Contamination and Persistence of *Salmonella* Enteritidis in Stressed and Unstressed Common Carp (*Cyprinus carpio* L.)

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Abstract

*Salmonella* is an important foodborne pathogen, causing a lot of different clinical syndromes and contamination of the human food can be a significant public health concern. The aim of this study was to assess the ability of *Salmonella* Enteritidis to penetrate and persist into the tissues of live common carps and the influence of stress on this ability. Four groups of fish were tested: control group, group of fish injected intraperitoneally with *S.* Enteritidis, group of unstressed carps and group of oxygen-deprived carps both immersed in water containing *S.* Enteritidis. For assessment of the stress level blood cortisol and glucose concentrations were measured. Carps, subjected to reduced dissolved oxygen concentration for four days, showed higher cortisol (*P* ≤ 0.05) and glucose (*P* ≤ 0.001) levels (283.46 ± 173.49 ng mL⁻¹ and 6.74 ± 0.93 mmol L⁻¹, respectively) compared to the unstressed fish (74.81 ± 46.51 ng mL⁻¹ and 3.61 ± 0.90 mmol L⁻¹, respectively). Intraperitoneally injected carps carried *Salmonella* up to 16 days after the inoculation. Stressed and unstressed fish contaminated with *S.* Enteritidis by immersion showed presence of the pathogen on the skin and gills 24 hours after the infection, but was not isolated at 4, 8 and 11 days. No difference was found in *Salmonella* persistence between these two groups.

Keywords: Stress, Common Carp, *Salmonella* Enteritidis, Contamination, Food Safety

1. Introduction

Genus *Salmonella* includes a number of serotypes; many of which are major foodborne pathogens and contaminant of the human food can present a risk for the human health. *Salmonella* spp. causes a number of different clinical syndromes, which can be grouped as enteritis and systemic disease. Gastrointestinal infections are predominantly caused by serotypes, widespread in animals and humans. Systemic disease is generally associated with host-adapted serotypes (*S.* Typhi and *S.* Paratyphi in humans) but many other serotypes can cause different types of systemic infections (Adams & Moss, 2000).

In 2010, foodborne outbreaks caused by *Salmonella* spp. constituted 30.5% of the total reported in the EU. Fish products were the source in 0.3% of the outbreaks, whereas *Salmonella* spp. was the causative agent in 2.3% of the strong evidence outbreaks. The predominant serovars were *S.* Enteritidis (61.3%) and *S.* Typhimurium (13.8%) (EFSA & ECDC, 2012).

FAO report (2010) concluded that the outbreaks of salmonellosis were rarely connected with products of aquaculture but there were a lot of pathways how *Salmonella* could enter the aquaculture environment. Mansilha et al (2010) in Portugal reported that *Salmonella* spp. was isolated from 23.1% of the total sampled waters with a distribution of 15.6% of coastal and 56.6% of inland and transitional *Salmonella* positive samples. Shabararith et al (2007) studied the prevalence of *Salmonella* in seafood collected from the southwest coast of India. By using Polymerase Chain
Fish and Sampling

A batch of 67 carps (body weight 1.0-1.5 kg) was obtained from a commercial fish farm in Nikolaevo County, Bulgaria. Immediately after arrival four fish were tested for presence of *Salmonella* spp. Five samples from each fish were taken, as follows: 1) Blood (1 mL) - by puncturing the heart; 2) Swabs from skin (50 cm²); 3) Swabs from gills; 4) Internal organs (total sample of 1 g from kidney, spleen and hepatopancreas); 5) Dorsal muscle (25 g). All samples, except the muscle, were placed in tubes containing 9 mL buffered peptone water (Biolife, Milan, Italy). The muscle samples were cut into small pieces, put in Stomacher bags with 225 mL buffered peptone water and homogenized for 1 min at 256 rpm².

The remaining 63 carps were divided into four groups - Control Group (n = 12), Group 1 (intraperitoneally infected fish; n = 19), Group 2 (unstressed fish; n = 16), Group 3 (oxygen-deprived fish; n = 16) and placed in four tanks with 800 Litre tap water (water temperature 16.7 ± 0.3 °C, pH 7.78 ± 0.04) and constant aeration.

2.2. Preparation of Bacterial Culture

Type strain *Salmonella* Enteritidis ATCC 13076 (Micro-BioLogics®, MN 56303, USA) was used as an experimental strain. Overnight bacterial culture was obtained on Tryptic Soy Agar (Biolife, Milan, Italy) after incubation at 37 °C for 24 h. Bacterial cell suspension was prepared by dissolving single *Salmonella* colonies in 0.9% saline solution (B. Braun Melsungen AG, Germany) to density equal to 0.5 McFarland (ca. 1.5x10⁹ cfu mL⁻¹). Concentration of bacterial cells was estimated by DEN-1 McFarland Densitometer (BIOSAN, Latvia). This concentration was used for intraperitoneal infection.

Overnight bacterial culture of *S. Enteritidis* in Tryptic Soy Broth (Merck, Darmstadt, Germany), containing about 10⁹ cfu mL⁻¹, was used for contamination of the water in the tanks of Group 2 and Group 3.

2.3. Stress Parameters and Experimental Infection

To analyze the influence of stress on the ability of *Salmonella* to pass through the body protective barriers, stress reaction was provoked in Group 3 only, by keeping the fish at low oxygen concentration. The dissolved oxygen level in the tank of Group 3 was lowered to 2.41 ± 0.33 mg L⁻¹ (for 8 hours per day) by using a system for intravenous infusion and in the other tanks this level was 3.65 ± 0.19 mg L⁻¹. Fish were kept for four days at these conditions. Blood glucose and cortisol levels were measured as stress parameters. Blood samples (2 ml) were taken from six unstressed and six stressed fish from Groups 2 and 3 by puncturing the caudal vessel.

The experimental infection with the prepared bacterial suspensions was then carried out. Control group was not contaminated with *S. Enteritidis*. Group 1 was infected by intraperitoneal injection with 1 mL bacterial suspension (0.5 MacFarland). Carps from Groups 2 and 3 were infected by pouring out 20 mL liquid bacterial culture (10⁹ cfu mL⁻¹) direct into each tank. Thus, the bacterial count into the tanks became 10⁹ cfu mL⁻¹. The fish were kept in the contaminated water until the end of the study.

2.2. Preparation of Bacterial Culture

According to Barton (2002); stress in animals can disturb the immunity and normal function of body protective barriers and thus enables the invasion of different microorganisms. When fish is exposed to stressors, the physiological reaction occurs in three steps. The first step (primary stress response) includes changes in circulating levels of cortisol and catecholamines. Secondary responses are mainly expressed by metabolic changes, such as changes in blood glucose, lactate, major ions and tissue levels of glycogen and heat-shock proteins (HSPs). Third step or tertiary responses are connected to alterations in growth, disease resistance and behaviour. Ndong et al (2007) found that subjection Mozambique tilapia (*Oreochromis mossambicus*) to temperature, stress reduces its immune capability and resistance against *Streptococcus iniae*. Chronic and acute stress can affect the normal intestinal barrier function. Disturbed integrity and increased paracellular permeability were observed in Atlantic salmon (*Salmo salar L.*) after prolonged exposure to low dissolved oxygen concentrations (Sundh et al, 2010). Similar findings are reported in Atlantic salmon (Olsen et al, 2002) and rainbow trout (Olsen et al, 2005) subjected to 15 min of acute stress.

2.3. Stress Parameters and Experimental Infection

The aim of this study was to assess the ability of *Salmonella Enteritidis* to penetrate and persist in the tissues of live common carps for sale and human consumption and the influence of stress on this ability.

2. Materials and Methods

2.1. Fish and Sampling

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days after the contamination. At each sampling time three fish from the Control Group and four fish from the other groups were tested. The remaining three fish from Group 1 were tested on 16 days because this group kept showing positive results even after 11 days. At 4 days water samples (1 mL) from each tank were taken and placed in tubes containing 9 mL buffered peptone water.

2.4. Analytical Methods

2.4.1. Biochemical Analysis

Blood for analyzing glucose levels was placed in tubes containing EDTA and centrifuged for 10 min at 20 °C and 8049 g for plasma separation. Blood glucose concentration was measured by automatic biochemical analyzer BS-120 (Mindray, China). For measuring cortisol levels blood samples were placed in tubes without anticoagulant and let coagulate at 20 °C for 30 minutes, until serum could be separated. Cortisol level in the blood serum was estimated by Cortisol ELISA kit EIA 1887® (DRG Instruments GmbH, Germany) using automatic ELISA reader SUNRISE® (Tecan, Austria).

Dissolved oxygen concentration and water temperature were measured using portable Multi meter 340i/SET (WTW, Germany) by immersing the probe direct in the water sample. Water pH was estimated by Sartorius Basic Meter PB-11 (Sartorius AG, Germany).

2.4.2. Microbiological Analysis

The cultural method for detection of Salmonella was performed according to ISO 6579:2005. For isolation Xylose Lysine Desoxycholate (XLD) Agar (Biolife, Milan, Italy) and Modified Semi-Solid Rappaport-Vassiliadis (MSRV) (Biolife, Milan, Italy) medium were used. Bacterial growth with typical cell and colonies was sub-cultured onto Tryptic Soy Agar and used for biochemical identification and serotyping.

Serotyping and biochemical tests were performed in the NRL “Salmonella, Campylobacter and Antimicrobial Resistance” in Bulgarian Food Safety Agency, according to Interlaboratory method No. 2, based on scheme of White-Kaufmann-Le Minor (for serotyping) (Grimont & Weill, 2007).

2.4.3. Statistical Analysis

StatMost 32™ for Windows XP, Dataxiom Software Inc. was employed to detect significant differences between the various means of data of blood cortisol and glucose levels. The data for quantity of blood cortisol and glucose were subjected to analysis of variance (ANOVA), where P < 0.05 was judged as indicative of a significant difference.

3. Results and Discussion

The results from our study showed that the four carps tested for the presence of Salmonella spp. immediately after arrival at our laboratory, as well as all fish from the control group, were not carriers of the pathogen. These findings confirm that the experimental carps were not contaminated with Salmonella spp. in the rearing ponds or during transportation and handling.

All intraperitoneally injected fish showed positive samples for Salmonella on the day after the experimental infection (Table 1).

The pathogen was isolated from the internal organs and blood of all tested carps, but only from two of the muscle samples. Eight days after the infection, Salmonella disappeared from the skin surface and was not isolated to the end of the study. 11th day after, the infection was the last sampling time when all fish showed presence of S. Enteritidis. The 11th day was the last sampling time after the infection when all fish showed presence of S. Enteritidis, as the pathogen was isolated from all of the tested internal organs, two of the muscle samples, one of the blood and one of the gill samples. At the last sampling time (16 days) only one positive sample (from internal organs) was found.

Table 1. Presence of Salmonella Enteritidis in the Intraperitoneally Infected Carps

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample</th>
<th>Positive Samples for Salmonella (n/n₀ %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Day</td>
<td>4 Days</td>
</tr>
<tr>
<td>Group 1 (n=19 carps)</td>
<td>Skin</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td></td>
<td>Gills</td>
<td>3/4 (75.0)</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td></td>
<td>Internal organs</td>
<td>4/4(100)</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>2/4 (50.0)</td>
</tr>
</tbody>
</table>

n/n₀: Salmonella positive samples/total samples

Both unstressed and stressed carps showed presence of Salmonella on the skin and gills 24 hours after the experi-
mental contamination. At 4 days the pathogen was isolated only from the internal organs of one fish from group 2. All other samples taken from both groups were negative for *Salmonella*. At 8 and 11 days of the experiment *S. Enteritidis* was not isolated from any of the tested samples from these groups (Table 2).

**Table 2. Presence of Salmonella Enteritidis in Unstressed and Stressed Carps, Infected by Immersion in Contaminated Water**

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample</th>
<th>Positive Samples for Salmonella (n/no %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 Day</td>
</tr>
<tr>
<td><strong>Unstressed</strong></td>
<td>Skin</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>(n = 16 carps)</td>
<td>Gills</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>0/4 (-)</td>
</tr>
<tr>
<td></td>
<td>Internal organs</td>
<td>0/4 (-)</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>0/4 (-)</td>
</tr>
<tr>
<td><strong>Stressed</strong></td>
<td>Skin</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>(n = 16 carps)</td>
<td>Gills</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>0/4 (-)</td>
</tr>
<tr>
<td></td>
<td>Internal organs</td>
<td>0/4 (-)</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>0/4 (-)</td>
</tr>
</tbody>
</table>

*n/no: Salmonella Positive Samples/Total Samples*

Autopsy of the infected fish did not show any pathological changes, caused by *Salmonella*, during the whole study.

All water samples taken from the tanks four days after the experimental infection were negative for presence of *Salmonella* spp.

Furthermore, we found significantly increased levels of blood glucose and cortisol in common carps subjected to reduced dissolved oxygen concentrations for 96 h. The stressed carps (Group 3) showed higher cortisol concentrations (283.46±173.49 ng mL⁻¹) than the unstressed fish from Group 2 (74.81±46.51 ng mL⁻¹) and this difference was statistically significant (P ≤ 0.05). Also, statistically significant differences (P ≤ 0.001) were found in the blood glucose levels between the two groups. The stressed fish had almost two times higher (6.74±0.93 mmol L⁻¹) levels compared to those of the unstressed carps (3.61±0.90 mmol L⁻¹).

*Salmonella* is a widespread pathogen and, it is often connected to faecal contamination of different aquatic habitats (Polo et al, 1998). *Salmonella* spp. was isolated from different samples, including water samples (Mansilha et al, 2010), freshwater fish (Budiati et al, 2011) and seafood (Kumar et al, 2009). These studies, however, do not show for how long *Salmonella* can persist in the water environment and if it is capable to invade the “sterile” tissues of fish. Our findings are in agreement with a previous study of Nesse et al (2005) on the persistence and dissemination of orally administered *Salmonella* in Atlantic salmon smolts. All fish fed with the lowest counts (10⁴ cfu g⁻¹) for one week were negative for the presence of *Salmonella*. In some of the samples (gastrointestinal tract and internal organs) taken from fish received 10⁶ cfu g⁻¹, the bacterium was found for at least 4 weeks. According to our results after intraperitoneal injection with *S. Enteritidis* can be isolated from the fish for at least 16 days after the infection. Doses up to 10⁸ cfu mL⁻¹ *S. Enteritidis* containing in the water lead to a short-term (24 h) contamination of the skin and gills.

The disappearance of *Salmonella* from the water and surface tissues is not very surprising, as there are previous studies confirming its short-term survival in the water environment. Chandran & Hatha (2005) reported fast reduction of *S. Typhimurium* in raw estuarine water. T₉₀ for this microorganism took less than 24 h. Baker et al (1983) observed fast decrease of the viable cells count (95 % during the first 6 h) after experimental contamination with *S. Typhimurium* of pools for culturing *Tilapia aurea*. The pathogen was isolated from water samples, internal organs and skin at 16 days, but not at 32 days after the infection. *Salmonella* was not found in muscle samples and there were no signs of septicemia. Furthermore, the short-term persistence of *S. Enteritidis* on the skin surface could be due to the mucus secretion, as there are studies demonstrating that the skin mucus of some fish species shows antimicrobial activity against different pathogens (Kuppulakshmi et al, 2008 and Balasubramanian et al, 2012).

To confirm higher stress level in the carps subjected to low dissolved oxygen, blood cortisol and glucose were measured. These two biochemical substances are widely used in

Available online at [www.scientific-journals.co.uk](http://www.scientific-journals.co.uk)
the scientific literature as stress indicators. EL-Khalidi (2010) reported a significant increase in cortisol levels after 24 and 72 h and significantly higher blood glucose concentrations after 72 and 144 h from the exposure of Nile tilapia to hypoxia. In our study we found significantly increased levels of blood glucose and cortisol in common carp subjected to reduced dissolved oxygen concentrations for 96 h. Our findings are similar to those reported by Dobšíková et al (2006) (201.6±36.38 ng mL⁻¹ blood cortisol and 9.4 ± 2.58 mmol L⁻¹ blood glucose) after long-distance transportation of common carp. Such high values confirm that our oxygen-deprived carps showed increased levels of stress despite the fact that this species is highly resistant to low dissolved oxygen concentrations.

There are studies showing the detrimental effect of stress on the immunity of different fish species. Acute short-term stress leads to stimulation of immune response, whereas chronic and prolonged stress has a suppressive effect (Tort, 2011). This pattern was confirmed by Fast et al (2008). Nevertheless, some experiments showed that even some short-acting stress factors can affect the normal immune function and thus may enable the growth and proliferation of different bacteria (Ndong et al, 2007 and Caipang et al, 2009).

On the other hand there is evidence that stress alters the intestinal barrier function in mammals (Meddings & Swain, 2000 and Cameron & Perdue, 2005). Similar changes are reported in fish subjected to acute or prolonged stress. Olsen et al (2002) observed increased intestinal permeability in midgut and decreased adherent bacteria count in midgut and hindgut of stressed Atlantic salmon. The latter, according to the authors, might be due to the sloughing off of mucus, which eliminated existing microflora, but could allow remaining bacteria to colonize the surface of the enterocytes. All these changes may lead to transcellular or paracellular translocation of different bacteria and let them enter into the venous or lymphatic system (Balzan et al, 2007). Contrary to these data our results did not show any effect of stress on the penetration of S. Enteritidis into the blood, internal organs and musculature of carps infected by immersion in water containing moderate bacterial concentrations (10⁹ cfu mL⁻¹).

4. Conclusion

It can be concluded that keeping live carps at low dissolved oxygen concentrations for four days causes stress reaction, as indicated by the high blood glucose and cortisol levels. Contamination of the water with low doses (10⁴ cfu mL⁻¹) of S. Enteritidis leads just to a short-term carriage (24 h) of the pathogen on the surface tissues of both stressed and unstressed fish. No significant effect of stress was found on the rate of microbial invasion. Longer persistence of S. Enteritidis was observed after intraperitoneal infecti-on with high bacterial count (~ 10⁶ cfu mL⁻¹). Our experiment showed that tap water was not an appropriate environment for long-term survival and persistence of S. Enteritidis in common carp, even when the fish were stressed considerably at the time of infection due to reduced oxygenation of the water.

Acknowledgements

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