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Recent applications of biotechnology to novel diagnostics for aquatic animals

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Summary

Improvement of the methods included in the World Organisation for Animal Health (OIE) *Manual of Diagnostic Tests for Aquatic Animals* and addition of novel techniques are dependent on the continual development and evaluation of both new and existing methods. Although conventional isolation and characterisation techniques for the diagnosis of many diseases still remain the methods of choice there is the potential to make significant progress in the development of rapid methods that will enhance the diagnosis of disease in aquatic animals. For example, serology, rapid kits based on immunochromatography, Luminex xMAP™ technology, adaptations of the polymerase chain reaction (PCR), polygenic sequencing and micro-array technology are all methods that merit validation. Each of the technologies has advantages and disadvantages that need to be considered. This is not a definitive list, and new methods being developed (or that are developed in the future) should continue to be validated if they provide potential benefit to the diagnosis of disease in aquatic animals.

Keywords

Aquatic animal – Biotechnology – Disease diagnosis – Luminex xMAP™ technology – Micro-array – Novel rapid method – Polygenic sequencing – Rapid kit – Real time PCR – Serology.

Introduction

Rapid, accurate diagnosis of disease is essential for effective outbreak control. Once the causative agent has been identified appropriate advice can be given to neighbouring farmers/trading partners. Prompt action in the early stages of any disease problem can have an enormous impact on the scale of the outbreaks. Rapid diagnostic methods therefore provide powerful tools during emergency management.

The World Organisation for Animal Health (OIE) *Manual of Diagnostic Tests for Aquatic Animals* 2006 (the *Aquatic Manual*) (34) contains a variety of diagnostic methods (traditional, immunological and molecular) for the identification of pathogens from aquatic animals. These are the most up-to-date methods that have been developed and validated for use in the identification of disease agents. Validation is the evaluation of a process to determine its fitness for a particular use and includes assay optimisation and demonstration of performance characteristics such as sensitivity and specificity. Although the aim of the *Aquatic Manual* is to provide a uniform approach to the diagnosis of the diseases, it is inevitable that the types of methods used differ between pathogens. Improvement of methods (and addition of new methods to the *Aquatic Manual*) is dependent on the continual development and evaluation of both new and existing methods. Conventional isolation and characterisation techniques for the diagnosis of many diseases still remain the methods of choice. However, these traditional methods tend to be costly, labour intensive, slow and might not lead to a definitive diagnosis, even when complemented with

histological evidence. The rapid progress made in biotechnology over the last decade has enabled the development and improvement of a wide range of molecular and immuno-diagnostic techniques (5, 12) and has made reagents and commercial kits more widely available. Although not all of these methods have been validated they do offer great potential and should be pursued and evaluated. This will enhance and widen the range of methods available for testing in reference laboratories and other recognised diagnostic facilities.

Existing methods

Pathogen detection in fish, molluscs and crustaceans can be achieved using a variety of methods – traditional, immunological or molecular (3). Some of these methods have been validated for use in the OIE *Aquatic Manual* for some diseases, but not for others.

Traditional methods

Traditional methods rely on bacteriology, virology, parasitology, and mycology skills, whereby the pathogens are cultured from specimen samples and then identified biochemically (e.g. API for bacteria), microscopically (e.g. parasites) or using the electron microscope (e.g. viruses). Skills in histology and histopathology are also very important in the diagnosis of disease. Histopathology is particularly useful where new diseases are emerging and the causative agent may not yet have been identified nor specific laboratory methods developed to detect the pathogen. Histology can be combined with immunological methods (e.g. immunohistochemistry using pathogen-specific antibodies) or molecular methods (e.g. *in situ* hybridisation).

Immunological methods

Many immunological methods have already been developed and a wide range of antibodies (4) and a variety of kits are now commercially available (Table I) to detect fish and shrimp pathogens.

Detection of pathogens

Immunological methods such as fluorescent antibody technique (FAT) and indirect fluorescent antibody techniques (IFAT) (Fig. 1a), immunohistochemistry (IHC) (Fig. 1b), enzyme-linked immunosorbent assay (ELISA) (Fig. 1c) and dot blot/Western blot (Fig. 1d and Fig. 1e) enable rapid, specific detection of pathogens without the need to first isolate the pathogen (3). Monoclonal antibodies (mAbs) provide ideal standardised reagents for such tests and many are now commercially available against a variety of fish pathogens (4).

The antibody-based test selected for the identification of a particular pathogen depends on a variety of factors because each method has its merits and disadvantages.

The FAT and IFAT are very simple, sensitive methods that can be performed within two hours. However, there is a requirement for specialised equipment (i.e. fluorescent microscope or confocal microscope) (Fig. 1f) and a skilled operator to read results, because there can be problems with non-specific background staining. Nevertheless, this method is widely used for the detection of fish pathogens in samples cultured from infected fish, formalin-fixed tissue sections, or on imprints made directly from infected tissue. It is particularly useful for the identification of viruses and bacteria that are difficult to culture. Immunohistochemistry, an extension of traditional histology, is an equally straightforward method (2). The method takes two to three days to complete as samples are formalised, paraffin wax embedded and then incubated with pathogen-specific antibody. A variety of substrates are available for IHC and the assay can be amplified using biotin-streptavidin (26), avoiding the need for a fluorescent microscope; only a simple light microscope is required. Although IHC is regarded as less sensitive than IFAT it has the advantage of being able to visualise the surrounding cells, and therefore the pathology associated with the infection.

The ELISA can be used in a variety of formats, both for the detection of pathogen and for serology (detection of antibodies to the pathogen) (2). In general, the sandwich ELISA used for the detection of pathogens is complex to set up because standards are required for quantification (1). This assay is useful for the detection of pathogens during clinical disease, but is limited in its application to sub-clinical infection. It does have the advantage of high throughput, automated equipment is available, and it is quantitative.

Western blot and dot blot are not used routinely as diagnostic methods, but their application can be useful in certain situations, e.g. they are used to confirm the presence of shrimp viruses (white spot shrimp virus [WSSV]; yellowhead virus; Taura syndrome virus) that cannot be cultured in cell lines. The Western blot is also used in mammalian diagnostics for the detection of prions and to discriminate between bovine spongiform encephalopathy and scrapie (31). In Western blot, proteins are first separated by size, using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in the gel are transferred to a membrane support such as polyvinylidene difluoride or nitrocellulose, and the membrane is then probed with pathogen-specific antibodies. Antibodies that bind to the proteins of interest can be visualised using chromogenic products, fluorescence, chemiluminescence or autoradiography.

Molecular methods

The possibility of amplifying very small amounts of defined sequences of DNA in the polymerase chain reaction (PCR) so that they can be detected in conventional methods has increased the potential of pathogen detection and identification. Molecular methods based on PCR are now widely used for the detection of pathogens in fish and shrimp (12). The PCR is exceptionally sensitive and is unaffected by the altered expression of antigens because DNA (RNA can also be used as a template) is detected rather than protein. However, false positive and negative results owing to contamination or inhibition can lead to problems in the analysis of results (21).

Molecular technologies are currently used for the confirmatory identification of pathogens isolated using traditional methods. However, the OIE *Aquatic Manual* does state that:

'With one or two exceptions, molecular techniques are currently not acceptable as screening methods to demonstrate the absence of a specific disease agent in a fish population for the purpose of health certification in connection with international trade of live fish and/or their products. There is a need for more validation of molecular methods for this purpose before they can be recommended in the *Aquatic Manual*'.

Novel methods with potential for inclusion in the OIE *Aquatic Manual*

Although immunodiagnostics and molecular biology have already made a significant impact on aquatic animal disease diagnosis there is potential for rapid advancements in the future, for example, through serology, the use of rapid kits based on immunochromatography, Luminex xMAP™ technology, adaptations of the PCR, polygenic sequencing and micro-array technology.

Detection of antibodies: serology

What is serology?

This technology enables detection of the host response to the pathogen. Detection of specific antibodies in the serum of animals is recognised as a useful indicator of previous exposure to pathogens, and such methods are regularly used in clinical and veterinary medicine (9, 16, 25, 35). They are capable of indicating infection before it is possible to detect the pathogen by culture, or other methods, and they have the advantage of being non-destructive. Serology can also be applied to the detection of pathogen-specific antibodies in fish. This may be in fish suspected of having been exposed to specific pathogens, e.g. in broodstock screening for exposure to viruses or following vaccination to monitor immune response. The ELISA is well suited to large-scale screening, and this can be performed in any species of fish where an anti-fish species antibody is available (4). The specificity of the test is dictated by the pathogen used to coat the ELISA plate. It is therefore very important that the most appropriate isolate is used. If the identity of the reacting epitope is known then a synthetic peptide could be used to coat the plate. Serological tests will not distinguish between exposed fish and vaccinated fish unless vaccines (in the future) are specifically designed to incorporate markers that can be identified. This should be considered, particularly for recombinant vaccines, as it would widen the availability of serology in diagnostic screening.

Serology as a screening tool

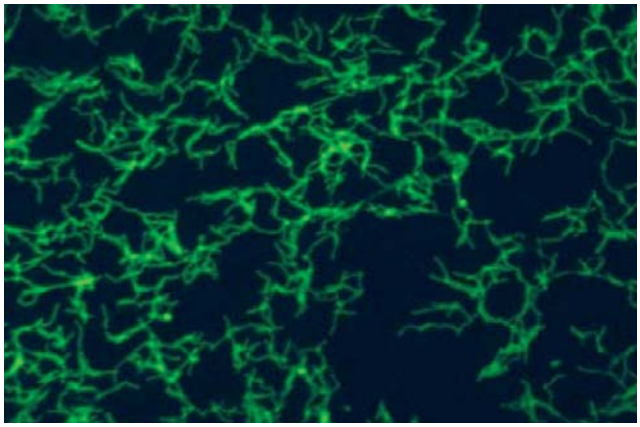
The diagnostic methods presented in the *Aquatic Manual* are all direct diagnostic methods, while serology offers an alternative, indirect approach to pathogen detection. Due to insufficient development of serological methodology, the detection of antibodies to pathogens in fish has not thus far been established as a routine method for assessing the health status of fish populations, or been widely accepted for use in the *Aquatic Manual* (mainly due to a lack of knowledge about the antibody responses of fish to viral infections), but it does have the potential for future use once validated, particularly for detecting pre-exposure of fish to viral pathogens.

In the latest edition of the *Aquatic Manual*, serology is included in some disease chapters as a method of confirming infection, e.g. Chapter 2.1.17 – koi herpesvirus (KHV) disease (34 [web version only]). It is known that an antibody response is produced in carp during KHV infection (6, 27), and detection of this response may help to establish previous exposure to KHV in apparently healthy fish, or possible latent infection by the virus.

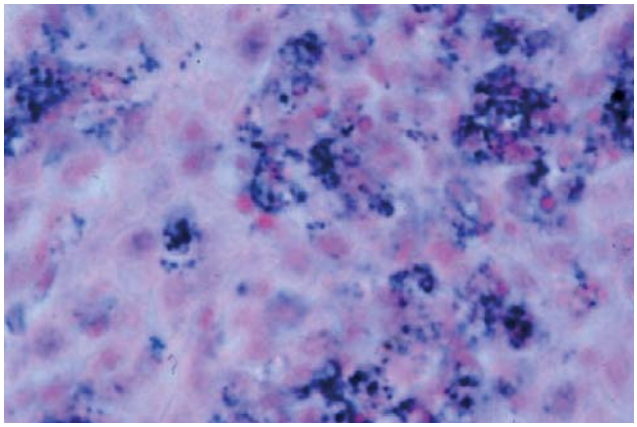
Molluscs and crustaceans do not produce antibodies as a response to infection and therefore this method cannot be used for these species.

Lateral-flow immunoassays

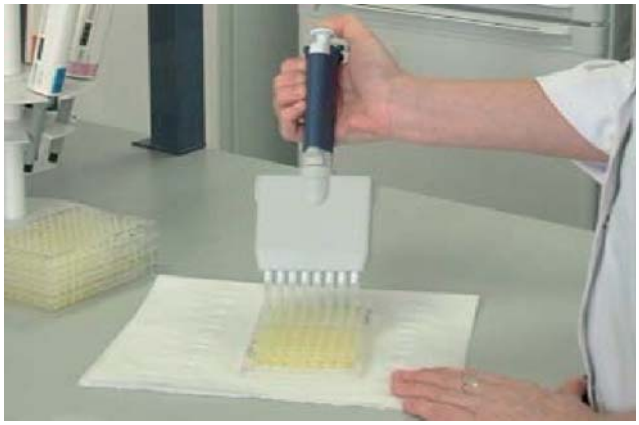
Lateral-flow immunoassay systems (based on immunochromatography) have been widely used to diagnose infections in animals and humans (7, 8, 9, 10, 15, 17) because they allow rapid and sensitive detection of pathogens. The assay is referred to as a lateral-flow immunoassay because it is based on the flow of the infected sample along a cellulose membrane by capillary action; it is also referred to as a gold-immunochromatographic assay, due to the application of



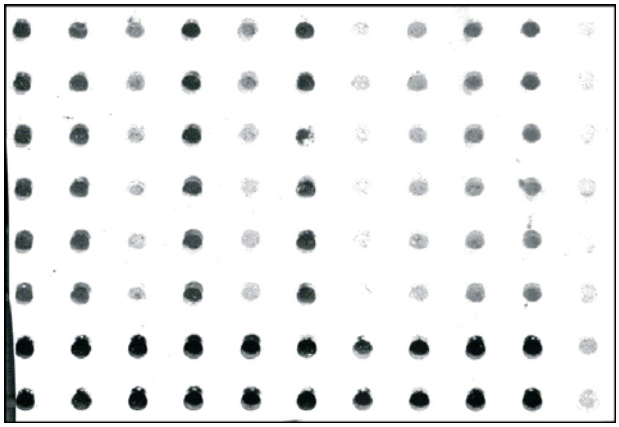
a) Indirect fluorescent antibody technique on a smear of *Flavobacterium psychrophilum*



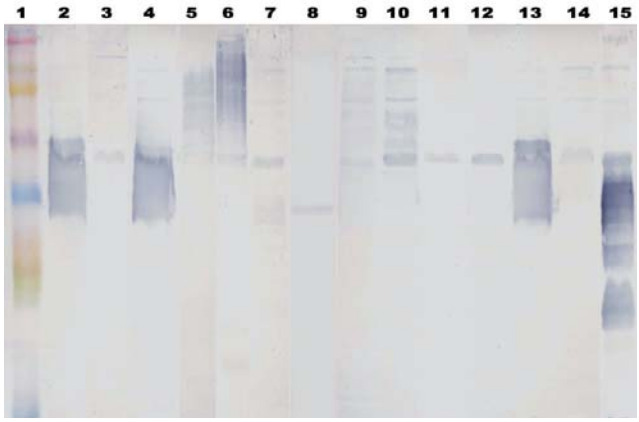
b) Immunohistochemistry of rainbow trout kidney infected with *Renibacterium salmoninarum*



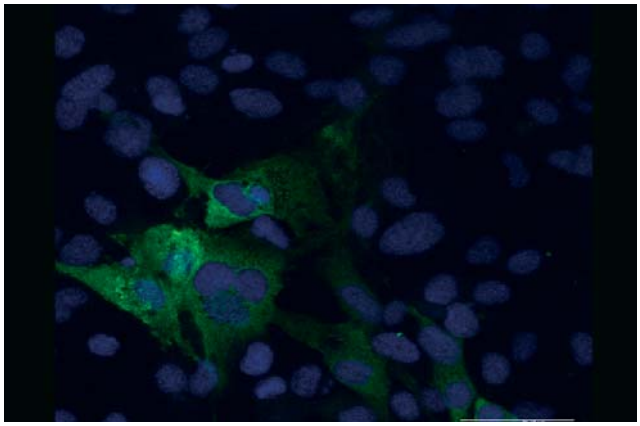
c) Enzyme-linked immunosorbent assay procedure



d) dot-blot assay with monoclonal antibodies against a variety of *Piscirickettsia salmonis* isolates



e) Western blot of immunised carp serum against different stains of *Aeromonas hydrophila*



f) Confocal microscopy of SHK-1 cells infected with infectious salmon anaemia virus

Fig. 1
Immunological diagnostic methods

colloidal gold-labelled antigen or antibody as the detector antibody.

Antibodies (usually mAbs) are immobilised onto the nitrocellulose membrane to form control (anti-mouse immunoglobulin mAbs) and test lines (pathogen-specific mAbs), where they act as capture antibodies, as shown in the example in Figure 2.

The test is performed by adding a small volume of sample (infected tissue homogenised in sampled buffer supplied in the kit) to a reagent pad that contains another pathogen-specific mAb labelled with colloidal-gold (i.e. this mAb is different from the one used to prepare the test line). If pathogen is present in the infected sample, it will react with the colloidal-gold-conjugate to form an antigen–antibody complex. This migrates along the nitrocellulose membrane by capillary action and, subsequently, reacts with the other pathogen-specific mAb immobilised on the test line to produce a band, the density of which is proportional to the concentration of pathogen present. The unbound conjugate continues to migrate and passes over the control line where it reacts with anti-mouse IgG antibody on the control line. Results of the assay are deemed positive if a band is present at both the test line and the control line, negative if the band appears only at the control line, or invalid if no band appears at either line or only one band appears at the test line (Fig. 3).

Rapid diagnosis and fast removal of infected fish are needed to implement effective control strategies during disease outbreaks. Lateral-flow immunoassays allow quick and sensitive detection of a pathogen, thus providing time to implement early control measures to avoid the spread of the disease. This technology has many advantages over traditional immunoassays; the assay is simple to use, very rapid (with results in minutes rather than hours or days), of low cost, does not require skilled operators or expensive equipment, and has similar sensitivities to PCR. Evaluation of the results is performed by the naked eye, and total assay time is less than 15 min. In addition, the kits are stable at room temperature. These assets make the assay very suitable for on-site (or laboratory) sampling to detect pathogens. The kits can also be set up to detect pathogen-specific antibodies (i.e. serology), but commercial application of the kits in this format may be restricted because of the need to include pathogen (antigen) in the kit. This could be overcome by using recombinant proteins or peptides to render the kits safe for transportation.

Adaptations in polymerase chain reaction

Polymerase chain reaction is a very versatile technique and variations in the PCR method include, reverse transcription PCR (RT-PCR) for amplification of RNA, and real-time quantative PCR (qPCR), which allows for quantification of the DNA (or RNA). The rapid development of molecular technologies such as real-time PCR and nucleic acid sequence-based amplification (24, 30) provides methods that reduce possibilities of contamination and offer high sample throughput.

The development of PCR technology has led to innovations in methods for the detection of pathogens in diverse environments such as water, soil, and food samples. The PCR, often in combination with other techniques, has opened up numerous possibilities in epidemiological studies for the identification of individual strains, and in particular the differentiation of closely related strains. Ribosomal RNA genes (for 5S, 16S and 23S rRNA) are often used as the target DNA for bacterial pathogens because they contain highly conserved regions and variable species-specific regions. Standard PCR might be sufficient for the detection of pathogens. However, if the pathogen needs to be identified to species level (26), differentiated from closely related species, or live pathogen distinguished from dead pathogen, then modifications and extensions to the method need to be made. Some of the most common PCR variations used in diagnostics are nested PCR (used to increase sensitivity, as shown in Figure 4), random amplified polymorphic DNA (RAPD), reverse transcriptase-PCR (RT-PCR) (12), reverse cross blot PCR (rcb-PCR) (26) and RT-PCR enzyme hybridisation assay (33).

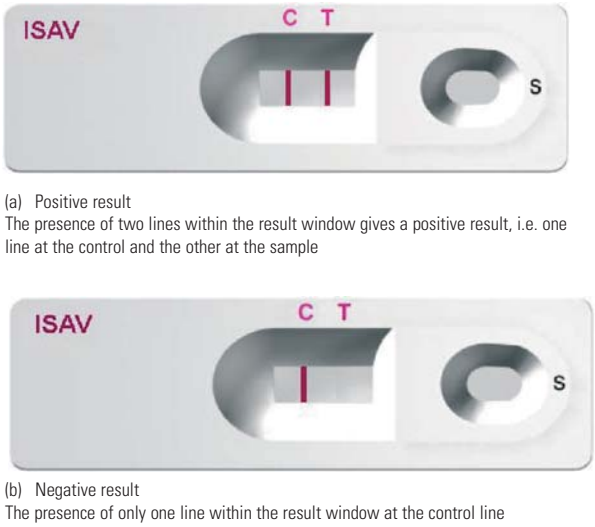
In the nested PCR, two different primer sets are used in two consecutive PCR reactions to increase the specificity of DNA amplification. The products generated in the first reaction are subjected to a second PCR reaction (the primer set of the second reaction amplifies DNA within the first reaction product). The RT-PCR enzyme hybridisation assay offers the advantages of detecting live pathogens and allowing large sample throughput. *In situ* hybridisation and *in situ* hybridisation-PCR are techniques for visualising specific DNA and RNA sequences within cells. They are mainly used for research purposes rather than diagnostics to identify and localise infections, map gene sequences to chromosomes, and identify sites of gene expression and portals of entry of pathogens (22). The quantitative polymerase chain reaction (qPCR) is used to quantify the amount of DNA present in the PCR products. Although other quantification methods are available that measure the level of DNA after the reaction is complete, the most sensitive qPCR is carried out in real-time, where the amount of DNA is measured after each cycle of PCR through the use of fluorescent markers. This is also referred to as real-time PCR (RT-PCR), not to be confused with reverse transcription PCR which is also referred to as RT-PCR, but is used to amplify RNA samples.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a relatively new method for amplifying DNA. It relies on

autocycling strand displacement DNA synthesis, and because it is carried out under isothermal conditions, it can be performed without the use of a thermacycler. The method uses *Bacillus stearothermophilus* DNA polymerase and a set of four specially designed primers (two inner and two outer primers) to recognise a total of six distinct sequences on the template DNA (23). The reaction time can be reduced using two further primers. LAMP is initiated by one of the inner primers, followed by strand displacement DNA synthesis by one of the outer primers. Loop mediated autocycling amplification then proceeds using the newly synthesised DNA as template and the other inner and outer primers for the next round of amplification. The products that result are stem-loop DNA structures which contain several inverted repeats in the target, and cauliflower-like structures with multiple loops as a result of annealing taking place between the inverted repeats on the same strand (29). Products of LAMP amplification can be seen by the naked eye with the addition of SYBR Green I to the mixture, which changes from orange to green in colour if the reaction is positive. The complete LAMP procedure can be performed in 90 min (15 min for DNA extraction; 60 min for the amplification and 2 min for colour detection) and is suitable as a field test if a water bath is available (29). The reaction is highly sensitive and capable of detecting as few as six copies of DNA in the reaction mixture (23).

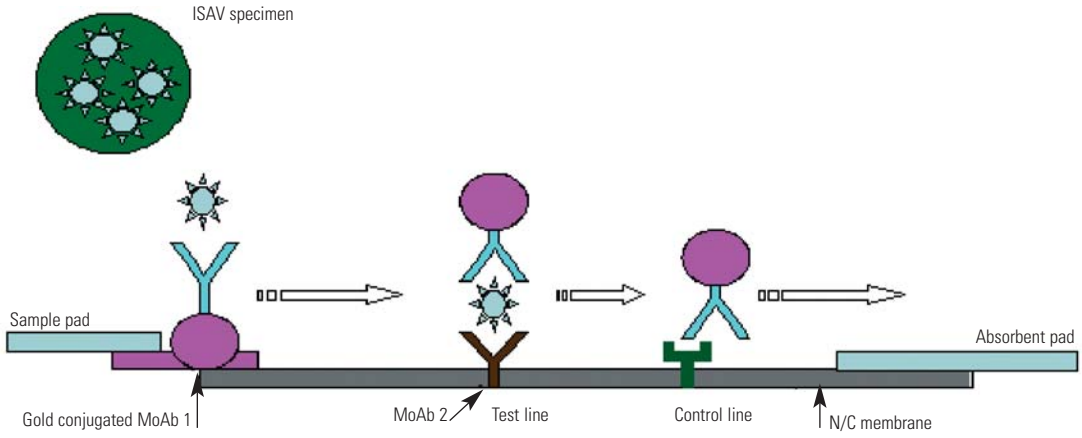
Ultimately, molecular methods used for routine diagnostics need to allow high-throughput of samples and must be rapid, specific, consistent, and cost-effective (32). Recent development in molecular methods includes analysis in solid phase (gels, DNA chips, glass slide arrays) and homogeneous solution assay formats (mass spectrometry, capillary electrophoresis); however, technologies permitting multiplexing (DNA micro-arrays, capillary electrophoresis, mass spectrometry, xMAP™ technology) have all of the advantages offered above together with simultaneous detection of multiple DNA sequences in a single reaction (13).



(a) Positive result
The presence of two lines within the result window gives a positive result, i.e. one line at the control and the other at the sample

(b) Negative result
The presence of only one line within the result window at the control line

Fig. 3
Immunochromatographic test kit showing (a) positive and (b) negative detection of infectious salmon anaemia virus (ISAV) in kidney samples from infected Atlantic salmon
The inscriptions 'C' and 'T' indicate control and test lines, respectively. 'S' indicates the sample well



MoAb: monoclonal Antibody against ISAV
N/C: nitrocellulose

Fig. 2
Diagrammatic representation of the infectious salmon anaemia virus (ISAV) rapid test kit
A small tissue sample is macerated in buffer and a sample is placed into the well (sample pad) on the kit. Antibody probes specific for ISAV (MoAb1) will then bind to ISAV in the sample and a second ISAV-specific antibody probe (MoAb2) will ensure that the ISAV is immobilised on the test line. Since the first antibody was linked to gold (gold conjugated MoAb1) this reaction results in precipitation of gold on the test line

Multiplex technologies

Luminex xMAP™ technology

Luminex xMAP™ technology is a multiplexed assay based on a platform using a microsphere suspension array in which up to 100 different reactions can be analysed in a single reaction (13, 14). The system allows high-throughput detection and quantification (in a 96 well format) of both proteins and nucleic acids; it can therefore be used in molecular and immunodiagnostics to detect pathogens directly from tissue samples or culture, or it can be used in serology to measure fish antibodies.

Small polystyrene beads containing two spectrally distinct internal fluorochrome dyes are used in the array. By adjusting the amount of each dye within each bead, every bead set in an array consisting of up to 100 different sets can be individually identified by a laser within the analyser. Because each bead set is distinct, the bead can be coated with different fragments of DNA or antibodies to detect pathogens, or proteins from a pathogen to detect antibodies. An appropriate reporter fluorochrome conjugate (i.e. to detect the test molecule) coupled with either *R*-phycoerythrin, Alexa 532, or Cy3 is then reacted with the bead. This label can be detected by a second laser. Thus, the system knows which bead set and analyte are being analysed.

The use of xMAP™ technology has been used increasingly in recent years both in clinical research and diagnostic laboratories (20, 32), and it is anticipated that it will become an integral part of fish diagnostic laboratories in the future.

Multiplex-PCR assays

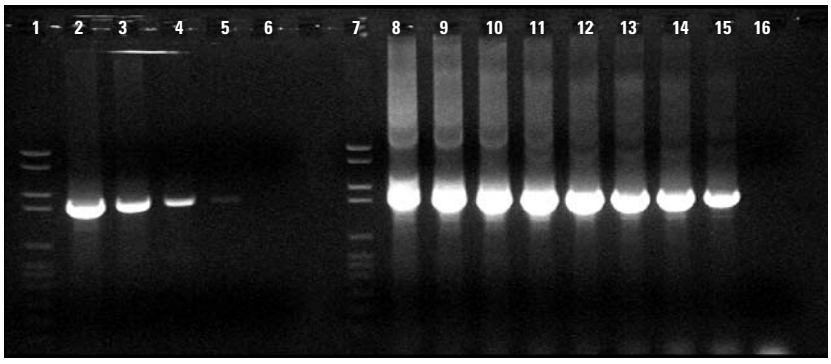
Multiplex-PCR methods are now being developed and used to screen samples for a variety of pathogens within a single reaction. Multiple primer sets, unique to the different pathogens under investigation, are subjected to a single PCR reaction to produce products of varying sizes specific to different DNA sequences of the various pathogens. However, the assay can be difficult to standardise because annealing temperatures need to be established for each of the primer sets, the size of each amplicon must be sufficient to be able to produce distinct bands by gel electrophoresis, and the specificity and sensitivity need to be established for each pathogen.

Micro-arrays

Micro-array technology, although complex and in its infancy with regard to aquaculture, offers a new dimension to diagnostics with possibilities for the detection of DNA, RNA or proteins (18).

DNA micro-arrays (also referred to as gene or genome chips, DNA chips, or gene arrays) are made from a collection of spots consisting of one or more DNA oligonucleotides, copy DNA (cDNA) or small fragments of PCR covalently attached to a solid support such as silicon chips or microscope slides. The advantage of this technology is that a large number of spots (100 µm in diameter) can be placed on a single slide allowing multiplexing for a number of different genes. Fluorescence is the most common method of detection for micro-arrays. Fluorescent dyes, commonly Cy3 and Cy5, are used for labelling dual cDNA samples (e.g. diseased versus healthy tissue) to look for up- and down-regulation of genes between the two samples, i.e. to identify which genes are being expressed during the disease process.

Arrays composed of molecules other than DNA are also possible. Protein arrays, and in particular antibody micro-arrays, may hold potential for the future (11, 19). Protein micro-arrays, for example (also referred to as biochips or proteinchips), are used to detect and/or quantify the concentration of proteins in a biological sample. When monoclonal antibodies are used as the capture agent in this technology (as they usually are) the array is referred to as an antibody micro-array. As with DNA, the mAbs are placed on a chip surface (e.g. glass, plastic or silicon) in a miniature array. The range of the protein concentrations



1% agarose gel
Lanes 1 & 7: markers
Lanes 2 to 6: *F. psychrophilum* suspensions 10^8-10^4 cells/ml (one-round PCR)
Lanes 8 to 16: *F. psychrophilum* suspensions 10^8-1 cells/ml (nested PCR)

Fig. 4
Comparison of the sensitivity of a one-round PCR and a nested PCR for *Flavobacterium psychrophilum*

Table I
Advantages and disadvantages of methods for aquatic animal disease diagnosis

Method	Advantages	Disadvantages
Conventional methods		
Culture	Useful because the pathogen is isolated and the aetiological agent can be confirmed	Labour intensive, can be expensive, not always possible to confirm identity of aetiological agent
Histopathology	Useful for assisting in the diagnosis of disease, particularly where the causative agents of new diseases have not yet been identified	Labour intensive; skilled personnel required, not always possible to identify agent
Microscopy	It is an important tool in many of the methods shown in this Table. Many different types of microscopes are now available	Can be labour intensive; skilled personnel required; can be expensive if using confocal microscope or TEM. Not always possible to identify agent
Biochemical analysis (commercial kits available [1])	Useful for identifying bacteria with characteristic biochemical profiles; commercial kits available for this purpose	Can be labour intensive; skilled personnel required. Not always possible to identify agent
Immunological methods		
Agglutination (commercial kits available [2])	Simple method, no requirement for specialised equipment	Not very sensitive in comparison to other immunological methods
ELISA-detection of pathogen (commercial kits available [3])	Versatile method that can be used to identify pathogens or antibodies depending on how assay is set up. Microassay – therefore small amounts of reagent needed. Quantitative; can be automated to analyse large sample numbers. Sensitive	Standardised reagents and specialised equipment needed. Need careful selection of controls and a skilled operator
Immunohistochemistry (commercial Abs available [4])	An extension of histopathology – the pathology can be observed around the infected tissue as the slide is counterstained. Can be amplified to increase sensitivity	Need formalin-fixed, wax embedded tissue sections, therefore procedure is labour intensive. Need a skilled operator to analyse results
Western blot	Particularly useful for serology to identify pathogen-specific proteins	Standardised reagents and specialised equipment needed. Need careful selection of controls and a skilled operator
Dot blot	Versatile method which can be used to identify pathogens or antibodies depending on how assay is set up. Microassay – therefore only small amounts of reagent needed. Protein not denatured in process unlike Western blotting	Standardised reagents need to be available to perform analysis. Need a skilled operator
FAT/IFAT (commercial Abs available [5])	Fast method if performed directly on infected tissue smears, takes longer if fixed tissue sections are used (i.e. need to process infected tissue). Sensitive. Useful for detection of viruses	Need a skilled operator to analyse results, autofluorescence on tissue sections can interfere with interpretation of results. Requires specialised equipment
Serology-ELISA detection of fish antibodies (commercial Abs available [6])	Non-destructive sampling method, uses ELISA format therefore can screen large numbers of samples	Indirectly detects the presence of the pathogen. Most suitable for viral infections as antibodies against Gram-negative bacteria may cross-react in assay. In order to perform the assay a specific anti-fish species antibody is required. Needs careful interpretation
Rapid kits (commercial kits available [7])	Fast (results obtained in minutes), inexpensive, suitable for field application. Easy to interpret results. Sensitive	Designed to be used with fresh tissue. Using frozen or fixed tissue may affect sensitivity of results

being measured in biological samples can be many orders of magnitude greater (and more diverse) than the levels of DNA being measured in DNA micro-arrays, and the chip must therefore reflect this.

Conclusions

A variety of novel rapid diagnostic methods are being developed that have potential for application in the diagnosis of aquatic animal health. These need to be fully validated to determine their fitness for a particular use, including assay optimisation and demonstration of performance characteristics such as sensitivity and specificity. The availability of standardised reagents (e.g. antibodies for immunodiagnostics) is crucial if comparable tests are to be run in different laboratories. A range of mAbs is now available commercially for the detection of fish and shrimp pathogens and for use in serology (to detect anti-fish species IgM). A limited but expanding range of kits is also commercially available.

Each of the new technologies has advantages and disadvantages that also need to be considered, as shown in Table I. In general, molecular methods are more sensitive than immunological methods, which are, in turn, more sensitive than traditional methods. However, the sensitivity and specificity of each assay need to be determined individually by direct comparison with known methods. The availability of equipment and skilled operators, the cost and speed of each test, as well as the ease of interpretation of results all need to be taken into account. For example, rapid kits and LAMP technologies require no equipment and can be run easily in the laboratory or in the field, while most of the other methods require a skilled operator and laboratory facilities. Rapid kits are currently commercially available to detect infectious salmon anaemia virus and WSSV in shrimp, and many more are under development. LAMP is an emerging technology for detection of fish and shellfish pathogens; research tests to detect *Edwardsiella tarda, E. ictaluri, Nocardia seriolae, Tetracapsuloides bryosalmonae* and infectious haematopoietic necrosis virus in fish, and WSSV in shrimp,

Table I (cont.)
Advantages and disadvantages of methods for aquatic animal disease diagnosis

Method	Advantages	Disadvantages
Molecular methods		
PCR (commercial kits available [8])	Very sensitive, can be automated to analyse large sample numbers	Only detects presence of DNA of pathogen, not the whole organism. False positive and negative results can occur
Nested PCR	Extremely sensitive method, more sensitive and specific than one-round PCR	Takes longer than the one-round PCR. False positive and negative results can occur
RT-PCR	Can detect live pathogens (i.e. detects RNA)	Care needed to ensure RNA is not degraded
Random amplified polymorphic DNA	Useful method for determining the identity of microorganisms at a strain level, assessing the genetic relationship of samples or analysing mixed pathogen populations in samples	Can be labour intensive. Skilled personnel required
Reverse cross blot PCR	Useful for distinguishing closely related species	Expensive. Labour intensive. Skilled personnel required
RT-PCR enzyme hybridisation assay	Can detect live pathogens. Large sample numbers can be analysed	Labour intensive. Skilled personnel required
In situ hybridisation	Detects DNA or RNA of pathogen, therefore there is no need for antibodies to detect protein	Labour intensive. Skilled personnel required. Expensive, sometimes difficult to see pathology in tissue sections after procedure
LAMP	Fast, with results obtained in a couple of hours. Suitable for field application. Does not require skilled operator. Results easy to interpret. Sensitive	Complex to set up initially
Quantitative PCR	Allows quantification of DNA that can be related to pathogen level in infected tissue. Extremely sensitive	Labour intensive. Requires specialised equipment. Skilled personnel required. Expensive
Multiplex methods		
Luminex xMAP™ technology	Versatile method that can be used to identify pathogens or antibodies depending on how assay is set up. Can detect proteins or DNA. Microassay – therefore only small amounts of reagent needed. Quantitative. Can measure several pathogens or analytes simultaneously. Sensitive	Labour intensive. Needs a skilled operator. Expensive. Standardised reagents need to be available to perform analysis. Requires specialised equipment
Multiplex-PCR assays	Can detect more than one pathogen with the assay. Sensitive	Difficult to standardise. Expensive
Micro-arrays	Can detect more than one pathogen with the assay. Allows up and down regulation of genes to be examined. Very sensitive	Needs a skilled operator, very expensive, labour intensive, designated software needed to analyse results. Requires specialised equipment

Availability of commercial reagents and kits*

1) Biochemical analysis: API & APzyme strips (Biomerieux, Inc.)
2) Agglutination: kits (Bionor; Microtek Inc.)
3) ELISA: kits (Cypress Diagnostics; DIATHEVA s.r.l.; TestLine Clinical Diagnostics, s.r.o)
4) Immunohistochemistry: anti-pathogen antibodies (Aquatic Diagnostics Ltd; Bio-X Diagnostics; Cypress Diagnostics; Microtek Inc.; Serotec)
5) FAT/IFAT: anti-pathogen antibodies (Aquatic Diagnostics Ltd; Bio-X Diagnostics; Cypress Diagnostics; Microtek Inc.; Serotec)
6) Serology (ELISA): anti-fish species IgM antibodies (Aquatic Diagnostics Ltd; Microtek Inc.; Serotec)
7) Rapid kits: kits (Aquatic Diagnostics Ltd; En Biotec Laboratories Co. Ltd)
8) PCR & related technologies: kits (Aqua Bounty Technologies Inc.; DiagXotics Inc.; Farming IntelliGene Tech Corp.; Shrimp Biotechnology Business Unit)

Abs: antibodies
ELISA: enzyme-linked immunosorbent assay
FAT: fluorescent antibody test
IFAT: indirect fluorescent antibody test
LAMP: loop-mediated isothermal amplification
PCR: polymerase chain reaction
RT-PCR: reverse transcriptase-PCR
TEM: transmission electron microscopy
*N.B. This is not a definitive list and reference to these products does not mean that they are endorsed by the authors or by the World Organisation for Animal Health

have been developed (28). However, rapid kits and LAMP technologies do not enable quantification of pathogens while the more technically demanding methods ELISA, qPCR and xMAP™ all do allow the level of pathogen to be determined. Multiplex technologies such as xMAP™ and micro-array both have huge potential for the future because simultaneous analysis of many analytes in one small sample can be achieved. These are currently expensive and labour intensive because assays are still being developed and optimised. However, these tests have the capability to analyse large sample numbers, so the cost per sample should eventually be economical.

Interpretation of results is sometimes difficult, both with existing and new technologies. It is therefore crucial that the appropriate positive and negative controls are included in the method. Serology, for example, only indirectly detects the presence of the pathogen by detecting the host antibody response to the pathogen; however, it does have the advantage of being a non-destructive test (i.e. using a blood sample rather than a tissue sample) and can accommodate large sample numbers, (ELISA format). The PCR, RT-PCR, and nested PCR in particular, are very sensitive assays, but false positive and negative reactions can occur and need to be carefully controlled.

If detailed information is required to species level or strain level for certain pathogens then specialised methods are required such as rcb-PCR or RAPDs. Although these tend to be expensive and labour intensive methods, they can provide extremely useful epidemiological information.

In conclusion, there is the potential to make significant improvements in diagnostic methods that will enhance the diagnosis of aquatic animal disease. Serology, lateral flow (immunochromatography), Luminex xMAP™ technology, adaptations of the PCR, polygenic sequencing and micro-array technology all represent such methods that merit validation.

■

Les applications récentes de la biotechnologie et les nouvelles techniques de diagnostic pour les maladies des animaux aquatiques

A. Adams & K.D. Thompson

Résumé

L'amélioration des techniques de diagnostic décrites dans le *Manuel des tests de diagnostic pour les animaux aquatiques* de l'Organisation mondiale de la santé animale (OIE) et l'introduction de méthodes innovantes dépendent des efforts déployés pour développer constamment de nouvelles méthodes et pour procéder à leur évaluation ainsi qu'à celle des techniques existantes. Bien que les techniques traditionnelles d'isolement et de caractérisation restent les méthodes de choix pour diagnostiquer un grand nombre de maladies, les progrès actuels permettent d'espérer que de nouvelles techniques rapides seront bientôt disponibles, qui amélioreront considérablement le diagnostic des maladies des animaux aquatiques. Par exemple, les techniques sérologiques, les trousses de diagnostic rapide par immunochromatographie, la technologie Luminex xMAP, les variantes de l'amplification en chaîne par la polymérase (PCR), le séquençage polygénique et la technologie du microdamier sont autant de techniques qui méritent d'être validées. Chacune de ces technologies comporte des avantages et des inconvénients, qu'il convient de prendre en compte. Dans le domaine du diagnostic, il n'existe pas de liste définitive : au fur et à mesure de leur développement, des méthodes nouvelles continueront d'être proposées et validées, pour autant qu'elles apportent une amélioration potentielle au diagnostic des maladies des animaux aquatiques.

Mots-clés

Animal aquatique – Biotechnologie – Diagnostic – Microdamier – PCR en temps réel – Séquençage polygénique – Sérologie – Technique de diagnostic rapide – Technologie Luminex xMAP – Trousse de diagnostic rapide.

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Aplicaciones recientes de la biotecnología a nuevos diagnósticos en animales acuáticos

A. Adams & K.D. Thompson

Resumen

El perfeccionamiento de los métodos que ya figuran en el *Manual of Diagnostic Tests for Aquatic Animals* (manual de pruebas de diagnóstico para los animales acuáticos) de la Organización Mundial de Sanidad Animal (OIE) y la inclusión en ese texto de técnicas novedosas dependen de un continuo proceso de desarrollo y evaluación de métodos tanto nuevos como ya existentes. Aunque las técnicas convencionales de aislamiento y caracterización siguen siendo la opción preferible para diagnosticar un gran número de enfermedades, hay mucho margen de progreso por lo que respecta a la elaboración de métodos que aceleren y mejoren el diagnóstico de enfermedades en los animales acuáticos. Por ejemplo, la serología, los kits de diagnóstico rápido basados en la inmunocromatografía, la tecnología de Luminex xMAP, las diversas variantes de la técnica de reacción en cadena de la polimerasa (PCR), la secuenciación poligénica o la tecnología de micromatrices son otros tantos métodos que merece la pena validar. Cada una de estas técnicas presenta ventajas e inconvenientes que es preciso tener en cuenta. Esta no es una lista definitiva, pues continuamente hay que valorar si los nuevos métodos que se describen (o se describan en el futuro) pueden resultar de ayuda para el diagnóstico de enfermedades de los animales acuáticos.

Palabras clave

Animal acuático – Biotecnología – Diagnóstico de enfermedad – Kit rápido – Micromatriz – Nuevo método rápido – PCR en tiempo real – Secuenciación poligénica – Serología – Tecnología Luminex xMAP.

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