Aquaculture and Biotechnology

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Foreword

This book is an attempt to focus attention on recent advances in the field of aquaculture biotechnology. Seafoods are a major export commodity for many countries in the Asian region and thus a valuable source of foreign exchange. Fish and fishery products have always been regarded as nutritious components of the human diet and with the recent crises in the meat sector caused by the fear of "mad cow disease", more and more people are turning to fish as an alternative to meat and meat products. The increasing demand for seafood has led to the over-exploitation of wild fish stocks in some regions of the world and has resulted in reduced catches for fishermen. In order to redress this balance, there has been dramatic global expansion in fish and shellfish farming over the past twenty years. Today, aquaculture is one of the fastest-growing sectors of food production, particularly in Asia, a region which accounts for about 90% of the total global production. In 1994, world aquaculture production supplied about 12 million tons of high quality food for direct human consumption that was valued at US $ 39 billion. The contribution that the aquaculture sector makes to global fish supplies is sure to continue its upward trend, and with this expansion, there will be the need for greater focus on the concept of sustainable aquaculture development. The importance of the aquaculture sector is reflected in this book, in that seven of the ten chapters deal with issues relating to the expansion of this industry.

Fish farming is an attractive alternative to commercial fishing, having the potential to decrease the dependence on dwindling stocks of wild fish. Aquaculture offers many advantages for the processor and the consumer when compared with capture fisheries. For instance, products of uniform size or age are a real advantage for automation in fish processing and the production of value-added products. Products from aquaculture tend to be less stressed and suffer from less physical damage

when compared to products that have been hauled in a net along the sea bottom or trapped in gill nets for extended periods. It is widely recognised that such products will be of better quality and will have a longer postharvest shelf-life. Aquaculture products will be fresher that capture fishery products, as harvests can be timed to corresponding schedules, and handling, icing and transportation are all easier to carry out on land when compared to on-board handling conditions and the long distances that some fishermen must travel to reach fishing grounds.

The increase in aquaculture production, particularly the expansion into intensive and semi-intensive methods of production, has been paralleled by an increase in fish diseases, resulting from high stocking densities and stress, conditions that favour the occurrence and spread of infectious disease. These issues are covered in four chapters of this book and specifically focus on antimicrobial chemotherapy and drug resistance in aquaculture; application of antibody probes in the diagnosis and control of fish diseases; immunoprophylaxis for sustainable aquaculture; and research and development needs of aquaculture. The need to prevent and treat diseases is presenting a major challenge to the aquaculture sector, which requires the development of new products to keep pace with the increased volume of production and the emergence of resistance in bacterial pathogens to approved veterinary drugs and biologicals. There are however, problems relating to new products coming in the market and these have been highlighted as: (i) regulatory constraints, where legislation and control measures at the national level may limit the availability of antibiotics for use in aquaculture; (ii) practical factors, such as how to apply antimicrobials; (iii) environmental factors, such as the build up of resistance in fish pathogens, problems relating to residues in products, and regulations preventing the discharge of biologicals in effluents from farms; (iv) economic factors relating to the costs of research and licensing of new antimicrobials; and (v) the absence of international harmonization regarding guidelines and standards for approval for use of aquaculture and biologics.

Scientific management of aquaculture involves improving somatic growth of fishes and maintainance of ecological balance by suppressing the multiplication of prolific breeders. One way to achieve this would be through manipulation of the sex of fishes. Advances in this field are covered in a chapter of this book.

Traditional methods for isolating, identifying and enumerating microorganisms in seafoods can be laborious, expensive and time consuming. Such methods do not provide immediate answers to the questions of microbial contamination, such as whether a product is safe to eat or is acceptable in export markets. With modern approaches to food safety that rely on the application of systematic approaches to safety assurance,

such as the Hazard Analysis and Critical Control Point (HACCP) system, rapid methods are required to detect microbial contamination and to confirm that critical limits for products are not exceeded. The rapid methods described in this textbook can be used when the HACCP system is being developed, implemented and maintained, in order to ensure consumer protection, from *farm to fork*. The successful combination of HACCP programmes and rapid microbiological methods will assist the seafood-processing industry to find new ways of obtaining reliable results more efficiently, and of ensuring seafood safety.

Foodborne diseases are one of the most widespread global public health problems in recent times and their implications for health and economy are being increasingly recognized. These diseases are attributable to a wide range of agents, with varying severity from mild indispositions to chronic or life-threatening illnesses. International trade in foods, particularly seafoods, has expanded dramatically over recent decades as a result of the globalization of world markets. Today, over 460 million metric tons of food move in international trade, valued at about US $ 200 billion. As a consequence, it is not unusual for an average meal to contain foods from many countries that have been produced and processed under different standards of food safety, increasing the risk of foodborne disease. When food safety management systems fail and contaminated foods are exported, the resulting outbreaks can span continents. A bacterial pathogen that has increased in importance is *Listeria monocytogenes*. This organism finds its victims among vulnerable groups such as immunocompromised individuals, pregnant women, neonates and the elderly. It is of particular concern to fish processors in that it can develop at chill-temperatures, particularly in minimally processed products. This textbook provides an excellent account of the occurrence and significance of *L. monocytogenes* in fishery products and the likely impacts for the seafood industry.

Marine environment is the source of not only fish, but also of innumerable life forms which are a reservoir of bioactive compounds. Marine biotechnology is an area, which has vast potential and this area has not received much attention in developing countries. Two chapters focus on this aspect, one of them showing how a fluorescent protein obtained from a marine organism is finding applications in a number of fields.

It is hoped that this book would be a valuable reference material for students and researchers in the area of fishery science and technology.

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Contents

1

Application of Antibody Probes in the Diagnosis and Control of Fish Diseases

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INTRODUCTION

Aquaculture is growing rapidly worldwide with respect to both the quantity and variety of fish species. Fish in culture suffer a wide variety of diseases including bacterial, viral, parasitic and fungal infections, as summarized by Austin and Austin (1993) and Adams *et al.* (1995a). Disease control is therefore an essential component of successful fish cultivation.

Disease control can be subdivided into the two main areas of treatment and prevention. Stress and disease which accompany intensive fish culture have led to treatment with antibiotics and chemicals. Disease prevention by means of optimal husbandry and use of biological control methods, such as vaccination and the use of immunostimulants should, however, be developed. These are essential as concerns for pollution associated with chemical treatments, and the emergence of multiple resistance to antibiotics makes control of infections increasingly more difficult.

Antibody probes have made a significant impact in fish disease control in both the areas of treatment and prevention (Adams *et al.*, 1995a, 1995b). In particular, the probes can be utilized as tools for the development of diagnostic tests, thus infections can be treated quickly and with the correct medication. Such probes have also proved invaluable in the development of vaccines.

Antibody Probes

Antibody probes can either be polyclonal or monoclonal in nature. Antiserum prepared in rabbits, for example, following a series of immu-

nizations, contains antibodies that are specific for many different antigens, i.e., polyclonal antibodies. Monoclonal antibodies on the other hand are produced in tissue culture by hybridoma cells. These are homogeneous, of defined specificity, and can be generated in unlimited quantities, making them powerful immunological tools.

Hybridoma production was first described by Köhler and Milstein (1975). In this technique antibody producing secretory cells, isolated from the spleen of an immunised animal, are fused with myeloma cells. These cell types complement each other because the cells from the immunized animal produce antibodies but cannot grow in culture, while the myeloma cells grow well in culture but do not produce antibodies. The hybrid cells can be maintained *in vitro* and will continue to secrete antibodies with a defined specificity. Antibodies that are produced by hybridomas are known as monoclonal antibodies (MAbs).

Initially, a series of immunizations is given to mice or rats in the production of MAbs (Campbell, 1984; Harlow and Lane, 1988). Once a positive response has been detected in the serum, the spleen from the animal, is removed. This organ contains the antibody-producing cells. The cells are then teased apart and fused to myeloma cells to prepare hybridomas.

Polyethylene glycol (PEG) is the chemical most commonly used to fuse mammalian cells. PEG fuses the plasma membranes of adjacent cells, forming a single cell with two or more nuclei. Even in the most efficient fusion, only about 1% of starting cells are fused and one cell in a hundred thousand cells forms viable hybrids (Harlow and Lane, 1988). This therefore leaves a large number of unfused cells still in culture. The cells from the immunized animal will not grow in tissue culture and will die within a few days. The myeloma cells, however, grow rapidly in tissue culture and must be removed. This is achieved by a process termed "chemical selection", using aminopterin in the culture medium. The hybridoma cells are cloned and cultured at 37°C, supplemented with 5% CO_2.

IDENTIFICATION OF FISH PATHOGENS

The majority of infections described for fish in culture are bacterial (Adams *et al.*, 1995a). Routine bacteriological screening by plating out onto agar involves a lag phase dependent on the growth cycle of the particular bacterium. This may vary from overnight, as in the case of *Photobacterium damsela* subs. *piscicida*, to two of three days for *Aeromonas salmoniciaa* and *Vibrio* spp. In some instances, where the growth is extremely slow, for example, with *Renibacterium salmoninarum* and *Mycobacterium* spp., culture on agar medium is rarely achieved due to overgrowth with

contaminants. In these cases, culture on selective medium and histological evidence for infection is used. The identification of bacteria once they have been cultured is normally done by biochemical analysis, using the API 20E rapid identification system, and may be productive even for those bacteria which grow quickly in culture. *P. piscicida,* for example, is a very unreactive pathogen only giving positive responses for arginine dihydrolase and weak acid production from glucose, with all other tests being negative (Austin and Austin, 1993). Serological tests can be particularly useful in these cases of unreactivity and slow growth.

Identification of viral, parasitic and fungal pathogens requires the skills of light and electron microscopy. Definitive recognition of specific pathogens can often not be made. The development of rapid serological methods is, therefore, of prime importance for the detection of such infectious agents.

RAPID SEROLOGICAL DIAGNOSTIC TESTS

A variety of antibody-based tests have been developed to detect mainly bacterial and viral fish pathogens, as shown in Table 1. These include slide agglutination, co-agglutination/latex agglutination, immunodiffusion, direct and indirect fluorescent antibody tests (FAT and IFAT), immunohistochemistry (IHC), enzyme linked immunosorbent assay (ELISA), dot blot/dip stick and Western blot (WB). Serological tests were first developed using PAbs. These tend to be stable probes which can be used in simple tests such as slide and latex agglutination, to achieve a result very quickly. Due to problems with cross-reactivity with related pathogenic species the antibodies very often have to be purified before use. Nevertheless, such probes have made a significant impact on the rapid diagnosis of fish disease. PAbs have been shown to achieve sensitivities of around 10^3–10^4 bacteria per ml in ELISAs (Adams and Thompson, 1990; Adams, 1992) and have been successfully used to detect carrier fish for *Aeromonas salmonicida* (Rose *et al.*, 1989). More recently antibody-based tests have been developed using MAbs. These probes assure a reliable source of antibodies and very often achieve greater sensitivity and specificity than PAbs. A combination of MAbs and PAbs in the same test can also prove useful (Adams and Morris, 1994, Adams *et al.*, 1995b).

In our laboratory IFAT, IHC, ELISA and Western blotting tests have been developed for the detection of one parasitic (PKX) and five bacterial fish pathogens (*A. salmonicida, Photobacterium damsela* subs. *piscicida, Mycobacterium* spp., *R. salmoninarum* and *Vibrio* spp.). The antibody-based method selected for the identification of pathogens depends on a variety of factors since each method has its merits and disadvantages.

Table 1: Summary of serological assays used to detect fish pathogens

Test type	Pathogen	Reference
Agglutination	*A. salmonicida*	McCarthy & Rawle (1975)
	A. hydrophila	Eurell *et al.* (1978)
	R. salmoninarum	Bullock *et al.* (1974)
	Streptococci	Kitao (1982)
	Vibrio spp.	Tajima *et al.* (1987)
Immunodiffusion	*R. salmoninarum*	Chen *et al.* (1974); Kimura *et al.* (1978)
Co-agglutination/ latex	*A. salmonicida*	McCarthy (1975), Kimura and Yoshimizu (1983; 1984)
	R. salmoninarum	Kimura and Yoshimizu (1983)
	Y. ruckeri	Hansen & Lingg (1976)
FAT/IFAT	*A. hydrophila*	Kawahara and Kusuda (1987)
	A. salmonicida	Sakai *et al.* (1986)
	Cytophaga spp.	Baxa *et al.* (1988)
	R. salmoninarum	Bullock *et al.* (1980), Bullock and Stuckey (1975); Laidler (1980), Lee and Gordon (1987), Mitchum *et al.* (1979), O'Halloran *et al.* (1995); Paterson *et al.* (1979)
	Streptococci	Kawahara & Kusuda (1987)
	Y. ruckeri	Johnson *et al.* (1974)
	IHN	Arnzen *et al.* (1991); LaPatra *et al.* (1989)
IHC/PAP	*A. salmoniarum*	Sakai *et al.* (1986)
	R. salmoninarum	Hoffman *et al.* (1989)
	Mycobacterium spp.	Gomez *et al.* (1993)
	Vibrio spp.	Evensen *et al.* (1991)
	Y. ruckeri	Jannson *et al.* (1991)
	myxosporeans	Bartholomew *et al.* (1990) Adams *et al.* (1992)
Dot blot	*R. salmoninarum*	Sakai *et al.* (1989), Sakai & Kobayashi (1992)
ELISA	*A. salmonicida*	Goerlich *et al.* (1984), Austin et al. (1986), Rose *et al.* (1989), Adams and Thompson (1990)
	Edwardsiella spp.	Rogers (1981); Ainsworth *et al.* (1986)
	Mycobacterium spp.	Adams *et al.* (1995b, 1995c)
	R. salmoninarum	Dixon (1987), Gudmundsdottir *et al.* (1993), Pascho et al. (1987), Hsu *et al.* (1991)
	Y. ruckeri	Cossarini-Dunier (1985), Austin *et al.* (1986)
	Vibrio spp.	Adams (1991); Campbell *et al.*, 1993
	IHN	Jorgensen *et al.* (1991)
	VHS	Jorgensen *et al.* (1991), Mourton *et al.* (1990); Olesen *et al.* (1991)
Western blot	*R. salmoninarum*	Sakai *et al.* (1990), Griffiths *et al.* (1991), Olivier *et al.* (1992)

IHC of formalin-fixed kidney sections enables the PKX parasite, which causes proliferative kidney disease (PKD) in salmonids, to be visualized, identified and subsequently counted under a light microscope. MAb12 binds to both intraluminal and interstitial stages (Adams *et al.*, 1992; Adams and Marin de Mateo, 1994, Marin de Mateo *et al.*, 1993, 1995). Counterstaining the tissue section also allows the cells surrounding PKX, and therefore host response, to be observed. IHC using PAbs has also proved useful for the detection of *Piscirikettsia salmonis* in Atlantic salmon, *Salmo salar* (Alday-Sanz *et al.*, 1994).

Immunofluorescent techniques offer the advantage of a more immediate result. For example, *R. salmoninarum* can be identified using IFAT on fresh kidney imprints within 30 minutes (Adams *et al.*, 1995a, 1995c). Rapid diagnosis can therefore be made avoiding lengthy culture of this slow-growing bacterium. The method, however, requires the use of a specialized microscope and the results cannot be stored for future examination.

Both IHC and IFAT are limited by the number of samples which can be processed, whereas the ELISA is ideal for screening large numbers of samples, as well as being quantitative and extremely sensitive. Sandwich ELISAs were developed to detect *Aeromonas salmonicida* in fish tissue (Adams and Thompson, 1990), *Vibrio anguillarum* in fish feed (Campbell *et al.*, 1993) and *Vibrio alginolyticus* in prawns using PAbs (Adams, 1991). More recently, MAbs have been produced to *A. salmonicida, Mycobacterium* spp., *P. damsela* subs. *piscicida* and *R. salmoninarum* for the development of rapid diagnostic tests (Neelam *et al.*, 1995; Adams *et al.*, 1995b; 1995c). The *A. salmonicida* ELISA was assessed for its effectiveness in the detection of carrier fish in comparison with the commonly used corticosteroid/heat protocol of McCarthy (1980) and plating out bacteria onto agar (Rose *et al.*, 1989). The ELISA appeared to be the most sensitive method and is a valuable tool for the rapid detection of *A. salmonicida* in clinically-diseased and asymptomatic carrier fish. However, ELISA cannot distinguish between living and dead cells. The culture of bacterial pathogens in parallel with serology is also recommended so that antibiogrammes, for example, may be determined. This may not always be possible with slow-growing microorganisms such as *R. salmoninarum* and *Mycobacterium* spp.

Mycobacteria are difficult to isolate from infected tissue despite the presence of large numbers of acid-fast bacilli. Diagnosis usually follows the histological rather than the bacteriological examination of granulomatous lesions, for example, using Ziehl Neilsen staining (Frerichs and Roberts, 1989). These methods are difficult and time-consuming, therefore Mab probes provide a useful alternative. A sandwich ELISA was developed using polyclonal antibodies to capture *Mycobacterium* spp. The bound pathogen was then detected using a cocktail of three MAbs. The

combination of antibody probes in the ELISA produced an effective rapid test for large numbers of samples. This methodology was applied to the screening of infected fish stocks in Thailand (Adams *et al.*, 1995b, 1995c) to aid in the control of mycobacteriosis. Western blot analysis of MAbs to *P. damsela* subs. *piscicida* and *Mycobacterium* spp. has provided useful information on the differences between strains (Bakopoulos *et al.*, 1995; Chen *et al.*, 1995).

This technique is often favoured for the detection of *R. salmoninarum* (Griffiths *et al.*, 1991).

VACCINE DEVELOPMENT

The primary considerations for the development of any successful vaccine in aquaculture are cost-effectiveness and safety. To accomplish this, the vaccine must provide long-term protection against the disease under the intensive rearing conditions found on commercial fish farms.

Numerous fish vaccines are currently being developed, however, the end products have had variable success (Adams *et al.*, 1995a). Failures may be due to the preparations containing the wrong antigens, because the protective antigens are only weakly immunogenic or the responses to them may be suppressed by other antigens.

Of the many infectious diseases affecting fish there are to-date, only eight for which effective commercial vaccines have been produced (Adams *et al.*, 1995a; Gudding et al. 1997). Four of these are against bacterial diseases which affect salmonids, i.e., enteric red mouth (ERM), vibriosis, cold water vibrosis and furunculosis, while the others are against a bacterial diseases (Pasteurellosis), are in catfish (enteric septicaemia of catfish, ESC) and sea the viral infections, spring viraemia of carp (SVC) and infectious pancreatic recrosis (IPN) of salmon.

All the commercial vaccines except one presently available comprise inactivated (killed) disease agents. When such an approach failed in the development of viral vaccines, for example (Leong and Fryer, 1993), then live attenuated vaccines were developed. However, when these are used there is always concern that the attenuated strain (usually as a result of gene deletion) may back-mutate and revert to the virulent wild type. Many of the successful vaccines against viral diseases in humans (e.g. rubella, measles, poliomyelitis) and in domestic animals (e.g. rabies, distemper) are live-attenuated organisms. Licensing of such vaccines may however prove to be very difficult in aquaculture. An alternative approach is to prepare subunit vaccines, where the specific components of the disease-causing agents are isolated and then used in vaccines. In order to increase the quantity of available antigens, the recent trend has

been to clone up the genes encoding for specific antigens and then to incorporate them into bacterial DNA where they are expressed, i.e., recombinant vaccines. The IPN vaccine presently used in Norway is a recombinant vaccine (Gudding *et al.*, 1997).

Antibody probes are extremely useful in the development of recombinant vaccines, as both PAbs and MAbs can be utilized to screen pathogen DNA libraries for potential vaccine antigens. Such vaccines have been developed for VHS (Thiery *et al.*, 1990; Lorenzen, 1991), IPN (Havarstein *et al.*, 1990; Manning and Leong, 1990; Lawrence *et al.*, 1989; Hah *et al.*, 1992), and channel catfish virus, CCV, (Awad, 1981). The development of bacterial and parasitic recombinant vaccines using MAbs as probes, is also underway for BKD and PKD, respectively (Adams *et al.*, 1995c).

Intimate knowledge of a parasite's life cycle plays a crucial role in the development of effective vaccines. Mab probes to PKX have revealed that antigens change during the development of PKX (Adams *et al.*, 1995c). The identification of common antigens to host and parasite alike have also been reported using the MAbs. In this instance, the MAb was shown to bind to the kidney tubule cells as well as to PKX (Adams *et al.*, 1992). Antigens from the fish sporozoan, *Myxosoma cerebralis* which mimic tissue antigens of rainbow trout (*Salmo gairdneri*) have been identified (Pauley, 1974). Such antigens would need to be avoided in the preparation of vaccines.

FUTURE PROSPECTS

With the expansion of aquaculture a more diverse range of fish species are being intensively cultured. Concurrently, there this is an increase in the variety of diseases encountered. For example, pasteurellosis was only recently described in Europe and Rickettsiosis in Chile, Ireland and Taiwan. Both are now recognized as highly significant pathogens. Antibodies, in particular MAbs, are powerful and versatile tools to aid in the control of fish diseases. These probes are being utilized in the development of a growing number of rapid diagnostic tests and vaccines.

The combination of immunology with molecular biology techniques, such as DNA probe detection (Hiney *et al.*, 1992), recombinant and DNA vaccines (Adams *et al.*, 1995a) provides an even more sophisticated array of diagnostic/vaccine methodologies for the future.

Acronyms

FAT: Fluorescent Antibody test
IFAT: Indirect Fluorescent Antibody test
IHC: Immunohistochemistry

PAP Peroxidase Anti Peroxidase
ELISA. Enzyme Linked Immunosorbent Assay
IHN: Infectious Haematopoietic Necrotic Virus
VHS Viral Hemorrhagic Septicemia

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