DETECTION OF MYCOBACTERIOSIS IN FISH USING THE POLYMERASE CHAIN REACTION TECHNIQUE

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Introduction
Infections by acid-fast bacteria are known to occur in both freshwater and marine fish, in warm-water as well as in cold-water species. The subject was reviewed in recent years by Conroy (1984) and Ghittino (1985), and many other cases have been reported since in species of fish of commercial value from various parts of the world (Verdonck et al., 1986; Hedrick et al., 1987; Lawhavinit et al., 1988; Chinabut et al., 1990; Noga et al., 1990). In Israel the disease was found to affect numerous stocks of European sea bass Dicentrarchus labrax grown both on the Mediterranean and the Red Sea coasts (Colorni, 1992), casting serious doubts over the future of commercial culture of bass in this country. The disease remains asymptomatic for a long time, stunts growth, is virtually impossible to eradicate with antibiotics and renders the affected fish unmarketable. Furthermore, cases of human infections by aquatic mycobacteria also have been reported with increasing frequency in both medical and ichthyopathological literature (several authors in Barksdale and Kim's 1977 review, Giavelli, 1979; Smith and Willett, 1980, van Duijn, 1981, Huminer et al., 1986).

As infections cannot be diagnosed by external visual inspection, current management options consist of histological examination of the spleens of randomly sampled individuals and destruction of whole stocks from which positive fish are detected. Histological examination requires the sacrifice of the fish, and is not normally sensitive enough to detect this pathogen in carriers or individuals at early stages of infection.

Recently, however, nucleic acid technologies, namely polymerase chain reaction (PCR) (Saiki et al., 1985) and direct sequencing (Böttger, 1989; Edwards et al., 1989), have held promise for the detection and identification of a variety of Mycobacterium species (Böddinghaus et al., 1990; Rogall et al., 1990).

In this communication, we report the application of PCR procedures for the detection of the disease in D. labrax.

Materials and Methods

Fish Stocks
The infected fish are the broodstock and their two offspring generations of European sea bass D. labrax originally imported to Eilat, Israel, as juveniles from France in 1984. They have been fed with pellets produced according to our own formulation. Sea water is pumped from the Gulf of Eilat and salinity and temperature fluctuations follow those measurable seasonally in the Gulf (40±1 ppt and 24±2°C, respectively).

Histopathology
As the spleen is consistently the most severely affected organ, histopathological examinations were carried out on spleen tissue fixed in neutral buffered formalin, embedded in paraffin, sectioned at 6μm and stained with Ziehl-Neelsen acid-fast reagents, according to standard procedures (Sheehan and Hrapchak, 1980).

Bacteriology
Mycobacterial cultures were grown on Löwenstein-Jensen medium (Difco) and incubated at 24(±0.5)°C.

DNA extraction and PCR reactions
DNA extraction and PCR reactions were carried out from both mycobacterial colonies and fish tissues following standard
methods (Innis et al., 1990; Knibb et al., in press; Maniatis et al., 1982.). Each PCR run for diagnosis included negative controls of no DNA template and human blood genomic DNA, extracted at the same time as other samples, and positive controls of 8 pg, 800 fg and 80 fg of mycobacterium total DNA with 20ng of calf thymus genomic DNA. Oligonucleotides used are identical or similar in sequence to those used by Böddinghaus et al., (1990) for mycobacteria, and the same nomenclature was used. They include:

#246 (5'-AGT TGA TCC TGG CTC AG-3')
reverse
#414 (5'-CAT CCC ACA CCG C[AT]A AAG-3')

Results and Discussion
The sequence of oligonucleotide #246 is highly conserved across the bacteria (Edwards et al. 1989), whereas r#414 is specific to, and conserved across, the genus Mycobacterium (Böddinghaus et al., 1990). Our analysis of all 16S rRNA sequences listed in the GenBank data base (December 1992) indicated that primer #246 with primer r#414 should direct the amplification from 16S rDNA only for species within the Mycobacterium genus. In our laboratory, these primers yielded a PCR product of approximately 220 bp from genomic DNA isolated from mycobacterial colonies grown on Löwenstein-Jensen medium (Fig 1). PCR analysis of spleen genomic DNA, isolated from an apparently uninfected D. labrax individual (as assessed by visual inspection of spleen), yielded two major products of approximately 400 and 1000 bp, whereas analysis of an apparently infected individual (as assessed by visual inspection of spleen) yielded the 400 bp product, the 1000 bp product (faintly), and one additional product of approximately 220 bp (Fig 1). Invariably the 400 bp product, and intermittently the

![Figure 1](image)

**Figure 1.** Mycobacterial DNA amplification from cultures and D. labrax spleens. Lanes one to three correspond to 16μl of PCR mixture after electrophoresis through a 4% agarose gel and ethidium bromide staining.

Lane 1: PCR amplification from mycobacterial genomic DNA using primers #246 and r#414

Lane 2: PCR amplification from 10ng of total DNA from uninfected* D. labrax spleen using primers #246 and r#414

Lane 3: PCR amplification from 10 ng of total DNA from infected D. labrax spleen using primers #246 and r#414

Lane 4: Molecular marker: MspI digested pBR322.

* assessed by visual inspection of spleens.
1000 bp product, were amplified from genomic DNA of all the different D. labrax individuals and tissues/samples tested (including spleen, fin, blood, mucus, semen), but products of the same size were not amplified from genomic DNA of several other teleosts (including Poecilia reticulata, Morone saxatilis, Oreochromis mossambicus, Oncorhyncus mykiss, Sparus aurata). DNA sequencing and restriction site analysis of the 220 bp PCR products showed they were derived from M. marinum (Knibb et al., in press).

In order to compare the sensitivity of PCR detection with conventional histological analyses, spleen samples were taken from eight D. labrax individuals from stocks with a prior history of infection with mycobacteria, and the presence of mycobacteria was tested using both methods. Three of the eight were assessed as positive by histology, whereas all eight were assessed as positive by PCR. These data point to a greater sensitivity of PCR compared with histology, and further analyses (Knibb et al., in press) have indicated the above PCR can detect as few as 20 mycobacterial cells. Analysis of spleens, however, requires autopsy of the fish examined, a serious drawback when dealing with valuable broodstock, as it precludes the option of screening out infected from among non-infected individuals. PCR was used to test for the presence of mycobacteria in the blood (Knibb et al., in press), which can be drawn without sacrificing the fish. Generally, for equal amounts of template DNA (10 ng), blood samples yielded weaker PCR products than from spleen samples of the above eight D. labrax individuals, and only six of the eight were assessed as positive. Further analyses (Knibb et al., in press) have shown that the sensitivity of PCR analysis of blood samples is comparable with that of spleen samples when the amount of blood sample DNA template is increased to 100 ng. Such procedures are more sensitive than bacteriological analysis of the same individuals (Colorni, unpubl.).

In conclusion, the extreme sensitivity of PCR should improve the reliability of a) screening newly imported fish stocks, b) detecting latent infections in existing stocks, and c) removing carriers from brood stocks, while at the same time may help to elucidate the mode of disease propagation, whether through feeds, water or vertically through infected eggs or sperm. Further, the PCR procedure should greatly facilitate the screening of samples collected in the field, since PCR analyses can be carried out on fish spleen tissue (0.5 mm³) preserved in 95% EtOH and stored at room temperature (Ankaoua, unpubl., also see Bramwell and Burns, 1988). Other potential advantages of PCR over classical histological and bacteriological procedures include rapidity of analysis, and accuracy of identification through DNA sequencing and restriction site analysis of PCR products (Knibb et al., in press).

Summary
Mycobacteriosis has become a major concern for the commercial mariculture of the European sea bass, Dicentrarchus labrax, in Israel. The disease remains asymptomatic for a long time, stunts growth, is virtually impossible to eradicate with antibiotics and renders the affected fish unmarketable. The polymerase chain reaction technique was evaluated as a diagnostic tool for detecting an ongoing Mycobacterium infection and found to be more reliable and sensitive than traditional histological and bacteriological procedures.

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References