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Bacterial Study of Fin Rot in Brown Trout by API20E

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Abstract: In order to study caudal fin rot with emphasis on *Aeromonas* sp. and *Pseudomonas* sp. in *Salmo trutta caspius* from the salmonids propagation and breeding center of Shahid Bahonar of kelardasht region, One hundred and eighty brood stocks having fin damage symptoms were chosen. Two bacterial samples from each fish were cultured on *Aeromonas* and *Pseudomonas* specific media. To identify isolated bacterial colonies by API20E diagnostic system, samples obtained from bacterial cultures 18 to 24 h were prepared and were inoculated into the cupules of test strips. At the end of incubation, after addition of reagents if they are necessary, the results recorded on the results sheets and were analyzed by Apiwe software. The results of API20E diagnostic systems showed that *Aeromonas* genus bacteria including *Aeromonas hydrophila* with 100% frequency while the bacterial genus of *Pseudomonas* including *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Pseudomonas putida* with 36. 84, 31.57 and 21.05% frequency, respectively. Also 10.25% of *Pseudomonas* samples were unidentified.

Key words: *Salmo trutta caspius*, fin rot, *Aeromonas* sp., *Pseudomonas* sp., API20E

INTRODUCTION

Fin rot in fish breeding, including problems that are involved with it aquaculture. Especially important among brood stock of salmonids well is evident. Since the role of management factors and health are effective in preventing the occurrence or have fin rot. Therefore, fin rot are mentioned as an important factor in evaluating the health level of the fish farms. Also In salmonids breeding there is a vast index of fin rot and this issue can be considered suitable for separation of wild and breeding fish together (Crick *et al*., 1987). Many factors are involved in the incidence of fin rot fish so that is named it as a syndrome, mainly because of special conditions intended rearing, more than one factor plays a role in the incidence. Among several factors in the incidence of infectious and noninfectious fin rot, bacterial factors in terms of being inclusive in aqueous environments and their presence as part of flora surfaces of fish are important, especially when environmental conditions is provided for growth and proliferation of these bacteria (Pickering, 1977). Some damage to the fins of fish lead to impaired access to swimming and hunting them. However, this issue also should be considered that fin rot may be increase possibility of catching systemic infections such as Furunculosis (Horak, 1969; Maheshkumar, 1995). Studies show that there are effective antibiotics against *Aeromonas* sp. and *Pseudomonas* sp. but some of them recently bacteria have become resistant to the bacteria and the number is growing. So coastal plant extracts, extract a kind of coral and algae as potentially rich sources of new drugs as an alternative aquaculture antibiotics commonly recommended (Choudhury *et al*., 2002).

MATERIALS AND METHODS

Sampling: For sampling of rotted brood stock's fins, 180 number of them based on a statistical evaluation were chosen and caught and then was transferred to canvas. Then, by using MS_{222} fish to be tranquillized and were carried in to the laboratory of salmonids propagation and breeding center of Shahid Bahonar of kelardasht region. Within laboratory samples of fins, the rotted fins were scraped by each edge of a sterile surgical blade and moved them to the specific medium of *Aeromonas* and *Pseudomonas*. When sampling finished, media were transferred to the laboratory for additional test.

API20E biochemical kit

Structure of API20E biochemical kit: API20E is a standard diagnostic method to identify family of

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Enterobacteriaceae bacteria and other gram negative rod shaped bacteria that are not fastidious. The system contains 21 biochemical tests on small models. Test strips of API20E, including 20 μ tube containing dehydrated materials and suspension of tested bacteria are inoculated into them. Produced metabolism lead to change color of micro tube which itself indicates the test result of test or by adding a reagent to be determined (Biomerieux Company).

Preparation of test's sample: Microorganism, at first must be isolated on culture medium to obtain pure form, so the clinical samples prior to purification on the medium cannot be used. New culture (18 to 24 h) is better than the others. For this purpose, colony of bacteria with enough quantity was taken, then mixed to the 5 mL 85% sodium chloride solution to obtain homogeneous and uniform suspension in test tubes. For this purpose it was necessary to perform it by vortex properly (Biomerieux Company).

Inoculation of bacteria's samples: Suspension before inoculation, the location test strip was required to prepare samples. For this purpose, a tray and the cap was embedded in the kit. On the floor of tray there were small cavities where pouring distilled water free from any contamination, minerals and gas. The water provides in the incubation time needed moisture in the reaction space. The next step was inoculation of prepared samples into the micro tube. Each micro tube containing of raw materials and a cupule at the end of it.

For inoculating of samples was performed to this method:

- Tests on citrate, VP and gelatin hydrolyze both parts of tube and cupule was filled with the suspension
- About other tests, only the tube was filled with the suspension
- Experiments on URE, H2S, ODC, LDC and ADH should be performed in anaerobic environment that was made by pouring paraffin on the cupule

Finally, tries were filled with distilled water and inoculated test strips were placed on and put the lid on them, numbered them, incubated for about 24 h at 37°C (Biomerieux Company).

Reading biochemical test strips and recording results: After incubation results according to the related tables were recorded in this way:

- If three or more tests (glucose positive or negative) were positive, all results are recorded and the tubes were need to reagent, it was added to the tubes and after the required time their result was registered. These reagents included the TDA reagent (iron chloride), Indol reagent (I, II), VP reagent (I, II), nitrate reagent (I, II) and zinc reagent (Zn)
- If the number of positive tests (including glucose test) before adding reagent were less than three tests in the test strip then were incubated again for about 24 h without adding any reagents at 37°C. It is necessary to mention that complete additional diagnostic tests including OF test, growing on the Mac Conkey medium (McC), Mobility test (MOB) and Oxidase test (OX) which there was no place for them on the test strips but they were recorded on the result sheets that might be needed (Biomerieux Company)

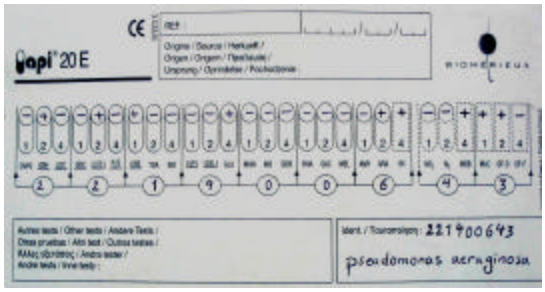

Fig. 1: Shows the result sheet and how to record positive and negative tests to achieve nine-digit code. (Biomerieux Company)

Analyzing test results: Considering positive or negative tests, results were recorded on result sheets. By adding every three positive tests together on the result sheet ultimately a nine-digit code was achieved. By using the software was built in the kit, intended code was identified and tested bacteria was described by the percentage probability (Fig. 1).

For example, whereas adding up positive numbers lead to a nine-digit code to be 221400643, using the relevant software, as the *Pseudomonas aeruginosa* with probability 5. 99 percent are diagnosed (Biomerieux Company) (Fig. 1).

RESULTS

After ending the tests, results were recorded on paper and ultimately a nine-digit codes were obtained. The codes were analyzed by Apiwe software and the results were achieved as follows (Table 1).

Table 1: Results achieved by API20E software

Samples	Nine-digit code	First diagnosis	Final diagnosis
A1	3.05E+08	*Aeromonas hydrophila*	*Aeromonas hydrophila*
A2	3.05E+08	*Aeromonas hydrophila*	*Aeromonas hydrophila*
A3	3.05E+08	*Aeromonas hydrophila*	*Aeromonas hydrophila*
A4	3.05E+08	*Aeromonas hydrophila*	*Aeromonas hydrophila*
A5	3.05E+08	*Aeromonas hydrophila*	*Aeromonas hydrophila*
A6	3.05E+08	*Aeromonas hydrophila*	*Aeromonas hydrophila*
A7	3.05E+08	*Aeromonas hydrophila*	*Aeromonas hydrophila*
A8	3.05E+08	*Aeromonas hydrophila*	*Aeromonas hydrophila*
P1	2.2E+08	*Pseudomonas aeruginosa*	*Pseudomonas putida*
P2	2.21E+08	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
P3	2.2E+08	*Pseudomonas fluorescens/putida*	*Pseudomonas putida*
P4	2.21E+08	*Pseudomonas fluorescens /putida*	*Pseudomonas fluorescens*
P5	2.21E+08	*Pseudomonas aeruginosa*	Unknown
P6	2.2E+08	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
P7	2.21E+08	*Pseudomonas fluorescens /putida*	*Pseudomonas fluorescens*
P8	6.24E+08	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
P9	2.2E+08	*Pseudomonas fluorescens /putida*	*Pseudomonas fluorescens*
P10	2.21E+08	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
P11	2.22E+08	*Pseudomonas aeruginosa*	*Pseudomonas putida*
P12	2.21E+08	*Pseudomonas fluorescens /putida*	*Pseudomonas fluorescens*
P13	2.2E+08	*Pseudomonas aeruginosa*	*Pseudomonas putida*
P14	2.2E+08	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*
P15	2.01E+08	*Pseudomonas aeruginosa*	Unknown
P16	2.21E+08	*Pseudomonas fluorescens /putida*	*Pseudomonas fluorescens*
P17	2.21E+08	*Pseudomonas fluorescens /putida*	*Pseudomonas fluorescens*
P18	2.21E+08	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
P19	2.21E+08	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*

From 27 samples of both *Aeromonas* sp. and *Pseudomonas* sp., eight samples belonged to *Aeromonas* sp. and nineteen samples were related to the *Pseudomonas* sp. *Aeromonas* samples, all were identified as *A. hydrophila*. *Pseudomonas* sample including *P. aeruginosa*, *P. fluorescens* and *P. putida* were 31.57, 36.84 and 21.05 per cent frequency, respectively.

DISCUSSION

Studies show that most samples obtained from water or fish skin, *Aeromonas* and *Pseudomonas* bacteria has to be isolated. Taylor (2003) in a study isolated 30 bacteria from common carp that, 13 isolates as *A. hydrophyla*, 11 isolates as *A. sobria* and two isolates as *A. caviae* were introduced . Neumann and Ploger (1980) also isolated in a study *A. hydrophyla* from fish farms of north western of Germany. In another study and Miranda and Zemelman (2002) were able to isolate *A. hydrophyla* and *P. fluorescens* from salmon farms of northern Germany. Saha and Pal (2002) in a study isolated 16 bacterial isolates from surface wounds of fish EUS Syndrome that Among them *Aeromonas* and *Pseudomonas* species were observed. In another study was performed by Pulmb *et al.* (1995) 200 isolates including *Edvardziella* sp., *Pseudomonas* sp., *A. hydrophyla* and *A. sobria* were identified. Although these bacteria are often found as skin flora but they can also be isolated from internal infection. For example, Lipton (1991) isolated *A. hydrophyla* from the wounds of a fish and isolated *P. aeruginosa* in breeding intensive condition. There are a few studies in this field within the country. Rafieepour, (1997) in serological study of shrimp and fish samples of different areas using the slide agglutination and Fluorescent antibody methods could isolate *Vibrio anguillarum* and *A. hydrophyla*. Also in investigating of morphological characteristics, physiological and biochemical of these organisms, identified species of Vibrio, *A. salmonicida*, *A. hydrophyla* and *A. caviae*. Naderi-Maivan (2003) also in a study on grass carp's fin rot stated that the most isolated from fin rot belong to the motile *Aeromonas* (*A. hydrophyla* and *A. caviae*) and then *Pseudomonas* genus. Aguilera-Arreola *et al.* (2005) to assess the genetic diversity of isolates of *A. hydrophyla* that were obtained from the different samples, including water and fish, used the technique of RAPD (Random Amplification of Polymorphism). Finally the results showed high genetic diversity. Also the manner of distribution of dependent virulence genes confirmed the genetic heterogeneity of *A. hydrophyla*. In another study Castro-Escarpulli *et al.* (2003), 82 isolates of *Aeromonas* species isolated from 250 fish were frozen, including *A. salmonicida*, *A. hydrophyla*, *A. caviae* and *A. veroni* (sobria biovar) in which *A. salmonicida*has the highest quantity and amount of others reduces, respectively. Whereas the genetic technique by PCR/RFLP based on 16SrDNA gene showed that these isolates were included *A. salmonicida*, *A. bestarium*, *A. Veroni* (sobriabiovar), *A. encheleia* and *A. Hydrophyla* . Thus, this study shows that all biochemical results were not confirmed by molecular study.

Xia *et al.* (2004) in a study isolates of silver carp fish isolated by the PCR method based on gene sequences of *A. hydrophyla* beta-hemolysin gene cloned which among them there were pathogenic species including *A. hydrophyla*, *A. caviae* and *P. fluorescens*. Also Biscardi *et al.* (2002) isolated isolates of *A. hydrophyla* by PCR based on Aerolysin gene from samples of water's bottles and hot water sources. In studies on *Pseudomonas* species were obtained from the water and

environmental samples and were identified by API20NE, were studied by molecular methods that there was *P. fluorescens* isolated from the river among them (Bodilis *et al*., 2004). Kong *et al*. (1999) isolated seven of *Aeromonas* species from water sources by molecular methods including *A. hydrophyla*, *A. caviae*, *A. veroni*, *A. trota*, *A. jandaei*, *A. Schubertii* and *A. entropelogenes*. Frahm *et al*. (2001) isolated *P. aeruginosa* by molecular methods. In a study related to sea water, samples were obtained from Tokyo Bay, after separation with selective media were evaluated by three methods: API20NE Kit, Genetic basis of lipoprotein genes in *P. aeruginosa* outer membrane and gene sequencing 16S rDNA. The results showed that most isolated samples were *P. aeruginosa* (Naderi-Maivan, 2003). Widmer *et al*. (1998) by using PCR method that it's primers were designed based on 16S rDNA gene could profitted to detect members of genus *Pseudomonas*. They intended to diagnose species of genus *Pseudomonas* by enzymatic digestion method using the enzyme HaeIII (RFLP technique) and could identify some species of *Pseudomonas* genus. However considering this method to identify all species of this genus was not efficient, so Porteous *et al*. (2002) took advantage of these studies using four types of enzyme RFLP method to identify species of this genus and put them in five separate branches. Accordingly Laganowska and Kaznowski (2004) also using the enzyme digestion pattern of *Aeromonas* samples by four types of enzymes, based on 16S-23S rDNA genes were amplified (PCR/RFLP), used to identify *Aeromonas* species. Various biochemical and molecular studies suggest that *Aeromonas* and *Pseudomonas* species in different water sources and fish exist as normal flora or pathogen. In this study there, some of these microbial agents that are associated more with fins rot indicated. We must say how much the role of microbial factors in the incidence of fin rot. It seems important to replicate this is necessary in the incidence of this complication as a syndrome can be so many factors are involved as primary causes. For instance deficiency of some essential nutrients such as vitamins and essential fatty acids, parasitic infections, physiological and behavioral changes during the reproductive, the physical damage caused by the high density of fish and even the existence of high suspended solids mud, especially in early spring due to floods and other environmental problems, stress management factors named. Therefore, because at least some of these factors affected farms and these bacteria also (according to numerous studies that show) is usually normal flora of fish's skin and water, it seems that after damaging the skin and the epithelium of fin by primary factors, the bacteria found in this location and then amplified. According to the capability they possess to secrete toxins and extracellular enzymes such as hemolysin, cytotoxin and proteases, cause damage and destruction of epithelial tissue and provide fin rot. Considering the above mentioned hypothesis is that the fish which samples are obtained from their rotten fins, do not show *Aeromonas* or *Pseudomonas* septicemia clinical signs that if there is a septicemia, primary bacterial agents will have more credibility. Whether these bacteria were either primary or secondary exist in fin rot and their role in the incidence of fin rot, depend on multiple factors including environmental conditions and their virulence. Considering what was described seems the best way to prevent fin rot is removal primary agents from fish farms.

CONCLUSION

Fin rot in fish is important problem in aquaculture especially among brood stock. Since the role of management factors and health are effective in preventing the occurrence or have fin rot. Many factors are involved in the incidence of fin rot. among several factors in the incidence of infectious and noninfectious fin rot, bacterial factors in terms of being inclusive in aqueous environments and their presence as part of flora surfaces of fish are important, especially when environmental conditions is provided for growth and proliferation of these bacteria. Result of this study showed that some *Aeromonas* and *Pseudomonas* species were isolated from fin rot; Therefore since these bacteria are considered potential sources for fin rot, sanitation management in the fish farm rearing is effective in preventing this problem. In the other words, fin rot are mentioned as an important factor in evaluating the health level of the fish farms.

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