Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland

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Ecological changes affect pathogen epidemiology and evolution and may trigger the emergence of novel diseases. Aquaculture radically alters the ecology of fish and their pathogens. Here we show an increase in the occurrence of the bacterial fish disease \textit{Flavobacterium columnare} in salmon fingerlings at a fish farm in northern Finland over 23 years. We hypothesize that this emergence was owing to evolutionary changes in bacterial virulence. We base this argument on several observations. First, the emergence was associated with increased severity of symptoms. Second, \textit{F. columnare} strains vary in virulence, with more lethal strains inducing more severe symptoms prior to death. Third, more virulent strains have greater infectivity, higher tissue-degrading capacity and higher growth rates. Fourth, pathogen strains co-occur, so that strains compete. Fifth, \textit{F. columnare} can transmit efficiently from dead fish, and maintain infectivity in sterilized water for months, strongly reducing the fitness cost of host death likely experienced by the pathogen in nature. Moreover, this saprophytic infectiousness means that chemotherapy strongly select for strains that rapidly kill their hosts: dead fish remain infectious; treated fish do not. Finally, high stocking densities of homogeneous subsets of fish greatly enhance transmission opportunities. We suggest that fish farms provide an environment that promotes the circulation of more virulent strains of \textit{F. columnare}. This effect is intensified by the recent increases in summer water temperature. More generally, we predict that intensive fish farming will lead to the evolution of more virulent pathogens.

Keywords: \textit{Flavobacterium columnare}; evolution of virulence; fish farming; salmon

\textbf{1. INTRODUCTION}

The emergence of infectious diseases are usually triggered by ecological changes, often associated with human interventions, such as transfer of organisms, environmental degradation, agricultural practices or technology (Schrag \& Wiener 1995; Patz \textit{et al.} 2000; Dobson \& Foufopoulos 2001; Murray \& Peeler 2005; Jones \textit{et al.} 2008). The agents of most emerging diseases in aquaculture have been shown to originate from another geographical area or from another host species. Introduction of parasites into naive populations can cause mass mortalities, as happened for example in the sturgeon population of the Aral Sea after introduction of \textit{Nitzschia sturionis} (Bauer 1961) and in salmon populations in Norwegian rivers after the introduction of \textit{Gynodactylus salaris} (Jansen \textit{et al.} 2007). However, native diseases may also cause unexpected outbreaks if there are changes in the environment (Rintamäki \& Valtonen 1991; Schrag \& Wiener 1995; Dobson \& Foufopoulos 2001). In aquaculture, circumstantial evidence suggests an increase in pathogenicity of furunculosis (Bakke \& Harris 1998), viral haemorrhagic septicaemia virus (Einer-Jensen \textit{et al.} 2004; Raja-Halli \textit{et al.} 2006) and infectious salmon anaemia virus (Cunningham \& Snow 2000; Nylund \textit{et al.} 2003). Whether pathogen evolution or an alteration in the epidemiology caused these changes is not entirely clear.

\textit{Flavobacterium columnare} is a bacterial pathogen of freshwater fish causing skin lesions, fin erosion and gill necrosis known as columnaris disease. During the last two decades it has become the most serious threat to salmon (\textit{Salmo salar}) and trout (\textit{Salmo trutta}) smolt and rainbow trout (\textit{Oncorhynchus mykiss}) production in Fennoscandia (Anon. 2009). In the US, columnaris disease causes annual losses of millions of dollars for the channel catfish (\textit{Ictalurus punctatus}) industry (Wagner \textit{et al.} 2002; Olivares-Fuster \textit{et al.} 2007). The reasons why this freshwater bacterium has become such a problem for industry is unclear.

Epidemiological models can be used to examine how different ecological factors affect the population dynamics of the pathogens and thus contribute to disease outbreak (Anderson \& May 1991). When considering fish farms as epidemiological units, changes that increase the lifespan of an infection and/or daily infectiousness can enhance the spread of parasites. Owing to the cost-effective nature of fish farming industry, fish may be cultured in densities more than 1000 times higher than under natural
conditions. This means an increase in the number of susceptible hosts available and the probability of a contact between infected and susceptible hosts once a pathogen is introduced in the rearing unit. The probability of transmission per contact might increase in homogeneous subsets of fish (Ebert 1998), and also in fish stressed by crowding or other environmental factors (Sniezko 1974). Exchange of contaminated material among rearing units may increase the number of available hosts further. Recently, we discovered that F. columnare may survive for months in sterilized lake water and can colonize fish carcasses (Kunttu et al. 2009). These abilities increase the potential for the pathogen to persist in fish tanks when suitable live hosts are temporarily removed, for instance at the end of a rearing cycle or during antibiotic treatments.

Fish farms could also be settings where the evolution of pathogen virulence is encountered. A number of factors, some of which are frequently encountered in aquaculture, may cause selection towards increased virulence at ecological time scales (Altizer et al. 2003; Galvani 2003). Our recent experiments revealed that F. columnare was not only able to colonize dead fish, but it had higher emission rates and more efficient transmission from dead fish to a living fish than from living fish to another living fish (Kunttu et al. 2009). Thus, contrary to the trade-off often assumed in the theory of evolution of virulence (Anderson & May 1982; Ebert & Mangin 1997; Mackinnon & Read 1999), death of the host on fish farms might entail little cost to F. columnare, and indeed might actually enhance transmission. In contrast, in natural populations, susceptible fish are far less likely to encounter dead fish or pathogen-contaminated water. Virulence is expected to evolve to higher levels when host death has little impact on pathogen reproductive success (Day 2002). In addition, the ability of these bacteria to persist in the environment could lead to enhanced transmission, and thus to increased virulence (Ewald 1994).

Treatment against disease may select more virulent forms, depending on the timing of treatment in relation to parasite transmission (Porco et al. 2005). Furthermore, the coexistence of several genetically distinct parasite strains in the same population may favour virulence if more virulent strains have a competitive advantage (Nowak & May 1994; Frank 1996; Gandon et al. 2001; Read & Taylor 2001), as they do in several systems (Ebert & Mangin 1997; de Roode et al. 2005a,b; Bell et al. 2006; Ben-Ami et al. 2008). Coexistence of genetically different strains of F. columnare has been detected during a disease outbreak at a fish farm (Suomalainen et al. 2006a).

In this paper we describe the emergence of columnaris disease caused by F. columnare in salmon fingerlings at a fish farm in northern Finland in mid–1980s and its occurrence, pathogenity and prevention during the subsequent 23 years. In addition, we summarize our published experimental data on virulence-related factors and transmission of this bacterium. We suggest that fish farms provide an environment where farming practices and dense populations of susceptible hosts have provided a setting for the emergence of this freshwater bacterium as the most serious contemporary threat to salmonid smolt production (Anon. 2008).

(a) Host–pathogen system
In Finland, fish farming started to increase by the end of 1960s. Currently, 13,000 tons of rainbow trout (O. mykiss) and 6 million salmon (S. salar) and trout (S. trutta) smolt are produced every year (Anon. 2009). At farms producing fish for stocking purposes, first summer fingerlings are mainly reared in indoor tanks, from which they are transferred into larger outdoor tanks in the beginning of their second summer. All tanks receive their inflow water individually from a lake or a river above the farm. At the age of 2 years, fish are stocked to the Baltic Sea or lakes.

Members of the genus Flavobacterium can be found ubiquitously in aqueous environments and most of the species are adapted to cold water (Bernardet & Bowman 2006). Flavobacterium columnare is commonly found in freshwaters (Rickard et al. 2003; Revetta et al. 2005), but it is mainly known as a fish pathogen occurring at freshwater farms worldwide. The pathogen is transmitted by contact or by propagules shed into the water. Outbreaks occur throughout the warmest summer months. Among untreated fish the disease spreads rapidly and can cause 100 per cent mortality (Suomalainen et al. 2005a). Antimicrobial drugs are usually administered when diseased fish are discovered in a tank.

2. MATERIAL AND METHODS
(a) Data collection from the fish farm
Fish diseases were intensively monitored at a farm in Northern Finland annually from the early 1980s as a part of a disease prevention monitoring programme. Flavobacterium columnare first appeared in 1984. Occurrence and mortality caused by the columnaris disease was monitored during 1984–2006. The farm studied produces Atlantic salmon (S. salar L.) and sea trout (S. trutta m. trutta L.) smolt for stocking. It is situated by the River Iijoki flowing into the Bothnian Bay, the northern part of the Baltic Sea (65°20’ N 25°24’ E). The salmon fingerlings are reared from another farm which maintains the broodstock of River Iijoki. River Iijoki is closed by power plants since 1961, and the stock is maintained by captive breeding and releasing programme (Säisä et al. 2003). The broodstock is replenished regularly with roe and sperm from salmon at the river mouth from the Baltic Sea. By 2003 there had been five generations since the founding of the broodstock in 1965–1969 (Säisä et al. 2003). Population genetic data indicate no decline in allele numbers in association with the breeding programme in the last 33 years and the mean heterozygosity remained unchanged in the Iijoki broodstock (Säisä et al. 2003). The data presented here were collected from first summer Atlantic salmon fingerlings at a rearing density of 800–1200 fish per square metre. Water temperature data during the study period is presented here as a sum of day degrees from the period when water temperature exceeded 18°C. At the latitude of the salmon farm, high temperature peaks in water are rare, so that this measure reflects the length of the favourable growth period for F. columnare. Monitoring and sampling was conducted by one of us (P.R.) two to four times per month from May to September for 23 years. Over this time, there were no marked changes in the farming practices, other than the introduction of antibiotics (oxytetracyclin) which, from 1993, were used against all columnaris outbreaks. The average number of treatments

per tank per year was recorded. Fish were observed for clinical signs of columnaris disease, and symptoms were recorded. Bacteria were isolated from infected tissues (fins, gills, skin and from 2004 onwards also from kidney) on Anacker and Ordal agar (Anacker & Ordal 1955). Tissue scrapings from infected tissue were also taken on microscopic slides. The slides were air-dried, Gram-stained and inspected for the presence of long rod-shaped bacteria using a microscope at 1000-fold magnification.

The symptoms of columnaris disease were divided into five categories according to their severity. The categories were in decreasing order of severity: necrosis, erosion or inflammation of tissue in (i) gills; (ii) jaws; (iii) saddleback area; (iv) tail; or (v) skin. Moribund or freshly deceased fish were collected for examination. All fish were studied before possible antibiotic treatment. The occurrence of columnaris disease was counted as proportion of tanks (n = ca 51, area 8 m²) infected each year. Yearly mortality owing to columnaris was counted as an average from all tanks exhibiting fish mortality.

(b) Experimental data
In order to present a thorough overview of factors affecting the transmission and virulence of F. columnare, we present here pooled and re-analysed data from our previously published papers, which detail the experimental methods (Suomalainen et al. 2006a,b). In brief, six genetically different F. columnare strains isolated from disease outbreaks from salmon, rainbow trout and arctic char at Finnish fish farms situated at four watershed areas and the F. columnare Type Strain NCIMB 2248T (National collection of Industrial, Marine and Food Bacteria) were used to study virulence and occurrence of disease symptoms in experimental challenge of rainbow trout fingerlings (Suomalainen et al. 2006b). Fish were exposed to each bacterial strain inoculated in Shieh broth (Shieh 1980) in a bath challenge containing 3.3 × 10⁵ CFU ml⁻¹ in 61 of water for 20 min. Fish were observed for signs of infection, clinical symptoms and mortality at 12 h intervals for 4 days and once a day thereafter for 3 days (Suomalainen et al. 2006b). The same strains were studied for their tissue-degrading enzyme activity (chondroitin AC lyase) at two temperatures. The degradation rate of chondroitin sulphate (ChS) extracted from shark cartilage (Sigma, St Louis, MO, USA) was determined as the change of ChS concentration in relation to total protein concentration of the culture (Suomalainen et al. 2006b). The strains were also studied for their population growth characteristics at different temperatures (Suomalainen et al. 2006a). A standard volume of bacterial culture cultivated in AO-broth at constant temperature and constant agitation until it reached 50 per cent of the maximum optical density value measured at 595 wavelength (50% saturation) were further cultured at different temperatures on microtitre plates. Population density measurements were continued until cultures reached 30–50% saturation (Suomalainen et al. 2006a).

3. RESULTS
Between 1984, when columnaris disease was first observed, and 1992, the proportion of rearing units infected with columnaris disease never exceeded 10 per cent (figure 1a). During the years when columnaris was present in this period, and before the use of antibiotics began, the columnaris-caused fish mortality increased significantly (figure 1b; r = 0.87, p = 0.02, n = 6). Fish suffered mainly from skin and saddleback symptoms and ulcers on the tail. In only 1 year jaw erosions were also found, but gill symptoms were not detected (figure 1c). In 1993 oxytetracyclin was introduced and from thereon used increasingly to treat columnaris outbreaks (figure 1a; r = 0.57, p = 0.03, n = 14). By the end of the study period, several rearing units had to receive antibiotic treatments several times within one season, indicating repeated outbreaks within the same tank. Repeated outbreaks may have become possible by the longer period of water temperature above 18°C (figure 1a), which is necessary for columnaris to develop. The sum of day degrees above 18°C increased during 1993–2006 (figure 1a; r = 0.76, p = 0.002, n = 14) indicating longer warm water periods. The number of antibiotic treatments per tank increased with the sum of day degrees above 18°C (r = 0.78, p = 0.001, n = 14). Thus, longer warm water period coincided with longer disease outbreak period. Even though antibiotics were administered whenever diseased fish were detected in any tank, medication did not prevent outbreaks from occurring and the proportion of infected tanks rose up to even 60 per cent (figure 1a). The disease-induced mortality could be controlled in most years below the level of the last years of the pre-treatment period. A transition towards more serious disease symptoms was observed. Necrosis of gills started to appear in 1993 (figure 1c) and were seen in most of the subsequent years (logistic regression for the presence of gill symptoms during the whole study period; Wald χ² = 6.7, d.f. = 1, p = 0.010).

In experimental infections with seven genetically characterized strains, some F. columnare strains caused 100 per cent mortality within 4 days post-infection (called highly virulent H1–H4) in rainbow trout fingerlings (figure 2a), while others did not exceed 25 per cent mortality within 7 days (called low virulence strains L1–L3; figure 2a). High mortality is not an artefact of experimental challenge: mortality from naturally occurring, non-treated columnaris infections in an outbreak at a rainbow trout farm in Central Finland reached 100 per cent mortality within 8 days (Suomalainen et al. 2005a). In experimental infections, necrosis of the gills was induced only by the virulent strains (figure 2b). Infectivity of high-virulence strains was higher than the infectivity of the low-virulence strains (figure 2c; χ² = 338, n = 467, p < 0.001). The chondroitin AC lyase activity, which measures the ability of the bacterium to degrade fish connective tissue, varied among strains (figure 2d; analysis of variance (ANOVA), strain nested within virulence Fₙₜₜ = 4.0, p = 0.006) with more virulent strains having higher lyase activity (ANOVA Fₙₜₜ = 14.4, p = 0.001), particularly at higher temperatures (figure 2d; ANOVA, temp × virulence Fₙₜₜ = 6.6, p = 0.015). Bacterial growth measured in vitro increased with temperature (figure 2e; analysis of covariance (ANCOVA), F₁,2₉ = 99.6, p < 0.001). There were strain differences in growth rate (ANCOVA, strain nested within virulence F₀,₂₇ = 6.9, p < 0.001), with virulent strains growing faster than two of the three non-virulent strains; one non-virulent strain, had a growth rate comparable to that of the virulent ones (figure 2e).

Figure 1. Columnaris disease in salmon (*Salmo salar*) fingerlings at a fish farm located in Northern Finland by River Iijoki. (a) Bars represent the proportion of infected rearing units in a given year and the line represents the sum of day degrees when the water temperature exceeded 18°C. (b) Mortality of fish owing to columnaris disease given as an average value (+ s.e.) among those tanks per year where infection by *Flavobacterium columnare* caused mortality. Data for 1986 have been removed because the mortality could not be separated from that of furunculosis outbreaks. In 1992, 1998 and 2000 there was no mortality owing to columnaris disease. White bars indicate the years when oxytetracyclin treatment against columnaris disease was not used. (c) Diversity of symptoms related to columnaris disease of moribund 0+ aged salmon. The symptoms (necrosis, erosion or inflammation of tissue) in skin, tail, saddleback area, jaws or gills. Number of fish studied is presented above each bar. (d) Use of oxytetracyclin treatments per infected tank owing to columnaris disease.
Here we report the emergence and increase in incidence, disease severity and mortality of *F. columnare* at a fish farm during a 23-year study period (figure 1). In the years before antibiotics were used, *F. columnare*-associated mortality increased. After 1992, antibiotics treatments began, but these failed to control mortality, even when the number of treatments administered per tank was increased. Moreover, fish began to suffer increasingly severe symptoms, particularly necrosis of gill tissue. Strains of *F. columnare* vary in lethality (figure 2a) and the severity of symptoms they induce (figure 2b). We suggest that ecological conditions associated with aquaculture have created the epidemiological conditions enabling virulent strains of *F. columnare* to spread and cause outbreaks, particularly during the warm water periods that have extended in length during the study period.

In addition to the rearing conditions at the fish farm increasing the potential for pathogen spread, the ability of *F. columnare* to propagate and transmit from dead fish, enhances greatly the transmission of this pathogen (Kunttu et al. 2009). Decaying fish tissue released from diseased or dead fish is commonly present in the tanks even though farm workers attempt to remove dead and moribund fish daily (Rintama¨ki & Valtonen 1991; Rintama¨ki-Kinnunen & Valtonen 1997). In addition, *F. columnare* is able to survive and maintain its infectivity in lake and distilled water for at least six months (Kunttu et al. 2009) and it is thus likely to persist in tanks even.
during periods when tanks are emptied during the yearly rearing cycle.

During the 23-year study period, water temperatures increased (figure 1a), part of a general summer warming trend in Finland (Altermatt et al. 2008). Longer warm water periods lengthen the period of growth for *F. columnare* and can thus extend the period for columnaris outbreaks. Higher temperatures cause physiological stress for cold-adapted salmonid fish, reducing their capacity to mount effective immune responses (Ilmari Jokinen 2009, unpublished data). In addition, *F. columnare* degrades tissue more rapidly at higher temperatures, an effect that is particularly marked for virulent strains (figure 2d; Kunttu et al. 2009, unpublished data).

Increased water temperatures cannot completely explain the increase of disease incidence and symptom severity. During 1984–1992 there were some summers when the sum of degree days was high (e.g. 1986, 1988), but the prevalence of columnaris disease was low and the gill symptoms were not observed (figure 1). This implies that during the early part of our study period, the strains then present had lower virulence than strains present later in the study period. Our experiments showed that low-virulence strains do not produce severe gill symptoms even at 25°C (figure 2b), a temperature higher than ambient conditions at Finnish fish farms (Suomalainen et al. 2005).

Thus, we hypothesize that the ecological changes associated with fish production, together with the ecological features typical to *F. columnare* (degradation of host tissues, ability to transmit from dead fish and to survive in water), have also resulted in the evolution of more virulent *F. columnare*. There are several reasons to expect that virulent *F. columnare* strains have greater fitness in fish farms than they would in natural fish populations. First, our experimental data show that virulent strains are more efficient in producing tissue-degrading enzymes than non-virulent strains (figure 2d) and that they have a higher growth rate (figure 2e), particularly at warmer temperatures. Thus with more frequent warmer and longer summers virulent strains are able to invade and transmit in a dense fish population better than the non-virulent strains. High growth rates have been previously shown to correlate with virulence in malaria parasites (Ebert & Mangin 1997; Vizoso & Ebert 2005).

Second, there is a positive relationship between virulence and infectivity for *F. columnare* (figure 2c). For a given level of exposure, highly virulent strains infect more fish than low-virulent strains and kill them rapidly because of their higher capacity of producing tissue-degrading enzymes and causing more severe symptoms. *Flavobacterium columnare* is able to propagate and transmit better from dead than from living fish and it is able to persist in water for long periods without fish host (Kunttu et al. 2009). Thus, the parasite has a strongly reduced cost when killing the host, when compared with a disease in which the pathogen dies with the host. As a consequence, virulence may evolve to high levels (Ebert & Herre 1996). However, in natural populations, the fitness advantage that the virulent strains achieve from rapid killing, growth and emission from dead hosts is likely to be much smaller. The lower density of natural populations goes hand-in-hand with a reduced contact rate. Under such conditions, long-living infected hosts (chronic infections) may be favoured. Infected animals are more prone to succumb to predation (Hudson et al. 1992; Seppälä et al. 2003). This effect is likely to be stronger with more severe symptoms. Thus in nature predation increases the costs of high virulence. Moribund fish are removed before transmission of the bacterium occurs.

Third, antibiotic treatments are used at farms to control for mortality caused by bacterial diseases. Antibiotics are administered into a tank whenever fish with columnaris disease are detected. Treatment is effective only when the antibiotic is consumed by fish (with fish food). Diseased and moribund fish cease feeding, so that the treatment neither affects the bacteria in them, nor can it affect the bacteria present in the water or in the fish already dead. Therefore, strains that infect and kill their hosts rapidly are most likely to survive the treatment. Also quick degradation of tissue might be favoured: fish tissue detached from host is not affected by antibiotics, either. The antibiotic treatment was only started after 1992 and usage increased since then (figure 1d). So far, *F. columnare* strains resistant to oxytetracycline have not been found (P. Koski, Finnish Food Safety Authority, Oulu 2009, personal communication). Selection by oxytetracycline may have favoured *F. columnare* strains with most severe symptoms (gill necrosis, killing quickly), that by the time the outbreak was noticed and the antibiotic treatment applied, the bacterium had already escaped into an antibiotic-free niche.

Fourth, within-host competition of co-occurring genetically different pathogen strains in *F. columnare* (Suomalainen et al. 2006a) can be a potent selective force of virulence (Nowak & May 1994; Gandon et al. 2001; Read & Taylor 2001; de Roode et al. 2005a; Bell et al. 2006). Preliminary results from a competition experiment between a virulent and a non-virulent *F. columnare* strain propagated in fish carcasses indicated a competitive advantage and increase in virulence for the virulent strain in only a few days (Suomalainen et al. 2009, unpublished data), as would be expected from relative growth rates (figure 2e). Our data to date suggest that competition could be a major force driving the evolution of virulence of *F. columnare*.

We consider that the arguments above make a strong case that the evolution of *F. columnare* towards higher virulence is one reason why columnaris disease has become such a problem for the industry. But our arguments are correlational or from first principles. Direct evidence that genetic change has occurred and is responsible for the increased incidence and disease severity would of course be more definitive. Unfortunately, attempts to culture *F. columnare* were not successful during the early years of the survey. This failure may in fact be a function of virulence evolution: even now, the isolation of non-virulent strains from fish caught in natural populations or even from experimentally infected fish does often fail, which prevented us from conducting cross-infection studies between *F. columnare* strains from farmed and wild fish. In the absence of such direct evidence other explanations for the increase *F. columnare* incidence are possible. It could be for example, that domestication has led to increased disease susceptibility in farmed fish (Naish et al. 2008). However, we note...
that in spite of the long-term captive breeding of the Ijoki salmon broodstock, no strong changes in genetic diversity have been detected (Säisä et al. 2003). In addition, increased susceptibility owing to domestication could be expected to increase the vulnerability of the fish towards other diseases as well. The incidence of four other bacterial diseases in salmon (Aeromonas salmonicida (furunculosis), atypical A. salmonicida, Versinia rucherii and Serratia sp.), followed during the disease-monitoring programme in 1984–2006 at the farm, have not increased (P. Rintamäki 2009, unpublished data). These bacteria are treated at the onset of the disease with disease-specific drugs as is \( F. columnare \), but to our knowledge they do not have the biological features that we have pointed to above. In particular, they do not transmit from dead fish, and there is no evidence for these diseases that virulence and infectivity are linked.

In summary, fish-farming creates a large number of epidemiological opportunities for virulent pathogen strains (see also Ganusov & Antia 2003). Our data show an increase in frequency and severity of outbreaks of \( F. columnare \) over 23 years at our study farm. We suggest that the ability of \( F. columnare \) to act as saprophyte increased the persistence of virulent strains, and the conditions and farming practices at the farm may have then further selected for virulence. More generally, aquaculture radically changes the ecology of pathogen transmission among fish by altering the epidemiology of the pathogen and by introducing a specific form of pathogen mortality (chemotherapy). These changes affect not only the disease dynamics, but also selection on virulence. We hypothesize that fish farming will in some cases lead to the evolution of more virulent pathogens, and an important challenge will be to identify which pathogens these will be, and whether farm practices can be developed, which usefully manage this evolution.

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REFERENCES


