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Francisella spp. infections in farmed and wild fish

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Abstract

Bacteria within the genus *Francisella* are non-motile, Gram-negative, strictly aerobic, facultatively intracellular cocco-bacilli. While the genus includes pathogens of warm-blooded animals including humans, and potential bioterror agents, there is also increasing evidence of a number of as yet unrecognised environmental species. Due to their nutritionally fastidious nature, bacteria of the genus *Francisella* are generally difficult to culture, and growth is also commonly inhibited by the presence of other bacteria within sample material. For these reasons, *Francisella*-related fish disease may be under-diagnosed. Following the discovery in 2004/2005 that a granulomatous disease in farmed and wild Atlantic cod (*Gadus morhua*) is caused by a previously undescribed member of this genus (*Francisella philomiragia* subsp. *noatunensis*), similar diseases have been identified in fish in at least seven countries around the world. These infections affect both freshwater and marine fish species and involve bacteria more or less closely related to *F. philomiragia* subsp. *philomiragia,* an opportunistic human pathogen. Recent work relating to characterisation of the disease/s, classification of fish pathogenic *Francisella* spp. and vaccine development will be presented. The potential impact of francisellosis in wild and farmed fish on a global perspective will also be discussed.

Francisella spp. infections in farmed and wild fish

Although infections in fish caused by Gram-negative intracellular bacteria refractive to culture on standard laboratory media have been recognised for many years, these have been commonly refered to as *Rickettsia*-like (RLO) and subsequently as *Piscirickettsia*-like organisms (PLO) due to their morphological similarities with the true *Rickettsia*.

Observation of intracellular bacteria within fish tissues was first recorded in Egypt in 1939 (Fryer and Mauel 1996) and observations of PLO infections have since been reported from both marine and fresh-water fish species world wide (Mauel and Miller, 2002). Nonetheless, *P. salmonis* was until recently the only described fish-pathogenic Gram-negative bacterium with an intracellular replication and infection with *P. salmonis*, piscirickettsiosis, has been confirmed in sea-farmed Coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar*), Rainbow trout (*Oncorhynchus mykiss)*, Chinook salmon (*Oncorhynchu tshawytscha*) and Pink salmon (*Oncorhynchus gorbuscha*) in Chile (Fryer and Mauel, 1997), Canada (Brocklebank et al., 1993), Norway (Olsen et al., 1997), Tasmania (Corbeil et al., 2005) and Ireland (Rodger and Drinan, 1993) as well as in the marine fish species European sea bass (*Dicentrarchus labrax*) (Comps *et al*., 1996; McCarthy *et al*., 2005) and white seabass (*Atractoscion nobilis*) (Arkush *et al*., 2005). The commercial losses as a result of piscirickettsiosis outside of Chile vary, but mortality levels are generally lower, which may be

due to varying virulence of the various genetic lineages of *P. salmonis*. Although the reservoir of *P. salmonis* is not known, the bacterium has been detected in plankton samples (Mauel and Fryer, 2001). While the bacterium has been identified in feral pacific salmon in Chile, infections in truly wild fish have not been identified.

Over recent years a degree of diversity within the PLO group been recognized and bacteria of the genus *Francisella* have "emerged" as serious pathogens of various fish species both farmed and wild, from various geographical regions world wide. This recent spate of diagnoses is, most probably more related to the increased awareness of intracellular infections and the widespread availability of non-culture based molecular techniques, than the appearance of truly novel disease infections. Molecular characterization of the 16s rRNA gene has demonstrated the existence of two different genetic lineages among the fish pathogenic *Francisella* isolates, both having a close relationship to the opportunistic human pathogen *F. philomiragia* subsp. *philomiragia* (Hsieh *et al*. 2006; Mikalsen *et al* 2007) (Figure 2). Despite morphological similarities both *Francisella* spp. and *P. salmonis* belong to the γ-proteobacteria and are therefore only distantly related to the true *Rickettsia* (*α*-proteobacteria) (Figure 1).

Figure 1: Un-rooted neighbour joining tree based on 16S rRNA data of selected bacterial species.

The genus *Francisella*

The genus *Francisella* consists of non-motile, Gram-negative, strictly aerobic, facultatively intracellular cocco-bacilli and includes 4 validly published species (Table 1.) The type species of the genus is *F. tularensis* which is the agent of tularaemia (Sjösted, 2005), a highly infectious disease of both animals and humans and a potential bioterror weapon. The genus can be divided into two major lineages on the basis of phylogenetic analysis of the 16S rRNA

gene (Figure 2), i.e. the *F. tularensis* lineage and the *F. philomiragia* lineage. A third human pathogenic lineage has recently been demonstrated (Kugler *et al*. 2008) and molecular studies of environmental samples have also demonstrated the existence of as yet undescribed members of this genus (Barnes *et al*. 2005).

Generally *Francisella* species are known to infect a large number of hosts, and are considered difficult to cultivate from the environment (Petersen *et al*. 2004). Although relatively little is known regarding the natural distribution and ecology of species within this genus (Petersen et al. 2004), transmission of *F. tularensis* in man has a clear association with exposure to rodents and blood-feeding arthropods (Goethert *et al*., 2004). An association of *Francisella* species with natural waters has been documented (Jensen *et al*., 1969; Friis-moller *et al*., 2004; Mailman and Schmidt, 2005) and *Francisella* species have been shown to enter a viable but non-cultivable (VBNC) state in cold water (Forsman et al. 2000). Moreover, a reservoir in aquatic protozoans has been proposed (Abd *et al*. 2003).

Table 1: Validly published species of the *Francisella* genus

***Francisella tularensis* lineage**	***Francisella philomiragia* lineage**
Francisella tularensis subsp. *tularensis*	*Francisella philomiragia* subsp. *philomiragia*
Francisella tularensis subsp. *holarctica*	*Francisella philomiragia* subsp *noatunensis**
Francisella tularensis subsp. *mediasiatica*	*Francisella piscicida**
Francisella novicida	

* Constitute heterotypic synonyms

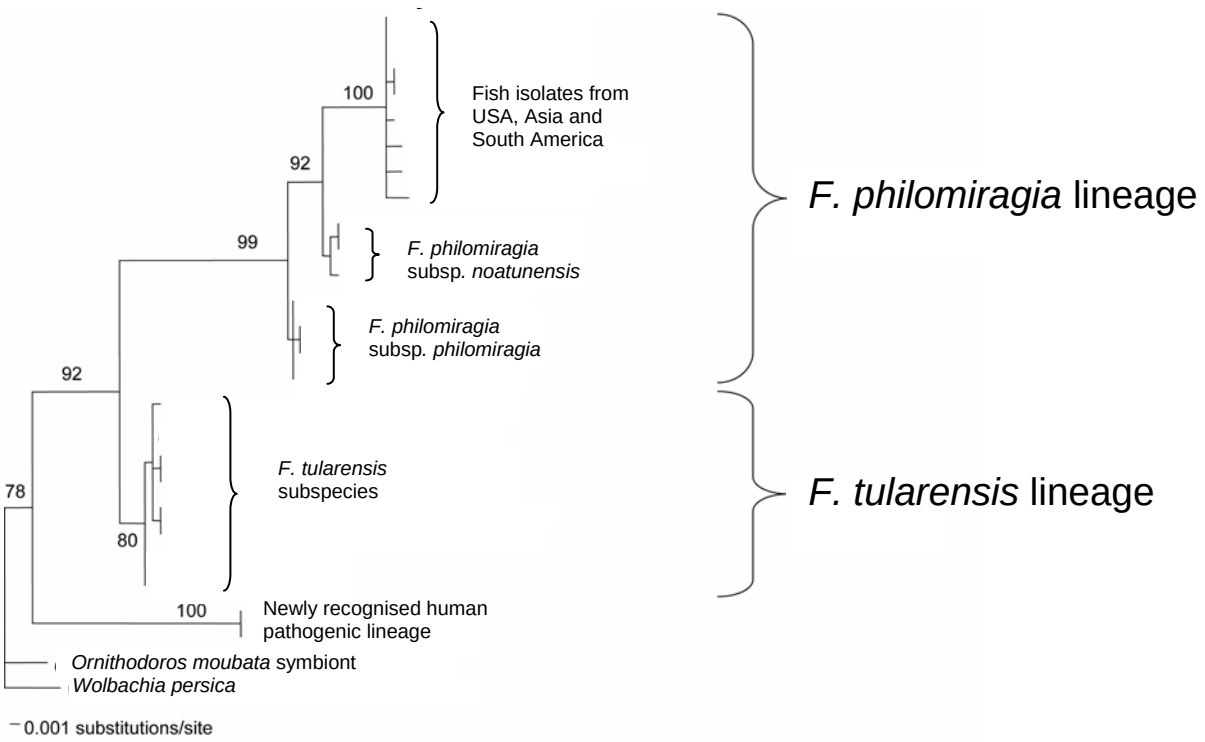

Figure 2: Maximum likelihood tree of 16S rRNA data showing the main relationships between species and strains within the genus *Francisella*. *Wolbachia persica* and *Ornithodoros* moubata symbiont constitute out-groups.

Francisellosis, infection with *Francisella* spp. in fish, affects both farmed and wild fish on a world wide basis. Reports of francisellosis have been made from fresh, brackish and marine water, including species such as Atlantic cod (Olsen *et al.*, 2006; Nylund *et al*. 2006), tilapia (*Oreochromis* spp.) (Hsieh *et al*., 2006; Mauel *et al*., 2006) three-line grunt (*Parapristipoma trilineatum*) (Kamaishi *et al*., 2005), hybrid striped bass (*Morone chrysops* x *M. saxatilis*) (Ostland *et al*., 2006) and Atlantic salmon (Birkbeck *et al*., 2007). Although most of the isolations reported have occurred in the last 10 years or so, the first confirmed (retrospectively) case of francisellosis occurred in pond-reared tilapia in Taiwan in 1992. The causal agent was then considered to be a PLO/RLO based on morphological characteristics (Chen *et al*., 1994), although the authors noted that this organism had different growth characteristics in fish cell lines than *P. salmonis* described as by Fryer *et al*. (1990). Subsequently francisellosis has been recorded in tilapia fish farms in Hawaii, Florida, California, and South Carolina (Mauel *et al*. 2005).. Further, PLO (uncultured) infections with pathology consistent with francisellosis have been reported in cultured Grouper (*Epinephelus melanostigma*) in Taiwan and wild-caught blue-eyed plecostomus (*Panaque suttoni*) (Khoo et al., 1995) from South America. Other, as yet unclassified PLO have been reported from dragonets (*Callionymus lyra* L) (Davies, 1986). Moreover, a PLO described in farmed salmon reared in freshwater in Chile in the 1990s (Cvitanich *et al*., 1995) was subsequently identified as a *Francisella* sp. (Birkbeck *et al*., 2007) with a close relationship to *Francisella* strains isolated from Atlantic cod in Norway.

So far, all fish pathogenic isolates have been identified as belonging to the *F. philomiragia* lineage, although the first report of *Francisella* as a fish pathogen by Olsuf'ev (1970) is somewhat unclear regarding the precise identification of the aetiological agent. The fish pathogenic isolates so far phenotypically and genotypically characterised, fall into two groups, comprising *F. philomiragia* subsp. *noatunesis* (heterotypic synonym = *F. piscicida* (Ottem *et al*,. 2007) isolates from Norwegian farmed Atlantic cod and Chilean farmed Atlantic salmon and isolates of an as yet un-named species from tilapia, three-lined grunt and hybrid striped bass (Mikalsen and Colquhoun, submitted manuscript).

Although closely related to *F. philomiragia subsp*. *philomiragia* (an opportunistic human pathogen most commonly associated with disease in immunocomprimised individuals or subjects of near-drowning incidents, fish pathogenic *Francisella* are considered unlikely to pose a significant zoonotic potential. These isolates do not grow at 37^{o}C on laboratory media and in contrast to *F. philomiragia* subsp. *philomiragia* require enhanced levels of cysteine in culture media, probably indicating a more restricted ecological niche. Injection of laboratory mice with large doses of the two different fish pathogenic species did not result in infection, disease or pathology (Mikalsen et al., in press). Genetically the two species can be clearly separated and while phenotypical similarities exist they can be separated on the basis of enzymatic activity and optimal growth temperature.

Freshwater vs. seawater

Although addition of salt to agar otherwise suitable for cultivation of *F. philomiragia* subsp. *noatunensis* reduces its effectivity as a medium for primary isolation of this bacterium, these bacteria have a reasonably high tolerance for salt. Both types of fish pathogenic *Francisella* have been identified in fish from both marine and freshwater.

Francisellosis: the disease

Generally the disease appears to have a chronic development in most affected fish species and may result in varying mortality. Affected farmed Atlantic cod are generally emaciated, and may display raised haemorrhagic nodules in the skin. Common observations in cod, three-lined grunt and tilapia include the extensive occurrence of white, partly protruding nodules (granuloma) of various size in the spleen, kidney and liver. Other organs which may be affected include the heart, testes, musculature, brain and eye. In cod the spleen is generally enlarged and sero-haemorrhagic ascites and thickened intestinal mucosa may be observed. Extensive chronic granulomatous inflammation with multiple granuloma in all organs is the main histopathological finding. Few to numerous small Gram-negative bacteria may be observed within granuloma.

Transmission of disease

From field observations it would appear that the disease is highly infective as prevalence of infection within affected stocks of farmed cod and tilapia is often extremely high. Ongrowing of juvenile cod in the sea in the South Western part of Norway has now largely ceased due to the threat of francisellosis. Laboratory trials have confirmed the rapid transmission and chronic course of disease. In a cohabitant challenge performed at 12^{o}C, all cohabitant cod sampled after 38 days were infected. At the end of the five month cohabitation period, 100% of cohabitant fish displayed severe macroscopic signs of disease and were culture positive. Interestingly no fish died during this period. Not surprisingly, water temperature appears to play a significant role in development of francisellosis in cod with increased mortalities in periods of high water temperature, although bacteria may also be readily cultured from infected fish during the winter months.

Prevalence in wild cod

Identification of wild fish suffering systemic bacterial infection is usually uncommon as affected fish generally quickly disappear from the population. However, *Francisella* spp., in common with other intracellular bacterial pathogens i.e. *Mycobacteria* spp., *Renibacterium salmoninarum*, due to the chronic nature of such infections and often visible macroscopic pathology, offer a greater chance of identification in wild stocks. Several PLO/RLO have been identified in wild fish but in most cases the precise identity of the aetiological agent has not been confirmed. *F. philomiragia* subsp. *noatunensis* infection has been identified in both farmed and wild cod.

Farmed cod in Norway are held in net cages in close contact with wild fish (including wild cod) which congregate around these structures. A recent screening (Ottem et al., 2008) of wild fish caught around the Norwegian coastline using Real Time PCR, performed in the wake of extensive outbreaks in farmed cod, reported the presence of low levels of *F. piscicida* (a.k.a. *F. noatunensis*) in several marine fish species i.e. saithe (*Pollachius virens*), pollock (*Pollachius pollachius*), mackerel (*Scomber scombrus*), European plaice (*Pleuronectes platessa*) and megrim (*Lepidorhombus whiffiagonis*) and other aquatic organisms such as blue mussels (*Mytilus edulis*) and edible crab (*Cancer pagurus*) in southern and south-western Norway. No positive findings were identified in wild Atlantic cod from the mid and Northern parts of Norway. The status of the disease/infection in wild fish prior to recent outbreaks and the effect of infection pressure from farmed fish to wild fish in these areas is not known. That the bacterium was pathogenic for wild cod previous to the outbreaks in farmed cod is clear. In 2004, a granulomatous condition was reported at a high prevalence in wild caught cod off the west coast of Sweden (Alfjorden *et al*. 2006), and the aetiological agent was subsequently confirmed as *F. philomiragia* subsp. *noatunensis*.

We have recently successfully traced francisellosis back to wild cod caught in the southern North sea during the late 1980s (Zerihun *et al*., manuscript in preparation). During a five year surveillance study of disease prevalence in wild caught cod between 1981 and 1985, a condition then described macroscopically as "presumptive mycobacteriosis" was identified annually with a maximum prevalence of 12.5% (van Banning 1987). During the summer of 1988, the condition, now termed "visceral granulomatosis" was again identified, apparently with a higher prevalence. These reports related to cod from the south-east coast of England, although individual cases were reported further west. Apparently only cod were affected and up to 30% of caught fish were affected. The internal abnormalities registered included discolouring of the liver, and multi-organ nodulation or cyst development, all consistent with Francisellosis. At the time fish suffering visceral granulomatosis were reported by fish processors, commercial fishermen and pleasure anglers. Bacteriological investigations and histopathological investigations carried out at that time did not reveal the aetiology of the disease. Subsequently the disease has been registered only sporadically in later years.

With formalin-fixed paraffin embedded material archived in 1988 as a basis for study we have confirmed, using immunohistochemical techniques and real-time polymerase chain reaction that the aetiological agent of "presumptive mycobacteriosis/visceral granulomatosis" in cod caught in the southern North sea during the 1980s was *F. philomiragia* subsp. *noatunensis*.

It is interesting to note that at the time fish displaying "presumptive mycobacteriosis/visceral granulomatosis" were not identified north of latitude 55^{o} during the 1980s yet francisellosis appeared in both farmed and wild fish almost simultaneously off the Swedish and Norwegian coastlines in the early part of the present decade. The reasons behind these outbreaks remain unclear but it may be tempting to speculate the involvement of either fish migration or increasing water temperatures in the Northern North Sea.

That the levels of bacteria identified in non-gadoid fish were low and that no clinical signs of disease were observed in non-gadoid fish in Norway (Ottem *et al*. 2008) or the previous survey in the southern North Sea (van Banning 1987) may indicate that this bacterium exibits a certain degree of host specificity for gadoid fish and Atlantic cod in particular.

Threat to wild fish

It is clear that under farming conditions with high concentrations of fish and subsequent high infection pressure, *Francisella* infections may be an extremely serious threat to farmed stocks. Whether *Francisella* infections present a significant problem for naive wild fish stocks given our present levels of knowledge is difficult to say, but the evidence indicates that Atlantic cod are a natural host for *F. philomiragia* subsp. *noatunensis* and that experiences in the southern North sea may indicate that the effect of infection in wild fish stocks may not necessarily be catastrophic, although this may depend on many factors.

Vaccination

No commercial vaccine is currently available against *Francisella* infections in fish, although several vaccine companies are involved in development work. Several trial vaccines, based on simple whole cell based preparations have been tested in the field in Norway, none have yet awarded a satisfactory degree of protection.

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