FREQUENCY OF BACTERIA *SALMONELLA ENTERICA* AND *LISTERIA MONOCYTOGENES* IN VEGETABLE IN THE REPUBLIC OF SRPSKA (BiH)

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Abstract: Recently, epidemics that have occurred as a result of the transmission of pathogenic microorganisms by consuming contaminated fresh vegetables have become more frequent. Among the most massive were poisonings by bacteria originating from spinach in the Unated States of America, from mung beans in Japan, as well as an epidemic caused by *Escherichia coli* from pumpkin in Germany. Unfortunately, hemolytic uraemic syndrome caused by *Escherichia coli* in Germany affected 3,950 people in 2011, of whom 53 died. Although vegetables do not represent a natural habitat for bacteria that cause diseases in humans, it is clear that certain groups of human pathogenic microorganisms can find their ecological niches in plant production systems. Ways of contaminating plants with these bacteria are poorly understood. It is assumed that fertilization and irrigation with untreated water are some of the possible ways of transmitting pathogenic bacteria to agricultural crops. The aim of the research was to indicate the possible presence of potentially pathogenic strains of bacteria on certain vegetable crops (lettuce, spinach, tomato, paprika, carrot, parsley, red onion) in the northeastern part of Republika Srpska (Bosnia and Herzegovina). Samples were collected over three months in 10 greenhouses and 10 vegetable gardens in the Semberija area. Extraction of bacteria from the samples was performed by immersing the macerated plant tissue in the extraction solution with sterile pepton water. Indirect culture methods on highly selective chromogenic media were used to isolate *Salmonella enterica* and *Listeria monocytogenes* strains. After comparing the obtained isolates with positive controls, suspicious colonies were isolated and the strains were confirmed by DNA extraction and AmpliTest PCR method with specific primers. The presence of *Salmonella enterica* was confirmed on tomato fruits, bell peppers, lettuce leaves and red onion bulbs, while *Listeria monocytogenes* was isolated on lettuce and spinach leaves. The presence of potential pathogens on fresh vegetables, especially those consumed without prior heat treatment, is alarming. In order to prevent negative consequences, it is necessary to specify the routes of their transmission as well as to examine their viability in non-specific ecological niches such as agricultural crops. Data on the viability of bacteria on agricultural crops can be found in the literature for only a small number of strains that are "most attractive", such as *Escherichia coli* strains, while for other pathogens data are very scarce or non-existent. Data on their infectivity is even harder to find.
Keywords: vegetables, *Salmonella enterica*, *Listeria monocytogenes*

1. INTRODUCTION

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. Hemolytic uraemic syndrome caused by *Escherichia coli* from pumpkin in Germany affected 3,950 people in 2011, of whom 53 died (Wiwanitkit, 2011). Massive poisonings have also been caused by bacteria originating from spinach in the United States, as well as from cantaloupes resulting in 147 people infected with 33 deaths. The last outbreak of *Salmonella* was linked to onion in USA (CDC, 2020). *Listeria* outbreak in Australia in 2018 transferred with rockmelont resulted in five deaths (Australian Institute of Food Safety, 2018). Although vegetables are not a natural habitat for disease-causing bacteria in humans, it is obvious that certain groups of human pathogenic microorganisms can find their ecological niches in plant production systems (Doyle, 1990). The question is how human pathogens reach vegetable crops in general. It is assumed that fertilization and irrigation with untreated water are some of the possible transmission routes (Pachepsky et al., 2011). Unfortunately, there are few municipal wastewater treatment systems in Bosnia and

Herzegovina (Vlada Republike Srpske, 2015). Sewage from all cities flows directly into rivers. Very often the arable land is irrigated with pumps directly with water from nearby watercourses, which also often flood the surrounding land. The northern part of Bosnia and Herzegovina is particularly prone to flooding: two major floods occurred in 2014 and 2019 which significantly increases the possibility of the presence of pathogens in this area. Precisely in this flooded area there are fertile arable lands on which 80% of the total cereal production and 60% of the total country production of vegetable crops are cultivated. On this way, during the flood , communal wastewater came directly to the arable lands.

Pathogenic bacteria cannot survive for long in an environment other than their natural habitat. In addition to their viability, the question arises as to how long they retain their infectivity and pathogenic properties. Data on the viability of bacteria on agricultural crops can be found in the literature for only a small number of strains that are "most attractive", such as *Escherichia coli* and *Salmonella spp*. (Ackers et al., 1998; Fenlon et al., 2000; Kljujev , 2012; Solomon et al., 2002) while data on their infectivity are even more difficult to find.

The aim of the proven research was to show the possible presence of potentially pathogenic strains of bacteria *Salmonella enterica* and *Listeria monocytogenes* on certain vegetable crops in the northeast area of the Republic of Srpska (Bosnia and Herzegovina). The research was initiated within the project "Transmission of human pathogenic microorganisms through vegetable crops", funded by the Ministry of Scientific - Technological Development, Higher Education and Information Society of the Republic of Srpska (Bosnia and Herzegovina).

2. MATERIAL AND METHODS

Salmonella spp. belong to the family Enterobacteriaceae and according to today's nomenclature include over 2600 different serovariettes. They are asporogenic, facultatively anaerobic, Gram-negative rods, 2-5 μm long and 0.7-1.5 μm wide which usually invade the gastrointestinal tract and cause salmonellosis (Murray, 2018). The most common routes of transmission are from animal to human, from human to human and through food of animal origin such as meat and eggs.

Listeria monocytogenes are asporogenic, facultatively anaerobic, motile Gram-positive rod bacilli, 2-3 μm long and 0.5 μm wide. They are one of the most virulent food borne pathogens which causes the infection listeriosis (Pizarro-Cerda, 2019).

Standard Test Method for *Salmonella spp*. in food includes nonselective pre-enrichment, followed by selective enrichment and selective isolation on solid substrates, followed by biochemical and serological confirmation of suspected colonies (ISO, 2017). A number of alternative methods, including real-time PCR protocols, have been developed and validated to detect *Salmonella spp*. (Dmitrić, 2018; Lee et al., 2015; Maurischat at al., 2015).

Vegetable samples were collected during three months (July, August and September) in 2020 in 10 greenhouses and 10 vegetable gardens in the northeastern part of the Republic of Srpska. Lettuce (60 samples) and spinach leaves (39 samples), tomatoes (60 samples) and bell peppers fruits (60 samples), carrot (60 samples) and parsley root (51 samples), as well as red onion bulbs (60 samples) were collected. From each plot, ten plants were sampled for each vegetable species, which are distant at least 2 meters from each other so as to obtain a representative sample. Parts of the sampled vegetables were placed in sterile bags and stored in transport refrigirator at 4 °C. In the laboratory samples were immersed in sterile buffered peptone water (Biomerieux, France) and homogenized on a shaker for 3 minutes. Indirect culture methods on highly selective chromogenic media were used to isolate *Salmonella enterica* and *Listeria monocytogenes* strains.

For detection of the presence and isolation of *Salmonella enterica*, 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 contol *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.

For detection of the presence and isolation of *Listeria monocytogenes* 10 ml of homogenized samples were enriched with 90 ml of medium Fraser broth. After incubation at 30 °C for 24 h, 0,1 ml of inoculum from blurred positive tubes were seeded on plates with CHROMagarTM Listeria (CHROMagar, France). The seeded plates were incubated at 37 °C for 24 h and presence of blue colonies with halo was detected. *L. monocytogenes* ATCC 13932 was used as positive control.

After comparing the obtained isolates with positive controls, suspicious colonies were isolated and the strains were confirmed by DNA extraction and AmpliTest PCR method with specific primers. For DNA exreaction a slightly turbid bacterial suspension was made (0.5 MF unit), and boiled for 5 minutes at 95 °C in a water bath. Strictly specific oligonucleotide probes used in these studies were: Salm-63-Cy3 for *Salmonella enterica* and Lis-1255-Cy3 for *Listeria monocytogenes*. The cyclical program consisted of primary denaturation 1 cycle at 94ºC –1 min and 45sec followed by 30cycles at 94ºC - 15 sec; annealing at 66ºC - 15 sec; elongation at 72ºC - 1 min; 1 cycle at 94ºC

- 15 sec, 66ºC - 15 sec and final elongation at 72ºC – 10min. The results of the PCR reaction were analyzed on an 1,2% agarose gel coloured by ethidium-bromide after horizontal electrophoresis.

3. RESULTS AND DISCUSION

After isolation of bacteria from collected samples on selective media at all 20 localities during three months and after confirmation of suspicious isolates using the PCR method, the presence of potential pathogens on vegetables was confirmed (Table 1).

The presence of *Salmonella enterica* was confirmed on tomato fruits, bell peppers, lettuce leaves and red onion bulbs, while *Listeria monocytogenes* was isolated from lettuce and spinach leaves. Bell pepper was positive on *Salmonella enterica* in 25%, tomato in 20%, red onion in 15% and lettuce in 13.3% of samples. Lettuce was positive on *Listeria monocytogenes* in 30%, and spinach was positive in 23% of samples. These two pathogens were not isolated from any sample of carrot and parsley root.

The incidence of pathogens was alarmingly high, but research by other authors has shown that the percentage of vegetables that are contaminated with pathogens varies depending on the type of pathogen and the country in which a study was done. In the USA, out of a total of 1,000 vegetable samples taken in supermarkets, *L. monocytogenes* was found in 11.4% of the analyzed samples (Heisick et al., 1989). *Salmonella spp*. was detected in 68% of a total of 120 lettuce samples from stores in Italy (Jerngklinchan & Saitanu,1993). In Croatia *S. enteritidis* was isolated from lettuce which was fertilized by manure. Enterobacteria were present in all analyzed samples (100% contamination), enterococci were detected in 48% of samples, while *E. coli* was isolated from 37% of samples of leafy vegetables, 25% of samples of fruit vegetables and 31% root vegetables (Pavić at al, 2005).

Table 1. Presence of Salmonella enterica and Listeria monocytogenes on vegetables in in the northeast area of the Republic of Srpska

bacteria	vegetable	*number of positive samples*	*number of negative samples*
Salmonella enterica	lettuce	8	52
	spinach	0	39
	tomato	12	48
	bell pepper	15	45
	carrot	0	60
	parsley	0	51
	red onion	9	51
Listeria monocytogenes	lettuce	18	42
	spinach	9	30
	tomato	0	60
	bell pepper	0	60
	carrot	0	60
	parsley	0	51
	red onion	0	60

Many authors point that irrigation with untreated water is possible transmission route for human pathogens to vegetables (Scott at al., 2004). Developing countries usually report much higher levels of pathogens in irrigation water than developed countries. In developing countries, untreated raw wastewater is often used for produce irrigation. Irrigation water from any surface source is likely to contain enteric pathogens at one time or another (Pachepsky et al., 2011). Islam et al. (2004) showed that the use of water for irrigation, contaminated with *S. tiphimurium*, results in contamination of carrots and that *Salmonella* can survive in the soil around 203 days. Poimenidou et al. (2016) showed that *Listeria monocytogenes* can survive and persist on lettuce leaf surfaces. Water for irrigation was the cause of epidemics caused by *E. coli* O157:H7 which is isolated from lettuce (Herwaldt, 2000). The northeastern part of the Republic of Srpska is rich in watercourses into which municipal wastewater is poured directly. There watercourses are used directly for irrigation, which can certainly be the cause of such a high frequency of human pathogens on vegetables.

4. CONCLUSION

Research conducted during 2020 proved the presence of human pathogenic bacteria on vegetables grown in the northeastern part of the Republic of Srpska. Because it is low-intensity production, these vegetables are usually grown for personal use or for selling in local markets without any additional processing. At this way consupmtion of fresh untreated vegetables increases the risk of outbreaks of human pathogens which can have catastrophic consequences. The most probably way of transmitting human pathogens to vegetables is through untreated irrigation water from a watercourses that directly receives communal wasatewater. The biggest attention should be paid when preparing lettuce, since it is consuming fresh, and it was infected with *Salmonella enterica* and *Listeria monocytogenes* in high percentage.

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