BACTERIOLOGICAL AGENTS IN FARMED CYPRINIDS FROM THE PRUT RIVER BASIN

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Abstract

The current paper aims to outline what could be the most common bacterial agents that can have a pathogenic effect on farm cyprinids form the Prut river basin in order to reduce fish health risks and even prevent future disease outbreaks that are bacterial in nature. Two field works expeditions to Rompescaris farm -Podu-Iloaiei from Iasi county and to Dracșani fish farm from Piscicola-Botoșani county were undertaken in May 2021 in order to collect biological samples. The following fish species were harvested: carp (Cyprinus carpio), silver carp (Hypophthalmichthys molitrix) and mirror carp (Cyprinus carpio var. specularis) using net fishing and the only the specimens with visible lesions or the moribund ones were collected. The fish that were apparently healthy were released and the ones that were harvested underwent a clinical, parasitological and bacteriological investigation.

Key words: *Cyprinus carpio, Hypophthalmichthys molitrix, bacteriological, diseases*

Aquaculture is one of the fastest growing food-producing sectors, supplying approximately 40% of the world's fish food. Besides such benefit to the society, the industry does have its problems. There are occupational hazards and safety concerns in the aquaculture industry. Some practices have caused environmental degradation. Public perception to farmed fish is that they are ''cleaner'' than comparable wild fish. However, some farmed fish have much higher body burden of natural and man-made toxic substances, e.g., antibiotics, pesticides, and persistent organic pollutants, than wild fish (Cole W. David et al., 2009). Farmed fish as a result of overcrowding can be more susceptible to diseases that are caused by one or more species of pathogen or opportunistic bacteria.

Consumers may select farmed fish for meal as a healthier and safer alternative to wild fish because aquaculture is presumably located away from industries that generate contaminated air and water (Cole W. David et al., 2009) and because it is presumed that in a controlled environment the risk of disease is greatly reduced. That being said now more than ever when it is estimated that by 2030 aquaculture will produce 60% of all fish destined for human consumption (FAO 2020) we must pay more attention to aquaculture systems similar to those found in Moldova county in which farmed fish are kept at high population densities in close proximity with wild fish reservoirs because it is an ideal situation in which spreading of wild type pathogens form the wild fish to the farmed ones and vice versa (Kibenge et. al 2012) is greatly enhanced.

Several fish bacterial pathogens have zoonotic potential, but transmission is much more common from ingestion of raw or inadequately cooked or processed fish than from contact with fish.

The highest risk from contact with fish is direct wound inoculation (e.g., fish spine injury or bite wound). Most fish bacterial pathogens are also common in the environment and transmission is possible through water contact with mucosal surfaces, open wounds, or food, although this appears to be rare.

Disease is more likely with immune suppression (Hadfield A. Catherine and Clayton Leigh Ann, 2021).

That being said we hope that through this set of investigations we can better understand what

bacterial diseases are affecting farmed fish in the Prut river basin.

MATERIAL AND METHOD

Following the external and internal clinical examination, characteristic lesions of erythrodermatitis were observed. This disease is caused by the development of a single bacterial species or several species of conditionally pathogenic bacteria, as follows: *Aeromonas hydrophila, Aeromonas caviae, Aeromonas sobria, Pseudomonas aeruginosa, Schewanella putrefaciens or Plesiomonas shigelloides.*

The bacteriological examination was performed by inoculation of bacterial strains sampled from the injured tissues but also from the uninjured organs, on specific media (Brain Austin and Dawn Austin, 2007)

Inoculations were made from the spleen, kidneys, hepato-pancreas, gills, skin, from areas adjacent to the injured tissue and not from the center of the wound.

Using a sterile Pasteur pipette and a sterile loop, the organ was deeply pierced and pathological material was harvested, which was then deposited on the surface of a non-selective medium (TSA agar, nourishing agar, BHI agar,) and incubated at 25 ° C for 24 to 48 hours.

After performing the cultural examination, morphological identification was performed by Gram staining, and biochemical identification by inoculating the bacterial strains on biochemical media and the use of API diagnostic tests.

Detection of virulence factors, hemolysin production. Blood-based agar (Bio-Rad) supplemented with 5% sheep's blood was used to produce hemolysin and incubated at 37 ° C for 24 hours.

In order to identify the species within the genus Aeromonas, mass spectrometry (MALDI-TOF MS bioMérieux system) was used.

RESULTS AND DISCUSSIONS

After the incubation time expired, the Petri dishes were examined for bacterial colonies, and the morphology of the colonies was assessed using an optical microscope or a magnifying glass.

We took into account: the type of colonies, smooth (S) or rough (R), their diameter, regular or irregular edges, pigmentation, tendency to confluence and the convexity or concavity of bacterial colonies.

On triple sugar iron agar, we found a series of morphological characteristics depending on the bacterial strain

Hemolysin which is a major virulence factor in Aeromonas spp. Infection was observed in all isolates. Hemolysin activity was observed on blood agar plates (figure 1 and 2) which confirmed its presence.

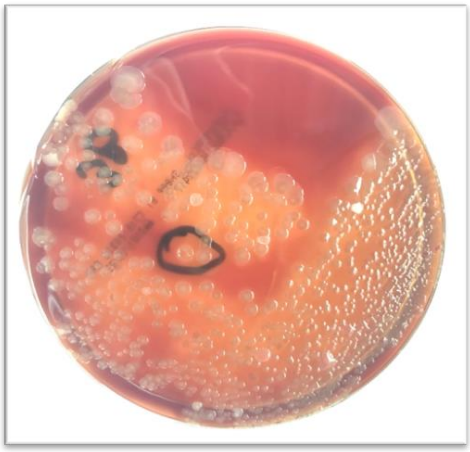

Figure 1. ***Aeromonas spp.*** **(Cultural aspect - blood agar)**

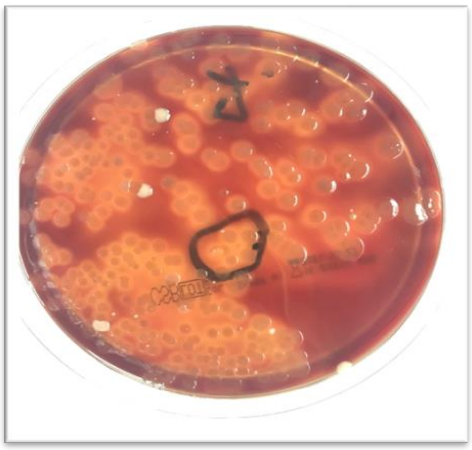

Figure 2. ***Aeromonas spp.*** **(Hemolysin activity)**

Testing the activity of bacterial strains against some sugars but also testing other characters is performed on TSI (triple sugar iron). It is a medium containing iron, 3 sugars in different concentrations (one monosaccharide = glucose and two disaccharides = lactose and sucrose) and a pH indicator (phenol red).

The medium poured into tubes was seeded using a loop with the bacterial strain to be tested in a column puncture or sloping grooves pattern.

The tubes were incubated at 25 and 37 ° C for 18, 24 and 48 hours, and the results were read.

Microorganisms that use glucose will induce an acidification of the medium until the sugar is depleted, causing the column of the medium to take on a yellow color. Bacteria that use lactose and / or sucrose will induce the same color change but in the slope of the tube - acidic PH.

If the bacteria don't use sucrose and lactose, then they degrade the protein (peptone) in the nitrogen-releasing substrate which will change the pH of the medium to alkaline and the color will shift to purple red.

Bacteria that ferment glucose with gas production will cause the accumulation of gas bubbles in the column, and bacteria that produce hydrogen sulfide, a compound that reduces iron sulfate from the environment to black iron sulfite, will cause blackening of the environment.

The results from testing the bacterial strains on TSI (triple sugar iron) are shown in table 1.

Table 1

Genus / species	Biochemical characters
Aeromonas hydrophila	Glu (+)/G(+) Lac (-) Suc (+)
Aeromonas caviae	Glu (+)/G(-) Lac (-) Suc (+)
Aeromonas sobria	Glu (+)/G(+)Lac (-)Suc (+)
Pseudomonas fluorescens	Glu (+)/G(+)Lac (-)Suc (-)
Schewanella putrefaciens	Glu (-)/G(-) Lac (-) H_2S (+)
Plesiomonas shigelloides	Glu (+)/G(-) Lac (+) H_2S (-)

Differentiation by growth at different incubation temperatures was also performed.

The pure bacterial cultures to be researched for 18-24 hours were seeded in tubes with non-differential medium (TSA) and incubated at 4°C, 25°C, 37°C and 41°C (table 2).

Biochemical testing with API tests was performed in order to highlight certain biochemical features and to identify the bacterial strain. The API test was chosen according to the species of bacteria that needed to be identified and the accuracy of the test.

The test was used according to the manufacturer's operating instructions and the identification was performed using the numerical profile on the test result sheet.

Table 2

Genus/ species	4°C	25°C	37°C	41°C
Aeromonas hydrophila	-	+	+	-
Aeromonas caviae	-	+	+	-
Aeromonas sobria	-	+	+/-	-
Pseudomonas fluorescens	+	+	-	-
Shewanella putrefaciens	-	+	-	-
Plesiomonas shigelloides	-	+	+	-

Antimicrobial susceptibility testing by diffusimetric method was also preformed and the interpretation consisted in appreciating the size of the zones of inhibition, induced by the antibiotic, area in which the microbial colonies are missing.

The diameter of the inhibition zones is measured in millimeters.

The results were marked with: "S" - sensitive, "R" - resistant bacteria and "MS" - moderately sensitive (table 3).

Table 3

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Antibiotic	The content in µg of the disc	Resistant area	Moderately sensitive area	Sensitive area
Oxytetracycline	30 µg	< 15 mm	15 -18 mm	≥ 18 mm
Enrofloxacin	5 µg	< 17 mm	18 – 21 mm	≥ 22 mm
Florfenicol	30 µg	≤ 16 mm	17 – 19 mm	≥ 20 mm
Flumequine	30 µg	< 21 mm	22 – 24 mm	≥ 25 mm
Erythromycin	15 µg	< 14 mm	14-17 mm	> 17 mm

During testing we got different results for each bacterial strain.

For *Aeromonas sobria* we found "S"-type colonies round with a diameter of 2-5 mm, regular, opaque, unpigmented edges (figure 3).

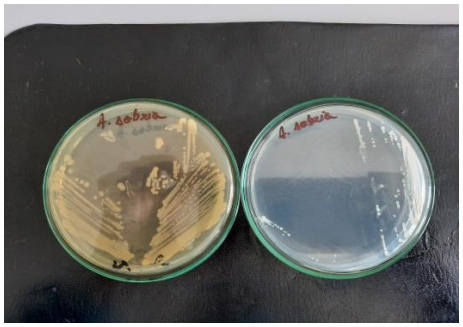

Figure 3. ***Aeromonas sobria*-cultural examination**

Antimicrobial susceptibility testing by diffusimetric method showed that *Aeromonas*

sobria was sensitive to florphenicol, doxycycline, enrofloxacin, trimethoprim and resistant to ampicillin, amoxicillin, erythromycin (figure 4).

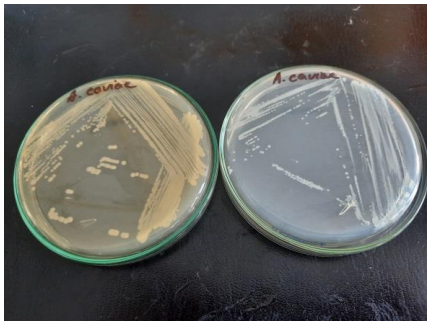

Figure 4. ***Aeromonas cavie*-cultural examination**

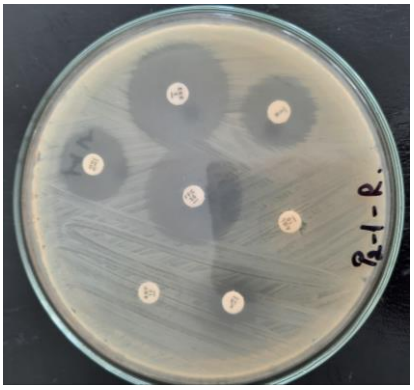

Figure 5. ***Aeromonas sobria*-susceptibility to antibiotics**

For *Aeromonas cavie* we found similar morphological characteristics (figure 5) and a slightly different susceptibility to antibiotics (figure 6).

Aeromonas cavie was sensitive to florphenicol, doxycycline, enrofloxacin, trimethoprim, erythromycin and resistant to ampicillin and amoxicycline.

CONCLUSIONS

Bacteria that are opportunistic and have the capability of causing a disease in fish can be found in water on a regular basis it is our aim to find out witch bacterial strains can cause problems more often and how to prevent or if necessary, treat these affections in fish populations.

Treatment using antibiotics can be efficient if done after a proper study or diagnostic but the actual administration of an active substance still poses some problems. The main obstacle is distributing the antibiotic in an even dose among the entire fish population, another problem is the resistance to antibiotics that can appear as a result of such treatments and the impact on the environment.

Most bacterial born diseases can be prevented in fish using a good management system but other pathogens such as parasites must also be managed very carefully because they can weaken the fish and make them more susceptible to bacterial strains that normally wouldn't cause any problems.

Through further studies we hope that in the near future we will better understand what bacterial strains found in the Prut river basin can cause diseases in fish and witch of them do so more often in order to reduce to a minimum the fish health risks in the area.

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REFERENCES

Austin Brain and Austin Dawn, 2007 - Bacterial fish pathogens. Diseases of fish farmed fish and wild fish Ed. 4

Cole David W., Cole Richard, Steven J. Gaydos, Jon Gray, Greg Hyland, Mark L. Jacques, Nicole Powell-Dunford, Charu Sawhney, William W. Au - Aquaculture: Environmental, toxicological, and health issues, 2009

Eissa Alaa Eldin, 2016 – Clinical and laboratory manual of fish diseases

FAO. 2020 - The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome. https://doi.org/10.4060/ca9229en

Hadfield Catherine A., Clayton Leigh Ann - Clinical Guide to Fish Medicine, 2021

Kibenge Frederick S.B, Marcos G. Godoy, Mark Fast - Countermeasures against viral diseases of farmed fish, 2012

Witman A. Kimberlley and Neil MacNair –Fish and sellfish- Bacteriology manual;