Diversity and virulence associated genes of Salmonella enterica serovars isolated from wastewater agricultural drains, leafy green producing farms, cattle and human along their courses.

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SUMMARY

From March to September 2014, the prevalence of Salmonella spp were investigated in; 60 water samples from two wastewater agricultural drains, 40 samples from each of water and sediment from irrigation canals of leafy green producing farms at villages along sides of these drains, 153 leafy green samples (60 lettuce, 25 cabbage, 48 Egyptian clover and 20 vegetative stage of corn) from the farms irrigated with agricultural drains’ water, 52 cattle faeces and 45 stool from leafy green farms workers at Sharkia province, Egypt. Salmonella spp were detected in wastewater agricultural drains samples, water and sediment of irrigation canals, cattle faeces and workers stool with the percentages of 18.3, 7.5, 12.5, 5.8, and 4.4%, respectively. On the other hand, the respective occurrence of Salmonella in lettuce, cabbage, and Egyptian clover were 3.3, 4, and 2.8%. However, vegetative stage of corn was found free from Salmonella. Serological identification of recovered Salmonella isolates (n=28) clarified that S. Typhimurium (67.86%) was the most prevalent, followed by S. Enteritidis (14.29%) and S. Newport (7.14%). However, each of S. Derby, S. Senftenberg and S. Virchow was detected with the percentage of 3.57%. Molecular investigation of S. Typhimurium, S. Enteritidis, and S. Newport, the more prevalent serovars in examined sources revealed widespread occurrence of invA, avrA, spvC, bcfC, and stn virulence genes in screened isolates. The respective virulence factors were detected with the overall values of 96, 68, 60, 88 and 76% in the examined Salmonella isolates.

Keywords: wastewater, irrigation, leafy greens, cattle, human

INTRODUCTION

Sharkia is the second province in Egypt after Cairo with a human population about 14 million people, 62% of whom live in country side, from which municipal, industrial and agricultural effluents were generated and disposed into the River Nile derivatives and agricultural drains through point and non-point source discharge [41]. Moreover, lack of adequate sanitation and waste disposal infrastructure in these places are among the direct causes of such pollution to these water derivatives and drains [38].

After mixing of wastewater with fresh water, the resulted diluted wastewater or polluted surface water is usually used by farmers for irrigating agricultural crops such as fruits and vegetables because of the scarcity of clean water resources and because of this water is seen by small-scale producers as a cheap means to improve soil fertility and adding essential nutrients for crops [19]. In a new review, about 46 countries including Egypt reported the use of polluted water for irrigation purposes [73]. However, the use of wastewater in irrigation involves many risks and negative impacts of great importance [13], among them the primary concern is to consumers using leafy green vegetables eaten uncooked and in raw salad dishes [29].

Over the past 10 years, there is an increasing demand for leafy green vegetables (lettuce, cabbage, and spinaches)
and their ready to eat (RTE) salads since people changed their eating habits because of healthier lifestyle interest. Nevertheless, fresh leafy green vegetables and their RTE salads are recognized as a source of food poisoning outbreaks in many parts of the world [47]. In USA, among 103 (54%) fresh-produce associated outbreaks with a known pathogen in the period from 1973 to 1997, 62 (60%) were caused by bacterial pathogen, of which 30 (48%) were caused by Salmonella [67].

Salmonella enterica is one of the most common causes of foodborne infection in human beings and still the main cause of acute diarrhea syndrome [45]. Farmers, their families and crop consumers might be at risk, because the contaminated leafy green vegetables grown in wastewater irrigated fields are mostly eaten uncooked [9]. In Chile [63] and Morocco [2], Salmonella infection rate was significantly higher among residents living in the wastewater-spreading fields than in control areas not practice wastewater spreading. In addition, as a result of usual occurrence of Salmonella organism in high concentration in wastewater and its long survival period in moist soil, cattle grazing on wastewater irrigated pasture could be infected with this microorganism [57]. Furthermore, people could take the infection when drinking milk or eating meat from such infected cattle [72].

The epidemiology and pathogenic process in salmonellosis are dictated by an array of factors that act in tandem and ultimately manifest in the typical symptoms of salmonellosis. Virulence genes encode products that assist the organisms in expressing its virulence in the host cells. Nucleic acid based techniques are being employed for the detection of various gene-encoded virulence factors viz., invA and avrA genes that associated with Salmonella pathogenicity islands (SPIs), the fimbrial related gene bfcC, the gene spvC from the spvC operon and stn involved in enterotoxin production. However, the distribution of these genes among various isolates obtained from biological sources is yet to be elucidated [51].

This study was carried out to determine the prevalence and diversity of Salmonella spp in two wastewater agricultural drains and in irrigation canals of leafy greens producing farms receiving their water at Sharkia province, Egypt. Other objectives were to study the potential hazard of leafy greens irrigated with diluted wastewater in dissemination of Salmonella to their human consumers and cattle and to assess the pathogenic potential of recovered Salmonella serovars using virulotyping PCR assay.

**MATERIALS AND METHODS**

**STUDY AREA**

This study was conducted at the vicinity and along the course of two wastewater agricultural drains located in Sharkia province, Egypt. The first one was El-Halabi drain about 8 km from Zagazig city and three villages along its sides and using its water for agriculture and maintenance of live stocks were sampled (Kafr El-Halabi, Houd El-Tarfa and Shinbaret El-Maymona villages, with a distance about 5 km between each other). The second was Equa drain about 12 km from Zagazig city and two villages along its course and using its water were sampled (Equa village and Saft Zreik village with a distance about 4 km in between). Both agricultural drains receive human, animal and agricultural wastes along their sides.

**SAMPLING**

From March to September 2014, a total of 390 samples were collected from El-Halabi and Equa wastewater agricultural drains and from five villages along their courses.

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>Type of sample</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wastewater agricultural drains (El-Halabi and Equa)</td>
<td>Water</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Irrigation canals</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Irrigation Water</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sediments</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Leafy greens for human</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Cabbage</td>
<td>25</td>
</tr>
<tr>
<td>Leafy green producing farms</td>
<td>Leafy greens for animal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Egyptian clover *</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Vegetative stage of corn (V5-V10) b</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Cattle faeces</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Farm workers’ stool</td>
<td>45</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>390</strong></td>
</tr>
</tbody>
</table>

*a* The scientific name of Egyptian clover is *Trifolium alexandrinum* and its local name in Egypt is Berseem.

*b* The local name of vegetative stages of corn in Egypt is Darawa. The vegetative stage of corn (V5-V10) was named according to [1].

Table I: Sources and types of samples.
at Sharkia province, Egypt. The collected samples and their sources were illustrated in Table I.

SAMPLES' COLLECTION AND PREPARATION

WATER SAMPLING FROM WASTEWATER AGRICULTURAL DRAINS

Water samples were collected approximately 1-2 m from bank by using sterile glass bottle one liter capacity, caged with a load and having two cords, one attached to the neck and the other to the stopper [3]. The bottle descends closed till a depth of 20-30 cm, then opened by jerking out the attached cord to the stopper. From each water sample, 100 ml was taken in a sterile plastic bottle of 150 ml capacity for bacteriological examination. Twenty five milliliter of each water sample was thoroughly mixed with 225 ml of buffered peptone water (BPW, Oxoid, CM509).

WATER AND SEDIMENT SAMPLING FROM IRRIGATION CANALS

The water samples were collected from various points along irrigation canals by using of sterile syringes and one sterile plastic bottle, 150 ml capacity was used for each sample. On the other hand, the upper 2-4 cm from the layer of sediment in contact with water was aseptically removed with a gloved hand and directly placed into sterile wide mouth screw capped plastic cup or polyethylene bag [6]. Twenty five milliliter/gm of each water or sediment samples were thoroughly mixed with 225 ml of BPW.

LEAFY GREENS

Leafy greens used for human consumption (lettuce and cabbage) and cattle feeding (Egyptian clover and vegetative stage of corn) were collected directly from investigated leafy green farms using gloved hands and placed separately in sterile polyethylene bags. Analytical portion (25 gm) of each leafy green was aseptically weighted using sterile scissor and spatula and blended for 2 minute with 225 ml of BPW using stomacher lab blender.

CATTLE FAECES AND FARM WORKERS’ STOOL

Fresh cattle faecal samples were collected from the ground in the vicinity of examined drains and as nears as possible to farms from which leafy greens were collected. Each sample was packed separately in a sterile polyethylene bag. For collection of farmers’ stool, sterile cups were distributed on farmers of the examined farms one day before collection and the persons were instructed to collect the next day’s stool [74]. Five grams of each cattle faecal and human stool samples were added to 45 ml of BPW and mixed together to form uniform slurry.

ISOLATION AND IDENTIFICATION OF SALMONELLA

After preparation of collected samples, all samples were incubated at 37 ± 1 ºC for 18 ± 2 hrs for resuscitation and pre-enrichment. For enrichment, 0.1 ml of each pre-enriched broth was transferred to 10 ml of Rappaport Vasiliadis medium (Oxoid, CM 669) and incubated at 41.5±0.5ºC for 24±3 hrs [14]. One loopful of each enriched broth was streaked aseptically onto Xylose Lysine Deoxycholate agar (XLD- Oxoid, CM 469) and incubated at 37±1ºC for 24±3 hrs. After incubation, the plates were examined and 3-5 black colonies with red back ground were selected and sub-cultured on nutrient agar for morphological and biochemical identification according to Koneman et al. [43].

SEROLOGICAL IDENTIFICATION

All biochemically identified Salmonella isolates from examined sources were serotyped at Serology Unit, Animal Health Research Institute, Dokki, Giza, Egypt. The serotyping was done by slide agglutination technique using polyvalent and monovalent antisera according to Kauffmann white Scheme [40].

MOLECULAR DETECTION OF VIRULENCE ASSOCIATED GENES IN ISOLATED SALMONELLA SEROVARS

To assess the virulence potential of Salmonella isolates from the examined sources, the presence of 5 virulence associated genes was determined in isolates of S. Typhimurium, S. Enteritidis and S. Newport because of their higher prevalence in the examined sources.

DNA EXTRACTION

DNA extraction from samples was done using the QIAamp DNA mini kit with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 200 µl of AL Buffer for 10 min at 56ºC. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

OLIGONUCLEOTIDE PRIMERS

Primers used were supplied from Metabion (Germany) are listed in Table II.

PCR AMPLIFICATION

Primers were utilized in a 25 µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in a T3 Biometra thermal cycler for 35 cycles of various temperature
ANALYSIS OF THE PCR PRODUCTS

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products were loaded in each gel slot. Gelpilot 100 bp, 100 bp plus ladders (Qiagen) and Gene ruler 100 bp plus DNA ladder (Fermentas) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software (Figure 1).

RESULTS

PREVALENCE AND SEROVARS OF SALMONELLA RECOVERED FROM THE EXAMINED SOURCES

Table III reveals that Salmonella spp were isolated from 11 water samples (18.3%) from wastewater agricultural drains. Three serovars were identified; S. Typhimurium (8 isolates), S. Enteritidis (2 isolates) and S. Virchow (1 isolate). Salmonella spp were detected in 3 (7.5%) water and 5 (12.5%) sediment samples from irrigation canals of leafy green farms. The serologically identified seovars from water samples were S. Typhimurium (2 isolates) and S. Senftenberg (1 isolate). However, those isolated from sediment were S. Typhimurium (3 isolates), S. Enteritidis and S. Derby (one isolate, each). S. Typhimurium was detected in; 2 (3.3%) lettuce, 1 (2.8%) Egyptian clover “Berseem”. One cabbage (4.0%) was contaminated with S. Newport. However, the vegetative stage of corn “Darawa” samples were found free from Salmonella. The prevalence of Salmonella spp in examined cattle faeces was 5.8% and the serovars of S. Typhimurium (2 isolates) and S. Enteritidis (1 isolate) were identified. Two stool samples (4.4%) from workers at leafy green farms were positive for

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Initial denaturation</th>
<th>Amplification Condition (35 cycle of denaturation/ annealing/extension)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>invA</td>
<td>GTGAAATTATGCGGCACGTTCGGGCAA TCATCGACCGTCGAAAGGACC</td>
<td>94˚C for 5 min.</td>
<td>94˚C for 5 sec./ 55 for 30 sec./ 72˚C for 30 sec.</td>
<td>284</td>
<td>[52]</td>
</tr>
<tr>
<td>avrA</td>
<td>CCT GTA TTG TTG AGC GTC TGG AGA AGA GCT TCG TTG AAT GTC C</td>
<td>94˚C for 10 min.</td>
<td>94˚C for 30 sec./ 58˚C for 30 sec./ 72˚C for 30 sec.</td>
<td>422</td>
<td></td>
</tr>
<tr>
<td>spvC</td>
<td>ACC AGA GAC ATT GCC TTC C TTC TGA TCG CCG CTA TTC G</td>
<td>94˚C for 10 min.</td>
<td>94˚C for 30 sec./ 53˚C for 30 sec./ 72˚C for 30 sec.</td>
<td>467</td>
<td>[34]</td>
</tr>
<tr>
<td>bcfC</td>
<td>ACC AGA GAC ATT GCC TTC C TTC TGC TCG CCG CTA TTC G</td>
<td>94˚C for 10 min.</td>
<td>94˚C for 45 sec./ 59˚C for 45 sec./ 72˚C for 35 sec.</td>
<td>617</td>
<td>[50]</td>
</tr>
<tr>
<td>Stn</td>
<td>TTG TGT CGT TCG TGG CAA CC ATT CGT AAC CCG CTC TCG TCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A final extension was carried out at 72˚C for 10 minutes for all genes except for invA gene; it was 72˚C for 7 minutes.

Table II: Primers sequence, target genes, amplicon sizes and cycling conditions.
Salmonella and serovars of S. Newport and S. Typhimurium (one isolate, each) were identified.

Table IV reveals that out of 28 recovered Salmonella isolates from different sources, 67.86% were S. Typhimurium, 14.29% were S. Enteritidis, and 7.14% were S. Newport, meanwhile, only one isolate (3.57%) was obtained from each of serovars S. Derby, S. Senftenberg and S. Virchow.

### DISTRIBUTION OF VIRULENCE GENES IN INVESTIGATED SALMONELLA SEROVARS

Molecular investigation of S. Typhimurium, S. Enteritidis, and S. Newport revealed widespread occurrence of invA, avrA, spvC, bcfC, and stn virulence genes in screened isolates. The respective virulence factors were detected with the overall values of 96, 68, 60, 88 and 76% in the examined Salmonella isolates (Table V and Figure 1A-E).

### DISCUSSION

The prevalence of Salmonella spp (18.3%) in wastewater agricultural drain samples collected from Sharkia province, Egypt in the present study agrees with those of Khedr [41] who isolated Salmonella spp from 45/216 (20.83%) water samples from three wastewater agricultural drains at the same province. Meanwhile, 10.6% of investigated wastewater effluent and River Nile water samples in Cairo, Egypt were found positive for Salmonella spp [53]. In Australia, Salmonella was isolated from 12% of polluted surface water

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. examined</th>
<th>No. positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water from agricultural drains</td>
<td>60</td>
<td>11</td>
<td>18.3</td>
</tr>
<tr>
<td>Water from irrigation canals</td>
<td>40</td>
<td>3</td>
<td>7.5</td>
</tr>
<tr>
<td>Sediment from irrigation canals</td>
<td>40</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>Leafy greens for human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lettuce</td>
<td>60</td>
<td>2</td>
<td>3.3</td>
</tr>
<tr>
<td>Cabbage</td>
<td>25</td>
<td>1</td>
<td>4.0</td>
</tr>
<tr>
<td>Leafy greens for cattle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egyptian clover</td>
<td>48</td>
<td>1</td>
<td>2.8</td>
</tr>
<tr>
<td>Vegetative stage of corn</td>
<td>20</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cattle faeces</td>
<td>52</td>
<td>3</td>
<td>5.8</td>
</tr>
<tr>
<td>Farm workers’ stool</td>
<td>45</td>
<td>2</td>
<td>4.4</td>
</tr>
</tbody>
</table>

**Table III: Prevalence and serovars of Salmonella recovered from the examined sources**

<table>
<thead>
<tr>
<th>Salmonella serovars</th>
<th>No. of isolates</th>
<th>Percent of isolation *</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Typhimurium</td>
<td>19</td>
<td>67.86</td>
</tr>
<tr>
<td>S. Enteritidis</td>
<td>4</td>
<td>14.29</td>
</tr>
<tr>
<td>S. Newport</td>
<td>2</td>
<td>7.14</td>
</tr>
<tr>
<td>S. Derby</td>
<td>1</td>
<td>3.57</td>
</tr>
<tr>
<td>S. Senftenberg</td>
<td>1</td>
<td>3.57</td>
</tr>
<tr>
<td>S. Virchow</td>
<td>1</td>
<td>3.57</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>100</td>
</tr>
</tbody>
</table>

* Percent of isolation was calculated to the total number of Salmonella isolates from different sources.

**Table IV: Diversity of Salmonella serovars in the examined wastewater agricultural drains as well as water and sediment of irrigation canals, leafy greens, cattle faeces and farm workers stool along their courses.**
examine the distribution of virulence genes in S. Enteritidis and Typhimurium, 24 (96) of which contained invA and avrA genes. In another study, nearly similar prevalence for Salmonella (6%) in water collected from agricultural canals to those reported in this study (7.5%) was previously recorded in water from irrigation system and ponds in farms on the Central California [6]. Blumenthal and Peasey [8] in Mexico and Duffy et al. [18] in Texas isolated Salmonella from irrigation water of produce farms with the percentages of 35.2 and 9.4, respectively. Moreover, despite the serovars (S. Typhimurium and S. Senftenberg) were commonly isolated from wastewater and surface water streams in other studies [39] but their occurrence in irrigation water is of public and veterinary health concern. In a recent study, irrigation with poor quality water increased the levels of Salmonella spp on leafy green produce [26]. Furthermore, as S. Typhimurium can survive in irrigation water up to 26 days in summer and 88 days in winter [75], the risk for Salmonella transmission was increased. Internalization of Salmonella in contact with plants could occur through stomata of leaves [37] or via damaged leafy tissues [25] or roots [32].

Lower prevalence for Salmonella spp (4.3%) than those recorded in our study (12.5%) was detected in sediment of irrigation system from leafy green farms in Central California, USA [6]. However, the examined sediment from six irrigation canals in Galveston, Texas showed much higher prevalence (47.2%) for the microorganism was recorded in water samples from oldman River watershed in Southern Alberta [48]. The frequent isolation and serovar diversity of Salmonella from water of agricultural drains in this study may reflect wide variance in sources of pollution and could be attributed to high human and animal stocking densities with subsequent heavy usage of drains water. In addition, absence of public sewers in such overcrowded villages results in exposure of such drains to municipal, industrial and agricultural effluents through point and non-point source discharges. Even more, serovars of S. Typhimurium, S. Enteritidis and S. Virchow recovered from water of agricultural drains in the present study were found most predominant among Salmonella serovars from wastewater in Portugal and Southern Spain [16] and [20].

Table V: Distribution of virulence genes in Salmonella enterica serovars Typhimurium, Enteritidis and Newport isolated from different sources

<table>
<thead>
<tr>
<th>Salmonella serovar</th>
<th>No. of examined isolates</th>
<th>invA No. (%)</th>
<th>avrA No. (%)</th>
<th>SpvC No. (%)</th>
<th>bcfC No. (%)</th>
<th>Stn No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Typhimurium</td>
<td>19</td>
<td>19 (100)</td>
<td>13 (68.4)</td>
<td>12 (63.2)</td>
<td>16 (88.9)</td>
<td>15 (78.9)</td>
</tr>
<tr>
<td>S. Enteritidis</td>
<td>4</td>
<td>4 (100)</td>
<td>3 (75)</td>
<td>3 (75)</td>
<td>4 (100)</td>
<td>3 (75)</td>
</tr>
<tr>
<td>S. Newport</td>
<td>2</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>0 (0)</td>
<td>2 (100)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>24 (96)</td>
<td>17 (68)</td>
<td>15 (60)</td>
<td>22 (88)</td>
<td>19 (76)</td>
</tr>
</tbody>
</table>

Leafy greens which are ready to eat have been implicated in many outbreaks of salmonellosis and foodborne illnesses worldwide. Outbreaks of salmonellosis associated with lettuce were previously recorded in Australia where S. Bovismorbidicans was incriminated [69] and in England and Wales where the incriminated pathogen was multi-resistant S. Typhimurium [33]. Contamination of leafy greens with Salmonella may occur during harvesting, transportation or washing with poor quality water [61]. Compared to the occurrence of different Salmonella serovars in leafy greens in this study (Table IV), similar frequencies were recorded by Froder et al. [22] and Simoes et al. [66] in Brazil. Whereas, lower frequencies were exhibited in the leafy vegetables examined by Sant’Ana et al. [62]. In Estonia, although the overall prevalence of Salmonella was 0.54% in examined food samples comprising mixed salads and vegetables, but 24 serovars were identified, where S. Typhimurium was the most frequent, followed by S. Enteritidis and S. Newport [44]. The recorded high occurrence of Salmonella on leafy greens in our study could be expected from the detected high prevalence for the microorganism in irrigation water and sediment from irrigation canals. Once contamination of leafy green takes place, all Salmonella serovars attached rapidly on intact and cut leaves surface and produce strong biofilms [56], causing gastroenteritis if not efficiently washed before consumption.

Salmonella transmission to cattle may be due to grazing on wastewater irrigated pasture especially in case of highly dense animal’s population and high concentration of Salmonella in irrigated wastewater [57]. Moreover, drinking contaminated water with such microorganism is another route of infection to cattle [72]. In Canada, slightly higher prevalence for Salmonella (7.46%) was detected in faecal samples from animals in the vicinity of Oldman River [39]. Whereas, in Iran, Salmonella was detected in 1.5% of cattle faeces examined [27]. The isolated Salmonella
serovars from cattle faeces in this study (S. Typhimurium and S. Enteritidis) were found most predominant among 68 Salmonella isolates from animals in Egypt [49]. On the other aspect, isolation of Salmonella from cattle in this study is of great concern and refers to dangerous role might be played by cattle in dissemination of salmonellosis to other animals and human. In a recent study in United Kingdom, cattle infected with S. Typhimurium was the principle issue of Salmonella contamination to cereals when cattle farms were used as a temporary plant and grain stores [15]. Also physical contamination of cow’s udder with such pathogen from wastewater irrigated pasture usually results in milk contamination and foodborne diseases [65].

The use of diluted wastewater for irrigation of growing crops is normally associated with some problems. One of such problems is the health hazards posed to farmers / farm workers, crop handlers, consumers and residents around the wastewater irrigated fields [36]. Among these health hazards, salmonellosis foodborne illness is more common and contaminated leafy green vegetables and their ready to eat (RTE) salads are important vehicles for their transmission [47]. In Mendoza, Argentina, a high incidence of Salmonella (23%) was recorded in exposed group of children living in wastewater spreading areas compared to 4% in children of control zone [7]. In Morocco, the prevalence of Salmonella was 32.56% among children living in wastewater spreading field of Marrakesh city (El Azzouzia), whereas in Sidi Moussa control area was only 1.14%. However, occupation of parents was a significant factor influencing the prevalence of Salmonella as children of agriculturists showed a significantly higher Salmonella rate than those from non-agriculturist families [2]. The comparatively lower prevalence of Salmonella in farmers stool (4.4%) in this study may be attributed to that the targeted groups included adults not children only and the use of diluted wastewater or polluted surface water for irrigation but not raw sewage or sludge spreading. In Santiago, Chile, human infection with Salmonella was totally disappeared when irrigation of vegetables with raw wastewater was stopped [64].

The predominance of S. Typhimurium, (67.86%) and S. Enteritidis, (14.29%) over S. Newport (7.14%), S. Derby, S. Senftenberg and S. Virchow (3.57%, each) in this study (Table IV) agrees with those of Khedr [41] who found that S. Typhimurium and S. Enteritidis accounted for 50 and 40% of Salmonella isolates from three wastewater agricultural drains at Sharkia, Egypt. In Southern Spain, despite, S. Enteritidis and S. Typhimurium were most predominant among Salmonella isolated from wastewater effluents (46.1 and 20.2%, respectively) but the serovar S. Virchow represented 3% of isolates [20]. Meanwhile, among 17 serotypes of Salmonella from wastewater in Portugal, the serovars S. Virchow (21.6%) and S. Senftenberg (8.1%) were found more frequent [16]. On the other aspect, in Estonia, S. Typhimurium, S. Derby, S. Enteritidis, and S. Newport were the most common serovars of Salmonella from non-thermally processed vegetables [44].

To assess the virulence potential of Salmonella isolates from the examined sources, the presence of 5 virulence associated genes was determined in the isolates of S. Typhimurium, S. Enteritidis and S. Newport because of their higher prevalence in the examined sources. The investigated genes comprised invA and avrA genes associated with Salmonella pathogenicity islands (SPIs), the fimbrial related gene bcfC, the gene spvC from the spvC operon and stn involved in enterotoxin production. These virulence determinants have been shown to be widely distributed among isolates from animals, humans and environment, but with some diversity [31]. The diversity in distribution could be explained by serovar specificity of virulence plasmid [60].

The occurrence values of invA gene (all S. Typhimurium and S. Enteritidis isolates and 50% of S. Newport isolates, Table V and Figure 1A) and bcfC gene (all S. Enteritidis and S. Newport isolates and 88.9% of S. Typhimurium isolates, Table V and Figure 1D) agrees with those reported by Osman et al. [54] who detected invA and bcfC in all S. Enteritidis and S. Typhimurium recovered from imported turkey pouls in Egypt. In Italy, all 13 S. Typhimurium isolates from water buffalo calves with lethal enteritis displayed the presence of invA and bcfC genes [10]. However, invA was detected only in 47.3% of S. Enteritidis and 50% of S. Typhimurium isolates from animals and human in Egypt [49]. The recorded high frequencies of invA (96%) and bcfC genes (88%) in the present study confirm the previous results that little or no variation occurred for most genes incorporated in SPIs (invA) and for the fimbrial markers (bcfC), thus these genes were present throughout most serotypes [55]. The invA is the Salmonella invasion gene which is essential for entry of bacteria into epithelial cells and is a putative inner membrane component of SPI-1 dependent type III secretion system (TTSS-1) virulence apparatus [35], whereas bcfC is bovine colonization factor and fimbrial usher [10].

Another investigated virulence gene in this study was avrA gene that controls Salmonella induced inflammation by inhibition of the proinflammatory, antiapoptotic NF- Kappa B pathway and is a genetic marker for presence of SPIs which is associated with enhanced invasion and intracellular survival within both phagocytic and non-phagocytic cells [34]. Higher occurrence of avrA gene in S. Typhimurium isolated from calves with gastroenteritis (100%) than that reported in this study (68.4%, Table. V and Figure 1B) was previously recorded by Boriello et al. [10], the same author detected this gene in the other Salmonella serovars with a frequency from 57-86%. In Egypt, Osman et al. [54] detected avrA gene in S. Enteritidis from turkey but not from S. Typhimurium from the same source.

The spvC (Salmonella plasmid virulence gene C) is located on virulence plasmid; mainly induced during infection of specific host [30]; was required for full expression of virulence in Salmonella [28] and promotes rapid growth and survival within the host [11]. This gene was determined with a total value of 60% in Salmonella isolates in this study.
(Table V and Figure 1C), where, it was detected in 63.2% of S. Typhimurium, 75% of S. Enteritidis but not present in S. Newport. Nearly similar spvC frequency (61.5%) was previously recorded in S. Typhimurium isolated in Japan [42], whereas, in Tehran, Iran, the gene was detected in both S. Typhimurium, and S. Enteritidis from calves [4]. Higher frequencies (92.5 and 100%) were respectively detected in Salmonella enterica isolates from calves in Japan and Mexico [23, 68]. On contrast, Salmonella serovars recovered from wastewater, poultry and human sources showed lower frequency (15.1%) for spvC gene [71].

Salmonella induced diarrhoea is a complex phenomenon involving several pathogenic mechanisms including production of enterotoxin [5]. This enterotoxin production is mediated by the stn gene [12]. This stn gene has been reported to be absent in S. bongori [58] strains and also the other members of Enterobacteriaceae or Vibrio, which have entero toxigenic potential [59]. In the present study, stn gene was detected respectively in 78.9, 75, and 50% of S. Typhimurium, S. Enteritidis, and S. Newport isolates (Table V and Figure 1E). In India, stn gene was respectively detected in 81.2 and 78.4% of S. Typhi and S. Paratyphi A but not in S. Typhimurium isolated from human [51]. However, Murugkar et al. [50] detected stn gene in all Salmonella isolates from five different serovars and four different sources.

From this study, it could be concluded that Salmonella spp were highly prevalent in the examined agricultural drains and in leafy green producing farms, cattle and farm workers along their courses. Serotyping of recovered Salmonella, however, clarified predominance of S. Typhimurium and S. Enteritidis in examined sources, but other four serovars were also encounter reflecting wide variance in pollution sources from municipal, industrial and agricultural effluents. Virulotyping of recovered Salmonella serovars verified widespread distribution of virulence associated genes among isolates and provided additional evidence on risk of virulent salmonellosis posed from wastewater agricultural drains and the use of their water in irrigation of leafy greens for human and animal use. In addition, the variety in number and distribution of different virulence markers among screened Salmonella serovars suggests that within those serovars there are different pathotypes potentially responsible for different clinical syndromes in the host.

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References


