observed.

Materials and Methods

Culture Based Detection of *S. Enterica*

Tomato accessions used were LO-2752(A), PB-017909(B), LA-2662(C), LA-1401(D), LA-2711(E), PB-017906(F), CLN-2418A(G), VRIT-47(H), 178556(I), PAKIT(J), HIT-9076-08(K), BL-1079(L), LO-4379(M), RIOGRANDI (N), BL-1077(O) and LO-3691(P). They were grown under field condition and irrigated with waste water till maturity. To detect the presence of *S. enterica* in the waste water, samples were collected in sterile plastic bottles in triplicates from three different sites of the source. The Xylose-Lysine-Tergitol 4 (XLT-4) agar was used as culture medium for *S. enterica* or any other bacterial growth. Waste water samples were streaked on XLT-4 solid growth media and incubated for 16–18 h at 37ºC. After 16–18 h visible bacterial growth occurred in the form of red colonies. Each presumptive colony was collected and transferred into XLT-4 liquid medium and kept at 37°C for 24 h for subsequent utilization of DNA isolation (Hintz *et al.,* 2010).

To trace the transmission of *S. enteric* from roots to fruits, fully ripened tomato fruits were picked in triplicates and collected in sterile plastic bags. These collected tomatoes were surface sterilized by 70% ethanol and dried under laminar flow hood until ethanol disappeared completely. Sterilized tomatoes were collected in sterile stomacher bags individually and stomached until a homogenous mixture was obtained. Later on the obtained homogenous mixture was streaked on XLT-4 solid growth media through sterile loop. The streaked plates were incubated for 16–18 h at 37ºC. After 16–18 h visible growth of bacteria occurred in the form of red colonies, each presumptive colony was collected and transferred into XLT-4 liquid growth medium and kept at 37°C for 24 h (Hintz *et al.,* 2010). After 24 h the DNA was extracted from XLT-4 liquid medium.

PCR Based Detection of *S. enterica* subsp. *enterica*

For PCR based diagnosis of pathogen transmission in tomato fruit primers of *phoP* gene were designed (Salem *et al*., 2010), which were specific only for *S. enterica* subsp. e*nterica* independent of any serovar. Their specificity for for *S. enterica* subsp. e*nterica* was also confirmed by nucleotide BLAST tool available at https://blast.ncbi.nlm.nih.gov. Liquid bacterial cultures were grown up to saturation, 1.5 mL culture was taken in 2 mL Eppendorf tube and spin for 2 min to get bacterial cell pellet. TE buffer @ 570 µL was added and vortexed to dissolve pellet thoroughly for 1 h at 37ºC. Then 100 µL of 5 M NaCl was added and mixed well. Later on 80 µL CTAB/NaCl solutions was added and mixed well and incubated at 65ºC for 10 min. Then 700 µL 24:1 Chloroform/Isoamyl alcohol was added and spun for 5 minutes. The supernatant, 500 µL was taken into new Eppendorf, 500 Phenol/Chloroform/Isoamyl alcohol was added and spun for 5 min. Supernatant was collected in new Eppendorf, and 600 µL of 100% ethanol was added and incubated at -20ºC for 20 min. Later on incubated sample was spun for 15 min and ethanol was removed. Pellet was washed with 70% ethanol. Ethanol was removed completely and pellet was dried at 37ºC and 50 µL of dH_2O was added to dissolve the pellet for further usage in PCR. Polymerase chain reaction (PCR) was performed using 100 ng of DNA and 0.05 mM of phoP-L1 and phoP-R1 primer. The PCR was set as initial denaturation at 94ºC for 5 min followed by 33 cycles of 94ºC for 1 min, 55ºC for 1 min, 72ºC for 1 min and final extension at 72ºC for 10 min. PCR products were electrophoresed on 1.5% agarose gel.

Results

Waste water samples showed positive results for bacterial growth on XLT-4 solid and subsequent liquid growth media (Fig. 1) proving the presence of pathogenic bacteria in the water. PCR based testing of these water samples using diagnostic primers of *S. enterica* subsp. e*nterica* also showed the amplification of target gene (Fig. 2). It depicts that the waste water, which is being used for irrigation purpose is contaminated with human pathogen that may cause fetal diseases and the consumer of such harvest along with farming community involved in this practice are at higher risk.

When a homogenized tomato fruit mixture was streaked on XLT-4 growth media, red bacterial colonies appeared in 14 out of 16 samples (Fig. 1). The 14 samples were further processed for PCR based detection of *S. enterica*. Good quality DNA was obtained from cultured media, which was used as template in subsequent PCRs. The PCR results showed the positive amplification of *phoP* gene confirming the presence of *S. enterica* subsp. e*nterica*. It confirmed that from these 14 infected genotypes only two tomato genotypes (178556 and LO-2752) or 12.5% were internally infected with *S. enterica* subsp.

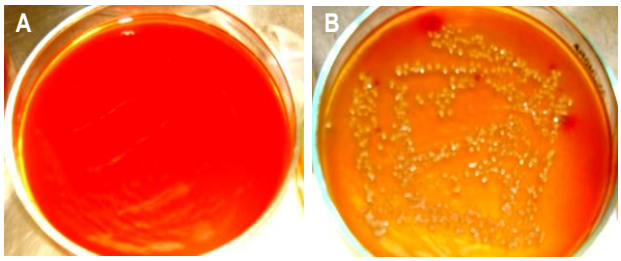

Fig. 1: Cultural detection of *S. enterica* on XLT-4 agar medium; (A) without bacterial colonies growth; (B) with bacterial colonies growth

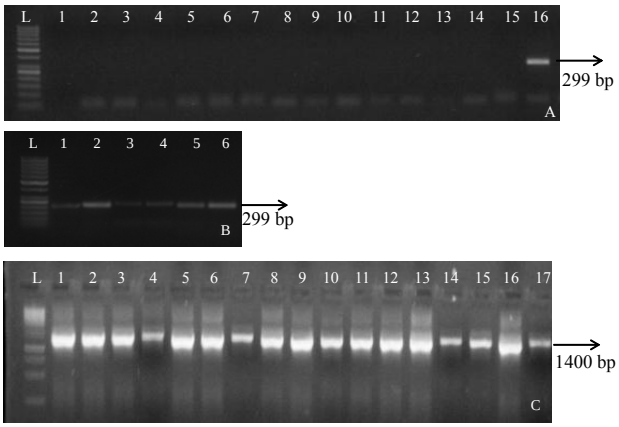

Fig. 2: PCR based detection of *S. enterica*. (A) PCR amplification of *phoP* gene using diagnostic primers for *S. enterica* subsp. *enterica* detection; L: 50 bp ladder; 1: Negative control; 2-15: tomato fruits of 14 genotypes; 16: positive control. (B) PCR based detection of *S. enterica* subsp. *enterica* in two tomato genotypes; L: 50 bp ladder; 1-4: two tomato genotypes in replicates; 5-6: waste water samples in replicate. (C) PCR amplification of 16Sr RNA gene from tomato (1-14) and three replicated samples of waste water (15-17); L: 1 kb ladder

Enterica, while 75% genotypes were infected with some other strains of bacteria. In PCRs, employing 16s ribosomal RNA gene primer as a positive control, 14 bacterial cultures showed amplification (Fig. 2 and Fig. 3), indicating that the 12 genotypes were also infected with some bacteria other than *S. enterica*. It also showed that the direct application of waste water without treatment is not safe for irrigating vegetables due to internalization of pathogen which cannot be removed by washing.

Discussion

The waste water is being opted for irrigation by large number of farmers due to scarcity of canal water and its enrichment of organic matter, which also serve as manure and reduce the amount of fertilizer to be applied. But the major drawback of applying waste water is the contamination of pathogenic bacteria and viruses. One of bacterial pathogens, usually present at high concentration in waste water, is *S. enterica* (Walczak *et al.*, 2009). But the dense and compact network of cellulosic fibres in plant cell walls usually do not allow microorganisms entry from waste water to the plant, but if the cell wall is ruptured then microorganisms can enter the plant and gain access to plant protoplast (Salem *et al.*, 2010; Gunasegaran *et al.*, 2011). The culture and PCR-based diagnostics showed that waste water used for irrigation was contaminated with *S. enterica* (Way *et al.*, 1993). While internal infection of *S. enterica* was detected only in two tomato accessions out of 16 genotypes. Of the 16 genotypes tested, 12 were found contaminated with bacterial growth other than *S. enterica*, as also shown by Jablasone *et al.* (2004). Previous studies endorsed our results regarding the variability of tomato genotypes for bacterial immobilization in tomato fruit (Miles *et al.*, 2009; Hintz *et al.*, 2010). Therefore, it is not necessary that if *S. enterica* is present in irrigation water it will also contaminate tomato fruit. The transmission of pathogen from roots to fruit might be the outcome of passive routing from wounds in the pericycle, mesophyl, and periderm of secondary lateral roots or natural openings resulting through damage during transplanting or growth (Hallman *et al.*, 1997; Dong *et al.*, 2003). Different opinions were found regarding entry, internalization, and transmission of bacterial pathogens to different plant parts when waste water was used for irrigation as a potential source of fruit contamination (Guo *et al.*, 2001; Guo *et al.*, 2002; Gu *et al.*, 2011) however, evidence that *S. enterica* is able to enter tomato plants through contaminated irrigation water remains inconsistent (Jablasone *et al.*, 2004; Miles *et al.*, 2009).

As 87.5% accessions were infected with one type of bacteria or another type. Amongst these infected samples only 14% were carrying *S. enterica* while rests were free of it. It depicted that most of the time *S. enterica* entry was blocked. The explanation of this blockage might be that plants have developed innate immune mechanisms against pathogens. The presence of structural, physical and physiological barriers limits the pathogen infection (Grennan, 2006). The presence of secondary metabolites, phenolic compounds (anthocyanins, flavonoids, tannins phytoalexins, lignin and furanocoumarins), enzymes and proteins (lectins, defensins, proteinase inhibitors and amylase inhibitors) might pose another layer of barrier for bacterial infection. These toxic molecules disrupt pathogen metabolism or cellular structure e.g. rishitin. Phenolic compounds are reported to be produced by tomatoes against pathogen attack (Wink, 1988; Guest and Brown, 1997). The variability in uptaking the pathogen might be related to the variation of genetic potential of these innate morphological and physiological barriers of tomato accessions.

Though fruits of only two tomato genotypes were found infected with *S. enterica*, risks of applying waste

Table 1: Primer sequences used for PCR based diagnosis of *S. enterica*

Primer	Sequence	Amplicon size (bp)	Reference
phoP-L1	5'ATGCAAAGCCCGACCATGACG3'	299	Salem *et al*., 2010
phoP-R1	5'GTATCGACCACCACGATGGTT3'		
16s-L1	5'AGAGTTTGATCMTGGCTCAG3'	1400	Shine and Dalgarno, 1974
16s-R1	5'CGGTTACCTTGTTACGACTT3'		

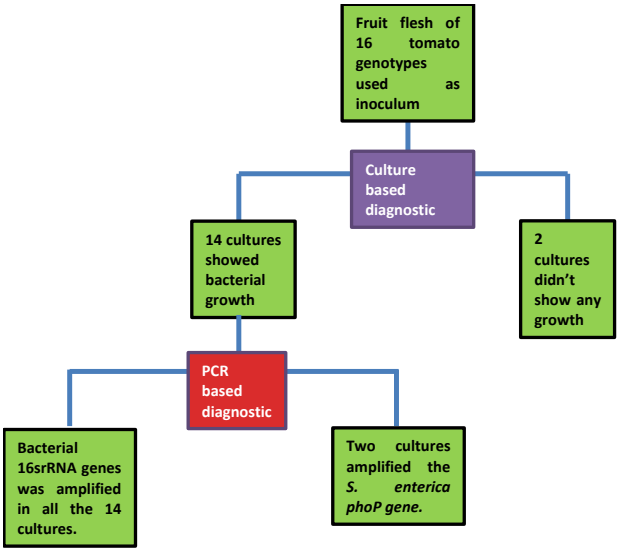

Fig. 3: Schematic layout of the experiment. The diagnosis of *S. enterica* in the tomato fruit, irrigated with waste water

water for a vegetable, which is also consumed un-cooked can't be afforded (Harris *et al*., 2003). Various treatments like lime coagulation, oxidation ponds, chlorination, activated carbon and sludge treatment can remove pathogens from waste water before it is used for irrigation (Cloette *et al.*, 1998; Schaub and Sorber, 1977). In addition, waste water should be applied primarily to industrial and non-edible crops (Shuval *et al*., 1986). Fruits or vegetables grown entirely for canning or processing, which destroys pathogens, are also good choices. Tomato should not be grown using waste water or if there is no other alternative then it should be cooked well before consumption, to minimize the pathogenic infection. The two genotypes (BL-1077 and LO-3691) which were found to be resistant against all bacterial infections including *S. enterica* can be used in the breeding programme for the development of genotypes which possess strong physiological barrier and defense system for the bacterial transmission to the tomato fruit as well as high yield to recommend for those areas where there is no other alternative instead of waste water.

Conclusion

Waste water irrigation is the need of the time, but one of the problems, associated with its application is the heavy contamination with pathogens, which can be removed by costly treatment. If not possible in a country like Pakistan this practice will lead to the produce contaminated with microbes like *S. entrica*. Another strategy to cope the situation is to screen such genotypes, which possess genetic capability attributed to physiological and molecular mechanisms to block their entry in the produce.

Acknowledgements

The authors acknowledge the financial support of Higher Education Commission (HEC) for Ph.D. studies of Ms. Shameem Raja. The technical help and productive suggestions of Mr. Usman Aslam, Dr. Waqas Malik and Dr. Abu Bakar Azmat are also gratefully acknowledged.

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(Received 12 May 2014; Accepted 25 September 2014)