

## Report about fish bacterial diseases

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# Report about fish bacterial diseases

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## About diagnostic laboratories for bacterial fish diseases

Almost all laboratories participating in the survey (51 out 54) from 14 different countries stated that they performed bacteriological studies.

Most reports about bacterial diseases, as has been seen for virus, come from finfish species produced intensively in Mediterranean countries, both from marine species (seabass, seabream and turbot, that represent about 22% of fish production) and from freshwater (trout, that represent 29% of the total fish production). Very scarce information was obtained on the diseases occurring in the main fish species produced in extensive and semi-intensive systems, i.e. tilapia, carp and mullet, that represent about 50% of the fish production in the region. It is here pointed out that only 1 laboratory from Egypt has participated in the survey.

As for all diseases or pathogens stated in the survey, the significance of a disease in a particular country can be overestimated by the high number of laboratories involved in fish diagnosis in the same country.

Discrepancies are noted between the diagnostic methods implemented in the surveillance efforts and the techniques available in the laboratories involved in the survey.

Although some of the commercial serological kits employed in the survey are appropriate for a rapid confirmative diagnosis of the diseases, they do not allow the serotypes to be distinguished. Therefore, the kits are not valid for epidemiological purposes.

Very scarce information is provided about the prevention measures implemented in the Mediterranean region and the efficacy of vaccination programmes. In fact, practically no data is available if the cases of mortality reported are from vaccinated or unvaccinated fish.

In the case of detection of diseases in several fish species, most of the laboratories report global data which makes it difficult to determine the incidence of the disease in a particular species.

## Main reported diseases

A total of 15 bacterial diseases are reported to be present in the area for the years 1998, 1999 and 2000 (Fig. 1).

The main diseases covered by the different laboratories (with reports from 6 or more countries and 14 or more laboratories) are vibriosis, pasteurellosis, enteric red mouth (ERM) disease, furunculosis and marine flexibacteriosis. As for vibriosis and pasteurellosis, in general, the highest incidence occurs in larval and juvenile stages of marine species, mainly seabass and seabream. ERM is stated as the most reported disease in trout farming in Mediterranean countries. The typical furunculosis is described in both fresh water fish (mainly cultured salmonids) and marine fish (mainly seabass, seabream and turbot). Marine flexibacteriosis is described in eleven marine fish species.

Other significant diseases, with lower reports are rainbow trout fry syndrome (RTFS), columnaris disease, motile *Aeromonas* septicemia, pseudomoniasis, streptococcosis, mycobacteriosis, epitheliocystis, and rainbow trout gastrointestinal syndrome (RTGS)

The less stated bacterial diseases in the survey are bacterial kidney disease (BKD), piscirickettsiosis, being reported in just 2 and 1 countries, respectively.

It is pointed out that within the OIE list of notifiable fish diseases there are no bacterial diseases. As for the OIE list of other significant diseases, only the presence of BKD and piscirickettsiosis was included. The other two diseases, ERM and furunculosis, are included in list III of the EU regulation.

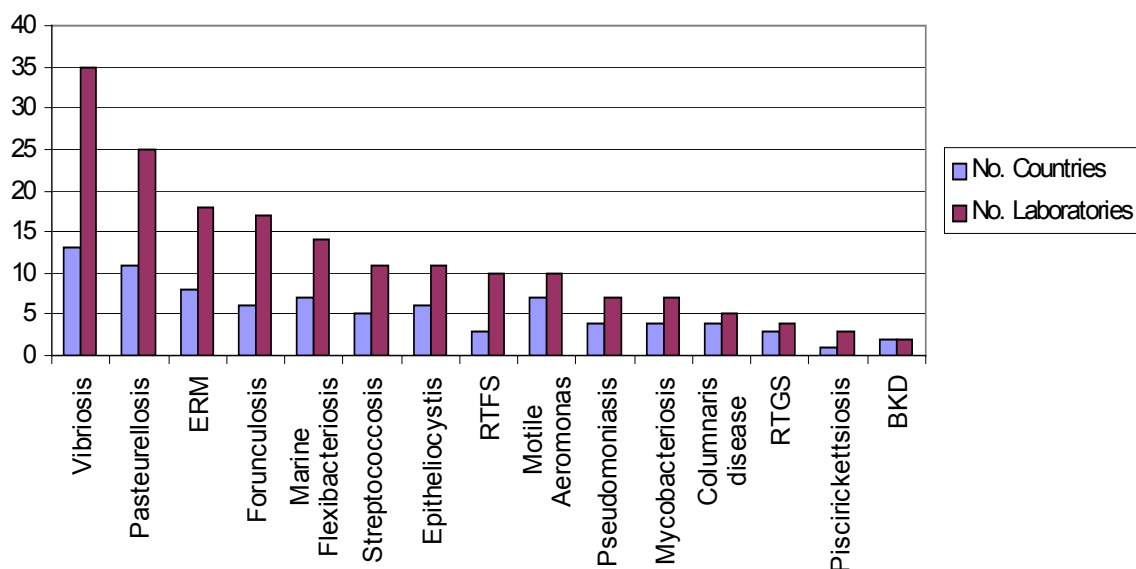


Fig. 1. Summary of reported bacterial diseases.

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## Vibriosis

Within the genus *Vibrio*, the species causing the most economically serious diseases in marine culture are *Vibrio anguillarum*, *V. ordalii*, *V. vulnificus* biotype 2 and *V. salmonicida*. However, vibriosis caused by *V. anguillarum* and *V. vulnificus* are the most significant diseases for the Mediterranean region.

*Vibrio (Listonella) anguillarum* possesses a wide distribution throughout the world causing a typical haemorrhagic septicaemia in a great variety of warm and cold water fish species of economic importance, including Pacific and Atlantic salmon, rainbow trout, turbot, seabass, seabream, striped bass, cod, Japanese and European eel, and ayu. Although up to a total of 23 O serotypes (O1-O23, European serotype designation) are known to occur among *V. anguillarum* isolates, only serotype O1, O2 and, to a lesser extent, serotype O3, have been associated with mortalities in farmed and feral fish throughout the world. The remaining serotypes are considered to be environmental strains and only on rare occasions are they isolated as the cause of vibriosis in fish. Whereas serotypes O1 and O2 have a wide distribution, serotype O3 affects mainly eel and ayu.

In contrast to serotype O1 which is antigenically homogeneous, serotypes O2 and O3 display antigenic heterogeneity showing the existence of two subgroups within each serotype named respectively O2 $\alpha$  and O2 $\beta$  and O3A and O3B. Interestingly, whereas subgroup O2 $\alpha$  occurs both in salmonid and non salmonid fish, subgroup O2 $\beta$  has only been detected in strictly marine fish. In the case of serotype O3, subgroup O3A is recovered from diseased fish and subgroup O3B comprises only environmental strains.

Genetic studies have also been performed to study the intraspecific variability within the major pathogenic serotypes of *V. anguillarum* (O1 and O2). Using ribotyping and pulsed-field gel electrophoresis (PFGE) the presence of two separated clonal lineages has been found within both serotypes corresponding respectively to the North European and South European isolates.

These serological and genetic studies possess a great epidemiological value in order to determine the possible origin of the *V. anguillarum* infections as well as to implement the adequate vaccination programmes in one particular country.

*Vibrio anguillarum* can be diagnosed on the basis of standard biochemical tests. However a serological confirmation employing specific polyclonal antisera is necessary. Although commercial

diagnostic kits based on slide agglutination or an ELISA test have been developed for a fast diagnosis of vibriosis, they do not distinguish between serotypes, and hence are not useful for epidemiological purposes. From 1989 several DNA probe-based detection protocols were developed but they were not specific and/or sensitive enough to be used in the diagnosis of vibriosis in the field. Only recently, a PCR-based approach was described for the accurate detection of *V. anguillarum* in infected fish tissues. The target gene was *rpoN*, a gene that codes for the sigma factor 54.

Although there are a great number of commercial vibriosis vaccines devised to be used mainly by bath or injection, most include only serotype O1 in their formulations or a mixture of serotypes O1 and O2 $\alpha$ . Only a bacterin developed by the University of Santiago (Spain) and marketed by Hipra Laboratories (Spain) (named GAVA-3) covers the three antigenic entities of *V. anguillarum* responsible for most epizootics (O1, O2 $\alpha$  and O2 $\beta$ ). Different polyvalent oil-adjuvanted vaccines including different combinations of *V. anguillarum* with other pathogens such as *Vibrio ordalii*, *Vibrio salmonicida*, *Aeromonas salmonicida*, and infectious pancreatic necrosis virus (IPN) are also available on the market to be used intraperitoneal (ip) in salmonids. In the case of strictly marine fishes such as turbot or seabass, *V. anguillarum* vaccines are being employed by bath in 1-2 g fish, two immersions being necessary in the vaccinal bath with a month's interval.

The species *Vibrio ordalii* has been established to accommodate strains formerly classified as *V. anguillarum* biotype 2. To our knowledge this species has never been documented in Europe, being described only in North America, Japan and Australia as affecting salmonids. In contrast to *V. anguillarum*, *V. ordalii* is antigenically homogeneous with no serotypes being detected.

*Vibrio salmonicida* is the aetiological agent of the "cold water diseases" or "Hitra diseases" which affects only salmonids and cod cultured in Canada and Nordic countries of Europe (mainly Norway and UK). As the name of the disease indicates, the pathogen only grows at temperatures below 15°C and in media supplemented with blood. This pathogen is biochemically and antigenically homogeneous, being a hydrophobic protein, called VS-P1, present in the surface layer, and being the dominant antigen in all the strains. Therefore, a confirmative serological identification of the pathogen based on the slide agglutination test using specific commercial polyclonal antiserum is usually employed for routine purposes. Despite the economic importance of this type of vibriosis in Nordic countries, to our knowledge no PCR-based approach has been developed for an accurate detection of the pathogen in the field.

As indicated above, salmonids in Nordic countries are systematically vaccinated with bacterins containing at least both *V. anguillarum* and *V. salmonicida*.

*Vibrio vulnificus* comprises two biotypes. Whereas biotype 1 is an opportunistic human pathogen causing disease generally associated with handling or ingestion of raw shellfish, biotype 2 is primarily an eel pathogen. However, this biotype 2 may also on some occasions cause infection in humans, representing a potential health hazard for fish farmers. Biotype 2 is biochemically homogeneous, indole production being the main trait which distinguishes both biotypes. Whereas biotype 1 is antigenically diverse, biotype 2 strains constitute a homogeneous O serogroup regardless of their geographic origin. This biotype is now considered a new serotype of *V. vulnificus* that is adapted to infecting eels and thus denominated serotype E. Therefore, to avoid possible misidentifications with strains of biotype 1, the confirmative identification of the eel pathogen *V. vulnificus* serotype E must be based on the use of agglutination tests using the specific antiserum. In addition, the use of a selective medium for *V. vulnificus* (VVM), recently designed, was proved useful for a preliminary differentiation of the eel pathogen in mixed bacterial populations. Genetic techniques such as ribotyping, randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) have also been described as powerful tools to discriminate eel-pathogenic strains from clinical and environmental isolates.

Several PCR-based methods for the diagnosis of this vibriosis have been developed using the 23S ribosomal gene (23S rDNA) or the cytolysin gene as target sequences of *V. vulnificus* allowing the successful detection of the pathogen in eel tissues, tank water and sediments.

Although until recently no commercial vaccines had been manufactured to prevent the vibriosis caused by *V. vulnificus*, a specific bacterin named Vulnivaccine, was developed by the University of

Valencia (Spain), which proved to be effective in field conditions. However, a triple exposure to the vaccine by prolonged immersion is needed to ensure an acceptable level of protection.

No type of vibriosis is considered by the OIE or the EU legislations.

## Current status based on answers received

Thirty-five laboratories corresponding to 13 countries (Croatia, Cyprus, Egypt, France, Greece, Israel, Italy, Malta, Morocco, Portugal, Spain, Tunisia and Turkey) are involved in the survey of vibriosis. Although the main causative agent reported is *V. anguillarum*, 18 laboratories do not specify the aetiological agent. Five laboratories do not perform biochemical identification and in 2 laboratories the description of the aetiological agent is made without identification procedures. In 18 laboratories agglutination tests (serology) are not employed to confirm the diagnosis or define the serotype of *V. anguillarum*, however, some of them claim that serological techniques are available in their laboratories. In fact, only 6 laboratories describe the most frequent serotype of *V. anguillarum* isolated. No molecular methods are employed in the diagnosis of vibriosis.

Routine surveillance of vibriosis is reported in only 8 laboratories corresponding to 4 countries (Spain, Greece, Italy and Croatia).

The disease is described in 12 fish species (*Dicentrarchus labrax*, *Sparus aurata*, *Anguilla anguilla*, *Dentex dentex*, *Solea* sp., *Scophthalmus maximus*, *Pagrus pagrus*, *Puntazzo puntazzo*, *Lethrinus pagellus*, *Mugil* sp., *Oncorhynchus mykiss* and *Salmo salar*), the species most affected being seabass and, to a lesser extent, gilthead seabream. The highest incidence of the disease caused by *V. anguillarum* corresponds to Turkey and Greece, which is in accordance with the highest production of gilthead seabream and seabass in these countries.

The specific eel pathogen *Vibrio vulnificus* is reported not only in *Anguilla anguilla* in 1 Spanish laboratory but also in 2 laboratories of Tunisia and Greece affecting seabass and seabream.

In general, the highest incidence of vibriosis occurs in larval and juvenile stages.

Only 7 laboratories give information about the use of vaccination programmes. With some exceptions, it seems that the *V. anguillarum* vaccines work properly because mortalities occur mainly in unvaccinated fish stocks.

## Interpretation comments

The precise incidence of vibriosis is difficult to evaluate because 10 laboratories do not describe the number of cases and, when the pathogen appears in both mortality and routine surveys, the majority of the laboratories give global figures meaning that it is not possible to determine the number of cases corresponding to each category.

The variability of the incidence of vibriosis in seabass among the laboratories within the same country is noteworthy. An example of this is the case of Greece, where very efficient vaccination programmes with a low number of cases are described in some laboratories, whereas a very high incidence with high mortality is reported in others.

Regarding the detection of vibriosis in several fish species most of the answers correspond to global data, which makes it difficult to determine the incidence of the disease in a particular species.

According to the answers related to the use of commercial serological kits (questionnaire B), some of the laboratories state that they use those from BIONOR (Norway). Although these kits are useful to confirm the diagnosis of *V. anguillarum*, they do not allow the identification of serotypes since the kits are based on a mixture of antisera against the three predominant serotypes (O1, O2 and O3).

Due to the absence of serological confirmation of vibriosis diagnosis in a high number of laboratories, misidentifications of *V. anguillarum* with *V. splendidus* biotype 1 is possible. Also, the reported isolation of *V. ordalii* in 1 laboratory is of doubtful credibility since this pathogen was not described as a causative agent of mortalities in Europe.

Although Italy is the main producer of the European eel, the laboratories of this country do not report the isolation of the specific eel pathogen *V. vulnificus*.

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## Pasteurellosis

Pasteurellosis, also currently described as photobacteriosis, is caused by the halophilic bacterium *Photobacterium damsela* subsp. *piscicida* (formerly *Pasteurella piscicida*), which was originally isolated from mortalities occurring in natural populations of white perch (*Morone americanus*) and striped bass (*M. saxatilis*) in 1963 in Chesapeake Bay. Since 1969, this disease has been one of the most important in Japan, affecting mainly yellowtail (*Seriola quinqueradiata*), and from 1990 it has caused economic losses in the marine culture of gilthead seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax*) and sole (*Solea* spp.) in the Mediterranean countries of Europe, and hybrid striped bass (*M. saxatilis* x *M. chrysops*) in the USA. Fish pasteurellosis was also known as pseudotuberculosis because it is characterised by the presence of white nodules in the internal viscera, particularly, spleen and kidney. Severe mortalities occur usually when water temperatures are above 18-20°C. Below this temperature, fish can harbour the pathogen as subclinical infection for long time periods.

Regardless of the geographic origin and source of isolation, all strains of this pathogen are biochemically and serologically homogeneous. However, DNA fingerprinting methods, such as ribotyping, AFLP and RAPD, proved to be valuable epidemiological tools since they allowed to detect two clear separate clonal lineages within *Ph. damsela* subsp. *piscicida*, the European and Japanese isolates.

Differences in susceptibility to pasteurellosis on the basis of fish age have been demonstrated in *Sparus aurata* as big fish are highly resistant to the infection. Histological observations and *in vitro* killing assays demonstrated that neutrophils and macrophages of bigger seabream efficiently phagocytise and kill the bacteria, while these cell types are not functional in small fish.

The presumptive identification of the pathogen is based on standard biochemical tests. In addition, although *Ph. damsela* subsp. *piscicida* is not included in the API-20 E code index, this miniaturised system can also be useful for a rapid presumptive diagnosis of the disease because all strains display the same profile (2005004). Slide agglutination test using specific antiserum is needed for a confirmative identification of the microorganism.

In the last years, the Norwegian company Bionor AS has marketed different kits based not only on direct bacterial agglutination (Mono-Pp) but also on ELISA tests (Aquarapid-Pp or Aquaeia-Pp), which allow a rapid detection of *Ph. damsela* subsp. *piscicida* in fish tissues. The evaluation of these ELISA kits in the field, demonstrated that the sensitivity of the Aquaeia-Pp (magnetic beads-EIA based method) was 100 to 1000 times higher than the standard ELISA kit (Aquarapid-Pp), which indicates its usefulness for the detection of asymptomatic carrier fish.



Although from 1997 a variety of DNA-based protocols have also been developed to attempt a fast and specific detection of the causative agent of pasteurellosis, only a multiplex PCR approach using as target the 16S ribosomal gene (16S rDNA) and *ureC* genes allowed the specific discrimination between *Ph. damselae* subsp. *piscicida* and *Ph. damselae* subsp. *damselae*.

The application of fast, specific and sensitive serological and molecular tools such as those based on ELISA or PCR is of crucial importance in the case of pasteurellosis since it has been demonstrated that the pathogen can be transmitted vertically through the ovaric and seminal fluids from the apparently healthy broodstocks and that this bacterium undergoes a viable -but non-culturable state which makes its detection very difficult in the farm environment.

In recent years several commercial vaccines against *Ph. damselae* subsp. *piscicida* have become available on the market but their efficacy is dependent on the fish species, fish size, vaccine formulation and use of immunostimulants. Only in the case of the toxoid enriched-whole-cell bacterin (DI vaccine) developed and patented by the University of Santiago (Spain) and marketed by Hipra Laboratories (Spain) was their effectiveness demonstrated in 50-day old gilthead seabream larvae. Therefore, bearing in mind that the majority of the pasteurellosis outbreaks occur from larval stages to fingerlings of 10-30 g, a vaccination programme which comprises a first dip immunisation at larval stage (average 0.05 g) and a booster vaccination by this Hipra vaccine when fish reach a size of about 1-2 g is encouragingly recommended to avoid the high economic losses caused by this disease.

Recently, different stable attenuated siderophore-deficient and *aro-A* deletion mutant strains have been constructed using an allelic replacement technique, which in experimental trials proved to be useful candidates as live vaccines.

This disease is neither included in the OIE international aquatic health code nor in the EU legislation.

## Current status based on received answers

Pasteurellosis caused by *Ph. damselae* subsp. *piscicida* is described in 25 laboratories corresponding to 11 countries (Croatia, Cyprus, France, Greece, Israel, Italy, Malta, Morocco, Portugal, Spain and Turkey).

The pathogen has been isolated in 9 fish species (*Dicentrarchus labrax*, *Sparus aurata*, *Dentex dentex*, *Pagrus pagrus*, *Pagrus major*, *Solea solea*, *Mugil cephalus*, *Lethrinus pagellus* and *Umbrina cirrosa*) the most susceptible of which are seabass and gilthead seabream. However, from the global data presented by most laboratories it is difficult to assess which of the 2 species is the most susceptible to pasteurellosis. Although the number of cases associated to mortality in these 2 species is very variable between the laboratories, even within the same country, the highest incidence of the disease corresponds to Greece.

In 12 laboratories serological confirmation of the diagnosis of pasteurellosis is not employed, although in practically all of them the biochemical identification of the pathogen is conducted.

Although in 6 laboratories corresponding to 4 countries (Spain, Italy, Croatia and Greece) the pathogen is isolated from routine surveys, only 1 laboratory in Spain reports the use of PCR and ELISA based methods to detect *Ph. damselae* subsp. *piscicida* in carrier fish including brood stocks.

Mortalities occur generally between 0.03 g and 40 g, although cases can appear in 100-200 g fish. No incidence of pasteurellosis is described in larger fish.

Only 2 laboratories (in Greece and Spain) provide information about the use of vaccination strategies, although only the Spanish laboratory indicates clearly that the majority of mortality cases correspond to unvaccinated fish.

## Interpretation comments

It is interesting that the susceptible fish age to pasteurellosis reported in the survey is in accordance with that reported in the scientific literature.

As reported in the scientific background of the disease, the application of specific and sensitive serological and molecular tools for the detection of *Ph. damsela* subsp. *piscicida* is crucial in the context of the sea farming. However, only 1 laboratory (in Spain) reports the use of ELISA kits and PCR-based methods to detect the presence of the pathogen in fish tissues. These sensitive procedures significantly improve its detection in healthy carrier fish including broodstocks, which is important to implement adequate measures to prevent the vertical and horizontal transmission of the disease in the farms. Then, the lack of the use of these methodologies in the surveillance of pasteurellosis can originate an underestimation of the presence of the aetiological agent of pasteurellosis in the Mediterranean countries.

From the information available about the prevention measures, it is not possible to draw a picture of the use of pasteurellosis vaccines in the Mediterranean region and their efficacy.

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## Enteric red mouth (ERM) disease

*Yersinia ruckeri* is the causal agent of yersiniosis or enteric red mouth (ERM) disease, which produces important economic losses in salmonid pisciculture all over the world. Moreover, sporadic isolations of this bacterium have also been documented in cultured non salmonid fish either in fresh- or seawater. *Yersinia ruckeri* was also recovered from wild fish, birds and mammals, which can act as potential vectors of the disease (Willumsen, 1989), and it has been demonstrated that this pathogen can persist in the environment (seawater and sediments) in a dormant but infective state.

The early signs of yersiniosis in the acute phase of infection are typical of many other Gram-negative bacterial haemorrhagic septicaemias. The reddening of the throat and mouth from which the disease received its name are commonly, but not invariably, present in the affected fish.

Classically, *Y. ruckeri* has been divided into two biotypes and five serovars. Biotype 1 corresponded with serovar I and included the non-sorbitol fermenting strains. Biotype 2 comprised the remaining serovars (II, III, V and VI) and contained the sorbitol-fermenting isolates. Further studies resulted in the acceptance of a new different serotyping scheme, consisting of four O serotypes: serotype O1 with two subgroups, O1a (former serovar I) and O1b (former serovar III); serotype O2 (former serovar II) with three subgroups O2a, O2b and O2c; and serotypes O3 and

O4, which correspond respectively to the former serovars V and VI in order to follow a logical chronological numerical order. However, for vaccination purposes, two groups (O1a and O2b) cause most epizootic outbreaks in cultured salmonids.

Plate culture is commonly employed to isolate *Y. ruckeri*, which is presumptively identified by conventional biochemical tests. Although commercial identification systems such as API-20E can be used, precautions must be taken since not all the possible generated profiles are included in the code index of the system. Even, misidentifications with *Hafnia alvei* can occur. Therefore, a confirmative serological identification of the isolates must be always be conducted to obtain an accurate diagnosis of the disease. Diagnostic commercial kits based on slide agglutination or on the ELISA test (Bionor Mono-Yr and Bionor Aquarapid-Yr, respectively) have been developed as they are useful for a fast diagnosis of ERM. However, they do not permit the distinction of serotypes and therefore lack epidemiological value.

Several molecular fingerprinting methods such as multilocus isoenzyme analysis, outer-membrane protein profiles, ribotyping, PFGE and interspersed sequences (IRS)-PCR, have been employed for epidemiological purposes allowing, in some cases, the differentiation of the *Y. ruckeri* isolates depending on the farm and/or season making it possible to follow the evolution of an outbreak.

Although two selective-differential media have been designed in order to simplify the isolation of *Y. ruckeri* from mixed populations usually present in water or tissues of carrier fish, these media lack enough sensitivity and/or specificity. To overcome these problems, PCR methodology based on the amplification of the 16S rRNA allowed the design of specific primers for a fast, sensitive and specific detection of the pathogen non only in fish tissues but also in non-lethal blood samples.

Although commercial ERM vaccines have been extensively used for decades with generally high efficacy, they do not eliminate the carrier state as the apparently healthy vaccinated fish is a vehicle through which ERM could be spread into non-endemic areas of the disease. Most of the commercial vaccines are based only on the Hagerman strain belonging to serotype O1a. However, it has been demonstrated that not all antigenic variants of *Y. ruckeri* can be effectively cross-protected by this serotype. Therefore, the inclusion of at least the predominant serovars (O1a and O2b) in the commercial vaccines is encouraged.

This disease is not listed in the OIE international aquatic health code but it is included in list III of the EU legislation.

## Current status based on answers received

Eighteen laboratories corresponding to 8 countries (Italy, France, Turkey, Spain, Portugal, Romania, Croatia and Greece) are involved in the survey of ERM. Although in all of them biochemical identification of the causative agent (*Y. ruckeri*) is accomplished, only in 9 laboratories is the identification confirmed by serological methods. However, only 4 report the serotype detected (serotype O1 seems to be the most predominant). Only in 2 laboratories (in Spain and France) are PCR-based methods employed in the diagnostics of ERM.

The disease is described only in 3 salmonid species including cultured (*Oncorhynchus mykiss*, *Salmo trutta* and *Salvelinus fontinalis*) and wild fish (*Salmo salar*). However, the significance of the ERM is only for rainbow trout. Only 1 French laboratory describes the isolation of *Y. ruckeri* from pike (*Esox lucius*).

*Yersinia ruckeri* is recovered in cases of routine monitoring in 6 laboratories corresponding to 5 countries (Spain, Italy, Portugal, Romania and Turkey).

Although based on the number of cases described, it seems that the highest incidence of ERM corresponds to Turkey and Spain, the poor answers of France and Italian laboratories makes it impossible to assess the true significance of the disease in these two important rainbow trout producers.

No data is provided about the most susceptible fish age to ERM.

Only 3 laboratories from 3 countries (Spain, France and Turkey) comment on the use of vaccines to prevent the disease, but no information is provided about whether the mortality cases correspond to vaccinated or unvaccinated stocks.

## Interpretation comments

Bearing in mind the answers related to the use of commercial serological kits (questionnaire B), some of the laboratories use the Mono-Yr (Bionor). Although this kit is useful for a rapid confirmation of the diagnosis of yersiniosis, it does not allow serotyping because the kit is based on a mixture of the three classical old serotypes I, II and III (now named O1a, O1b and O2).

As reported in the scientific literature, some *Y. ruckeri* profiles are not included in the database of the API-20E systems or some misidentification with *H. alvei* can occur. If this happens, an underestimation of the incidence of ERM can be produced if serological confirmation is not carried out.

Although it is widely known that commercial *Y. ruckeri* bacterins have been employed worldwide for several years, with general success, a general conclusion of the use of ERM vaccines and their efficacy in the Mediterranean region cannot be made, based on the scarce information provided in this survey.

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## Furunculosis

*Aeromonas salmonicida* subsp. *salmonicida* is the causative agent of the so-called "typical" furunculosis which causes economically devastating losses in cultivated salmonids in fresh and marine waters. It also affects a variety of non-salmonid fish, and shows a widespread distribution. The impact of the "typical" furunculosis may even become a limiting factor in the continued survival among certain threatened or endangered populations of fish, such as the Atlantic salmon. In fact, it has been demonstrated that Atlantic salmon harbour covert *A. salmonicida* infections when they return from ocean migrations. Typical furunculosis develops as a chronic or acute haemorrhagic septicaemia, often with extensive liquefactive necrosis. In the acute cases, deep ulcerative lesions usually appear. The "atypical" strains of *A. salmonicida* are included within 3 subspecies, *masoucida*, *achromogenes* and *smithia* and cause ulcerative diseases in a variety of fish species such as goldfish, carps, eels, marine flat fish and salmonids mainly in Europe and Japan.

Although *A. salmonicida* subsp. *salmonicida* can be isolated in conventional microbiological media, the appearance of the typical brown-pigmented colonies takes often more than 48 hours. For the primary recovery of fish tissues especially in the case of carrier fish, a pre-enrichment of the samples in culture broth is recommended. It has been demonstrated that the mucous is a useful site for a non-lethal detection of *A. salmonicida* from asymptomatic fish. To recover *A. salmonicida* from the mucous samples in which mixed bacterial population usually occurs, the use of the selective medium Coomassie Brilliant Blue (CBB) agar is recommended.

*Aeromonas salmonicida* subsp. *salmonicida* is biochemically and antigenically homogeneous with no biotypes or serotypes being detected, which simplifies the identification of the typical pigmented strains. Using sensitive DNA-based fingerprinting methodologies such as RAPD analysis, certain genetic heterogeneity can be evidenced but no correlation between the generated profiles and the country of origin or host species could be established. All typical *A. salmonicida* strains possess a consistent and distinctive pattern of three or four cryptic plasmid bands, which have been employed for confirmative identification of this pathogen as well as to design gene-probes or PCR-based methods for rapid diagnosis of furunculosis.

Although for several years it has been widely accepted that a correlation exists between virulence and the possession of a cell-surface protein array, the A-layer, this was further questioned by the isolation of virulent strains lacking this A-layer as well as avirulent strains which retain the A-layer. Now it is widely accepted that although cell-surface characteristics can play a role in the pathogenesis of furunculosis, they are not the sole virulent determinants of *A. salmonicida*.

The absence of an efficient selective media, the slow growth characteristics of this bacterium, the existence of a viable but not-culturable state as well as the possibility of vertical transmission of

this pathogen (Austin and Adams, 1996) support the need of culture-independent, molecular diagnosis protocols. Many DNA probes and PCR primers have been designed for a rapid and specific detection of *A. salmonicida* subsp. *salmonicida* in pure cultures and in fish tissues. Most of these molecular protocols are based on the use of plasmid sequences, A-layer or 16S rDNA as target genes. Although the highest specificity in the detection of *A. salmonicida* is obtained when the PCR assay is directed to the amplification of the surface A-layer gene (Gustafson *et al.*, 1992), precautions must be taken because some cross-reactions with motile *Aeromonas* species can occur.

Although many furunculosis vaccines have been developed and marketed from 1980, to be used by injection, immersion or orally (Newman, 1993; Midtlyng, 1997), their efficacy has been questioned because of the lack of repetitive results and/or the short protection period. The best results in terms of protection have been reported in salmonids with the mineral oil-adjuvanted vaccines. However, these bacterins had several adverse side effects such as the induced formation of granulomatous lesions adherent to the viscera and reduction in weight gain (Ellis, 1997). To avoid these drawbacks, new non-mineral oil adjuvanted vaccines have been recently developed and are now on the market. Polyvalent vaccines including different *Vibrio* species and *A. salmonicida* as antigens are also available for salmonids, which seems to be more effective than monovalent furunculosis bacterins. However, in the case of strictly marine fishes such as turbot, the protection covered by the furunculosis vaccines is very short (about 3 months) even by the ip route. Therefore, new vaccines and/or strategies are being investigated to improve the efficacy of the furunculosis immunisation in marine fishes.

Recombinant DNA technology allowed the construction of attenuated mutant strains, which were employed experimentally as safe live vaccines with great success, but their approval to be used in the field remains to be seen in the future.

This disease is not listed in the OIE international aquatic international health code but it is included in list III of the EU legislation.

## Current status based on answers received

Seventeen laboratories corresponding to 6 countries (France, Italy, Spain, Portugal, Romania and Turkey) are involved in the survey of furunculosis. Although identification is carried out in practically all the laboratories, no serological confirmation is carried out in 10. No DNA-based detection methods are used in the survey of furunculosis.

Although the majority of the reports should correspond to the typical furunculosis caused by *A. salmonicida* subsp. *salmonicida*, only 3 laboratories specify the aetiological agent.

The typical furunculosis is described in cultured salmonids (*Oncorhynchus mykiss*, *Salmo trutta* and *Salvelinus* spp.), and marine fish (*Sparus aurata*, *Dicentrarchus labrax* and *Scophthalmus maximus*), as well as in wild fish (*Salmo salar*, *Petromyzon marinus* and *Chondrostoma polylepis*). Unfortunately, from the global data presented it is not possible to define the most susceptible cultured species to furunculosis (salmonids or marine fishes). The isolation of atypical *A. salmonicida* strains is reported only by a French laboratory from *Anguilla anguilla*, *Esox lucius*, *Cyprinus carpio* and *Carassius auratus*.

In 6 laboratories from 4 countries the pathogen is also recovered from cases of routine survey.

The highest incidence of furunculosis seems to be in Italy, France and Spain. However, whereas Italy only reports cases of furunculosis in salmonids, in France and Spain both salmonid and marine fish are affected by the disease.

With the exception of turbot where it is indicated that all ages can be affected by furunculosis, no information is provided about the most susceptible age to this disease in other species.

Only 1 laboratory in France reports an efficient vaccination programme in marine fish.

## Interpretation comments

The incidence of furunculosis reported in cultured species is not in accordance with the production data. In fact, the lack of reports of furunculosis in rainbow trout in France and Turkey is surprising, as well as the absence of reports of this disease in marine fish (seabass and gilthead seabream) in Greece and Italy.

The characteristic slow growth of *A. salmonicida* which allow other species to overgrow, together with its proved vertical transmission makes the use of DNA-based detection methods very important for epidemiological studies of furunculosis. Therefore, the lack of use of molecular protocols in this survey, can originate an underestimation of the incidence of furunculosis in the Mediterranean region.

Although vaccines against *A. salmonicida* are widely employed over the world especially in salmonids, from the scarce information provided in this survey, a general conclusion of the use of furunculosis vaccines in the Mediterranean region cannot be made.

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## Marine flexibacteriosis

*Flexibacter maritimus* (formerly, *Cytophaga marina* and *Flexibacter marinus*) is the causative agent of flexibacteriosis in marine fish. Several other names, such as "gliding bacterial diseases of sea fish", "eroded mouth syndrome" and "black patch necrosis", were used to designate the disease caused by this bacterium. It is important to report that on the basis of recent phylogenetic, chemotaxonomic and phenotypic studies it was proposed that *Flexibacter maritimus* should be transferred to the new genus *Tenacibaculum*, as *Tenacibaculum maritimum*.

Marine flexibacteriosis is widely distributed in cultured and wild fish in Europe, Japan and USA. In Europe, the disease has been reported in sole (*Solea solea* and *S. senegalensis*), seabass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*) and coho salmo (*Oncorhynchus kisutch*). In Japan, *F. maritimus* has been isolated from red sea bream (*Pagrus major*), black sea bream (*Acanthopagrus schlegeli*) and flounder (*Paralichthys olivaceus*). In USA, marine flexibacteriosis has been described in white seabass (*Atractoscion nobilis*), Pacific sardine (*Sardinops sagax*) and northern anchovy (*Engraulis mordax*).

Although both adults and juveniles may be affected by marine flexibacteriosis, younger fish suffer a more severe form of the disease. An increased prevalence and severity of the disease has been reported at higher temperatures (above 15°C). In addition to water temperature, the disease is influenced by a multiplicity of environmental (stress) and host-related factors (skin surface condition). In general, the affected fish have eroded and hemorrhagic mouth, ulcerative skin lesions, frayed fins, and tail rot. A systemic disease can be also established involving different internal organs. The loss of epithelial fish surface, typical of this disease, is also a portal of entry for other secondary bacterial or parasitic pathogens.

The clinical signs, along with the microscopical observation of accumulations of very long rods in wet mounts or Gram-stained preparations obtained from gills or lesions, can be used as an initial step for the presumptive diagnosis of marine flexibacteriosis. This preliminary diagnosis must be supported by isolation of the pathogen in the appropriate medium or by the use of specific molecular DNA-based methods applied directly to fish tissues. This bacterium only grows in specific media since it needs an absolute requirement of seawater as well as low concentration of nutrients. Although several media (i.e. Anacker & Ordal, Marine Agar, FMM) have been devised to isolate *F. maritimus*, the FMM medium proved to be the most effective for the recovery of this pathogen from fish tissues. Typical colonies of *F. maritimus* are pale-yellow, flat with uneven edges. Although the bacterium is biochemically homogeneous at least two major O serotypes can be detected which seem to be related to the host species.

One of the major problems in the study of this bacterium is the difficulty of distinguishing it from other phylogenetically and phenotypically similar species, particularly those of the genera *Flavobacterium* and *Cytophaga*. Therefore, the application of the PCR methodology is very important for an accurate identification of the pathogen. Different PCR protocols have been published using the 16S rRNA gene as target, which demonstrated its efficacy in field conditions.

Although until recently no vaccines were available to prevent the disease (Bernardet, 1997), a flexibacteriosis vaccine ("FM-95") patented by the University of Santiago (Spain) and commercialized by the Hipra Veterinary Laboratory (Spain) is the only bacterin currently on the market to prevent mortalities caused by *F. maritimus* in turbot. Divalent formulations to simultaneously prevent flexibacteriosis and vibriosis or flexibacteriosis and streptococcosis are also available.

Marine flexibacteriosis is neither listed in the OIE nor in the EU legislation.

## Current status based on answers received

Fourteen laboratories corresponding to 7 countries (Cyprus, Spain, France, Greece, Malta, Croatia and Turkey) are involved in the survey of flexibacteriosis. In 4 laboratories the diagnosis is based only on clinical signs, microscopic observation of fresh smears and/or histopathology but bacterial isolation is not attempted. Only in 4 laboratories from 4 countries (Spain, Greece, Croatia and Malta) biochemical identification of the causative organism is achieved. However, the serological and PCR confirmation of the assignation of the isolated bacteria to the species *F. maritimus* has only been conducted in 1 laboratory (in Spain).

The disease is described in 11 species of marine fish (*Dentex dentex*, *Dicentrarchus labrax*, *Scophthalmus maximus*, *Solea senegalensis*, *Solea solea*, *Sparus aurata*, *Lethrinus pagellus*, *Mugil cephalus*, *Pagrus pagrus*, *Puntazzo puntazzo* and *Umbrina cirrosa*). However, considering the species of high commercial value in the Mediterranean countries, the disease has an important significance for seabass and gilthead seabream cultured in all the countries involved in the survey and in turbot cultured in Spain. Among the other species, *Solea senegalensis*, *Solea solea* and *Umbrina cirrosa* are described as very susceptible fish to marine flexibacteriosis.

In 5 laboratories from 4 countries (Cyprus, Spain, France and Greece) the disease is reported also in routine cases.

The highest incidence of the disease occurs in the small fish, associated in some cases with stress induced by handling, transport, high densities, external injuries caused by parasites or cannibalism.

## Interpretation comments

Based on the answers of the survey, the correct incidence of marine flexibacteriosis in the Mediterranean region caused specifically by *F. maritimus* cannot be properly evaluated and, therefore, its significance can be underestimated or overestimated. Some of the reasons are indicated below:

(i) The diagnosis is conducted in some laboratories without bacterial isolation or without bacterial identification procedures in others.

(ii) As cited in the scientific background of the disease, using only the specific medium for *F. maritimus* FMM, the recovery of the pathogen is efficient. If the laboratories involved in the survey employed other classical media reported in the literature, the recovery of *F. maritimus* from diseased fish is low.

(iii) The difficulty to recognise the colonies of the pathogen among the mixed population that usually overgrow in the plates because of the slow growth characteristics of *F. maritimus*. In addition, there are several filamentous bacteria belonging to other *Flexibacter*, *Cytophaga* or *Flavobacterium* species, which also produce pigmented colonies and, therefore, can be misidentified as *F. maritimus* if the appropriate identification is not performed.

(iv) Only in 1 laboratory (in Spain) has serological and PCR confirmation of the diagnosis been conducted. The application of these methodologies in the survey of *F. maritimus* is of crucial importance because of the existence of other phenotypically similar gliding bacteria which cannot be easily differentiated based only on biochemical tests.

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## Cold water disease or rainbow trout fry syndrome (RTFS)

*Flavobacterium psychrophilum* (syn. *Cytophaga psychrophila* and *Flexibacter psychrophilus*) has been known as the causative agent of bacterial cold-water disease (BCWD) or peduncle disease in salmonids since 1948. The same bacterium has been shown to be the agent involved in the rainbow trout fry syndrome (RTFS) since the 1980s. The disease has been reported in USA, Europe, Japan, Tasmania and Chile. Although farmed salmonids (especially coho salmon and rainbow trout) reared in fresh water are particularly susceptible, the pathogen has been isolated from non salmonid fish such as eels and cyprinids in Europe, and ayu in Japan.

The disease usually occurs in very young fish in which the pathogen provokes an acute septicaemia with spleen hypertrophy. Some fish display abdominal swelling and become abnormally dark. In fingerlings, external lesions may also appear. The severity of the disease occurs typically when water temperatures are between 4 and 13°C.

Although there is some evidence of horizontal transfer of the *F. psychrophilum*, direct vertical transmission of the pathogen from infected brood fish to the offspring has been demonstrated. In fact, imported infected eggs from the USA have been responsible for the outbreaks of BCWD in coho salmon hatcheries in Japan.

To isolate the pathogen from infected fish, the use of specific oligotrophic media based on modifications of the *Cytophaga* agar formulation is necessary. Colonies are bright yellow with a thin spreading margin. Deeply coloured nonspreading variants with an entire edge have also been reported. Identification of *F. psychrophilum* is generally based on the studies of its phenotypical and serological characteristics. Although this microorganism is a biochemically homogeneous pathogen, the identification is usually delayed or impeded by its rather fastidious growth and weak reactivity in most biochemical tests. In addition, although classically three main serotypes were defined, several minor intermediate serogroups were also shown to exist in this bacterium but no correlation was apparent between serotypes and geographical origin of strains, the species of host fish, or the virulence of the isolates. Therefore, although slide agglutination, immunofluorescence and ELISA tests applied to the presumptive isolated colonies or directly to the infected spleen tissues can be employed for a confirmative diagnosis of the disease, they require the preparation of specific antisera against the main pathogenic serotypes.

Because the cited conventional methods are relatively low in sensitivity or time-consuming for the detection of *F. psychrophilum* especially in carrier fish, PCR methodology based on the amplification of 16S rDNA segments has become the fastest, most specific and sensitive technique for the successful identification of the pathogen not only in infected tissues and fish eggs but also in wild fish and water samples. Because it has been reported that some of these methods can amplify non-specific PCR products under certain conditions, a multiplex PCR based on the combined use of 16S rDNA based primers with *gyrB* based primers has been recently described as a more reliable and accurate assay than the PCR using only a primer set.

Several DNA fingerprinting methods such as ribotyping and RAPD proved to be very useful epidemiological tools for a rapid differentiation of the *F. psychrophilum* isolates since they allowed to detect distinct genetic profiles clearly associated with the fish species from which the strains were isolated.

Few vaccination attempts have been published to prevent the disease caused by *F. psychrophilum*. This was due, in part, by the difficulties in culturing this gliding bacterium as well as by the lack of an experimental challenge model resulting in well-controlled and quantitatively reproducible effects. Recent vaccination experiments performed in rainbow trout demonstrated that only significant protection is achieved using oil adjuvanted ip vaccines. However, this route is impracticable for the early-live fish stages in which these diseases usually occur. In addition, no cross protection among serotypes is evidenced. Therefore, it is important to consider the inclusion in the RTFS vaccines of all the serotypes of *F. psychrophilum* occurring in a particular geographical area. Although no commercial vaccines against this disease are available, some countries are using autogenous bacterins made from single farm isolates.

The RTFS is not included in the OIE or EU legislations.

## Current status based on answers received

RTFS caused by *F. psychrophilum* is reported in 10 laboratories corresponding to only 3 countries (Spain, France and Turkey). The diagnosis in 4 laboratories is based only on clinical signs and histopathology. Although in the remaining 6 laboratories bacterial isolation is conducted, biochemical identification is reported only in three of them. Only in 1 laboratory (in Spain) are serological and PCR methods employed in the diagnosis of the disease.

The disease is described mainly in rainbow trout (*Oncorhynchus mykiss*) and, on the basis of the number of reported cases, the highest incidence of RTFS corresponds to Spain and Turkey. Only in 2 laboratories of France is the disease described also in *Anguilla anguilla* and *Salvelinus maycush*. All the RTFS reports are associated to mortality cases. No information is provided about the use of vaccines.

## Interpretation comments

The lack of reports of RTFS in Italy is surprising, despite its high production of rainbow trout.

The difficulty to recover *F. psychrophilum* in plates, even using specific media, together with the scarce use in this survey of identification procedures makes it possible that the incidence of this disease was underestimated.

Because of the demonstrated vertical transmission of *F. psychrophilum*, the use of PCR based methods is of crucial importance for the detection of this microorganism especially in routine surveys of broodstocks.

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## Columnaris disease or saddleback disease

"Columnaris" disease is caused by the chromogenic gliding bacterium *Flavobacterium columnare* (syn. *Chondrococcus columnaris*, *Cytophaga columnaris*, *Flexibacter columnaris*). This disease exists worldwide in fresh and brackish waters especially in America, Europe and Asia and affects mainly ictalurids, eels, salmonids, cyprinids, centrarchids and ornamental fish such as golden shiner and goldfish.

Columnaris disease usually occurs when the water temperature exceeds 15°C. The clinical signs and lesions are usually restricted to the body surface causing skin erosions and gill necrosis. Characteristic "saddleback" lesions can be found in advanced cases especially in catfish. A systemic infection may occur in severe cases, depending on the virulence of the strains. The disease can be easily complicated by dual infections in which other bacterial or protozoan parasites can be involved.

A preliminary, presumptive diagnosis can be based on the observation of long slender, flexing rods forming "hay stacks" or columns in wet mount preparations made from lesions. The microorganism grows poorly in conventional media, the use of oligotrophic media being preferable. Typical colonies of *F. columnare* are yellow, flat, thin, rhizoid and adhere tightly to the agar. Columnaris diseases can be confirmed by biochemical tests together with serological assays like slide agglutination using specific antisera or by the direct fluorescent antibody test. Although this pathogen is biochemically homogeneous, the strains are not antigenically identical since four major serological groups and several minor ones were evidenced by reciprocal absorption, which can complicate the serological typing. At the genomic level, an intra-species variation was shown among strains based on RFLP analysis of the 16S rDNA, and three distinct genomic groups were established, which may be of epidemiological value.

PCR methodology targeted to the 16S rDNA has been developed for a fast confirmative identification of the microorganism avoiding a possible misidentification with other chromogenic microorganisms of the gliding bacterial group. However, this molecular approach has not been evaluated in the detection of the bacterium in fish tissues, which is necessary for the diagnosis of the diseases in the field.

Several vaccination experiments against *F. columnare* have been performed on several fish species using different routes of administration (injection, bath, oral) but the results in the field trials were inconsistent maybe due to the intimate association of stress with the disease process. No commercial vaccines are available.

Columnaris disease is not included in the OIE or in the EU legislation.

## Current status based on answers received

Only 5 laboratories from 4 countries (Egypt, Greece, France and Spain) reported the diagnosis of columnaris or saddleback disease caused by *F. columnare* affecting tilapia (*Oreochromis* sp.), seabass (*Dicentrarchus labrax*), salmonids (*Oncorhynchus mykiss* and *Salvelinus mamaycush*) and ornamental fish. No biochemical identification was conducted in these laboratories.

By the data reported, it seems that the disease does not represent any threatening problem in the Mediterranean region. Although the incidence in tilapia cultured in Egypt is elevated, all the cases reported corresponded to routine survey.

## Interpretation comments

The assignation of *F. columnare* as the aetiological agent of the saddleback disease is not clear since no identification procedure was performed, the diagnosis being based in some cases only on external signs and histopathology.

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## Motile *Aeromonas* septicaemia

Motile *Aeromonas* of the *Aeromonas hydrophila* complex cause a haemorrhagic septicaemia in numerous species of cultured and wild freshwater fish such as rainbow trout, brown trout, coho salmon, eels, carp, channel catfish, tilapia, ayu and goldfish. Although classically 3 species, *A. hydrophila*, *A. sobria* and *A. caviae*, were included within the motile *Aeromonas*, further taxonomic data including genetic studies allowed the identification of at least 10 new motile *Aeromonas* species. However, still *A. hydrophila* is regarded as the predominant fish pathogen, although its importance may have been overestimated in the past. Isolates differ greatly in their pathogenicity with some strains being highly virulent and others non-virulent.

Although motile *Aeromonas* species are typically recognised as opportunistic pathogens or secondary invaders, cases have been reported of *A. hydrophila* acting as a primary fish pathogen. In fact, *A. hydrophila* is widely distributed in the intestinal tract of fish as well as in the water and sediment of freshwater ponds, which are rich in organic matter. Virulent strains of *A. hydrophila* in these environments are a possible source of infection.

Outbreaks of *Aeromonas* septicaemia are usually associated with a change in environmental conditions. Stressors, including overcrowding, high temperature, a sudden change of temperature, handling, transfer of fish, low dissolved oxygen, poor nutritional status and fungal or parasitic damage of the epidermis, contribute to physiological changes and heighten susceptibility to infection. Motile *Aeromonas* species are transmitted horizontally but not vertically.

Diseased fish usually display cutaneous haemorrhages of the fins and trunk, and the condition is often referred to as "red fin disease". In the case of European carp infected with *A. hydrophila* fish show severe tail and fin rot and visible haemorrhaging and ulceration of the body surface.

Motile *Aeromonas* can be easily recovered in conventional purpose media. In addition, numerous selective media have been developed for the isolation and presumptive identification of *A. hydrophila* in mixed populations, but the majority of them lacked enough specificity and/or sensitivity (Joseph and Carnahan, 1994). Although commercial systems such as API systems are used widely for identification of motile *Aeromonas*, some biochemical characteristics must be backed up by standardised tests in order to obtain an accurate species identification (Toranzo *et al.*, 1986). Serological methods such as slide agglutination, immunofluorescence or ELISA are of limited value to confirm the diagnosis of the disease because almost 100 serotypes have been reported to exist within the motile *Aeromonas* group. However, DNA fingerprinting methods such as ribotyping, pulse field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), and amplified length polymorphism (AFLP) proved to be valuable high-resolution genotype tools for classification of *Aeromonas* species.

The development of DNA-based technology for the rapid diagnosis of motile *Aeromonas* septicaemia has been impaired for the elevated number of common DNA fragments between motile *Aeromonas* and *A. salmonicida*. However, some PCR assay using 16S rDNA targeted primers have been published for the specific identification of *A. hydrophila*.

Although experimental vaccination to prevent infections by *A. hydrophila* in different fish species has been examined, the development of an appropriate commercial vaccine is hampered by the great phenotypic and serological heterogeneity existing within the group of mesophilic motile *Aeromonas* species. Prophylactic measures such as good hygiene, avoidance of overcrowding and excessive handling are the best methods of prevention.

Motile *Aeromonas* septicaemia is not considered a significant disease by the OIE or in the EU legislations.

## Current status based on answers received

Bacterial septicaemia associated to motile *Aeromonas* is reported in 10 laboratories belonging to 7 countries (Egypt, Greece, Italy, Portugal, Spain, Tunisia and Croatia). Although the presence of motile *Aeromonas* is described in 10 fish species (*Oreochromis* sp., *Acipenser* sp., *Anguilla anguilla*, *Cyprinus carpio*, *Barbus barbus*, *Dicentrarchus labrax*, *Mugil* sp., *Liza* sp., *Oncorhynchus*



*mykiss* and *Carassius auratus*), the highest incidence seems to correspond to tilapia (*Oreochromis* sp.) and carp (*Cyprinus carpio*) cultured in Egypt but only associated to routine survey. However, reports in carps associated to motile *Aeromonas* are also described in 4 laboratories from other countries such as Croatia, Italy and Greece.

In all the laboratories biochemical identification of bacteria is conducted but no serological or molecular methods are performed. The assignation of the diseases to a particular species (*A. hydrophila* or *A. sobria*) is only described in 4 laboratories

## Interpretation comments

The precise assignation of the disease to a particular species such as *Aeromonas hydrophila* is not always easy because the motile *Aeromonas* group includes a high number of phenotypically related species which are difficult to differentiate on the basis of only using standard biochemical tests.

Although Israel is the second producer of tilapia and carp, no reports of the incidence of this septicemia were provided in this country.

When the disease affects several species, some laboratories give only global data and, therefore, the incidence in a particular species is difficult to evaluate.

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## **Pseudomonadiosis (*Pseudomonas anguilliseptica*)**

Among the *Pseudomonas* species recovered from diseased fish (*P. chlororaphis*, *P. anguilliseptica*, *P. fluorescens*, *P. putida*, *P. plecoglossicida*), *Pseudomonas anguilliseptica* is considered the most significant pathogen for cultured fish.

*Pseudomonas anguilliseptica* was originally described in 1972 as the aetiological agent of "Sekiten-bio" or "red spot disease", which caused massive mortalities in pond-cultured Japanese eel (*Anguilla japonica*) in Japan. From this year onwards, this bacterium was recorded in European eel (*Anguilla anguilla*) reared in Taiwan, Scotland and Denmark. The pathogen was subsequently isolated from other fish species such as black sea bream (*Acanthopagrus schlegeli*) and ayu (*Plecoglossus altivelis*) in Japan, salmonids in Finland, wild herring in the Baltic sea, and from 1995 was considered as responsible agent of the "winter diseases syndrome" characteristic of gilthead seabream (*Sparus aurata*) cultured in the Mediterranean area. Very recently, *P. anguilliseptica* was also recovered as an emerging pathogen of turbot (*Scophthalmus maximus*) cultured in Spain.

The disease occurs at low temperatures (below 16°C) during the winter months. The main clinical signs of the fish affected by this septicaemia are abdominal distension and hemorrhagic petechia in the skin and internal organs, but the lesions in eels are always more severe than those observed in gilthead seabream.

*Pseudomonas anguilliseptica* grows very slowly and weakly in conventional media, its growth being favoured in blood agar. In addition, the brain is the recommended organ to recover this pathogen in pure culture from diseased and carrier fish. *Pseudomonas anguilliseptica* seems to be a biochemically homogeneous pathogen regardless of the source of isolation. However, regarding the serological characteristics, recent studies indicated the existence of two major O serotypes related to the fish host, one characteristic of the eel isolates and another typical of the gilthead seabream and turbot isolates. In addition, genetic characterisation studies employing RAPD techniques revealed the presence of two genetic groups, which were coincident with the two serological groups. All this information is very useful in order to develop an adequate vaccine against this disease.

Two PCR protocols, based on the amplification of the 16S rRNA gene, have been recently described for a rapid identification of *P. anguilliseptica*. However, only one of them was shown to be sensitive enough to the direct detection of the pathogen in the fish tissues and has become a powerful tool for the diagnosis of fish pseudomonadiosis under field conditions.

Recent research efforts by the group of the University of Santiago, in collaboration with Hipra Veterinary Laboratory (Spain) led to the development of aqueous and non-mineral oil-adjuvanted bacterins (including the two major serotypes detected) which proved to be effective in experimental trials in gilthead seabream and turbot (unpublished results).

No diseases caused by *Pseudomonas* species are included in the OIE or in the EU legislation.

### **Current status based on answers received**

Although the disease is reported in 7 laboratories from 4 countries (Greece, Turkey, France and Portugal) affecting 5 fish species (*Anguilla anguilla*, *Dicentrarchus labrax*, *Sparus aurata*, *Puntazzo puntazzo* and *Oncorhynchus mykiss*), only Turkey describes *P. anguilliseptica* as the species responsible for the disease in gilthead seabream and seabass. In other countries the identification remains at the genus level.

No serological or PCR based methods are employed in the diagnosis of the disease. However biochemical tests are conducted in all the laboratories.

## Interpretation comments

The characteristic slow growth of *P. anguilliseptica* which allow other species to overgrow may be the cause of the scarce reports describing the isolation of this pathogen. In addition, on the basis of only biochemical tests, the assignation of *Pseudomonas* sp. to the species *P. anguilliseptica* cannot be made properly if serological and/or PCR confirmation is not performed. Therefore, based on the answers received, a conclusion of the importance of the disease caused by *P. anguilliseptica* in the Mediterranean region cannot be drawn because in most of the laboratories the *Pseudomonas* species are not identified at species level.

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## Streptococcosis

Streptococcal infection of fish is considered a re-emerging pathology affecting a variety of wild and cultured fish throughout the world (Kitao, 1993; Bercovier *et al.*, 1997; Romalde and Toranzo, 1999, 2002). Classification of Gram-positive cocci based on DNA-DNA hybridisation coupled with 16S sequencing has shown that at least 5 different defined species are considered of significance as fish pathogens: *Lactococcus garvieae* (syn. *Enterococcus seriolicida*), *Streptococcus iniae* (syn. *S. shiloi*), *Streptococcus agalactiae* (syn. *S. difficile*), *Streptococcus parauberis* and *Vagococcus salmoninarum*. Therefore, streptococcosis of fish should be regarded as a complex of similar diseases caused by different genera and species capable of inducing central nervous damage characterised by suppurative exophthalmia ("pop-eye") and meningoencephalitis. Whereas "warm water" streptococcosis (causing mortalities at temperatures above 15°C) typically involves *L. garvieae*, *S. iniae*, *S. agalactiae* and *S. parauberis*, "cold water" streptococcosis (occurring at temperatures below 15°C) is caused by *V. salmoninarum*. It is important to report that the aetiological agents of "warm water" streptococcosis are considered also as potential zoonotic agents capable of causing disease in humans.

*Lactococcus garvieae* is capable of infecting saltwater fish species such as yellow tail in Japan and fresh water species like rainbow trout mainly in Italy, Spain and France and, to a lesser extent, in UK and Australia.

*Streptococcus iniae* is the main aetiological agent of streptococcosis in tilapia hybrids in USA and Israel and rainbow trout in Israel. Also, it was isolated from marine fish as yellow tail and flounder (*Paralichthys olivaceus*) in Japan, European seabass and red drum (*Sciaenops ocellatus*) in Israel, and barramundi (*Latex calcarifer*) in Australia.

*Streptococcus agalactiae* affects mainly tilapines and striped mullet (*Mugil cephalus*) cultured in Israel, although it was also isolated from yellowtail in Japan.

*Streptococcus parauberis* is endemic in turbot (*Scophthalmus maximus*) cultured in Spain. *Vagococcus salmoninarum* was recovered from diseased salmonid fish (rainbow trout, brown trout and Atlantic salmon) in France and Australia.

Gram-positive cocci can be isolated in general-purpose media but growth is enhanced in blood agar, the brain being the organ of choice to recover the cocci in pure culture from infected fish. Biochemical characterisation can be accomplished by traditional tube and plate procedures as well as by using commercial miniaturised systems. Although some of them, especially the API-32 Strep, proved to be useful for a fast presumptive identification of some of the aetiological agents of streptococcosis, misidentification of *L. garvieae* with *L. lactis* subsp. *lactis*, or *S. iniae* with *S. uberis* can occur. It should be emphasised that identification to species level remains difficult based only on phenotypic traits. Therefore, serological confirmation must be performed by slide agglutination test or fluorescent antibody procedures using the appropriate specific antisera. In the case of *L. garvieae* the existence of two serotypes associated with the presence (serotype KG<sup>-</sup>) or absence (KG<sup>+</sup>G) of a capsule were observed.

In recent years molecular techniques such as ribotyping, RAPD and PFGE have been usefully applied in epidemiological studies to study the heterogeneity within some of the aetiological agents of fish streptococcosis. With regard to *S. iniae*, although no differences between tilapia and rainbow trout isolates were found by ribotyping, this technique allowed the American and Israeli fish strains to be demonstrated, showing a lack of epidemiological links between infections in the 2 countries. In the case of *L. garvieae*, the RAPD and PFGE methods were able to differentiate distinct genogroups closely related with the host of origin (rainbow trout, yellow tail and cat fish) and, in addition, within the rainbow trout strains it was possible to evidence the existence of three genetically distinct clones associated to the geographical origin of the isolates. Regarding *S. parauberis* isolated from turbot in Spain, whilst all the strains exhibited the same ribopattern, the RAPD analysis allowed isolates to be discriminated on the basis of their farm of origin.

With respect to the application of molecular techniques to the diagnosis of fish streptococcosis, only two aetiological agents have received attention, *L. garvieae* and *S. iniae* (Romalde and Toranzo, 2002). To our knowledge, only two PCR-based protocols have been published for each of these species. Among them, the techniques based on amplification of 16S rDNA seem to be of choice as a standard method for diagnosis of these Gram-positive cocci.

Several attempts have been made to develop appropriate vaccination programmes for fish streptococcosis. However, considerable variability in the protection was observed depending on the fish and bacterial species, the route of administration, the age of the fish, as well as the use of immunostimulants. All the streptococcosis vaccines rendered good levels of protection only when they were administered by intraperitoneal injection. However, whereas *L. garvieae* and *S. iniae* experimental vaccines conferred protection in rainbow trout for only 2-3 months and 4-6 months respectively, the *S. parauberis* bacterin displayed high levels of the protection in turbot for more than two years.

Precautions must be taken in the antigenic formulation of rainbow trout lactococcosis vaccines because several failures have recently been reported in both licensed and autogenous vaccines (which caused heavy losses in the farms). The antigenic composition of these bacterins corresponded to avirulent non-capsulated strains of *L. garvieae*, which gives little protection against a natural infection by virulent capsulated strains.

No streptococcal infection is included in the OIE or in the EU legislation.

## Current status based on answers received

Eleven laboratories corresponding to 5 countries (Spain, Italy, France, Turkey and Israel) are involved in the survey of streptococcosis. Whereas *L. garvieae* is reported as the causative agent in 9 laboratories of 3 countries (Spain, Italy and France), only in 3 of them, belonging to France and Italy, are cases of streptococcosis caused by *V. salmoninarum* also described. Only 1 laboratory of Turkey reported streptococcosis by *S. iniae*. No information of the aetiological agent is provided in Israel.

Biochemical identification and PCR procedures are widely employed in the survey of the different agents causing streptococcosis, especially in Spain, France and Italy. However, only in 1 Spanish laboratory is serological identification conducted.

The streptococcosis is described mainly in salmonids (*Oncorhynchus mykiss* and *Salmo trutta*). The majority of isolates were from rainbow trout. Only 1 laboratory from Israel reports the disease in seabass (*Dicentrarchus labrax*) without specification of its incidence.

*Lactococcus garvieae* and *V. salmoninarum* are also recovered in routine surveys conducted in rainbow trout by 5 laboratories corresponding to 3 countries (Spain, France and Italy).

With the exception of 1 laboratory that reported mortalities occurring only in fish over 150 g, no data is provided about the most susceptible fish age to streptococcosis.

Vaccination programmes are implemented in the majority of the countries involved in the survey, but no information is provided if the mortality cases correspond to vaccinated or unvaccinated stocks.

## Interpretation comments

The extended use of DNA-based methods for the diagnosis of streptococcosis, as well as the generalised use of vaccines to prevent the disease, indicate that streptococcosis, especially that caused by *L. garvieae* (named also lactococcosis), can be considered as one of the most threatening bacterial diseases affecting rainbow trout cultured in the Mediterranean region.

Despite the general use of vaccines against streptococcosis, no information of their efficacy is reported.

Although the scientific literature widely reports that streptococcosis, especially that caused by *S. iniae* have a great incidence in tilapia and rainbow trout cultured in Israel, no reports of recovery of this pathogen was provided in this survey.

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## **Bacterial kidney disease (*Renibacterium salmoninarum*)**

Bacterial kidney disease (BKD), caused by the Gram-positive diplobacillus, *Renibacterium salmoninarum*, is a chronic systemic disease of salmonids which causes mortality in cultured fish in fresh and marine environments. The pathogen has also been found in wild fish populations. The disease has been reported to occur in North America, Japan, Western Europe and Chile. It is of economic importance especially with regard to Pacific salmon (*Oncorhynchus* spp.), because of its widespread distribution in fresh and saline waters, its chronicity, which does not allow the disease to be suspected before late clinical signs, its vertical transmission through sexual products, and the inefficacy of the main therapeutic measures used in treating fish. In fact, the intracellular occurrence of the pathogen inside phagocytic fish cells could contribute to the chronic nature of the disease by protecting it from circulating antibodies and chemotherapeutic agents. The overt disease only appears in advanced cases of infection, when the fish have completed their first year of life. The gross external signs are exophthalmia, abdominal distension and petechial haemorrhages. The infection is characterised by a systemic infiltration of the viscera by the bacterium causing

granulomatous lesions especially in the kidney. Greyish abscesses tend to multiply resulting in enlargement and necrosis of the whole kidney, which appears swollen and bloated with irregular greyish areas. In any case, clinical diagnosis only provides a suspicion of BKD because other Gram-positive bacteria, namely lactic bacteria, have been demonstrated to produce similar infections in salmonids.

*Renibacterium salmoninarum* isolates are biochemically and antigenically homogeneous, which favours the use of specific antisera in identification procedures. The main common antigen is the heat-stable p57 protein, which is present in the cell surface and is also released to fish sera and tissues during the infection. The detection of this 57 kDa major soluble antigen was the basis to the development of serological and genetic methods to the diagnosis of this disease.

*Renibacterium salmoninarum* has also been described as a highly conserved genospecies, which makes the differentiation of the isolates from distinct geographic areas or biological sources difficult. In fact, the DNA fingerprinting technique RAPD, applied to a great number of strains from USA, Canada and different countries of Europe, allowed only a weak correlation of the RAPD profiles obtained with the geographic origin of the isolates to be detected. Therefore, the epidemiology of BKD still remains unclear.

Although isolation of *R. salmoninarum* from fish tissues, followed by serological identification by slide agglutination or immunofluorescence, is considered a definitive diagnosis, the bacterium is a fastidious growing organism that requires prolonged incubation (from 2-3 weeks to more 2 months in subclinical cases) at 15°C to produce colonies. In addition, cysteine and serum or serum substitutes such as charcoal are requisite growth factors, and different media (i.e. KDM-2, KDM-C, SKDM) have been proposed to improve its growth or reduce the development of associated fast-growing microorganisms. Primary isolation can be enhanced by a heavy inoculum of a "nurse culture" in the centre of a petri dish or the addition of sterile spent media to the culture plates.

Since culture of *R. salmoninarum* is difficult and time-consuming, several immunodiagnostic assays are currently used for the detection of the agent in infected tissues. The most widely used serological assays are the direct or indirect immunofluorescent antibody tests and ELISA using polyclonal antisera or monoclonal antibodies (MAbs) directed against different epitopes on p57 antigen. However, to obviate the risk of cross-reaction with other bacteria (Bandín *et al.*, 1993; Brown *et al.*, 1995), the use of MAbs is recommended. Different commercial ELISA kits such as Aquarapid-Rs (Bionor, A/S, Norway) and K-Dtect or Kwik-Dtect (DiagXotic, Inc., USA) are available for the specific detection of the microorganism in field conditions. However, the detection limit of these kits is about 10<sup>6</sup> bacteria/g tissue, which indicates that their sensitivity is not good enough to detect carrier fish.

In the last years, PCR or nested reverse transcriptase PCR (RT-PCR) based methods using either primers to the 16S rRNA or the p57 gene proved to be the most sensitive approaches to detect *R. salmoninarum* in kidney tissues, ovarian fluids, and salmonid eggs as well as in fish lymphocytes. Since it was demonstrated that kidney tissue could produce some inhibitory effects reducing the sensitivity of the assay, the use of lymphocyte lysates rather than crude tissues was suggested in the PCR technique. In addition, the nested RT-PCR assays means an important advancement in *R. salmoninarum* detection protocols since this molecular approach allows the detection of viable *R. salmoninarum* cells.

Although vaccination trials using classical bacterins, recombinant vaccines or attenuated live vaccines have been reported, and there is evidence that under some conditions *Renibacterium* elicits an immune response in fish, the protective ability of a vaccine is questionable because the intracellular nature and vertical transmission of the pathogen as well as by the possible immunosuppressive role of the protein p57. Recently, a commercial aqueous live vaccine developed by Novartis S.A. has been licensed under the name of "Renogen" for BKD prevention in the field. This vaccine is constituted by live cells of *Arthrobacter* sp. strain, a bacterium which possesses common antigens with *R. salmoninarum*.

BKD is included in the group of significant diseases in the OIE legislation (OIE, 2000) as well as in list III of the EU legislation.



## Current status based on answers received

BKD attributed to *Renibacterium salmoninarum* is reported in only 2 laboratories from 2 countries (Croatia and Spain) affecting *Oncorhynchus mykiss*. However, the diagnosis can be regarded as preliminary because it was only based on histopathology but no confirmative serological (immunofluorescence or ELISA) or PCR methods were employed.

## Interpretation comments

The apparent low incidence of this disease in the Mediterranean area may be due to technical difficulties regarding the recovery of *R. salmoninarum* in culture medium and/or the lack of application of specific serological and molecular methods to detect the pathogen in diseased and asymptomatic carrier fish.

If the diagnosis is based only on histopathology, a misidentification of BKD with infections by other Gram-positive bacteria can occur.

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## Mycobacteriosis (fish tuberculosis)

Mycobacteriosis in fish (or fish tuberculosis) is a sub acute to chronic wasting disease known to affect near 200 freshwater and saltwater species. Although *Mycobacterium marinum* is considered the primary causative agent of fish mycobacteriosis, a great number of *Mycobacterium* species associated with tubercle granulomas in cultured, aquarium and wild fish populations have been described: *M. marinum*, *M. fortuitum*, *M. chelonae*, *Mycobacterium smegmatis*, *M. abscessus*, *M. neonarum*, *M. simiae*, *M. scrofulaceum*, *M. poriferae* and *M. triplex*-like. All these species cause disease in humans.

Although in cultured fish, mycobacteriosis was documented in Pacific and Atlantic salmon (*Oncorhynchus* spp., *Salmo salar*), pejerrey (*Odonthestes bonariensis*), snakehead fish (*Chana striatus*), turbot (*Scophthalmus maximus*), tilapia (*Oreochromis mossambicus*), European seabass (*Dicentrarchus maximus*) and red drum (*Sciaenops ocellatus*), since 1990 the disease has represented a significant threat, especially for seabass cultured on the Mediterranean and the Red Sea coasts of Israel. Recently, this disease is a matter of concern for the turbot culture in Europe.

Among the wild fish that have been reported to suffer mycobacteriosis are cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*), striped bass (*Morone saxatilis*), Atlantic mackerel (*Scomber scomber*) and yellow perch (*Perca flavescens*).

As mycobacteriosis is a chronic disease, it seems likely that the fish maintained in aquaria will show a higher incidence of this disease than cultured or wild species, because aquarium fish are often kept for long periods of time compared with fish raised for commercial purposes.

Internal signs of mycobacteriosis vary according to the fish species but typically include greyish-white nodules (granulomas) in the spleen, kidney and liver. External manifestations include scale loss accompanied by haemorrhagic lesions penetrating the musculature in advanced cases.

Diagnosis of the disease depends on clinical and histological signs and identification of the bacterial pathogen. Smears from spleen and kidney tissues should be made and stained with Ziehl-Neelsen based stains in order to visualise the acid-fast short bacilli characteristic of *Mycobacterium* species. An immunocytochemical method using the avidin-biotin complex was also recommended to demonstrate the presence of a small number of mycobacteria in affected tissues. In any case, a specific diagnosis of mycobacterial infection requires the isolation and identification of the microorganisms. Specific media devised for clinical *Mycobacterium* must be employed.

Because the disease remains asymptomatic for a long time, stunts growth, is virtually impossible to eradicate with chemotherapeutic agents, renders the affected fish unmarketable, together with the fact of the slow and poor growth exhibited by the majority of the *Mycobacterium* species, it is essential to develop reliable DNA-based methods for a fast identification of the main pathogenic *Mycobacterium* species in fish tissues. PCR approaches using the 16S rDNA as target gene, coupled with restriction enzyme analysis of the amplified fragment, were already reported and proved to be highly specific and sensitive for the detection of mycobacteria not only in fish tissues but also in the blood. Therefore, this methodology can constitute a useful non-destructive method to screen carrier broodstocks.

Mycobacteriosis is not considered a significant disease by the OIE or EU legislations.

### Current status based on answers received

Fish tuberculosis attributed to *Mycobacterium* species is reported in 7 laboratories corresponding to 4 countries (Greece, Israel, Italy and Spain). Although the disease is described in 4 fish species (*Carassius auratus*, *Dicentrarchus labrax*, *Epinephelus aeneus* and *Sciaenops ocellatus*), the most susceptible fish seems to be *Dicentrarchus labrax* and the ornamental species *Carassius auratus*. The diagnosis is not confirmed in 2 laboratories because only microscopic observation of fresh imprints or histopathology was performed. However, biochemical identification and confirmation with PCR methods are employed in 3 laboratories (in Greece and Italy).

In 3 laboratories the disease was described only in routine surveys of seabass.

## Interpretation comments

Because the reports of most laboratories are limited to the presence or absence of cases but no quantitative data are provided, a clear conclusion of the prevalence of the fish mycobacteriosis in the Mediterranean countries cannot be drawn.

Unfortunately, the description of the aetiological agent of the fish tuberculosis was not reported in any laboratory despite the use of PCR methods in 3 laboratories that may have allowed specific identification of *M. marinum*.

Although it is known from the scientific literature that mycobacteriosis, mainly caused by *M. marinum*, is a problem of concern for European seabass cultured in Israel and that researchers from this country developed and published adequate PCR procedures to identify this microorganism, this information was not provided in this survey.

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## Piscirickettsiosis

Piscirickettsiosis is a septicaemic condition of salmonids. The causative agent of the disease is *Piscirickettsia salmonis*, a non-motile Gram-negative, obligatory intracellular bacterium. The disease was described for the first time in 1989 affecting coho salmon (*Oncorhynchus kisutch*) cultured in Chile where mortalities between 30-90% were reported. From 1992, the disease was also described in Ireland, Norway, Scotland, and both the west and east coasts of Canada. Although *P. salmonis* has been detected in different species of Pacific salmon, Atlantic salmon, and rainbow trout, the most susceptible species seems to be coho salmon. Natural outbreaks of piscirickettsiosis typically occur a few weeks after smolts are transferred to the sea. However, the disease has also been observed in fresh water facilities.

Although horizontal transmission is one of the main routes of infection, in certain cases, the existence of vertical transmission of *P. salmonis* has been shown. Therefore, to avoid the possible risk of congenital transmission of the pathogen, the Chilean salmon farming industry has implemented the elimination of carrier broodstocks. Intermediate vectors such as external hematophagous isopods may also play a role in the natural transmission of piscirickettsiosis.

Reported clinical signs of fish affected by piscirickettsiosis are lethargy, anorexia, darkening of the skin, respiratory distress, and surface swimming. The first physical evidence of the disease may be the appearance of small white lesions or shallow haemorrhagic ulcers on the skin. The most characteristic gross internal lesions are off-white to yellow subcapsular nodules, measuring up to 2 cm in diameter, scattered throughout the liver.

*Piscirickettsia salmonis* can only be isolated in fish cell lines commonly employed in virology (CHSE-214 or EPC) where it produces a cytopathic effect. However, the technique is time consuming and difficult since culture has to be performed without antibiotics, which makes the appearance of contaminants very easy. Therefore, a preliminary diagnosis of the disease is normally made by examination of Gram, Giemsa or acridine orange-stained kidney or liver imprints, with confirmation by serological methods such as immunofluorescence or immunohistochemistry employing specific antiserum. Although an ELISA assay is commercially available (Microteck International Ltd., Canada, or DiagXotics, Inc., USA) there is scarce information of its use on *P. salmonis*. In addition, the identity of the aetiological agent of piscirickettsiosis can be confirmed by PCR-assays. Until present, two different PCR-based protocols have been published for the fast diagnosis of the disease in infected tissues. Whereas one of them is based on a nested PCR assay employing the 16S DNA as the target gene, in the other protocol part of the internal transcribed spacer (ITS) of the rRNA operon is amplified. This last PCR assay was further employed in phylogenetic studies of strains of *P. salmonis*. Both serological and molecular methods must also be utilised to confirm the positive isolation of *P. salmonis* in fish cell-lines.

It is noteworthy that although kidney and liver tissues are the recommended sources for the isolation of *P. salmonis* (OIE, 2000), it was recently reported that the brain might represent an important residence site of the pathogen, as its bacterial load is about 100 times higher than the loads observed in liver and kidney.

Although at present in Chile some commercialised vaccines are available against *P. salmonis*, the efficacy of these bacterins is questioned because there is insufficient protection data in experimental and field conditions.

Salmonids have not been the only target fish of rickettsial organisms, and several reports have been published describing rickettsial infections as being responsible for epizootic outbreaks in non-salmonid fish such as different cultured species of tilapia in Taiwan, imported blue-eyed plecostomus (*Panaque suttoni*) in USA, and juvenile seabass (*Dicentrarchus labrax*) in Europe. Although in the majority of cases no comparison between these *Rickettsia*-like organisms (RLOs) and the *P. salmonis* isolates have been made, recent immunohistochemistry studies demonstrated antigenic similarities between the RLOs from European seabass and *P. salmonis*. However, genetic studies are needed in order to know if the RLOs from seabass belong to *P. salmonis* species or whether they are a new undescribed species of rickettsial fish pathogens.

Piscirickettsiosis caused by *P. salmonis*, is listed in the group of significant diseases in the OIE legislation, but is not considered in the EU legislation.

## Current status based on answers received

Piscirickettsiosis attributed to RLOs is reported only in Greece (3 laboratories) affecting *Dicentrarchus labrax*. However, in 2 laboratories the diagnosis can be considered with precaution since neither isolation in fish cell lines of the causative agent nor application of serological and/or PCR procedures were conducted. Only in 1 laboratory was immunohistochemistry applied to confirm the diagnosis.

## Interpretation comments

Without the implementation of the appropriate available methodology to isolate and characterise the rickettsial organisms observed in the fish it is not possible to make the taxonomical placement of these agents.

Because of the difficulty in isolating the rickettsial organisms in cell culture without contamination problems, the use of available specific PCR based methods applied directly to fish tissues is recommended as a valuable tool for the specific identification of *P. salmonis*. Since in this microorganism the existence of vertical transmission was shown, the application of molecular tools to asymptomatic brood stocks is encouraged.

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## Epitheliocystis

Epitheliocystis occurs as a benign or proliferative disease, characterised by cysts in the branchial epithelia of the host. The causative agent(s) of the disease are morphologically diverse and may represent a group of related organisms that produce similar pathology in different hosts (Paperna and Sabnai, 1980; Lewis *et al.*, 1992; Turnbull, 1993; Lannan *et al.*, 1999). The taxonomic placement of these diverse intracellular microorganisms is undefined but is considered to fit within the genus *Chlamydia*.

Clinical signs of epitheliocystis may include lethargy, flared opercula and rapid respiration. Cysts

may appear as transparent white to yellow capsules on the gill filaments. Generally the host response is limited, and there is little or no mortality associated with infection.

Characteristic cysts are hypertrophic host cells filled with the causative bacterium. The enlarged host cells range from 10 to 400 µm in diameter and are frequently surrounded by squamous or cuboidal epithelial cells.

Although the documental host range of epitheliocystis includes species in more than 20 families of freshwater, marine and anadromous fishes from both warm- and cold-water environments, *Chlamydia*-like organisms (CLO) have only been associated with mortalities in a few fish species namely *Sparus aurata*, *Morone saxatilis*, *Cyprinus carpio*, *Seriola dumerilii*, *Liza ramada*, *Mugil cephalus*, *Pagrus major*, *Oncorhynchus mykiss* and *Salvenilus mamaycush*.

Natural transmission of the CLO is not understood, but horizontal transmission apparently occurs within some host species. Contaminated nets or other equipment can be a cause of spreading the infection in culture facilities (Paperna, 1997). There is little information on interspecies transmission and, therefore, it is not known if differences in the morphology of the CLO relate to different host specificities or to the CLO itself.

Due to the inability to isolate and culture the microorganism, preliminary diagnosis of epitheliocystis is made by observation of the white to yellow cysts on the gills or skin of affected fish. It was suggested that the pseudobranch must also be examined for cysts (Crespo *et al.*, 1990). The thick capsule and granular contents, which are characteristic of the cysts, are easily seen in wet mounts. No serological techniques are available for identification of the CLO or for the diagnosis of infection. Electron microscopy is required for definitive diagnosis of infection (Paperna *et al.*, 1978, 1981; Grau and Crespo, 1991). With this technique, the intracellular forms can be observed clearly and differentiated from cysts having a viral aetiology.

Future studies of epitheliocystis should be addressed toward isolation and *in vitro* propagation of the aetiological agents, biochemical characterisation, determination of taxonomic placement and clarification of the relationship among the morphologically diverse group of these CLO. This will be helpful for the development of more rapid and precise diagnostic procedures for the identification of the disease as well as for the implementation of vaccination strategies.

Epitheliocystis is not considered as a significant disease by the EU or OIE legislation.

## Current status based on answers received

Eleven laboratories from 6 countries (Cyprus, Spain, Greece, Israel, Italy and Malta) are involved in the survey of epitheliocystis caused by CLO. The diagnosis of the diseases is based on a microscopical observation of fresh smears and histopathology. In 5 laboratories from 3 countries (Greece, Israel and Cyprus), the presence of the pathogen in the gills is reported only in routine surveys.

Although epitheliocystis is described in 5 species of marine fish (*Sparus aurata*, *Dicentrarchus labrax*, *Dentex dentex*, *Pagrus pagrus* and *Scophthalmus maximus*) and in ornamental fish (i.e. *Carassius auratus*), the disease is only significant for *Sparus aurata*, in which all the laboratories reported the presence of CLO.

The highest incidence and severity of the disease is associated with elevated stock densities, poor environmental conditions and multiple infections with other pathogens. Mortalities occur mainly in juvenile fish only when they are affected by heavy infections. In other cases, a loss of growth is observed but without associated mortality.

The application of some husbandry practices as a preventive measure to reduce the cases of epitheliocystis is not reported.



## Interpretation comments

Despite the wide distribution of epitheliocystis in *Sparus aurata*, the disease was not reported in Turkey, an important producer of this fish species.

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## Rainbow trout gastrointestinal syndrome (RTGS)

The first cases of the rainbow trout fry syndrome (RTGS) were described in France in 1992 and two years later the disease appeared in Spain. The disease occurs mainly during the warm months (from April to September) when the water temperature is above 15°C. One of the first external signs of the affected fish is a loss of appetite. Usually, diseased fish show a normal external aspect and only in some cases is there a slight abdominal swelling. Internally, the unique organs affected are the stomach and intestine, which are filled with a viscose and opaque fluid with a total absence of feed. All fish ages are susceptible to this syndrome, and mortalities can reach 0.3% of the affected stock.

Although all attempts to isolate any microorganism from the diseased fish failed, segmented filamentous bacteria (SFB) were microscopically observed in some of the smears prepared from the intestinal mucous material.

Recently, using fluorescence "in situ" hybridisation and 16S rRNA sequencing it was demonstrated that the filamentous bacterium which is usually present in the intestinal mucous fluid of diseased trout corresponding to a Gram-positive, endospore-forming microorganism closely related to *Clostridium* which was provisionally named "*Candidatus* Arthromitus". However, the role of SFB in diseases requires clarification, since these organisms have been reported as commensal or associated with intestinal illness in a great variety of vertebrate and invertebrate animals. At the moment, a cause-and-effect relationship cannot be established and the presence of other pathogens cannot be ruled out.

## Current status based on answers received

This syndrome is reported only in 4 laboratories from 3 countries (Spain, France and Croatia) affecting, as the own name indicates, *Oncorhynchus mykiss*. The diagnosis is based only on the observation of external and internal clinical symptoms, microscopical examination of fresh smears and histopathology.

## Interpretation comments

Until the putative role of SFB in the disease is clearly demonstrated, the procedures employed in the survey are the only available methods to diagnosis the RTGS.

Despite the known high incidence of this syndrome in France, where the first cases appeared, only 1 laboratory from this country reports this disease but the number of cases is not indicated.

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