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Addressing the problems of using the polymerase chain reaction technique as a stand-alone test for detecting pathogens in aquatic animals

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Summary
The polymerase chain reaction (PCR) test is increasingly being used as the diagnostic tool of choice and with pathogens of aquatic animals, particularly molluscs and crustaceans, a PCR test is often the only laboratory diagnostic test available. When a diagnostic decision is required in the absence of clinical disease (e.g. health certification or a survey for disease freedom) limitations with the PCR technique may become apparent. A review of validated PCR tests demonstrates that most are imperfect assays with diagnostic sensitivities and/or specificities less than 100%. False positive and false negative results will thus occur and large numbers of samples need to be tested to achieve statistically significant results. The results from a survey for disease freedom from white spot syndrome virus in Australia are used to illustrate the problem and to assess various solutions. The use of pooled samples or the use of two independent PCR tests can substantially reduce the number of tests required and the associated costs of such a survey.

Keywords
Aquatic animal – Health testing – Pathogen – Polymerase chain reaction.

Introduction

Recently, polymerase chain reaction (PCR) has become the 'tool of choice' for detecting pathogens in infected animals and is available for 31 of the 34 diseases listed in the *Manual of Diagnostic Tests for Aquatic Animals (Aquatic Manual)* of the World Organisation for Animal Health (OIE) (32). It is the most commonly used technique other than histological examination because it is applicable to all aquatic animal hosts. Cell culture techniques are not available for many viruses due to a lack of host cell lines and immunology-based techniques are not applicable to species that do not produce antibodies. The PCR technique is rapid, can be used to examine simultaneously large numbers of samples, and is extremely sensitive, with some PCR tests claiming to be able to detect a single copy of the target DNA.

However, there are problems with the PCR technique. The *Aquatic Manual* (32) states 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'.

In disease investigation, PCR is used as part of a panel of investigative techniques that includes examination, gross

pathology and histopathology. PCR then becomes a tool to confirm the findings of the initial examination and diagnosis is based on the consistent findings from the panel of techniques. Used in this fashion, problems rarely arise, because the PCR results must agree with the findings of the other diagnostic techniques before a definitive diagnosis can be reached.

In other situations, however, when PCR is used as a stand-alone technique, e.g. for health certification of stock or population surveys to demonstrate freedom from disease, the limitations of PCR become apparent. Hoet *et al.* (16) compared the use of three tests – immunoelectron microscopy, enzyme-linked immunosorbent assay (ELISA) and PCR – for the diagnosis of bovine torovirus infection and concluded that no single test should be used alone in an epidemiological survey because of the observed limitations of each assay. Thus, the question arises, how can health certification be provided or disease freedom established when only a single test is available? This paper highlights the problems with the use of PCR for diagnostic testing for diseases of aquatic animals and assesses some potential solutions. Because there are few papers on validation of PCR tests for pathogens of aquatic animals, examples of the use of PCR for pathogens of terrestrial animals and humans will also be discussed.

Fitness for purpose

Validation of a test is 'the evaluation of a process to determine its fitness for a particular use' (32). The concept that a diagnostic test may be applicable to one use but not another is highlighted in the *Aquatic Manual*: 'For example, a particular method may be highly suitable to diagnose clinical cases of disease in individual animals of a certain age group, but the same method may be unsuitable for assessing the infection status of large numbers of clinically healthy animals'.

Fitness for purpose also applies to the types of samples being tested. Smith *et al.* (28) attempted to validate a PCR test for *Aeromonas salmonicida* for use with samples of fish kidney and river sediment. The test had previously been validated for use with laboratory samples and had a diagnostic specificity (D-SP) of 100% and a diagnostic sensitivity (D-SN) of 93% (4). In the absence of a 'gold standard' method to categorise the field samples, the experimental design aimed to determine an estimate of D-SP and D-SN using a comparison of performance with an ELISA and direct bacterial culture. No positive results were obtained with the culture technique and the concordance between the positive samples identified by PCR and ELISA was only 26%, a level of agreement no greater than that expected by chance alone. The authors commented that 'the conclusion that all three methods lack any validity for the applications studied here is also consistent with the results of this study'.

Validation of polymerase chain reaction tests

Validation evaluates a test to determine its applicability to a particular task. In the process of validation, estimates of the test's specificity and sensitivity are obtained through assessment against a range of standard samples. The OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual)* refers to formula and tables for determining the number of samples that must be tested to make initial estimates of D-SN and D-SP (see chapter 1.1.4. of the *Terrestrial Manual* [33]). The number will vary depending on certain variables. Given this requirement and the additional requirement that each of these samples be categorised by a 'gold standard' method, it is not surprising that very few PCR tests have been validated. In many cases, only limited numbers of standard samples are available (or none at all). Logically, if a PCR test is the only laboratory diagnostic technique available for a particular pathogen, it is impossible to validate the test – there would be no independent technique to classify the reference samples and the situation reduces to a circular argument of a sample being positive because it is positive to the PCR test.

The problems associated with the absence of a 'gold standard' method are highlighted by Crowther *et al.* (5) who discuss the problems associated with validating PCR tests, including the 'prevalence paradox', where a population with a known prevalence of disease is needed to determine the D-SN and D-SP of a test but the true prevalence cannot be determined without a validated test.

Of those PCR tests where D-SN and D-SP have been determined, few have been found to have a 100% D-SN or 100% D-SP and only a small proportion of these have been tested on the number of samples required by the OIE (19, 33). Table I lists published D-SNs and D-SPs for a range of PCR tests, and of the 20 tests listed, only those for *Bacillus anthracis* and *Myxobulus cerebralis* had both a sensitivity and specificity of 100%.

Throughout this article, reference will be made to the use of the OIE-recommended PCR technique for detection of white spot syndrome virus (WSSV) (32) and a second PCR technique developed at the Long Pocket Laboratories of the Commonwealth Scientific and Industrial Research Organisation (the LPL technique), which amplifies a different 480-base-pair region of the WSSV genome, and their use in a survey for WSSV in Australia (10). Neither test has been validated. The D-SP of a diagnostic test can

Table I
The diagnostic sensitivity and specificity of selected polymerase chain reaction tests

Target pathogen	Sensitivity (%)	Specificity (%)	Required sample size	Maximum number of positive results allowed	Reference
Actinobacillus pleuropneumoniae	95	83	NP	NP	12
Aeromonas salmonicida	93	100	160	0	4
Bacillus anthracis	100	100	149	0	17
Cowdria ruminantium	88-97	98	861	24	26
Helicobacter spp.	95	90	NP	NP	24
Malignant catarrhal fever virus	95-97	94-100	153-2,183	0-149	23
Trichomonas vaginalis	90.8	93.4	2,626	194	20
Mycobacterium avium paratuberculosis (Idexx kit)	67.5	100	221	0	30
Mycobacterium avium paratuberculosis (Adiagene kit)	60	100	248	0	30
Mycobacterium avium paratuberculosis (Institut Pourquier)	61.3	100	243	0	30
Maedi–visna virus	98	100	152	0	11
Mycobacterium avium subsp. *paratuberculosis*	94.1	92.3	2,842	242	14
Mycobacterium avium subsp. *paratuberculosis* (immunomagnetic PCR)	100	95	1,671	98	15
Turkey coronavirus	93	92	3,021	266	3
Serpulina intermedia	100	94.3	1,888	124	29
Feline infectious peritonitis virus	91.6	94	2,354	160	13
Mycoplasma synoviae	82	100	182	0	22
Myxobolus cerebralis	100	100	149	0	1
Actinobacillus pleuropneumoniae (Adiavet App test)	83	100	179	0	12
Actinobacillus pleuropneumoniae (om1A test)	93	100	160	0	12

NP: it is not possible to achieve the desired confidence level using a test with these D-SP and D-SN values even if all samples were tested

be estimated in the absence of a 'gold standard' method if one assumes that all the test samples were obtained from a disease-free area and that all positive results obtained are false positive results (9). Using this approach, the D-SP of the two techniques was estimated to be 95.8% for the OIE-recommended technique and 94.4% for the LPL technique.

Impact of imperfect tests on surveillance programmes

Population surveys must take into account the D-SN and D-SP of the diagnostic test used (32). Values of D-SN and/or D-SP of less than 100% will increase the number of samples required to meet the desired confidence level. In addition, if D-SP is less than 100%, a number of false positive results will be obtained. Table I shows the number of samples that must be assayed for each of the PCR tests listed and the number of positive results that could be expected in a disease-free population when using these tests. The figures quoted are those required to enable detection with 95% confidence of a pathogen at a prevalence of 2% or more (the OIE standard for establishing disease freedom) in a population of 10,000 animals (32). The results indicated that D-SN has only a modest effect on sample size and reducing the D-SN to 60% only increases the sample size required from 149 to 248. However, reducing the D-SP has a major effect on the sample size. For example, reducing D-SP from 100% to 95% increases the sample size needed to 1,671 and will also result in 98 false positive samples being observed in a disease-free population.

In the Australian WSSV survey, the assumed minimum prevalence of WSSV if present was 10% (10). Table II

Table II
Sample size required to detect, with 95% confidence, white spot syndrome virus present at 10% prevalence, using a range of techniques with different diagnostic specificities (D-SP) at a range of different diagnostic sensitivities (D-SN)

Test	D-SP (%)	Sample size for different D-SN			
		100%	95%	90%	85%
PCR (in 2 labs) – bioassay combination	100	29	30	32	34
OIE-recommended PCR	95.8	93	96	110	125
LPL PCR	94.4	110	122	135	149

PCR: polymerase chain reaction
LPL: Long Pocket Laboratories of the Commonwealth and Scientific Industrial Research Organisation

shows the sample sizes required for this survey (for a range of D-SN), using either the OIE or the LPL PCR tests separately. The results are compared to the sample size required using a theoretical, perfect test. The lower D-SP of either PCR test used individually increases the required sample size 3 to 4-fold.

This increase in sample size has several effects. An unacceptably large number of animals may be lost to sampling and the cost of the testing will increase. In the Australian WSSV survey (10), PCR tests were costed at AU$112 each. Thus, to demonstrate a prevalence of <10% would cost AU$10,416 for 93 tests and to demonstrate a prevalence of <2%, would cost AU$161,616 for 1,443 tests. A testing cost of AU$10,000 is clearly not sustainable and alternative surveillance strategies need to be developed. Several potential strategies are discussed below.

Testing strategies

Pooling samples

Pooling samples reduces the total number of tests conducted and thereby results in cost-savings. However, as samples are pooled, less DNA from each individual is included in the test and if only a proportion of the population is infected then the concentration of pathogen DNA in the pooled sample will be less than that in individual positive samples. As the total number of samples in each pool increases, the amount of pathogen DNA may decrease to below the test's analytical sensitivity (A-SN) (33). This results in false negative reactions and a decrease in D-SN. Despite these theoretical arguments against pooling samples, Currie *et al.* (6) compared the performance of a PCR test for *Chlamydia trachomatis* when samples were assayed either individually or in pools of five. The relative D-SN for pooled samples compared to individual samples was 89.5% for vaginal swabs, 92.7% for endocervical swabs, and 100% for urine specimens. Most importantly, the relative specificity was not reduced and was measured as 100%, 99.9% and 100%, respectively, for these three classes of samples. Initial pooling of the specimens resulted in a 60% reduction in the number of tests performed and a 39% reduction in the cost of testing. The limit of pooling that can be achieved depends on the A-SN of the assay and the concentration of the pathogen nucleic acid within the infected host. One extreme example detected one infected animal in a pool of 100 samples using a reverse transcriptase PCR(RT-PCR) test for bovine viral diarrhoea virus (BVDV) (31).

The effect of sample pooling can be estimated using dilutions of nucleic acid preparations. Deregt *et al.* (7) found that a PCR test using 1/10 dilutions of DNA samples was as effective as isolation of virus for detecting BVDV, and Weinstock *et al.* (31) were able to dilute samples by at least 100-fold and sometimes as much as 100,000-fold in their RT-PCR test for BVDV.

A moderate dilution of sample DNA is often used to improve A-SN because it reduces the effect of PCR inhibitors while still maintaining the pathogen DNA level above the A-SN (2, 21). Pooling samples may prove effective in a uniformly infected population, but may not be effective when infection is present at a low prevalence.

However, care must be taken when pooling samples to screen clinically healthy animals, because these animals have much lower levels of infection than those found in diseased animals and it is much easier to dilute samples below the A-SN. Peng *et al.* (25) tested *Penaeus monodon* brooders for WSSV and found a proportion that tested negative before spawning but positive after spawning. The stress associated with spawning had allowed amplification of the virus in these animals to a level above the A-SN for this particular PCR test.

Given the ability of pathogenic viruses to exist at very low levels in healthy animals, pooling samples for health screening or surveillance is not recommended as a routine practice. Although pooling may reduce the costs and time associated with testing large numbers of samples, it does not address the problem of having to sample large numbers of animals destructively.

Repeat testing of samples

Zenilman *et al.* (34), when screening samples for gonorrhoea, required any initial positive test to be confirmed by a second test on the same sample. Using this protocol, 6 of their 36 initial positive cases of gonorrhoea could not be confirmed and were classified negative. They concluded that 'repeat testing of positives will increase specificity and reduce the incidence of false positive test results'. Unfortunately, in the absence of an independent confirmatory test, such an interpretation of results is controversial and may result in false negative results with adverse consequences for misdiagnosed patients. Unlike the serial use of two different tests, repeated use of the same test has little effect on the D-SP because, although two different tests are independent (or are likely to be to some degree) and combining them provides additional information, two iterations of the same test are not independent. Repeated use of the same test is an assessment of the test's repeatability rather than a strategy to increase the D-SP. Hsu *et al.* (18) tested 25 *Penaeus monodon* spawners for the presence of WSSV. Each test was replicated five times and only 6 of the 25 animals returned consistent results across the five tests – the remaining 19 animals varied from having one of five tests positive to

having four of five tests positive. The authors were attempting to identify WSSV-free animals to produce WSSV-free larvae, and thus concluded that animals with a minimum of one positive test were truly infected with WSSV. This decision highlights that the aim of the testing has the potential to introduce significant bias to interpretation of the results. Although a seller of stock that tested positive on one occasion from a total of five tests may be happy to declare the stock as disease-free, it is unlikely that a potential purchaser would have the same degree of confidence in the claims of disease freedom.

Repetition of the same test has no effect on the D-SP or D-SN of a diagnostic test and thus cannot be used to reduce sample size. However, repetition of the same test may identify any operator error in the conduct of testing, especially if the retesting includes preparation of a new DNA template from the samples. Repetition of the same test may also be of value if a particular test has a low repeatability. Certainly, the example of Hsu *et al.* (18) suggests that the particular PCR test has a low repeatability, although repeating the tests did not clarify the disease status of any individual showing some positive and some negative results, and assignment of the disease status was thus purely subjective.

Increasing annealing temperature

The specificity of PCR may be increased by increasing the annealing temperature. However, too great an increase will result in all samples testing negative and the procedure becomes self-defeating. Dingemans *et al.* (8) increased the specificity of a PCR test for cytokeratin-19 in cancer patients by increasing the annealing temperature from 72°C to 78°C and avoiding amplification of a related pseudogene. Rahman *et al.* (27) increased the specificity of a PCR test for microsatellite analysis and achieved maximum specificity using a touchdown protocol of starting the amplification with a high annealing temperature and reducing the temperature in subsequent cycles.

The problem with increasing the annealing temperature to increase the technique's D-SP is that, in the absence of a confirmatory diagnostic technique, it is not possible to determine the optimum annealing temperature because known positive and negative samples are not available.

Use of two different polymerase chain reaction tests

Two different PCR tests may be independent if they amplify different regions of the pathogen genome. During the Australian WSSV survey, 230 sample pools were tested with the OIE-recommended test and with the LPL test (Table III). The extent of dependence of the two tests and a D-SP for combined use of the two tests in series was calculated (9). When used in series, the D-Sp increased to 98.2%. Use of these two tests in series will decrease the D-SN, but without known positive samples, the magnitude of this effect cannot be estimated. However, because a reduced D-SN has less effect on sample size than that of decreased D-SP, the sample size can be reduced significantly even with conservative estimates of D-SN. If the D-SN is arbitrarily set at 85%, the number of samples required to achieve 95% confidence is only half that required using the LPL test alone and approximately two-thirds of that required using the OIE test alone (Table IV).

Table III
Comparison of the results obtained from testing 230 samples for white spot syndrome virus with two different polymerase chain reaction tests

	LPL test positive	LPL test negative	Total
OIE test positive	11	6	17
OIE test negative	2	211	213
Total	13	217	230

LPL: Long Pocket Laboratories of the Commonwealth and Scientific Industrial Research Organisation

Table IV
The effect on sample size for a range of diagnostic sensitivities of use of the OIE-recommended and LPL polymerase chain reaction tests in series, compared to use of either test alone
Sample size required to detect, with 95% confidence, a pathogen present at 10% prevalence

Test	D-SP (%)	Sample size for different D-SN			
		100%	95%	90%	85%
OIE-recommended PCR	95.8	93	96	110	125
LPL PCR	94.4	110	122	135	149
Two tests used in series	98.2	65	68	71	75

D-SP: diagnostic specificities
D-SN: diagnostic sensitivities
LPL: Long Pocket Laboratories of the Commonwealth and Scientific Industrial Research Organisation
PCR: polymerase chain reaction

Byers *et al.* (4) assessed two different PCR techniques for the detection of *A. salmonicida*. When used individually, the AP-PCR test (amplifying the surface array protein gene *vapA*) had a D-SP of 100% and a D-SN of 93.3%, and the PAAS-PCR test (probe assay for *A. salmonicida*) had a D-SP of 100% and a D-SN of 93%. When used in parallel, the D-SN of the testing system increased to 99.4%. Given the small effect that imperfect D-SN has on sample size, there would be no economic gain in this instance. However, this example demonstrates that two different PCR tests may be independent and provide a testing scheme with potential to reduce sample numbers and costs.

Conclusions

The use of the PCR technique as a diagnostic tool is hindered because most PCR tests have not been validated, are imperfect tests and may require large sample numbers to achieve statistical significance.

There are several potential approaches to address this problem; one is pooling samples. This is effective for populations where individuals have a low value, population numbers are large and the prevalence of disease is high. In these circumstances, pooling of samples in batches of 10, 20 or more will reduce costs without loss of D-SN.

The second approach is a combination of two PCR tests targeting different regions of the pathogen's genome. This reduces the number of samples required and is advantageous where destructive sampling is required or the net value of individuals is higher.

Solutions aux problèmes posés par l'utilisation de l'amplification en chaîne par polymérase comme test unique de diagnostic chez les animaux aquatiques

I.J. East

Résumé

L'amplification en chaîne par polymérase (PCR) est de plus en plus la technique de choix pour le diagnostic des maladies animales ; dans le cas des maladies des animaux aquatiques, particulièrement des mollusques et des crustacés, la PCR est souvent le seul test de diagnostic disponible. Lorsque le diagnostic ne répond pas à l'apparition de signes cliniques mais qu'il s'inscrit, par exemple, dans une démarche de certification sanitaire ou d'établissement de statut vis-à-vis d'une maladie particulière, les inconvénients de cette technique deviennent rapidement perceptibles. Un bilan des épreuves PCR validées montre que les performances diagnostiques de la plupart d'entre elles sont imparfaites, avec une sensibilité et une spécificité inférieures à 100 %. En raison des résultats faussement positifs et faussement négatifs qui sont alors produits, il devient nécessaire de tester un grand nombre d'échantillons afin d'obtenir des résultats significatifs au plan statistique. Les résultats d'une étude conduite pour établir le statut indemne d'infection par le virus de la maladie des points blancs en Australie permettent d'illustrer ce problème et d'évaluer les diverses solutions. L'utilisation d'échantillons composites et la réalisation de deux épreuves PCR indépendantes sont deux possibilités permettant de réduire significativement le nombre d'épreuves à réaliser ainsi que les coûts associés à ces enquêtes.

Mots-clés

Agent pathogène – Amplification en chaîne par polymérase – Animal aquatique – Test pour le contrôle sanitaire.

Resolución de los problemas derivados del uso de la reacción en cadena de la polimerasa como prueba única de detección de patógenos en animales acuáticos

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Resumen

La técnica de reacción en cadena de la polimerasa (PCR), cada vez más utilizada como medio de diagnóstico preferente, suele ser la única prueba disponible para detectar en laboratorio patógenos de los animales acuáticos, en especial moluscos y crustáceos. Cuando hay que emitir un diagnóstico en ausencia de síntomas clínicos (por ejemplo para expedir un certificado sanitario o como parte de una investigación para declarar la ausencia de una enfermedad), las limitaciones inherentes a la técnica de PCR pueden quedar patentes. Previo análisis de las pruebas de PCR validadas, se concluye que la mayoría de ellas son imperfectas y presentan una sensibilidad y/o especificidad de diagnóstico inferior al 100%. Es inevitable, pues, que se produzcan falsos negativos y positivos, lo que obliga a analizar un gran número de muestras para obtener resultados estadísticamente significativos. El autor utiliza los resultados de un estudio realizado en Australia para certificar la ausencia del virus del síndrome de la mancha blanca para ilustrar el problema y valorar distintas soluciones. El uso de mezclas de muestras o de dos pruebas de PCR independientes puede reducir sustancialmente el número de ensayos requeridos y con ello los costos asociados a este tipo de estudios.

Palabras clave

Animal acuático – Patógeno – Prueba con fines sanitarios – Reacción en cadena de la polimerasa.

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