Detecting Yersinia ruckeri on the death of carp (Cyprinus carpio) farmed in southern Russia

A Kazarnikova\textsuperscript{1,2}, A Trishina\textsuperscript{3}, M Galeotti\textsuperscript{4}, M Manzano\textsuperscript{4}, N Abrosimova\textsuperscript{1}, and A Ermakov\textsuperscript{1}

\textsuperscript{1}Don State Technical University, 344000, Rostov-on-Don, Russian Federation
\textsuperscript{2}Federal State Budgetary Institution of Science "Federal Research Centre The Southern Scientific Centre of The Russian Academy of Sciences", 344006, Rostov-on-Don, Russian Federation
\textsuperscript{3}Rostov-on-Don Antiplague Institute of Rospotrebnadzor, Rostov-on-Don, Russian Federation
\textsuperscript{4}University of Udine, 33100, Udine, Italy

E-mail: derezinasovet@mail.ru

Abstract. Yersinia ruckeri was first discharged from carp brood fishes (Cyprinus carpio) farmed in ponds in southern Russia. The death of fish was observed at a temperature of 15°C in March-April 2015. Ten fish with clinical signs of the disease were taken for research. Water samples were taken for hydrochemical analysis. The research results indicated a high level of permanganate index (2.4–2.8 MAC), nitrite nitrogen (1.5–2 MAC), nitrate nitrogen (1.4–1.5 MAC), and a low oxygen content in the bottom layers (2.0 mg/dm$^3$). A parasitological study of fish revealed an invasion by monogenes (Dactylogyrus extensus) and mollusks (Unio pinctorum). As a result of bacteriological studies, 62 isolates were discharged, 30 from water and 32 from fish. The determination of bacteria was carried out according to morphological and enzymatic properties. The biochemical characteristics of the Yersinia ruckeri strains made it possible to classify them as serotype 1. All Yersinia ruckeri strains were sensitive to ciprofloxacin, tetracycline, chloramphenicol and resistant to furazolidone. MALDI TOF mass spectrometry and PCR were used for additional identification of bacteria. Serious pathological and histopathological changes in the fish tissues were not detected.

1. Introduction

Yersinia ruckeri is a Gram-negative bacterium. The disease caused by Y. ruckeri is known as «enteric red mouth – ERM» and is yersineosis, accompanied by mouth redness. The incubation period of yersiniosis is 5-10 days at a temperature of 13-15°C. The disease proceeds in acute, subacute and chronic forms.

Factors affecting the occurrence and spread of the disease are stress (handling, etc.) and adverse growing conditions (low oxygen content, high concentration of organic substances, etc.). Most often, the disease was recorded in Salmoniformes, such as salmon, Salmo salar, mykiss, Oncorhynchus mykiss, sockeye salmon, O. nerka, pink salmon, O. gorbusha, coho salmon, O. kisutch and chinook salmon, O. tshawytscha, pale, Salvelinus fontinalis [1-4], channel catfish, Ictalurus punctatus, atlantic cod, Gadus morhua [5]. In addition to salmon fish, the Yersinia ruckeri bacterium, the causative agent of yersineosis, was also isolated from fat-mined minnow, Phoxinus phoxinus, Amur sturgeon,
Acipenser schrencki, Siberian sturgeon, A. baerii, perch, Micropterus salmoides, whitefish, Coremonusunota peled, C. muksun, burbot, Lota lota and other fish [6]. The disease was characterized by inflammation and erosion on the head, especially around the oral cavity, gill covers and at the base of the fins.

The aim of research was to analyze the causes that led to death of carp producers in fish ponds of the Rostov Region, as well as to discharge and describe the potential causative agent of yersineosis, the Yersinia ruckeri bacterium, discharged from carp for the first time in this region. In addition, sensitivity of discharged microorganisms to the antibiotics was investigated, and a molecular genetic analysis of the discharged isolates using PCR was carried out.

2. Materials and Methods
The death of carp brood fishes (P=0.4-3.7 kg, L=28-58 cm, l=20-49 cm) was recorded in wintering ponds (depth - 1.6, area - 1 ha) in March-April 2015 at a temperature of 15°C and at the time of the study reached 300 units (15%). The fish-holding density in the ponds was 8 t/ha. The year before, ponds contained rainbow trout imported from the Krasnodar Territory.

Water analyzes were carried out according to the chemical analysis manual for the land surface waters [7]. The results of the study were compared with the maximum allowable concentrations (MAC) for fishery reservoirs in order to determine, which of them could affect the death of fish.

Each fish was examined clinically, the attention to damage on the surface of the body was paid. Internal organs were examined for pathological changes and then selected for bacteriological studies. Scrapes from the gills and body surface, as well as internal organs and the gastrointestinal tract, were examined by compression under a microscope. In total, 10 fish with signs of the disease were examined ichthyopathologically. The collection, fixation and further processing of parasites was carried out according to methods generally accepted in parasitology [8, 9]. The species affiliation of the parasites was determined using the “Key to parasites of freshwater fish” [10].

For the manufacture of histological preparations, tissue pieces (liver, spleen, heart) from 5 fish were fixed in 4% neutral buffered formalin. After that, the samples were kept at room temperature and placed in an automatic histoprocessor (TISBE histoprocessor, Diaphath). Prepared units were poured into paraffin (ParaplastPlus, Diaphath). 5 μm thick sections were obtained using a microtome (Reichert-Jung 2050), then they were stained with hematoxylin – eosin according to Giemsa and Gram. The preparations were examined under a light microscope (Leica DMRB) and photographed using the Nikon system.

Water for bacteriological studies was taken from the reservoir surface and the total bacterial count (TBC), coli titer and coli index were determined [11,12].

Bacterial isolates were discharged from 10 infected fish. Parenchymal organs (liver, spleen, kidneys) were excised and emulsified in physiological saline followed by plating on the following solid nutrient media: Soyabean Casein Digest agar, blood agar, MacConkey agar, Aeromonas Isolation Medium Base, Potato Dextrose agar (HiMedia Laboratories P, Limited. India). Platings were incubated at 25°C for 36 h. Identification of the discharged bacterial cultures was carried out by studying the morphology of bacteria, their cultural, biochemical, and other traits relevant for each species [13]. Manual microorganism identification systems API20 NE, RapiD 20 E (bioMeieux, France) were used for biochemical studies.

Additional identification of microorganisms was performed using MALDI TOF mass spectrometry. For processing and analysis of mass spectra, the following software of BrukerDaltonics (Germany) was used: flexControl 2.4 (Build 38), mass spectra were processed using the FlexAnalysis 2.4 software package (Build 11).

The sensitivity of the isolated microorganisms to antimicrobial agents was determined by the disk diffusion test. Standard disks with various antibacterial substances manufactured by Hi Media Laboratories Pvt. Limited, India were used when studying antibiograms. The interpretation of the results was carried out in accordance with international standards NCCLS.
The Yersinia ruckeri strains were placed in physiological saline tubes and stored in a refrigerator at 4°C until the start of the study. Then, replating on a broth with a cardiac extract (Oxoid, Milan, Italy) was carried out and incubation at a temperature of 20°C for 48 h.

DNA was extracted from pure colonies grown on MBP using DNA genetic purification equipment (Promega, Milan, Italy) according to the existing protocol. Purification and concentration of DNA samples was monitored using a 2000 s spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA from Pseudomonas fluorescens (Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Milan, Italy, DISTAM), Escherichia coli (Dipartimento di Scienze AgroAlimentari, Ambientali e Animali, Udine, Italy), Vibrio ruber DSM 14hemulmund Dermon GmbH, Braunschweigh, Germany), Yersinia enterocolitica (DISTAM), Bacillus subtilis DSM 1092, Kokuria christinae DSM 232, Enterobacter cloacae DSM 30054 were used as a control in PCR analysis. DNA samples were standardized to a concentration of 250 ng/μl using sterile distilled water and then molecular biology studies were started.

Two specific Yersinia ruckeri primers YER8F (5’-GCG AGGAGGAAGGGTTAAGTG-3’) and YER10 R (5’-GAAGGCACCAAGGCATCTCTG-3’) were used for amplification of the 575 base pair [14]. Amplification was carried out in a total volume of 50 μl containing 1.5 mmol/liter (mM) MgCl₂, 1.25 units. Taq polymerase (Promega, Milan, Italy), 1 µl of each primer with a concentration of 10 mM, 1 µl of 10 mM dNTPs mix, 1 µl of DNA with a concentration of 250 ng/μl, distilled water was used as a negative test. The following parameters were used: pre-denaturation for 5 min at 95°C, then 35 one-minute cycles at 95°C, 1 min at 61°C, 1 min at 72°C, and the final stage at 72°C for 7 min.

PCR products were determined on a 1.5% agar gel using electrophoresis and staining with 0.5 μg/ml ethidium bromide and visualization with ultraviolet light. Amplicons were purified using the QIA quick® PCR Purification Kit (Qiagen, Milan, Italy), dried and sent for sequencing to Germany (Eurofins MWG GmbH, Martinsried, Germany), where the NCBI/BLAST program was used to identify them.

3. Results

The water temperature in the ponds was 15°C when the fish died. A number of following hydrochemical indicators exceeded the MAC: permanganate oxidizability (2.4–2.8 MAC), nitrite nitrogen (1.5–2 MAC), nitrate nitrogen (1.4–1.5 MAC). The pH_{sur}/pH_{bot} ratio was 1.02 and indicated the oxygen concentration in the bottom layers in the range of 0.0-2.0 mg/dm³ and hypoxia [7]. Exceeding the MAC of sulfates by 2 times rather characterizes the natural background and corresponds to the norm for this region.

Sick fish kept near the surface of the water. 50% were found to have anemic gills on clinical examination, covered with a white coating at the base. The gills were swollen, anemic, abundantly covered with mucus and algae. The white cotton-like coating was observed on the body surface of some specimens (30%), caused by parasitization of pore fungi Saprolegniales. Point hemorrhages were noted on the ventral side of the body.

Postmortem examination revealed an increase in kidney size, hemorrhage on the serous membrane of the abdominal cavity and swim bladder, and hyperemia of the posterior intestine.

A parasitological study of sick fishes (n=10) revealed their infection with 2 types of parasites – monogenes (Dactylogyrus extensus) and mollusks (Unio pictorum). Greater extensiveness and intensity of invasion was recorded for D. extensus (100%; 14–40 units) compared with U. pictorum (40%; 2–4 units).

As a result of bacteriological research, 62 isolates (30 from water and 32 from fish) belonging to the genus Yersinia were discharged. TBC of water in the examined stores was 3×10³ CFU/ml; coli index – 900; coli titer – 1.11, which allows them to be attributed to the 2nd category of pollution. Bacterial contamination of parenchymal organs was found in all examined fish. The qualitative composition of the microflora was represented by gram-negative oxidase-negative bacteria identified.
as Yersinia ruckeri. Indicators of microbial contamination of fish parenchymal organs were $2 \times 10^3$ CFU/g.

Bacteria on the MPA formed round, whitish, merging colonies, convex on the Endo, rounded with a smooth edge, with a diameter of 0.1-0.2 mm. Gram-negative sticks are in smears. The research results are presented in Table 1.

**Table 1. Biochemical characteristics of Yersinia ruckeri strains discharged from carp.**

| Size, microns | 1-3 |
|---------------|-----|
| Tinctorial properties | Gr- |
| Urease | - |
| Ramnose | - |
| Melibiosis | - |
| Sucrose | - |
| Glucose | + (K) |
| Lactose | - |
| Raffinose | - |
| Arabinose | - |
| Sorbitol | - |
| Dulcete | - |
| Inositol | - |
| Salcin | - |
| Esclun hydrolysis | - |
| Xylose | - |
| Maltose | + |
| Sorbose | - |
| Ornithine decarboxylase | + |
| Lysine decarboxylase | + |
| Arginine dihydrolase | - |
| Indole | - |
| Voges-Proskauer reaction at 25°C | - |
| Methyl reaction | + |
| Mobility at 25°C | + |
| Indophenol Oxidase Production | - |
| Catalase | - |
| Gelatinase | + |
| Denitrification | + |
| H2S Products | - |
| Phenylalanine desaminase | - |

Note:

Legend: "+" - positive reaction; "-" - negative

The study of clinical samples and identification results by MALDI-TOF mass spectrometry (2.238) completely coincided with the results of laboratory studies.

The sensitivity to antibiotics of various microorganism types discharged in this study is presented in Table 2. All A. hydrophila and Y. ruckeri strains discharged from fish parenchymal organs were sensitive to ciprofloxacin, tetracycline, and chloramphenicol. 90% of aeromonad isolates and 100% of yersinia strains were resistant to furazolidone.

**Table 2. Sensitivity (%) of Yersinia ruckeri to the studied antibiotics.**

| Antimicrobial content | The drug | The number of sensitive Aeromonas hydrophila strains (%) | The number of sensitive Yersinia |
|-----------------------|----------|----------------------------------------------------------|---------------------------------|
|                       |          |                                                          |                                 |
Using the protocol for indicating the test sample with YER8F and YER10R primers [14], followed by analysis of the 16S rRNA fragment (Figure 1) allowed determining that the studied cultures corresponded to the general profile of the Yersinia ruckeri strain numbered JQ657818.1, CP011078.1, KJ606914.1, CP009539.1, LN681231.1, KM220889.1, KM220888.1 (99% match) in the existing GenBank database.

|                  | disk, mcg | ruckeri strains (%) |
|------------------|-----------|---------------------|
| Furazolidone     | 300       | 10                  | 0                   |
| Ciprofloxacin    | 5         | 100                 | 100                 |
| Tetracycline     | 30        | 100                 | 100                 |
| Chloramphenicol  | 30        | 100                 | 100                 |

No obvious pathological changes were detected in the examined fish taken for histological analysis. Accumulations of low density lymphocytes were observed in the spleen (Figure 2). In the liver, small accumulations of leukocytes were located around the vessels (Figure 3). In the heart, such clusters were recorded around myofibrils (Figure 4). In the kidneys (Figure 5), no changes were registered either in the hematopoietic or excretory parts. No bacteria were found in the studied organs.

**Figure 1.** 575 bp amplicons obtained using YER8F and YER10 specific primers for Yersinia ruckeri. Line 1: Molecular weight of the marker is 100 bp (Sigma, Milan, Italy); line 2: isolate DNA; line 3: Yersinia enterocolitica DISTAM **; line 4: Pseudomonas fluorescens DISTAM; line 5: Enterobacter cloacae DSM 30054; line 6: Vibrio ruber DSM 14379; line 7: negative test.

*DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH, Braunschweig,
**DISTAM: Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Milan, Italy

**Figure 2.** Spleen. Small accumulations of medium density lymphocytes. HE.

**Figure 3.** Liver. Accumulation of medium density white blood cells and slight inflammation. HE.
4. Discussion

The conducted research indicates a negative effect of the complex of abiotic factors on the fish organism. Those factors are low oxygen concentration in the bottom layers (2.0 mg/dm$^3$) and high level of organic pollution, as evidenced by permanganate oxidation indices (2.4 - 2.8 MAC), nitrite (1.5-2 MAC) and nitrate nitrogen (1.4-1.5 MAC).

A complex of unfavorable abiotic factors affects the ability of the fish organism to adapt to the environment. Often, low oxygen content in the water and temperature caused the death of fish from bacterial diseases. pH is one of the most important factors that regulate most biochemical processes. It is known that the pH level, CO$_2$ content, and organic pollution are stress factors for fish and affect their survival more than a separately taken low content of dissolved oxygen [14,15].

Detection of Saprolegniales mushrooms on the surface of the fish body indicates a weakening of the fish organism and the poor sanitary state of the ponds. A parasitological study showed that the level of invasion by monogenes (Dactylogyrus extensus) and mollusks (Unio pictorum) was not high, but in combination with unfavorable breeding conditions could contribute to the death of fish.

The identification of isolates from water and fish on MPA, Hoinger's medium, Endo, and blood agar allowed them to be classified as Yersinia ruckeri. According to published data [16], the Y. ruckeri strains discharged during the yersiosis outbreak did not ferment sorbitol. These isolates belong to serotype 1, same as ones found in this study. At the same time, isolates belonging to serotype 2 ferment sorbitol and are not so pathogenic for fish.

Oxytetracycline is used for treating the salmon yersiniosis most often in Europe. However, Y. ruckeri is able to form biofilms, which affects the development of epizootics in fish farms [16]. Biofilms have shown high resistance to oxolinic acid, an antibiotic often used in the treatment of yersiniosis. The use of trimethoprim/sulfamethaxosole gives the best results. In addition, enrofloxacin and doxycycline are used for treatment. The study of antibiotic sensitivity of the discharged microorganisms in the research showed the sensitivity of Y. ruckeri to ciprofloxacin, tetracycline, chloramphenicol and resistance to furazolidone.

Two specific primers YER8F (5’-GCGAGGAGGAAGGTTAAGTG-3’) and YER10 R (5’-GAAGGCACCAAGGCATCTCTG-3’) were used for Yersinia ruckeri throughout the research [17]. The production of specific 575 bp amplicons confirmed the presence of Yersinia ruckeri in the test sample. This result coincided with the data of biochemical studies.

In order to confirm the specificity of the amplicons synthesized in the reaction, sequencing was additionally used. The 99% coincidence of the studied cultures with Yersinia ruckeri strains under the numbers JQ657818.1, CP0011078.1, KJ606914.1, CP009539.1, LN681231.1, KM220889.1, KM220888.1 (in the existing database (GenBank database) was determined.
Pathological and histological examination did not reveal changes in the structure of internal organs in this study, except of the accumulation of low density white blood cells in many organs and the leukocyte infiltration in fish liver. These observations are contrary to bacteriological and PCR analyzes, which determined the presence of Yersinia ruckeri in all studied carps. We were not able to identify histological changes typical of yersiniosis, such as hyperemia with an inflammatory process in all tissues, with bacterial colonies in the kidneys, spleen, heart, liver and gills. The results of the study do not allow Yersinia ruckeri to be considered as the causative agent of the disease and as the cause of fish death. The results of bacteriological studies and PCR allow us to consider this species as a carrier state.

The conducted research shows that adverse breeding conditions, such as low oxygen content and high concentration of organic substances in this case, could affect the death of fish. It is also known that the formation of biofilms is an important feature of the survival of bacteria on the surface and bottom of water bodies.

The results of the study cause interest because the Yersinia ruckeri infection of carp in southern Russia was recorded for the first time. Prior to this, ponds contained rainbow trout brought from the Krasnodar Territory. Most likely, subsequent infection of carp with Yersinia ruckeri occurred from trout, in which this pathogen is registered quite often. It is known that Yersinia ruckeri can survive for a long time in fresh and sea water [14]. Thus, the pathogen could remain in the pond of this fish farm after the realization of rainbow trout. The research was carried out as part of the state assignment of the Ministry of Science and Higher Education of the Russian Federation No. 01201354245.

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