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# Studies on the marine fish infection and antibiotic sensivity test selected pathogens

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**Abstract**
This approach worked but not as well or for as long as one would have wished. Additionally, it has been at least two decades since any new class of antibiotic has been discovered. We now have the opportunity to understand the fundamental factors that microbes use to cause infection and disease. It is from this understanding that eventually we will devise better means for the control of microbial disease. However, we also must remember that human life has evolved to live in a constant sea of microorganisms, and it is doubtful (if not impossible) that a genotobiotic life is human destiny; instead, by understanding virulence mechanisms, we can attempt to design strategies to control microbial infection and disease. However infected fish isolated three pathogens *Escherichia coli, Salmonella sp. and Staphylococcus sp* future studies carried Antibiotic Sensitivity Testing of Pathogens Isolated from Spoiled Fish which is giving more zone *Staphylococcus sp.* Streptomycin encountered 15 µg 27≤. Thus our present studies mainly focused marine fish infection and antibiotic sensivity test selected pathogen.

**Keywords:** marine fish, infection, antibiotic sensivity test, pathogens

## Introduction

Fish is widely recognized as a very good source of protein in the -human diet. This protein is high in essential amino acids, and has a good amino acid structure. The fish is also a good source of mineral and vitamin. About 80% of weight of fresh fish consists of water, 18% of protein, carbohydrate content of 1% and lipid content of 1% (Hobbs, 1983) [1]. The muscle tissue of flesh live healthy fish is truly sterile. Seafood is well known for its nutritive values, minerals, and vitamin contents such as vitamin A, B, D, and omega-3-fatty acid. Biological agents, particularly bacteria and viruses are recognized as the etiological agents of infectious diseases in a wide range of marine animal species. The appearance and development of a fish disease is the result of the interaction among pathogen, host and environment. Fish carry a variety of microorganisms from both aquatic and terrestrial sources. The most prominent Gram-negative bacterial fish pathogens are distributed across the phyla Proteobacteria and Bacteroidetes, and a high number of identified and widely studied pathogens belong to the phylum Proteobacteria. The range of diseases found in fish reflects the diversity of virulence factors and virulence mechanisms utilized by these microbes. In general, bacterial infection is successful when the pathogen can successfully adhere to the host tissue, multiply and invade. The most common diseases seen in temperate fish are fungal infections as the fungal spores easily spread through water, colonise and cause infection. Particular significance is whether the water is sewage polluted in which case the fresh water food is potentially capable of transmitting various pathogenic microorganisms (Pelczar*et al*., 1998) [2]. Microorganisms are the major cause of spoilage of most seafood products. At a time when there is so much interest in emerging pathogens, along with the failure of anti-infective therapy to control some of the most common disease-causing microbes, there is more interest than ever in the field that has become known as microbial pathogenesis. The infectious bacterial diseases in marine fish affected by the following: (i) Significant economic losses in cultured fish are caused by a relatively small number of pathogenic bacteria; (ii) Several typical of fresh water aquaculture diseases turns as marine culture serious problems; (iii) The disease clinical signs depend on the host species, age; (iv) Absence of any correlation between the external and internal signs; and (v) The lack of the stressful conditions, resulted in increasing the mortality and the severity of the disease in cultured fish than that in wild fish. The pathogenic agents described in the culture systems are usually present in wild fish populations. However, in natural environments, they rarely cause mortality due to the lack of the stressful conditions that usually occur in the culture facilities (Toranzo *et al*., 2005) [3]. Fishes by reasons of their habitat are continually bathed in aqueous suspension of microbes; their external surface therefore is in constant contact with these organisms. Some of these organisms may colonize the surface of the fish becoming part of the resident microflora. The development production of fish can trigger the disease attack due to unbalanced interactions between the host, the pathogen, and the environment. They are found in marine, estuarine and fresh ecosystems, constituting about 60% of total heterotrophic bacteria. Moreover, they represent a part of the normal fish flora in the aquatic organisms that could act as vectors for the disease transmission. Therefore, only multidisciplinary studies involving the characteristics of potential pathogenic microorganisms for fish, aspects of the biology of the fish hosts as well as a better understanding of the environmental factors affecting such cultures, will allow the application of adequate measures to prevent and control the main diseases limiting the production of marine fishes. Regarding the infectious diseases caused by bacteria in marine fish, although pathogenic species have been described in the majority of the existing taxonomic groups, only a relatively small number are responsible of important economic losses in cultured fish worldwide.

The presence of these micro floras inhibits the arrival and subsequent colonization by other organisms that may be pathogenic to the fish. Bacteria inhabits other parts of the

fish such as gill, mouth and gut (Jalal *et al.,* 2010) [4], while, a large stable population of bacteria inhabits the fish gut (Hamid *et al.,* 1979) [5]. These populations are able to survive the harsh conditions of the gastrointestinal tract. The microbial flora of freshly caught fish and other aquatic specimens is largely a reflection of the microbial quality of the waters from where they are harvested or stored. Microorganisms are the major cause of spoilage of most seafood products. Moreover, *A. hydrophila* infection in humans induces gastroenteritis and extra-intestinal disease (i.e. meningitis and endocarditis; (Zhang *et al.,* 2012) [6]. The unbalanced interaction resulted in stress on the fish thus weakening the mechanism of self-defence and the disease attack the fish. Bacterial pathogens rely on the synergistic action of different virulence determinants and on specialized secretion systems to cause disease in susceptible hosts (Finlay and Falkow, 1997; Thanassi and Hultgren, 2000) [7, 8]. Initiation of infection is often triggered by adherence of the pathogen to the skin or mucosal surface of the host tissue using attachment mechanisms such as non-fimbrialadhesins (Ostland *et al.,* 1997; Weber *et al.,* 2010; Guardiola *et al.,* 2019) [9, 10, 11], pili or fimbriae (Mattick, 2002; Gerlach and Hensel, 2007; Craig *et al.,* 2019) [12, 13, 14] which recognize specific receptors. A successful uptake of the pathogen into host cells is then mediated by specific invasion factors (e.g., invasins) which are either membrane anchored proteins of the pathogen or are secreted through specialized secretion systems. Invasins promote translocation of pathogens in host cell. The visible symptoms of disease such as inflammation, bleeding, or lethal shock is typically induced by different toxins. An important toxin is a structural component of Gram-negative pathogens, the lipopolysaccharides, that are a key component of the cell membrane and that exert intense biological effects on the host which may be lethal (Sampath, 2018) [15].

The use of antibiotics in the aquaculture leads to a variety of public health hazards. The greatest potential risk to public health is thought to be the transfer of resistant organisms through consumption of contaminated fish, the development and spread of antimicrobial resistant bacteria and resistance genes and the dissemination of the resistance genes by horizontal gene transfer (Lukkana *et al.,* 2012) [16]. In addition to these so-called endotoxins, extracellular toxic proteins known as exotoxins, are produced and secreted by pathogens via specific secretion machinery. Unlike the extensive systemic damage of endotoxins that is mainly based on an adverse immune reaction, exotoxins typically target local tissues and are often restricted to particular cell types or receptors (Cavaillon, 2018) [17]. For other virulence factors, their identification and in-depth characterization is fundamental to the development of specific diagnostics and treatment tools. For instance, molecular, structural and biochemical characterizations of genes and proteins involved in secretion systems are useful for discovery of novel treatments for combating pathogenicity in bacteria (Costa *et al.,* 2015; Alteri and Mobley, 2016) [18, 19]. Nevertheless, to obtain proper epidemiological models, animal health surveillance and biosecurity programs must integrate environmental information and information from different areas like pathogenesis, disease diagnosis, disease resistance, physiological response to pathogens, pathogen characterization, host immune system responses characterization, disease biomarkers and organism response to disease treatment products (Rodrigues *et al*., 2018; Cash. 2009) [20, 21].

## Materials and methods

### Sample collection

A total of 10 fresh fish samples of marine water fish were collected from Mandapam, fish landing site, Ramanathapuram, Tamil Nadu, India. After the collection, samples were aseptically and immediately transported in a sterile polypropylene bag placed in a cooler box. The cooler box contained crushed ice, and the temperatures were between 4°C and 8°C during transportation. The samples were transported to the PG Department of Zoology, Syed Ammal Arts and Science College, Ramanathapuram (Figure 1).

### Enumeration of total microbial population

The electroplating effluent contaminated soil samples were collected from the electroplating industrial effluent discharged sites and used for isolation of total microbial population like bacteria, fungi and actinomycetes using standard procedures (Edward *et al.,*2009; Subba, 1995; Kannan, 1996) [22, 23, 24] as shown in Table 1.

**Table 1:** Procedure followed for enumeration of total colony forming units (CFU) of bacteria, fungi and actinomycetes

| Organism studied | Medium used | Dilution tested | Growth temperature (°C) | Incubation Period (Days) |
|---|---|---|---|---|
| Bacteria | Nutrient | $10^{-6}$ | 37 | 1 |
| Fungi | Martin's Rose Bengal agar | $10^{-4}$ | 28 | 5 |
| Actinomycetes | Kenknight's agar | $10^{-4}$ | 37 | 7 |

### Enumeration of total bacterial population

A soil sample (1g) was dissolved in 100 ml of normal saline (85% NaCl) in 250 ml Erlenmeyer flask and serially diluted up to $10^{-9}$ dilutions. Diluted samples (0.1 ml) from the dilution $10^{-6}$ were pipette out and spread on the nutrient agar (Peptone 5 $gL^{-1}$, Beef extract 3$gL^{-1}$, NaCl 5$gL^{-1}$, Agar 15$gL^{-1}$) plates. All the plates were incubated in the microbiological incubator (NSW-151, Narang Scientific Works, India) at 37°C for one day. The total Colony Forming Units (CFU) of bacterial population were counted and expressed as $CFUg^{-1}$ of the soil sample. The total numbers of CFUs were counted after incubation i.e., one day for bacteria, five days for fungi and seven days for actinomycetes. The microbial loads between the substrates and between the microbial groups were analyzed using One-way ANOVA method. Based on the higher density, the bacterial isolates alone were taken for screening studies for their metal tolerance.

### Pure culture and identification of metal tolerant bacterial isolates

The sixteen predominant and morphologically distinct bacterial colonies grown on nutrient medium were isolated and pure cultured through repeated streaking on nutrient agar plates. All the sixteen bacterial isolates were identified through morphological and biochemical characteristics using standard procedures (Apun*et al.,* 2000). The morphological characteristic studies include bacterial

staining and motility test. The bacterial staining procedure was carried out using gram's staining kit to identify the gram nature of the organism. The motility test was performed by hanging drop method to determine the motility of the organism. The biochemical characteristic studies includeindole production, methyl red reaction, voges-proskauer reaction, catalase activity, starch hydrolysis, citrate utilization, urease reaction, glucose fermentation and gelatin liquefaction. The results of both morphological and biochemical studies of the sixteen bacterial strains were compared with Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) [26] and thus identified all the sixteen bacterial isolates at genus level.

**Antibiotic sensitivity test by disc diffusion method**

The above identified bacterial colonies were study the antibiotic sensitivity test. The strains are following commercially available antibiotic discs used for the Antimicrobial sensitivity studies. The test was carried out by disc diffusion method on Muller Hinton Agar medium (MHA) following the method of NCCLS, 1999.

**Results**

The spoiled fish sample was crushed and serially diluted with normal saline (85% Nacl) up to $10^{-9}$ dilutions. Sample (0.1ml) from $10^{-5}$ and $10^{-6}$ dilutions were spread on the nutrient agar plates and incubated at 37°C for 24 hrs. The three predominant colonies grown on nutrient agar medium were selected and maintained as pure cultures. All the three bacterial strains were identified through morphological and biochemical characteristics through Gram's staining and Motility Test, Indole Production, Methyl Red reaction, Voges Proskauver reaction, Citrate Utilization test, Urease test, Starch Hydrolysis, Catalase test. All the strains were authenticated by Bergey's manual of determinative bacteriology (Holt *et al.,* 1984). The identified strains were confirmed as *Escherichia coli*, *Salmonella* sp. and *Staphylococcus* sp. by the growth of strains in selective media *viz*., EMB agar, HE agar and MacConkey agar (Table 2). These identified strains are undergoes the anti sensitivity testing against streptomycin, ampicilin,

**Table 2:** Morphological and biochemical characteristics of isolated bacterial strains

| Strain No | S - 01 | S - 02 | S - 03 |
|---|---|---|---|
| Colony Morphology | Colorless Mucoid Colony | Whitish Pink Colony | Whitish Colony |
| Simple staining | Rod | Rod | Cocci |
| Motility test | Motile | Motile | Motile |
| Gram's reaction | Gram Negative | Gram Negative | Gram Positive |
| Indole production | Positive | Negative | Positive |
| Methyl Red reaction | Positive | Positive | Negative |
| Voges Proskauer reaction | Negative | Negative | Negative |
| Catalase activity | Positive | Positive | Positive |
| Starch hydrolysis | Negative | Negative | Positive |
| Citrate utilization test | Negative | Negative | Positive |
| Urease reaction | Negative | Negative | Negative |
| Glucose fermentation | Positive | Positive | Negative |
| Gelatin liqufication | Negative | Positive | Negative |
| Growth in Selective Media | Metallic Sheen Colony in Eosin Methylene Blue Agar | Colourless Colony in Hektoen Enteric Agar | NLF Colony in MacConkey Agar |
| Name of the Strain | *Escherichia coli* | *Salmonella* sp. | *Staphylococcus* sp. |

**Antibiotic Sensitivity Testing**

The activity of selected antimicrobial agents against *Escherichia coli, Salmonella sp. and Staphylococcus sp.* were determined with the agar disk diffusion test to determine the diameter of the zone of inhibition. The *Escherichia coli, Salmonella sp. and Staphylococcus sp*.strains used in this study were isolated from spoiled fish. Two antibiotics, which are used to found the antibiotic sensitivity of isolates. According to the evaluation criteria, 65.2 (Erythromycin) to 93.5% (Streptomycin) *Escherichia coli, Salmonella sp. and Staphylococcus sp.* strains were susceptible to the antibiotics tested. *Escherichia coli and Salmonella sp.* strains reveal a lower resistance rate than *Staphylococcus sp.*

**Table 3:** Antibiotic Sensitivity Testing of Pathogens Isolated from Spoiled Fish

| Strain Name | Antibiotic | Zone of Inhibition (mm) | | | |
|---|---|---|---|---|---|
| | | concentration | Resistant | Intermediate | Susceptible |
| *Escherichia coli* | Erythromycin | 15 µg | ≤16 | 17-21 | 22≤ |
| | | 10 µg | ≤14 | 15-18 | 19≤ |
| | | 5 µg | ≤11 | 12-14 | 15≤ |
| | Streptomycin | 15 µg | ≤18 | 19-24 | 25≤ |
| | | 10 µg | ≤14 | 15-18 | 19≤ |
| | | 5 µg | ≤10 | 11-15 | 16≤ |
| *Salmonella* sp. | Erythromycin | 15 µg | ≤17 | 18-23 | 24≤ |
| | | 10 µg | ≤14 | 15-18 | 19≤ |
| | | 5 µg | ≤11 | 12-15 | 16≤ |
| | Streptomycin | 15 µg | ≤19 | 20-24 | 25≤ |
| | | 10 µg | ≤15 | 16-19 | 20≤ |
| | | 5 µg | ≤12 | 13-16 | 17≤ |
| *Staphylococcus sp* | Erythromycin | 15 µg | ≤20 | 21-25 | 26≤ |

| | | | | | |
|---|---|---|---|---|---|
| | | 10 µg | ≤17 | 18-21 | 22≤ |
| | | 5 µg | ≤13 | 15-18 | 19≤ |
| | Streptomycin | 15 µg | ≤21 | 22-26 | 27≤ |
| | | 10 µg | ≤18 | 19-24 | 25≤ |
| | | 5 µg | ≤15 | 16-19 | 20≤ |

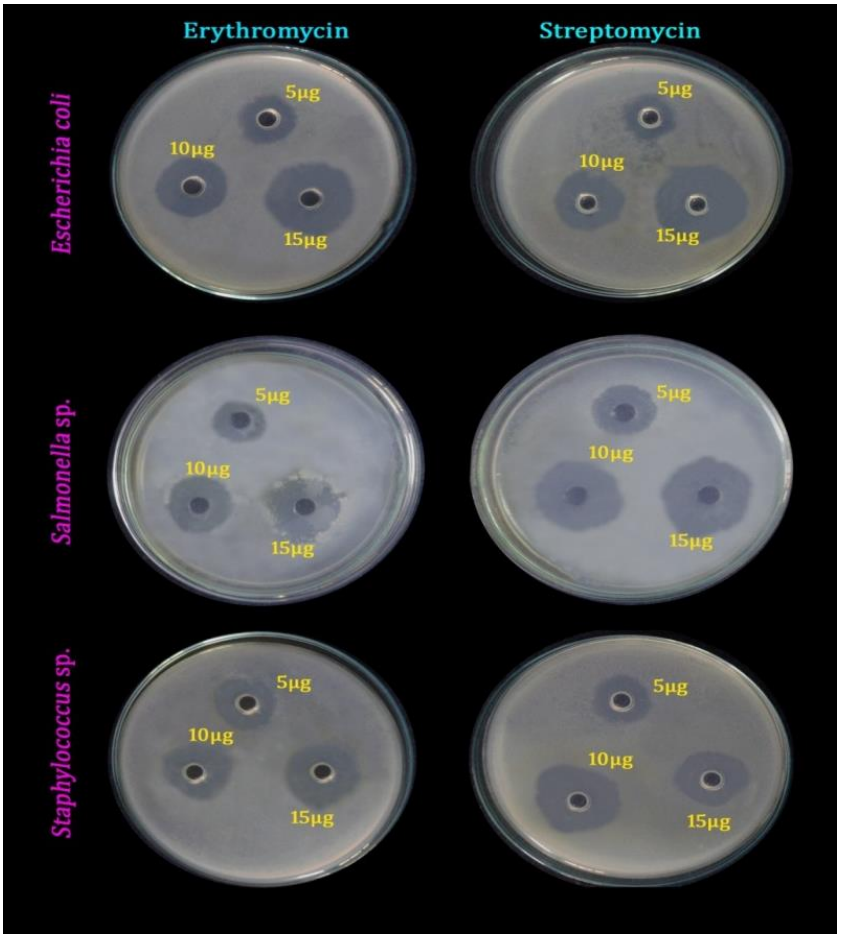


**Plate 1**: Antibiotic sensitivity testing of pathogens isolated form spoiled fish

The total numbers of CFUs were counted after incubation i.e., one day for bacteria, five days for fungi and seven days for actinomycetes. The microbial loads between the substrates and between the microbial groups were analyzed using One-way ANOVA method. Based on the higher density, the bacterial isolates alone were taken for screening studies for their metal tolerance. The sixteen predominant and morphologically distinct bacterial colonies grown on nutrient medium were isolated and pure cultured through repeated streaking on nutrient agar plates. All the sixteen bacterial isolates (Plate 1) were identified through morphological and biochemical characteristics using standard procedures (Apun *et al.,* 2000) [25]. The morphological characteristic studies include bacterial staining and motility test. The bacterial staining procedure was carried out using gram's staining kit to identify the gram nature of the organism. The motility test was performed by hanging drop method to determine the motility of the organism. The biochemical characteristic studies in cludeindole production, methyl red reaction, voges-proskauer reaction, catalase activity, starch hydrolysis, citrate utilization, urease reaction, glucose fermentation and gelatin liquefaction. The results of both morphological and biochemical studies of the sixteen bacterial strains were compared with Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994) [26] and thus identified all the sixteen bacterial isolates at genus level. The above identified bacterial colonies were study the antibiotic sensitivity test. The strains are following commercially available antibiotic discs used for the antimicrobial sensitivity studies. The test were carried out by disc diffusion method on Muller Hinton Agar medium (MHA) following the method of NCCLS, 1999. The spoiled fish sample was crushed and serially diluted with normal saline (85% Nacl) up to $10^{-9}$ dilutions. Sample (0.1ml) from $10^{-5}$ and $10^{-6}$ dilutions were spread on the nutrient agar plates and incubated at 37°C for 24 hrs. The three predominant colonies grown on nutrient agar medium were selected and maintained as pure cultures. All the three bacterial strains were identified through morphological and biochemical characteristics through Gram's staining and Motility Test, Indole Production, Methyl Red reaction, Voges Proskauver reaction, Citrate Utilization test, Urease test, Starch Hydrolysis, Catalase test. All the strains were authenticated by Bergey's manual of determinative bacteriology (Holt *et al.,* 1984). The identified strains were confirmed as *Escherichia coli*, *Salmonella* sp. and *Staphylococcus* sp. by the growth of strains in selective media viz., EMB agar, HE agar and MacConkey agar (Table 3).

## Discussion

The skin is one of the largest organs in fish and a primary barrier to the external environment (Noga 2010) [27]. For that reason, it plays an important role in the first immune defence mechanisms (Roberts 2012) [28]. Disruptions of the skin, such as erosions or ulcerations, are often correlated with loss of barrier functions and disease development (Sveen, Karlsen, *et al. 2020*) [29]. A wide variety of bacterial pathogens are also responsible for the major losses to the freshwater aquaculture. The details of common bacterial diseases are reported in aquaculture are present.In most cases, several types of bacteria were isolated from one individual. In this situation, determination of the causative agent of disease can be difficult without conducting a challenge test. The biodiversity of microorganisms inhabiting aquarium tanks in Lublin and infecting fish is high and includes many strains of bacteria. Many experiments using animal models have tested the relationship between bacteria and viruses over the course of infection. To cause disease, microorganisms must cross the mucosal barrier, penetrate the tissues, multiply in the tissues, inhibit the host's defenses, and damage the host's cells. In the case of fish diseases, studies that would determine the correlation between infection with viral and bacterial pathogens are limited, and there are no studies that would determine whether IPNV infection affects the pathogenicity of *Y. ruckeri*. Future studies should consider all skin conditions, so that potential infectious agents are considered as a community, and not in isolation, which may lead to mistaken inferences (Telfer *et al*. 2010) [30]. There is no available information about its pathogenicity for aquatic organisms; however, the genus is classified as a fish pathogen. The epidermal changes are the principle pathology observed but in addition, the absence of inflammation in the dermis and underlying tissues appears characteristic. If we assume an infectious aetiology, it is possible that mutation increased the infectivity (and pathogenicity) of the causative agent(s). The study primarily explains about prevalence of different fish diseases, host/size preference, and seasonality of infections that occurred during 2014-2018 mainly at different parts of eastern India in the major freshwater aquaculture farms. The results obtained seem to be a reflection of generalized picture for the whole country although it was beyond the scope of the present study in our laboratory. The collected/received samples analyzed during the study period gave a clear indication that parasites play critical role in aquaculture being the major share to the list of pathogens. It has been suggested that streptococcal infections in fish have become increasingly important because of overcrowding in farms and transport (Eldar. A*et al.,1995* and Kusuda. R 1991) [31, 32]. We do not believe that *S. iniae* has gone unrecognized in the greater Toronto area because of a failure of identification, since most hospitals routinely refer viridans streptococci isolated from sterile sites to a reference laboratory. Although isolates of *S. iniae* obtained from the surfaces of tilapia and other species of fish are genetically diverse, only two distinct, highly related clones (with PFGE patterns A and A') caused invasive disease. There have been similar findings with regard to other bacteria that cause infectious diseases (Musser JM 1996). [33] This suggests that a virulence factor or factors that are not present in all strains may be important for pathogenicity in humans and fish. Our surveillance for cellulitis associated with injuries during the handling of fish was not population-based, and we do not know the sensitivity of the reports made by the emergency departments during the survey period. Furthermore, current diagnostic tests are not adequate to define the bacterial cause of cellulitis when no cultures are obtained from a sterile site or such cultures are negative. Our data suggest that cellulitis may occur in association with injuries received during fish preparation, but they do not allow an estimate of frequency or of the proportion of cases that may be associated with a given pathogen. The development of suitable preventive and control measures, specific therapy for fish diseases assumes paramount significance, for the farmers to protect their crop against pathogens. The implementation of Better Management Practices (BMP) is most important to prevent frequent occurrence of disease and production loss in aquaculture. Further enhancement in knowledge about the disease process, host – pathogen and the environment interaction leading to disease occurrence, are very much essential for development of scientific methods of disease control (Subasingha, R.P., 2001) [34]. This needs due attention by the farmers for attaining of higher yields. Such infections may not have been recognized in the past as a cause of cellulitis, for several reasons. Cellulitis occurring after local injury or spontaneously is by far most often due to *S. pyogenes* or *Staphylococcus aureus*. Even if identification to the species level were performed with current commercial systems of identification, *S. iniae* would probably not be correctly identified, since it is not found in those data bases. Molecular information is extremely scarce in many cases, despite the importance of the diseases caused. Part of this problem is that in most cases, the researchers working with fish diseases by tradition are not molecular biologists, but veterinarians more interested in the treatment of acute conditions using traditional antimicrobial therapies. Vaccine development in the aquaculture industry is also not based on molecular targets and studies, but rather relies on heat-killed bacterial extracts for cost reasons. This is especially true for fish diseases with very broad and general symptoms that are hard to connect to a single pathogenic species or strain, such as streptococcosis, that is caused by diverse *Streptococcus* species depending on the host fish. A notable exception is *Mycobacterium marinum*, the causative agent of fish tuberculosis that has received a lot of attention because it infects the model species zebrafish and thus makes a formidable model system for the study of human tuberculosis disease mechanisms. Although antibiotics, brief salt dips (3%), formalin and increased levels of vitamins in the diet have been used, no specific effects were observed (Bruno, Noguera, and Poppe 2013; Maddocks*et al. 2015*) [35, 36]. Hence, further work is needed to determine the aetiology and develop effective control strategies to reduce the impact of this condition in rainbow trout aquaculture (Cano, Verner-Jeffreys, *et al. 2016*) [37].

## Conclusion

It is possible that the condition had resolved or that environmental conditions at the time of the visit, that is, water temperature, meant that the disease was subclinical. Economic losses from diseases are likely to increase as aquaculture expands and intensifies. The lack of awareness about fish diseases and reporting places and diagnostic laboratory, can lower the reporting of the fish diseases by the farmers.

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