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Development and validation of DNA-based diagnostic techniques with particular reference to bivalve mollusc pathogens

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Introduction

The International Aquatic Animal Health Code of the OIE (the World Organization for Animal Health) includes serious pathogens that have been causing important losses in the mollusc aquaculture industry throughout the world. This list also meets the ones established by the European Union regulation (Annex B of Directive 91/67/EC; Annex D of Directive 95/70/EC). In fact, one of the very few ways to reduce the impact of such pathogens on commercially exploited molluscs, is to establish effective programmes to prevent the transfer of infected stocks. Consequently, an area where molluscs are infected with any of these pathogens should not be allowed to export into another area free of this pathogen. Obviously, both country imports and abnormal mortality outbreaks in mollusc stocks should be examined for the presence of listed pathogens. This includes the detection of exotic diseases as well as emerging diseases. As a matter of fact, the effective control of diseases of bivalve molluscs requires an access to diagnostic tests that are rapid, reliable, accurate and sensitive. Techniques applicable to molluscan pathogens are limited and most investigations are based on histological and ultrastructural examinations. Arising from this, the development of molecular diagnostic tools will probably be one of the most important areas for research in the near future.

Potential detrimental consequences of transfers of molluscs and the need of accurate diagnostic tools

There are very few ways to limit the detrimental effect of mollusc pathogens. Molluscs are usually reared in the open sea and this strongly limits the use of chemotherapy, because of the quantity required and thre consequent impact on the environment. On the other hand, vaccination is also limited, due to the fact that molluscs do not produce antibodies. Consequently, one of the few methods of controlling mollusc diseases is likely to be the establishment of effective programmes to prevent the transfer of infected stocks. This is of utmost importance if we consider that the introduction of molluscs from other geographic areas for aquaculture has frequently resulted in the introduction of devastating pathogens in native stocks. The risk associated with transfers of molluscs particularly serious when they occur over long distances or overseas. Unforseen dramatic consequences, due to pathogens described as being of no concern, may result from exposure of a naive population. For example, in the early 1970s, the Portuguese oyster, *Crassostrea angulata*, was dramatically affected by an iridovirus (Marteil, 1976). It has been speculated that uncontrolled transfer of *Crassostrea gigas* introduced this iridovirus to *C. angulata*, which was highly susceptible. *Crassostrea angulata* and *C. gigas* are two taxa of the same species (Boudry *et al.*, 1998).

Another example of particular interest is *Bonamia ostreae* which, in 1979, dramatically affected the flat oyster (*O. edulis*) industry in France (Pichot *et al.*, 1979). This pathogen rapidly spread to almost all oyster farming areas in Europe, including Spain, Netherlands, Ireland and United Kingdom (Van Banning, 1982; Banister and Key 1982; Polanco *et al.*, 1984; McArdle *et al.*, 1991). A microcell disease similar to bonamiosis was also described in California in the 1960s and was known to occur in several populations of flat oysters from the western coast of North America (Elston *et al.*, 1986). *Bonamia ostreae* was later identified as the causative agent of this disease on the basis of host susceptibility and ultrastructural characteristics. Moreover, the use of monoclonal antibodies

demonstrated no antigenic differences between *B. ostreae* isolates originating from Europe and the USA (Mialhe *et al.,* 1988). More recently, bonamiosis was recorded from the east coast of the USA, New Meadows River, Quahog Bay and Damariscotta River (Barber and Davis, 1994; Friedman and Perkins, 1994). These results and historical commercial records lead to the hypothesis that the Californian microcell disease was actually bonamiosis and that bonamiosis spread from California to Europe because of transfers of *B. ostreae* infected flat oysters (Elston *et al.*, 1986; Cigarria and Elston, 1997).

Decision makers responsible for supervising translocations of molluscs deal with a high risk situation. Risk analysis prior to transfers should help to minimise this risk. However, a serious limiting factor is a lack of scientific information on even basic biology of mollusc pathogens (Hine, 1996).

Surveillance for mollusc pathogens is routinely performed by histology. This technique is time consuming and dependant of visula observation. In 1998, the Community Reference Laboratory proposed a ring test for the detection of two parasites (*Bonamia ostreae* and *Marteilia refringens*) by means of histology which is currently the standard method. The goal of this profeciency test was to establish that examination of a given sample lead to the same conclusions in any of the eight participating laboratories. The ring test was based on an itinerant collection of stained histological sections of *Ostrea edulis*. Statistical analysis of the results was based on the test of symetry and kappa coefficient. Significant discordance among the results obtained by participating laboratories was evident from the study. This clearly illustrated the need for training in histological diagnosis, particularly for exotic diseases, and the need for epidemiological surveillance programmes in order to prevent the transfer of diseases. However, using histological methods many pathogens are difficult to detect when present in low numbers.

Recent efforts to overcome these problems have led to the development of immunoassay techniques and nucleic acid-based diagnostic methods. Serological methods for diagnostic purposes obviously cannot be applied to molluscs as they do not produce antibodies. Molecular probes, such as monoclonal antibodies or nucleic acid probes, may be used for direct detection of pathogenic agents. These techniques are expected to find increasing use in routine disease monitoring programs in aquaculture, in field epidemiology and in efforts to prevent the international spread of pathogens. Therefore, it is extremely important to develop, validate and standardize this type of diagnostic technique for major mollusc diseases and pathogens.

Few prerequisites to the development of molecular diagnostic methods

When mortalities occur, various presumptive diagnostic methods can be used in addition to histology. This led us to consider three different levels of investigation which are: i) diagnostic procedures (standard methods for the assessment of a disease free status in a zone); ii) detection procedures (presumptive methods for the quick detection of a suspected pathogen); and iii) confirmatory procedures (methods for the specific identification of an encountered pathogen).

Obviously, the required quality criteria of the selected method depend on the level of investigation for which it is to be used. For example, detection procedures require techniques that are easy to perform (e.g. smears or tissue imprints) or sensitive techniques that usually are based on an amplification step (e.g. culture of the pathogen or polymerase chain reaction- PCR). Choice of technique may then be based on the time required to obtain a result and this can range from few hours to few days. In the case of diagnostic procedures, specificity of the selected methods is obviously the most important criterion. Confirmatory procedures currently in use are ultrastructural observations by transmission electron microscopy. One should say that in the near future, with the increasing use of molecular technics, diagnostic procedures will increasingly be confirmatory.

Increased sensitivity is often used as an argument for the use of new diagnostic methods. However, we should stress here that sampling strategy is of a central importance to ensure detection of a pathogen whatever detection method is used. The timing and frequency of sampling should be determined by the cycle of infection and pre-patent period. Also, the intensity of infection may increase following spawning due to loss of host condition and therefore molluscs should be sampled post-spawning. The recommanded sample size for each sampling site is 150 or a sufficient number to ensure detection at a 95% confidence level of pathogens at a prevalence of 2%. However, if molluscs are to be moved from natural beds onto a farm site or between natural beds in different zones, large numbers of molluscs must be sampled because of low parasite prevalence. In Western Australia, *Perkinsus* sp. occurs in isolated beds sometimes at very low prevalences (Hine, 1996). However, the probability of detecting an infection may be increased by holding the molluscs in quarantine for a long period, subjecting them to stress and examination of cohabitant species of molluscs that are highly susceptible to the infection. The use of PCR could help in such situations. However, widely recognized limitations of PCR methods include the false positive results (due to inhibiting substances in marine organisms, lack of target organs, pre-patent periods, etc) and false positive results (cross-reaction with closely related organisms, laboratory contamination of samples, etc). This leads us to consider specificity as one of the main imput of molecular diagnostic methods currently developped.

The problem of specificity in pathogen diagnosis is clearly illustrated by difficulties in dfferentiating *Marteilia* species. In Europe, *Marteilia refringens* has been observed in *Ostrea edulis* (Grizel *et al.*, 1974) and also in *Mytilus edulis* and *M. galloprovincialis* (Tigé and Rabouin, 1976; Claver-Derqui, 1990; Villalba *et al.*, 1993). However, *Marteilia maurini* has also been described in both *Mytilus edulis* and *M. galloprovincialis* from France (Comps *et al.*, 1982; Auffret and Poder, 1985). In spite of numerous papers published on the genus *Marteilia*, the question of taxonomic relationships of these species remains unresolved. Differential diagnosis of *M. refringens* and *M. maurini* was based on ultrastructural characteristics and host specificity (Grizel *et al.*, 1974; Comps *et al.*, 1982) but host specificity was discarded when *M. refringens* was described in *Mytilus galloprovincialis*. Indeed, the species parasitizing mussels may not be truely different from *M. refringens*. Recently, the small subunit of the rRNA gene was sequenced and sequences confirmed that both *Ostrea edulis* and *Mytilus edulis* are hosts of *M. refringens* (Berthe *et al.*, 1999). Current work is directed towards establishing the existing species among the genus *Marteilia*. Clarification of taxonomy of the targeted pathogens is of a central importance but is often underestimated as a problem in diagnosis.

Perkinsus atlanticus is another well documented example of data gap in the field of taxonomy prior to the development of molecular tools. This organism is known to occur in both Europe (Azevedo, 1989) and Asia (Hamaguchi *et al.*, 1998). In fact, more than 50 species of molluscs may harbour *Perkinsus* species from temperate to tropical waters of the Atlantic and Pacific oceans and Mediterranean Sea, apparently without harmful effect. Nucleotide sequence analysis of the internal transcribed spacers (ITS) of the ribosomal gene cluster (rDNA) has indicated that the Australian organism *P. olseni* is probably conspecific to *Perkinsus atlanticus* (Goggin, 1994). Taking this into account, the geographical distribution of the mollusc pathogen *P. osleni* could be wider than currently accepted. This should be urgently investigated because of the obvious consequences of such considerations.
In summary, we would like to pinpoint the need of adequacy of the methods to be developed and validated, as well as the absolute need of a clear taxonomy of pathogens under consideration.

Development of DNA-based methods for the detection of mollusc pathogens: the example of *Marteilia refringens*

In a preliminary study, the 18S gene of *M. refringens* was sequenced (Berthe *et al.*, 1999). Apart of clarifying the controversial taxonomy of *Marteilia refringens* and its relatives, this gene is interesting from a detection point of view because it is present in a high copy number in the genome, and so provides increased sensitivity of detection when targeted. Furthermore it contains conserved and non-conserved regions interspaced in the sequence which allows the design of universal and specific PCR primers.

After alignement of the *Marteilia refringens* rDNA SSU sequence with various eukaryotic organisms, PCR primers were designed (Le Roux *et al.*, 1999). Specific primers were used to amplify DNA extracted from purified *Marteilia refringens* and infected hosts. The detection was also possible from paraffin embedded tissues which is a frequent source of biological material in the field of mollusc pathology. The specificity of amplified fragments was confirmed by Southern blotting with an oligoprobe. Furthermore, the sensitivity of the detection was increased by this method. In brief, the designed primers allow rapid and specific screening of numerous samples from different sources for the presence of *Marteilia refringens* with a good sensitivity.

Universal primers provide an internal control for amplification experiments. Working with marine organisms, such an internal control of the PCR reaction is of a central importance as the reaction efficiency depends on various parameters including the presence of inhibitory factors and the quality and quantity of targeted DNA. In the present study, universal primers were designed and successfully used to amplify DNA from both *Marteilia refringens* and its hosts. These primers should be included in further use of PCR for *M. refringens* detection.

For *in situ* hybridization, four probes were tested by Northern blotting for the specific detection of 18S RNA isolated from *Marteilia refringens* and other eukaryotic cells. The most specific probe was used successfully to detect *Marteilia refringens* by *in situ* hybridization. The selected probe produced consistent strong reactions when used for *Marteilia refringens*-infected *Ostrea edulis* and *Mytilus edulis*, as well as for *Marteilia maurini*-infected *Mytilus galloprovincialis*. A similar result was obtained with *Marteilia sydneyi* in *Saccostrea commercialis*. However, no cross-reaction was noted when the probe was tested against *Marteilioides chungmuensis* in *Crassostrea gigas*. It was concluded that the sequence of Smart 2 is shared partially, if not completely, by *Marteilia* spp.

Similarly, specific primers designed from the 18S sequence of *Marteilia refringens* led to the amplification of specific fragment from European *Marteilia*-infected bivalves. No amplification was obtained when *M. sydneyi* DNA was targeted. Although the taxonomic relationships among the European species are not clearly established, PCR could be used to specificity discriminate *Marteilia refringens* from *M. sydneyi*.

Repeatability and reproduceability were successfully tested. A study was commenced to validate the *in situ* hybridization as a confirmatory method. Oysters originating from three different European zones (highly infected originating from Marennes-Oléron, France (n = 200); medium infection from Brittany, France (n = 200); and free of marteiliosis (n = 200) originationg from Lake Grevelingen, Netherlands) were processed by this method and compared with classical histology which is considered to be the standard method. Statistical analysis indicated a strong validation of the *in situ* hybridization method for the detection of *Marteilia refringens*. However, most of these resulsts were obtained on laboratory material stored in good condition, small size samples and were conducted by trained staff. Further work is underway in our laboratory to further validate of these tools.

Potential use of these new diagnostic tools

The results presented here clearly demonstrate the growing interest in molecular methods. However, in the case of molluscs, histology provides a large amount of information and should be used initially, before and beside any other type of examination. It is particularly important because macroscopic examination usually gives no pathognomonic signs. Also, mortality may be due to several pathogens, or loss of condition following spawning, and this can only be determined by histology.

In the case of *Marteilia refringens*, it is possible to recommand selected methods for the three different levels of investigation described above. The detection of *Marteilia refringens* by *in situ* hybridization could be used in addition to classical histological examination as a confirmatory method at a genus level. Histology and *in situ* hybridization can thus be used as a two step diagnostic procedure, and

could become a standard method for the assessment of a disease free status in a zone. Detection procedures that require presumptive methods for rapid detection of a suspected pathogen could be cunducted by using digestive gland imprints. It is very important to keep in mind the multiple advantages of such a method (which can be applied in the course of sample preparation for histology) as it is cheap and provides an immediate answer. PCR tests, because of their specificity, could be proposed for the specific identification of encountered pathogens as a confirmatory procedure. However, standardization of protocols, including negative and positive controls, is required. Compared to transmission electron microscopy (the currently used confirmatory procedure), PCR provides a quick and specific answer.

In the near future, the number and diversity of available methods should increase. In the case of *Marteilia* spp., oligoprobes targetting the ITS region have been developed and should be used for diagnostic purposes at a species level. Similarly, the sequencing of this region of the ribosomal gene cluster is currently demonstrating the possible existance of different strains within the species *refringens*. Further development in the knowledge of these parasites could lead to an increasing number of molecular methods at different levels of specificity (i.e. genus, species and strain). These could include techniques such as restriction fragment lengh polymorphism (RFLP) and reverse blot PCR.

At a national and regional level, reference laboratories will provide sequences of primers and oligoprobes to be used. The role of these laboratories in the validation of molecular reagents as diagnostic tools is obvious. Furthermore, these laboratories will have a growing responsibility in providing standardized protocols including positive and negative controls. Proficiency evaluations such as ring tests should also be organized for these diagnostic procedures in order to avoid mis-interpretation of results.

It should be said here that some of the DNA-based methods presented in this paper were aiming the study of life-cycle of *Marteilia refringens* which may include intermediate hosts or free-living stages (Berthe *et al.*, 1998). With similar goals, a number of research laboratories are already engaged in developing DNA-based diagnostic techniques for mollusc pathogens. Therefore, several new diagnostic tools for mollusc diseases should be available in the future.

Another potential use of molecular diagnostic tools is for detection of *Haplosporidium nelsoni*, one of the causative agent of haplosporidiosis - a disease of eastern oyster (*Crassostrea virginica*). A parasite morphologically similar to *H. nelsoni* was described in the Pacific oyster (*Crassostrea gigas*) on the west coast of the USA (Friedman *et al.*, 1991). This parasite was identified as *H. nelsoni* by the use a specific DNA probe (Stokes and Burreson, 1995). Furthermore, some of the *C. gigas* stocks were traced back to Japan where the examination of native *C. gigas,* demonstrated infection by a *Haplosporidium* sp. indistinguishable from *H. nelsoni* described in *C. gigas* from the USA (Friedman, 1996). A *Minchinia* sp. (*Haplosporidium*-like organism) has also been known to occur in *C. gigas* in Korea since the mid-1970s (Kern, 1976). In France, several authors have reported the occurance *Haplosporidium* spp. in several species of molluscs (Bonami *et al.*, 1985; Chagot *et al.*, 1987; Comps and Pichot, 1991). There is some confusion in the taxonomic relationship of the two pathogens *H. nelsoni* and *H. costale*. This should be investigated in the near future. This example illustrates an unforseen consequence of the use of DNA probes and reveals how taxonomy is an underestimated key point in mollusc pathology.

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References

Azevedo C. (1989). Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsea) parasite of clams, *Ruditapes decussatus,* from Portugal. *J. Parasitol.*, 75, 627-635.

Auffret M. and Poder M. (1985). Recherches sur *Marteilia maurini*, parasite de *Mytilus edulis* sur les côtes de Bretagne nord. *Rev. Trav. Inst. Pêches marit.* 47 :105-109.

Bannister, C. and Key D. (1982). *Bonamia* a new threat to the native oyster fishery. *Fish. Nat. MAFF Direct . Fish. Res.* 71,7.

Barber, B.J. and Davis C. (1994). Prevalence of *Bonamia ostreae* in *Ostrea edulis* populations in Maine. *J. Shellfish Res.* 13, 298.

Berthe, F.C.J., Pernas, M., Zerabib, M., Haffner, P., Thébault, A. and Figueras, A.J. (1998). Experimental transmission of *Marteilia refringens* with special consideration of the life cycle. *Diseases of Aquatic Organisms* 34, 135-144.

Berthe, F.C.J., Le Roux F., Peyretaillade E., Peyret P., Rodriguez D., Gouy M. and Vivarès C.P. (1999). The existence of the phylum Paramyxea Desportes and Perkins, 1990 is validated by the phylogenetic analysis of the *Marteilia refringens* small subunit ribosomal RNA. Submitted in *Molecular and Biochemical Parasitology*.

Bonami, J.R., C.P. Vivares and M. Brehelin. (1985). Étude d'une nouvelle haplosporidie parasite de l'huître plate *Ostrea edulis* L.: morphologiė et cytologie de différents stades. *Protistologica* 21: 161-173.

Boudry, P., Heurtebise S., Collet B., Cornette F and A. Gérard (1998). Differentiation between populations of the Portuguese oyster *Crassostrea angulata* (Lamark) and the Pacific oyster, *Crassostrea gigas* (Thunberg), revealed by mtDNA RFLP analysis. *J. Exp. Mar. Biol. Ecol.* 226, 279-291.

Chagot, D., Bachère, E., Ruano, F., Comps, M. and Grizel, H. (1987). Ultrastructural study of sporulated instars of a haplosporidian parasitizing the clam *Ruditapes decussatus. Aquaculture* 67: 262-263.

Cigarria, J. and Elston, R. (1997). Independent introduction of *Bonamia ostreae*, a parasite of *Ostrea edulis*, to Spain. *Dis. aquat. Org.* 29, 157-158.

Claver-Derqui A. (1990). Datos sobre Marteiliosis en la provincia de Cadiz. Acta III Congreso Nac. Acuicult. 885.

Comps M., Grizel H. and Papayanni Y. (1982) Infection parasitaire causée par *Marteilia maurini* sp. nov. chez la moule *Mytilus galloprovincialis*. Cons. Int. Explor. Mer, F:1-3.

Comps, M. and Y. Pichot. (1991). Fine spore structure of a haplosporidan parasitizing Crassostrea gigas: taxonomic implications. *Diseases of Aquatic Organisms* 11: 73-77.

Elston, R.A., Farley, C.A., and Kent, M.L. (1986). Occurrence and significance of bonamiasis in European flat oyster *Ostrea edulis* in North Amerinca. *Dis. aquat. Org.* 2, 49-54.

Friedman C.S. (1996). Haplosporidian infections of the Pacific oyster, *Crassostrea gigas* (Thunberg), in California and Japan. Journal of Shellfish Research 15 : 597-600.

Friedman C.S., Cloney D.F., Manzer D. and Hedrick R.P. (1991). Haplosporidiosis of the Pacific oyster, *Crassostrea gigas. J. Invertebr. Pathol.*, 58, 367-372.

Friedman, C.S., and Perkins, F.O. (1994). Range extension of *Bonamia ostreae* to Maine, U.S.A. *J. Invertebr. Pathol.* 64, 179-181.

Grizel H., Comps M., Bonami J.R., Cousserans F., Duthoit J.L., and Le Pennec M.A. (1974). Recherche sur l'agent de la maladie de la glande digestive de *Ostrea edulis* Linne. *Sci. Pêche. Bull. Inst. Pêches marit.* 240:7-29.

Goggin, C.L. (1994). Variation in the two internal transcribed spacers and 5.8S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). *Molecular and Biochemical Parasitology* 65: 179-182.

Hamaguchi, M., Suzuki, N., Usuki, H. and Ishioka, H. (1998). *Perkinsus* protozoan infection in short-necked clam *Tapes (=Ruditapes) philippinarum* in Japan. *Fish Pathology* 33, 473-480.

Hine P.M. (1996). Southern hemisphere mollusc diseases and an overview of associated risk assessment problems. *Rev. Sci. Tech. Off. Int. Epiz.*, 15(2) 563-577.

Kern F.G. (1976). Sporulation of *Minchinia* sp. (Haplosporida, Haplosporidiidae) in the Pacific oyster *Crassostrea gigas* (Thunberg) from the Republic of Korea. *J. Protozool.*, 23, 498-500.

Le Roux, F., Audemard, C., Barnaud, A., and Berthe, F. (1999). DNA probes as potential tools for the detection of *Marteilia refringens*. Submitted in *Mol. Mar. Biol. Biotechnol*

Marteil L. (1976). La conchyliculture française. 2. Biologie de l'huître et de la moule. *Rev. Trav. Inst. Pêches marit.*, 40, 149-345.

Mc Ardle, J.F., McKiernan, F., Foley, H., and Jones D.H. (1991). The current status of *Bonamia* disease in Ireland. *Aquaculture* 93, 273-278.

Mialhe, E., Boulo, V., Elston, R., Hill, B., Hine, M., Montes, J., Van Banning, P., and Grizel, H. (1988). Serological analysis of *Bonamia* in *Ostrea edulis* and *Tiostrea lutaria* using polyclonal and monoclonal antibodies. *Aquat. Living Resour.* 1, 67-69.

Pichot, Y., Comps, M., Tigé, G., Grizel, H., and Rabouin M.A. (1979). Recherche sur *Bonamia ostreae* gen. n., sp. n., parasite nouveau de l'huître plate *Ostrea edulis* L. *Revue Trav. Inst. scient. tech. Pêch. marit.* 43, 131-140.

Stokes, N. A., and Burreson, E. M. (1995). A sensitive and specific DNA probe for the oyster pathogen *Haplosporidium nelsoni*. *J. Euk. Microbiol.*, 42 (4) : 350-357.

Tigé G., and Rabouin, M.A. (1976). Etude d'un lot de moules transférées dans un centre touché par l'épizootie affectant l'huitre plate. *Cons. Int. Explor. Mer*, K:1-10.

Van Banning, P. (1982). The life cycle of the oyster pathogen *Bonamia ostreae* with a presumtive phase in the ovarian tissue of the European flat oyster, *Ostrea edulis*. *Aquaculture* 84, 189-192.

Villalba A., Mourelle S. G., López M. C., Carballal M. J., and Azevedo C. (1993). Marteiliasis effecting cultured mussels *Mytilus galloprovincialis* of Galicia (NW. Spain). I. Etiology, phases of the infection, and temporal and spatial variability in prevalence. *Dis. aquat. Org.* 16:61-72.