

TRANSMISSION OF HUMAN PATHOGENIC BACTERIA TO VEGETABLES THROUGH IRRIGATION WATER

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Abstract: In the last 50 years, there have been several outbreaks of epidemics caused by pathogenic bacteria that have been transmitted by consuming contaminated fresh vegetables. The greatest consequences were caused by an epidemic caused by *Escherichia coli* O157:H7 from pumpkin, which affected almost 4,000 people in Germany in 2011, over 50 of whom died. Massive poisonings in United States have also been caused by bacteria that have been transmitted through spinach and cantaloupes resulting with 147 people infected and 33 deaths. *Listeria* outbreak in Australia in 2018 transferred with rockmelont resulted in five deaths. A large number of poisonings have been recorded around the world, which fortunately did not end fatally. In all cases, the diseases were caused by pathogenic bacteria that cannot normally be found at vegetables. It is obvious that that certain types of bacteria that cause diseases in humans can survive for a certain time in environments that do not represent their natural environment, such as different species of vegetables. Since they have to somehow reach agricultural crops, irrigation with untreated water is singled out as one of the possible ways of their transmission. Namely, in the Republic of Srpska (entity of Bosnia and Herzegovina) there are only three facilities for the purification of municipal wastewater: in Bijeljina in the northeast, and in Trebinje and Bileća in the south of the country. In all other cities, municipal waste water is poured directly into watercourses. In rural areas, sewage is poured into septic tanks, through which it is further drained and partially purified reaches the groundwater. In a large number of cases, water from watercourses or wells is used directly for irrigation of vegetable crops. The aim of the conducted research was to determine eventually presence of potentially pathogenic microorganisms *Escherichia coli* and *Salmonella spp.* in the water used for irrigation, as well as their possible presence on certain vegetable crops that were irrigated with that water. Sample collection was carried out at 20 small vegetable producers, 10 of which use irrigation water from local wells, and 10 from nearby watercourses. For isolation of bacterial strains from irrigation water and vegetables extract indirect culture methods on specific selective media were used. The collected plant material was previously macerated and submerged in sterile peptone water in order to extract the bacteria. After that, they were inoculated on highly specific nutrient media and the obtained isolates were compared with positive controls. In well water used for irrigation, *Salmonella spp.* was isolated on two farms, while *Escherichia coli* was isolated from 4 wells. In watercourses used for irrigation, *Escherichia* was isolated in all cases, while *Salmonella* was not isolated in any of them. Bacterium *Escheria coli* was isolated from spinach and lettuce leaves, tomato, potato tuber and red onion bulbs while *Salmonella spp.* was isolated from lettuce, tomato, red onion and bell peppers as well. The fact that potentially pathogenic bacteria have been isolated in several different locations on different types of vegetables, including vegetables that are used fresh, is extremely worrying. In order to prevent new outbreaks, it is necessary to determine their origin, respectively the ways of their transmission to agricultural crops, as well as how long they stay viable on them. The conducted research showed that irrigation with untreated water can be one of the ways of transmission of *Escherichia* to vegetables, while the way of transmission of *Salmonella* has yet to be determined.

Keywords: irrigation water, vegetables, *Escherichia coli*, *Salmonella enterica*

1. INTRODUCTION

Lately there have been several outbreaks around the world caused by human pathogenic bacteria introduced by consumption of contaminated fresh vegetables. In Germany in 2011 an epidemic of *Escherichia coli* transmitted through pumpkin caused hemolytic uraemic syndrome which affected almost 4,000 people, of whom more than 50 died. Several poisonings have also been caused by bacteria introduces with spinach leaves and cantaloupes in the United States, when 147 people were infected, of whom 33 died. *Listeria* outbreak in Australia in 2018 transferred with rockmelont resulted in five deaths. In the USA in 2020 more than 1100 people were infected by *Salmonella*

transmitted with onion, but fortunately they did not end fatally (CDC, 2020). Every year, a significant number of cases of infection with bacteria transmitted through the consumption of fresh vegetables are recorded around the world. These bacteria are human pathogens and vegetables do not represent their natural habitat. The question arises how these bacteria get to the vegetables since they do not represent their ecological niche. One of the possible ways of their transmission is through irrigation with untreated water. There is a growing interest in investigating routes of contamination of vegetables by waterborne pathogens. Several pathogens of distinct significance are: *Escherichia coli* O157: H7, *Campylobacter spp.*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella spp.*, *Shigella spp.*, *Listeria monocytogenes* and *Yersinia enterocolitica*. However, there is still a lack of knowledge on many issues related to their transmission and viability (Pachepsky *et al.*, 2011).

In the Republic of Srpska, there is no special legislation that refers to the quality of irrigation water. The Regulation on water classification and categorization of watercourses stipulates that water used in agriculture must be at least of the third quality class. Such water can contain up to 200 N/ml of *Escherichia coli* and up to 200 N/ml of fecal streptococci, while monitoring the presence of other potentially pathogenic microorganisms in surface waters is not legally prescribed at all (Official Gazette of the Republic of Srpska 42/2001). On the other hand, in order to avoid the high costs of water, many farmers use directly water from watercourses or wells for irrigation of agricultural crops. Unfortunately, in the Republic of Srpska there are only three wastewater treatment plants: in Bijeljina in the northeast, and in Trebinje and Bileća in the south of the country. In all other cities, municipal waste water is poured directly into watercourses. In rural areas, sewage is poured into septic tanks, through which it is further drained and partially purified reaches the groundwater. The largest city in the region with more than 200,000 inhabitants, Banja Luka, does not have a wastewater treatment plant at all. All sewage water is poured directly into the river Vrbas (Vlada Republike Srpske, 2015), which flows downstream through the field Lijevo polje, where agricultural production is highly developed.

The aim of the proven research was to determine eventually presence of potentially pathogenic microorganisms *Escherichia coli* and *Salmonella enterica* in untreated water from watercourses and wells which is used for irrigation, and possibly to link their presence in irrigation water with the presence of the same strains on fresh vegetables.

2. MATERIAL AND METHODS

Escherichia coli is a rod-shaped Gram-negative, nonsporulating, facultative anaerobic bacterium which belongs to the family Enterobacteriaceae. Cells are approximately 2.0 µm long and 0.25–1.0 µm wide with peritrichous flagella's arrangement (Madigan *et al.*, 2006). They usually live in the lower intestine of warm-blooded organisms and reach into environment together with fecal matter. Most strains are part of the normal gut microbiota (Eckburg *et al.*, 2005) but several can cause serious diseases in humans such as urinary tract infections, meningitis, hemorrhagic colitis, hemolytic uremic syndrome (HUS) etc.

Salmonella spp. are rod-shaped Gram-negative bacteria from the family Enterobacteriaceae. According to present nomenclature, genus *Salmonella* includes over 2600 different serovarieties. They are asporogenic, predominantly motile, facultatively anaerobic rods, 2-5 µm long and 0.7-1.5 µm wide. *Salmonella spp.* species are intracellular pathogens which usually invade the gastrointestinal tract, of which certain nontyphoidal serotypes cause illness and cause salmonellosis (Murray, 2018). The most common routes of nontyphoidal transmission are from animal to human, from human to human and through food of animal origin such as meat and eggs (Jantsch *et al.*, 2011).

Parallel sampling of vegetables and the corresponding water with which they are irrigated was carried out during the summer 2021 at 20 small vegetable producers, 10 of which use irrigation water from local wells, and 10 from nearby watercourses. Lettuce and spinach leaves, tomatoes and bell peppers fruits, potato tuber as well as red onion bulbs were collected. In the laboratory sampled vegetables were homogenized on a shaker for 3 minutes after which isolation was performed.

Isolation of *Escherichia coli* from irrigation water was performed in MacConkey Broth (MPN method). Inoculated tubes were incubated at 44 °C for 24 hours. From blurred test tubes or tubes with gas production, inoculum was transferred onto petri dishes with Eosin Methylene Blue Agar (EMB Agar) (Biomerieux, France). The seeded petri dishes were incubated for 24 hours at a temperature of 37 °C. Green-metallic colonies with a diameter of 2-3 mm, which reflect light and have a dark purple center that transmits light, were evaluated as *E. coli*.

Isolation of *Salmonella spp.* from irrigation water was performed by enrichment in Selenite Broth (Biomerieux, France). Tubes with 10 ml of Selenite Broth were seeded with 1ml of inoculum and incubated at 35 °C for 24 hours. From blurred test tubes, inoculum was transferred onto petri dishes with Salmonella-Shigella Agar (SS Agar) (Biomerieux, France). The seeded petri dishes were incubated for 24 hours at a temperature of 35 °C. Colorless colonies with a black center are typical for *Salmonella spp.*

For isolation of *Escherichia coli* from vegetables 10 ml of homogenized samples were enriched with 90 ml of peptone water (Biomerieux, France). Enriched samples were inoculated on highly selective Eosin Methylene Blue Agar (Biomerieux, France). Incubation and detection of *E. coli* is carried out in the same way as in samples of irrigation water. As positive control *Escherichia coli* ATCC 25922 was used.

For isolation of *Salmonella spp.* from vegetables 10 ml of homogenized samples were enriched with 90 ml of medium Selenite Broth (HiMedia, India). After incubation at 35 °C for 24 h, 0,1 ml of inoculum from blurred positive tubes were seeded on plates with ChromID™ *Salmonella* Agar SX2 (Biomerieux, France). As positive control *Salmonella enterica* ATCC 14028 was used. The seeded plates were incubated at 35 °C for 24 h and presence of pale pink or purple colonies was detected.

3. RESULTS

The results of the conducted research are presented in Table 1. In watercourses used for irrigation, high number of *Escherichia coli* was isolated in all 10 samples, while *Salmonella* was not isolated in any of them. In well water, *Escherichia coli* was isolated in 4 samples, while *Salmonella* was isolated in two of them.

Table 1. Presence of *Escherichia coli* and *Salmonella spp.* at irrigation water and vegetables

type	sample	bacteria in water (N/100 ml)		lettuce leaves		spinach leaves		tomato		bell pepper		potato		onion	
		E.c.	Sal.	E.c.	Sal.	E.c.	Sal.	E.c.	Sal.	E.c.	Sal.	E.c.	Sal.	E.c.	Sal.
watercourse	Povelic01R	700	<1	+	+	+	-	+	+	-	-	+	-	-	-
	Povelic02R	250	<1	+	-	-	-	+	-	-	+	+	-	-	-
	Bajinci01R	150	<1	-	-	-	-	+	-	-	-	+	-	+	-
	Brezovljani01R	100	<1	+	-	-	-	-	-	-	-	-	-	-	+
	Brezovljani01R	220	<1	-	-	-	-	-	+	-	+	-	-	-	+
	Brezovljani01R	180	<1	-	+	-	-	-	-	-	-	-	-	-	-
	Sitneši01R	310	<1	+	+	+	-	-	+	-	-	+	-	-	-
	Sitneši02R	45	<1	-	-	-	-	-	-	-	-	-	-	-	-
	Inadjol01R	650	<1	+	+	-	-	+	+	-	-	+	-	-	+
	Resavac01R	85	<1	+	-	-	-	-	-	-	-	+	-	-	+
well water	Povelic01B	85	+	+	-	-	-	+	+	-	-	+	-	-	-
	Povelic02B	<1	<1	-	-	-	-	-	+	-	+	-	-	-	-
	Povelic03B	35	<1	+	+	+	-	-	-	-	-	+	-	-	+
	Bajinci01B	28	+	+	+	+	-	-	+	-	-	-	-	-	-
	Bajinci02B	<1	<1	-	-	-	-	-	-	-	-	+	-	-	-
	Crnaja01B	<1	<1	-	-	-	-	-	-	-	-	-	-	-	-
	Crnaja02B	<1	<1	-	+	-	-	-	-	-	-	-	-	-	+
	Brezovljani01B	110	<1	+	-	+	-	+	-	-	-	-	-	-	-
	Sitneši01B	<1	<1	-	-	-	-	-	-	-	-	-	-	-	-
	Sitneši02B	<1	<1	-	+	-	-	-	+	-	-	-	-	-	-

*E.c. – *Escherichia coli*; Sal.-*Salmonella spp.*

At different localities *Escheria coli* was found on spinach and lettuce leaves, tomato, potato tuber and red onion bulbs while *Salmonella spp.* was isolated from lettuce, tomato, red onion and bell peppers as well.

4. DISCUSSIONS

Numerous studies have shown that in developing countries, the water used for irrigation contains a significantly higher number of pathogenic microorganisms than in developed countries because they often use untreated raw surface water (Thurston-Enriquez *et al.*, 2002). A significant number of potentially pathogenic bacteria can also be found in groundwater. It is also shown that water from unprotected wells contains much more bacteria than groundwater from protected wells (Shortt *et al.*, 2003). Kljujić tested the quality of water from watercourses, opened

and closed wells and from piezometers which is used for irrigation in Serbia (2012). Due to the lack of adequate legislation he compared the obtained results with German standard DIN 19650 and U.S. Environmental Protection Agency Standard. According to the number of *E. coli* in water for irrigating vegetables that are consumed fresh, only one of 30 samples met the prescribed standard. He found both *Escherichia* and *Salmonella*, in most of the tested samples. He also found *Escherichia* and *Salmonella* at potato tuber, tomato, bell pepper, cabbage, carrot, onion and parsley. Research conducted during 2020 proved the presence of human pathogenic bacteria on vegetables grown in the northeastern part of the Republic of Srpska (Lolić *et al.*, 2020). They found *Salmonella enterica* on different types of vegetables: tomato, bell peppers, lettuce and red onion bulbs, while *Listeria monocytogenes* was confirmed on lettuce and spinach leaves. However, there is very little research showing that bacteria isolated on vegetables actually originate from irrigation water. In 2008, Greene *et al.* showed that the *Salmonella* outbreak in Newport (USA) was caused by the consumption of contaminated fresh tomatoes. Söderström *et al.* (2008) pointed that the epidemic in Sweden with specific strain of *E. coli* which produces verotoxin was caused by consumption of fresh lettuce which was irrigated by untreated water from a near small watercourse. These are rare studies in which it was proven that the bacteria got to tomatoes from underground water that was used for irrigation. In our research, the presence of *Escherichia coli* was proven in all watercourses used for irrigation, as well as in 4 out of 10 tested well waters. The significant number of this bacteria in water can certainly lead to contamination of vegetables that are watered with it. *E. coli* can survive in surfacewater for over 30 days (Maule, 2000). On the other hand, *Salmonella* was isolated from two well waters, while its presence in watercourses was not proven. Since *Salmonella* was isolated in many cases on different vegetables in gardens that were irrigated with water from streams, its presence on vegetables in most cases cannot be linked to the water used for irrigation. A possible way of transferring it to vegetables is via manure applications.

5. CONCLUSIONS

Conducted research proved the presence of *Escherichia coli* and *Salmonella spp.* both in untreated water used for irrigation and on different fresh vegetables. These vegetables are usually grown for personal use or for selling in local markets without any additional processing which increases the risk of human contamination. It has been shown that irrigation with untreated water can be one of the ways of transmission of *Escherichia* to vegetables. The absence of *Salmonella* in irrigation water in some localities where it was isolated on vegetables shows that other possible ways of its transmission should be investigated. In order to accurately determine the origin of potentially pathogenic bacteria on vegetables in the next phase of research, it is necessary to use molecular analyzes to show whether they are identical strains. In any case, the fact that potentially pathogenic bacteria have been isolated in several different locations on different types of vegetables, including vegetables that are used fresh, is extremely worrying. In order to prevent new outbreaks, it is necessary to determine their origin, respectively the ways of their transmission to agricultural crops, as well as how long they stay viable on them. The conducted research showed that irrigation with untreated water can be one of the ways of transmission of *Escherichia* to vegetables, while the way of transmission of *Salmonella* has yet to be determined.

ACKNOWLEDGEMENTS

The research was carried out as part of the project “Transmission of human pathogenic microorganisms through vegetables - their viability and infectivity” financed by Ministry of Scientific and Technological Development, Higher Education and Information Society (Republic of Srpska, BiH).

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