Assessment of Various Subtypes of *Salmonella serotypes* and *Salmonella enteritidis* as Important Human Pathogens According to Standard Microbiological Methods

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Abstract

The principal objective of this study was to evaluate and identify *Salmonella serotypes* and *Salmonella enteritidis* as important human pathogens according to standard microbiological methods. Fish samples were collected during March-October 2016-2017 applying the electrofishing method alongside Sitnica, Lepenci, and Lumbardhi I Prizrenit rivers. In addition, the bacteriological analyses involved bacteria genus *Salmonella serotypes* and *Salmonella enteritidis*, on the following fish species Squalius cephalus and Carassius gibelio. Analyses and serotyping *Salmonella serotypes* and *Salmonella enteritidis* species were conducted according to ISO 6579:2002 methods. The analyses of various serovars of Salmonella spp. from different sources indicates that *Salmonella serotypes* and *Salmonella enteritidis* in the gastrointestinal organ of the mentioned fishes was not present. As we know, the variability of subtypes of bacteriological aspects reflected for research, risk management, and public health.

Introduction

The purpose of this study consists of analyzing some of the microbiological parameters as an indicator of microbiological pollution, specifically the bacterias *Salmonella serotypes and Salmonella enteritidis*. Besides many other resources, Kosovo has the perspective potential for the fisheries sector. Lots of rivers and suitable conditions make fishery the promising sector in the field of economic development. However, potential rivers for fish farming in the country are the Drini I Bardhë, Lumëbardhi I Pejës, Lumëbardhi I Prizrenit, Lepenci, Lumi i Brodit, Lumi i Restelicës and other rivers with lower water capacity. *Salmonella spp., Shigella spp.,Coliform and Streptococci* and

enteroinvasive *Escherichia coli* (EIEC) are important human pathogens, responsible for the majority of cases of endemic bacillary dysentery prevalent in developing nations *Shigella spp., Salmonella, Coliform and Streptococcii* a genus of Gram-negative, facultative anaerobic, nonspore-forming, nonmotile, rod-shaped bacteria closely related to Salmonella.[1,2] The genus is named after Kiyoshi Shiga, who first discovered it in 1897 the causative agent of human shigellosis, *Shigella spp., Salmonella, Coliform and Streptococci* causes disease in primates, but not in other mammals.[3,4] It is only naturally found in humans and apes during infection, it typically causes dysentery.[3,10,11] *Shigella spp., Salmonella spp., Coliform and Streptococci* is one of the leading bacterial causes of diarrhea worldwide. Insufficient data exist, but conservative estimates suggest *Shigella spp., Salmonella spp., Coliform and Streptococcicauses* about 90 million cases of severe dysentery, with at least 100,000 of these resulting in death each year, mostly among children in the developing world.[5]

Shigella spp., Salmonella, Coliform and Streptococci species are classified by four

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serogroups: Serogroup A: S. dysenteriae (10 serotypes) Serogroup B: S. flexneri (six serotypes), Serogroup C: S. boydii (19 serotypes) Serogroup D: S. sonnei (one serotype).[15,5]

Groups A-C are physiologically similar; S. sonnei (group D) can be differentiated on the basis of biochemical metabolism assays.[2] Three *Shigella spp., Salmonella, Coliform and Streptococci* groups are the major disease-causing species: S. flexneri is the most frequently isolated species worldwide, and accounts for 60% of cases in the developing world; S. sonnei causes 77% of cases in the developed world, compared to only 15% of cases in the developing world; and S. dysenteriae is usually the cause of epidemics of dysentery, particularly in confined populations such as refugee camps.[8] Each of the *Shigella spp., Salmonella, Coliform and Streptococci* genomes includes a virulence plasmid that encodes conserved primary virulence determinants. The *Shigella spp., Salmonella, Coliform and Streptococci* chromosomes share most of their genes with those of E. coli K12 strain MG1655.[8] Phylogenetic studies indicate *Shigella spp., Salmonella, Coliform and Streptococci* is more appropriately treated as subgenus of Escherichia, and that certain strains generally considered E. Coli- such as E. coli 0157:H7 are better placed in *Shigella spp., Salmonella, Coliform and Streptococci.*

Shigella spp., Salmonella, Coliform and Streptococci infection is typically by ingestion (fecal–oral contamination); depending on age and condition of the host, fewer than 100 bacterial cells can be enough to cause an infection.[9] *Shigella spp., Salmonella, Coliform and Streptococci* causes dysentery that result in the destruction of the epithelial cells of the intestinal mucosa in the cecum and rectum. Some strains produce the enterotoxin shiga toxin, which is similar to the verotoxin of E. coli O157:H7 and other verotoxin-producing E. coli.[10] Both shiga toxin and verotoxin are associated with causing hemolytic uremic syndrome. As noted above, these supposed E. coli strains are at least in part actually more closely related to *Shigella spp., Salmonella, Coliform and Streptococci* than to the "typical" E. coli. *Shigella spp., Salmonella, Coliform and Streptococci* species invade the host through the M-cells interspersed in the gut epithelia of the small intestine, as they do not interact with the apical surface of epithelial cells, preferring the basolateral side.[10] *Shigella spp., Salmonella, Coliform and Streptococci* uses a type-III secretion system, which acts as a biological syringe to translocate toxic effector proteins to the target human cell.

The effector proteins can alter the metabolism of the target cell, for instance leading to the lysis of vacuolar membranes or reorganization of actin polymerization to facilitate intracellular motility of *Shigella spp., Salmonella, Coliform and Streptococci* bacteria inside the host cell. For instance, the IcsA effector protein triggers actin reorganization by N-WASP recruitment of Arp 2/3 complexes, helping cell-to-cell spread. After invasion, *Shigella spp., Salmonella, Coliform and Streptococci* cells multiply intracellularly and spread to neighboring epithelial cells, resulting in tissue destruction and characteristic pathology of shigellosis.[11,12] The most common symptoms are diarrhea, fever, nausea, vomiting, stomach cramps, and flatulence. It is also commonly known to cause large and painful bowel movements. The stool may contain blood, mucus, or pus. Hence, *Shigella spp., Salmonella, Coliform and Streptococci* cells may cause dysentery. In rare cases, young children may have seizures. Symptoms can take as long as a week to appear, but most often begin two to four days after ingestion. Symptoms usually last for several days but can last for weeks. *Shigella spp., Salmonella, Coliform and Streptococci* is implicated as one of the pathogenic causes of reactive arthritis worldwide.[10]

The diagnosis of shigellosis is made by isolating the organism from diarrheal fecal sample cultures. *Shigella spp., Salmonella, Coliform and Streptococci* species are negative for motility and are generally not lactose fermenters, but S. sonnei can ferment lactose.[13] Hand washing before handling food and thoroughly cooking all food before eating decreases the risk of getting shigellosis. Severe dysentery can be treated with ampicillin, TMP-SMX, or fluoroquinolones, such as ciprofloxacin, and of course rehydration. Medical treatment should only be used in severe cases or for certain populations with mild symptoms (elderly, immunocompromised, food service industry workers, child care workers). Antibiotics are

usually avoided in mild cases because some *Shigella spp., Salmonella, Coliform and Streptococci* species are resistant to antibiotics, and their use may make the bacteria even more resistant. Antidiarrheal agents may worsen the sickness, and should be avoided.[6] For *Shigella spp., Salmonella, Coliform* and *Streptococci*-associated diarrhea, antibiotics shorten the length of infection.[14]

Salmonella is a member of the Enterobacteriaceace, Gram negative, motile, with peritrichous flagella and nonsporeforming rods (the rods are typically 0.7-1.5 μm x 2.5 μmin size). *Salmonella spp.,* is facultatively anaerobic (can grow with or without oxygen) catalase positive and oxidase negative bacteria. However, *Salmonella spp.*, is not included in the group of organisms referred to as coliforms.[15] These mesophilic organisms are distrubuted geographically all over the world, but principally occurring in the gastrointestinal tracts of mammals, reptiles, birds, and insects and environments polluted with human or animal excreta.[16] The damage in liver due to infecting the animals with STm indicated as noticed in the histological sections is due to the toxins secreted by the microorganism which caused the observed damage. Moreover, there was noticed some damage to liver following the whole-body exposure of healthy animals to SMP which were repaired after 15 days post exposure. The damage thus occurred can be analyzed depending on the interaction mechanism of magnetic fields with biological systems.[17] The bacterium was first isolated from pigs suffering hog cholera by an American scientist, Dr. Daniel Elmer Salmon, in 1885.[17] Enteric bacteria (nonindigenous bacteria) that are present due to fecal contamination (*Salmonella spp., Shigella spp., pathogenic Escherichia coli, Staphylococcus aureus*). Human infections caused by organisms transmitted from fish or the aquatic environment are quite common depending on the season, the patients' contact with fish and related environment, dietary habits, and the immune system status of the exposed individual.[19] However, quantification of the occurrence of these diseases is difficult because many manifestations, typically gastrointestinal illness, go unreported since the symptoms usually do not last long and are self-limiting in healthy people. Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include mycobacteria, *Streptococcus and Salmonella spp.*[20] (Figure 1).
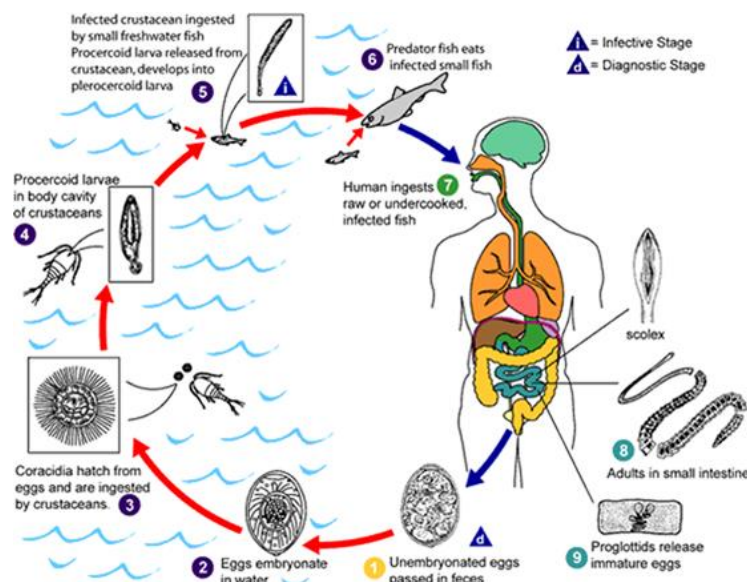

Figure 1. The life cycle of pathogenic enterobacteria in humans.

The United States Centers for Disease Control and Prevention reported that fish and shellfish account for 5% of the individual cases and 10% of all foodborne illness outbreaks, with most of the outbreaks resulting from the consumption of raw molluscan shellfish.[21] *Salmonella spp.,* and is responsible for more than 40.000 cases of food-borne illness every year. The incidence of Salmonella infections has risen dramatically since the 1980s, leading to

high medical costs, a loss of wages for workers who become ill, and a loss of productivity for the companies whose workers do become ill. In all, these financial losses can cost more than $3.6 billion each year. Salmonella infections have long been a concern to scientists, doctors, and the U.S. Food and Drug Administration (FDA).[2]

Material and methods

Subjects

The procedure of diagnosis of *Salmonella spp.,* in the fish intestinal tract. The samples were obtained from March-October 2016-2017. An average of 60 live fishes (Squalius cephalus and Carassius gibelio) were used from rivers located in three different Kosovo Country. Then samples were transported to analyze in the Kosovo Food and Veterinary Agency.

Experimental procedure

Analysis and identification were carried in the Laboratory of Kosovo Food and Veterinary Agency. For the analysis of Salmonella was used method according to ISO:6579:2002, with diagnostic material BPW, MSRV, BG, XLD, Brilliance™ Salmonella, O.B.I.S, test, Rapid™ One System, XLT, Merck, Salmonella antiserum Sifin Berlin, Salmonella antiserum Staten's Serum Institute-Denmark.[22, 23, 24] Weight out 25g

intestine with a sterile wood spatula, was put it into sterile flash and was added 225 ml buffered peptone water to obtain 1 part sample + 9 part buffer. Mix. Organs (intestines) 25gr, 225ml BPW (ratio 1:10) incubation at 37°C for 18-24 h, followed whith 100 microliter transferred into selective enriched agar semisolid MSRV 41.5C 24 + /-3h, streaking were made in plate solid agar BG, XLD, XLT4 and Brilliance ™ Salmonella for 24h at 37°C after has become the description of the colonies and then the transfer of colonies in TSI slant agar and incubation at 37°C for 24h.[25,26,27,28] During experimentation if typical colonies of bacteria *Salmonella spp.* or those suspected colonies would given a good isolated result through solid agar XD, we will continued with biochemical confirmation. In the case of our analyzed of fish samples research there are not found Salmonella spp, therefore it was not necessary to go further in the phase of biochemical and serological confirmation. In our research analyzed of fish samples research there were not found *Salmonella spp*, therefore it was not necessary to go further in the phase of biochemical and serological confirmation. Also, to have a wide range about this research we have analyzed physical-chemical water quality carried out according to the method ISO 5667-5: 2000 water samples are taken at three different points of the mentioned rivers (Table 1-3).

Pathogen	Min.Aw (using salt)	Min.pH	Max.ph	Max.%water phase salt	Min.temp	Max.temp	Oxygen requirement
Salmonella spp.	0.94	3.7	9.5	5°C	5°C	47°C	fakultative anaerobe

Table 1. Conditions for *Salmonella spp.* Growth.

Food vehicle	nr. of outbreaks	nr. of Salmonella outbreaks	% of outbreaks associated with Salmonella
Fish and fishery products	130	3	2.3
Crustaceans, shellfish, molluscs, and products	75	2	2.7
All food vehicles	2025	590	29.1

Table 2. Fishery Product Associated Outbreaks in the European Union.

Organism	Clostridium botulinum; non-proteolytic types B, E, F	Quantitative levels
Clostridium botulinum; non-proteolytic types B, E, F	Temperate and Arctic aquatic environment; multiplication in aquatic carrion (type E)	Generally low (<0.1 spores/g fish) but up to 5.3 spores/g fish has been recorded
Pathogenic Vibrio spp. incl. V.cholerae V.parahaemolyticus V. vulnificus	Ubiquitous in warm (>15°C) seawater environment	Up to 102-103 cfu/g in shellfish; up to 104-108 cfu/g in intestines of shellfish-eating fish
Plesiomonas shigelloides	Warm aquatic environment Freshwater fish (animals)	
Aeromonas spp.1	Aquatic environment	Generally low, but up to 104 cfu/ml in seawater; 107 cfu/ml in sewage and 106 cfu/g in raw seafood

Table 3. Pathogenic Bacteria Indigenous to the Aquatic Environment and Naturally Present on Fish.

River	N1	Fishes	Level of Human Pathogenic Bacteria in Fish	C^2	Bacteria Salmonella spp. (gr or cm2)
Lumbardhi Prizren	20	S.cephalus C.gibelio		0	_ (gr or cm2)
Lepenci	20	S.cephalus C.gibelio	104-108 cfu/g	0	_ (gr or cm2)
Sitnica	20	S.cephalus C.gibelio		0	_ (gr or cm2)

n1Number of representative sample units; c2 Analyzed on samples of the fish are not resulted of Salmonella Spp.

Table 4. Result of evaluation and serotyping of Salmonella Spp (*Salmonella serotypes and Salmonella enteritidis*)

Parameters	Standard method	Permissible limits		March-October 2016-2017
		Unit	Value	Results
Zinc (Zn)	APHA 3111B	mg/l	3.0	0.037
Copper (Cu)	APHA 3111B	mg/l	2.0	≤0.020
Cadmium (Cd)	APHA 3111B	mg/l	0.05	**≤ 0.019**
Lead (Pb)	APHA 3111B	mg/l	0.5	**≤ 0.049**
Cobalt (co)	APHA 3111B	mg/l	/	≤ 0.030
Nickel (Ni)	APHA 3111B	mg/l	0.001	≤ 0.019
Potassium (Na)	APHA 3111B	mg/l	150	38.4
Kalium (K)	APHA 3111B	mg/l	12	1.60

Table 5. The results of heavy metal concentrations in the water samples (realized with APHA 3111B methods). Ambient temperature: 8-23-13 (°C) March-October 2016-2017.

Results and discussion

The principal objective of this study was to determine and identify Salmonella spp. analyzed from fish species in three different Kosovo rivers. Fish samples were collected during March-October 2016-2017 applying the electrofishing method alongside Sitnica, Lepenci, and Lumbardhi I Prizrenit rivers. In addition, the bacteriological analyses involved Bacteria genus *Salmonella serotype of S. Enteritidis and S Typhimurium,* on the following fish species: Squalius cephalus and Carassius gibelio.

Analysed from this study (as shown in table 3) from 60 samples were tested with ISO 6579:2002 standard. Our analyzes showing that *Salmonella serotypes* and *Salmonella enteritidis* was not found in the same fishes. Comparing with some EU countries it was shown that in Kosovo the prevalence of *Salmonella serotypes* and *Salmonella enteritidis* is not similar. The analyses of various serovars of Salmonella spp. from different sources indicates the distribution of *Salmonella enteritidis* fortunately in the gastrointestinal organs of the fish was not present. As we know, a variability of subtypes of

different *Salmonella spp.* reflected for research, risk management, and public health strategies (Table 4).

The lack of bacteria *Salmonella Spp.*, in the gastrointestinal tract fishes can justify the water purity of these rivers according to a microbiological aspect, even though there was the presence of sewage waste collector. Also, these results could have been impacted by the presence of heavy metals such as Cadmium (Cd) and Plumb (Pb). It is worth mentioning that the values were a bit higher than allowed standards according to the World Health Organization (WHO). However, they did not show a critical impact on the quality of water in the above-mentioned rivers. The water samples from Sitnica, Lepenci, and Lumbardhi I Prizrenti River, had a good physic-chemical results except the level of Cd and Pb which were higher than standard values, while for other metals (Cu, Mn, Ni, and Zn) were inside permissible standard, according to Food and Agriculture Organization (United Nations) FAO. It's know that heavy metals can be a significant risk for humans and animals.[6] High levels of pollutants in river water systems cause an increase in biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS), total suspended solids (TSS), toxic metals and fecal coliform and hence make such water unsuitable for drinking, irrigation, and aquatic life (Table 5).[2, 20, 4]

Conclussion

Assessment of various subtypes of *Salmonella spp.*, (*Salmonella serotypes and Salmonella enteritidis*) as important human pathogens on the intestinal tract of fish species Squalius cephalus and Carassius gibelio, were not isolated. The lack of *Salmonella Spp.*, on the intestinal tract samples of fishes could justify the quality of these fishes and purity of water according to a microbiological aspect, even though there was the presence of sewage waste collector. The lack of this enterobacteria could have been impacted by the presence of heavy metals such as Cadmium (Cd) and Plumb (Pb). The best model to prevent the possibility of fish infection by *Enterobacteriaceae* is regular and professional supervision of the wastewater, biosecurity measures as well as the improvement of the quality of the fish at the national level.

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