Survey for the presence of White Spot Syndrome virus in Australian crustaceans

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Objective To examine populations of Australian crustaceans for evidence of White Spot Syndrome virus (WSSV).

Design A national survey was designed to provide 95% confidence of detecting at least one infected crustacean population (site) in Australian waters assuming that at least 5% of sites would be infected and that at least 10 % of crustaceans at those sites would be infected with virus that was detectable by polymerase chain reaction (PCR).

Procedure A two–stage sampling regimen was used. All samples were tested by the OIE-recommended PCRs. If positives were found, they were retested at the Australian Fish Diseases Laboratory. Any sample that tested positive in PCR tests at both laboratories was to be subjected to a bioassay.

Results 3051 samples from 64 sites throughout Australia were tested. No mortalities, clinical signs of disease nor evidence of WSSV were detected at any site during the survey.

Conclusion The results of the survey support the case that Australia's crustacean populations are free of WSSV.
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AAHL	Australian Animal Health Laboratory
AFDL	AAHL Fish Diseases Laboratory
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DAC	Darwin Aquaculture Centre
LPL	Long Pocket Laboratories
NTU	Northern Territory University
OIE	Office International des Épizooties
PCR	Polymerase chain reaction
TNA	Total nucleic acid
WSSV	White Spot Syndrome virus
WSD	White spot disease

White spot disease is the major disease affecting the prawn farming industry throughout southeast Asia, India and central and South America. Outbreaks of WSD usually progress rapidly and can result in 100% mortality within a few days. The causative agent of WSD is WSSV, a double-stranded DNA virus that is potentially lethal to most of the commercially cultivated penaeid shrimp species. It is thought that most crustacean species including prawns, crabs and lobsters are susceptible to infection with WSSV but may not develop clinical signs.[1]

The first recorded cases of WSD occurred in Chinese Taipei and the Chinese mainland between 1991 and 1992. Subsequently, the virus spread to Japan through importation of prawns from China.[2] Since that time, WSSV has spread throughout all prawn farming regions of south and southeast Asia, and, in 1999, spread to central and South America. Australia, New Zealand and the islands of the South Pacific are reported to be free of WSSV.[3] In the USA and Australia, imported commodity green prawns have been shown to contain viable WSSV[4,5] and this finding has led to concern regarding the importation of green prawns to Australia.

In November 2000, staff at the DAC noticed that commodity green prawns purchased as diet for cultured mud crabs, *Scylla serrata*, were labelled as "Product of Indonesia". Although this particular batch had not been used for feeding the mud crabs, the supply of imported prawns as aquaculture feed (in contravention of the Centre's agreement with the supplier) raised concerns that previous batches may have included some imported prawns. The Centre was immediately destocked and disinfected. Samples of both the prawns purchased for feed and mud crabs maintained at DAC were sent for laboratory testing for WSSV. Further investigations also indicated that the aquaculture facility at the NTU was using imported Indonesian green prawns as feed for tiger prawn, *Penaeus monodon,* broodstock. This facility was also destocked and disinfected, and samples of both the Indonesian prawns and the *P monodon* broodstock were tested for WSSV. Both a proportion of the mud crabs from DAC and all the *P monodon* from the NTU tested positive for WSSV with the PCR test. In addition, five of 12 shore crabs collected adjacent to the outfall of the DAC were also positive for WSSV with the PCR test.

Although no clinical evidence of WSSV existed in Australia, the Consultative Committee on Emergency Animal Diseases considered it prudent, due to the possible presence of viable WSSV in commodity prawns, their use as aquaculture feed and the possible spread of WSSV to Darwin Harbour, to conduct a national survey to determine whether WSSV existed in crustacean populations within Australia. This survey was designed to focus on wild crustaceans and thus supplement an earlier WSSV survey, conducted on Australian farmed prawns in August 2000, that had not detected WSSV.[6] Since there are no standard procedures for the in vitro culture of WSSV, the survey relied on the detection of WSSV by PCR tests.

Materials and methods

A national survey of wild-caught crustaceans was undertaken to look for evidence of WSSV in Australia. Virus detection was by means of PCR. Selection of survey sites was not random but was biased towards inclusion of sites with a high usage of green prawns for recreational bait fishing. These sites were identified by State/Territory fisheries patrol officers based on experience and observation of recreational anglers. In addition, samples were also collected and tested from 17 crustacean farms and research facilities. Since these captive populations are sourced directly from wild-caught broodstock, these animals were included in the survey.

Expected prevalence of disease

It was assumed for the purposes of this survey, that if WSSV were present in Australia, then it would be present in 5% of the crus-

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tacean populations (sites). The problem in attempting to estimate an expected prevalence in populations that are not necessarily consistently isolated in space or time is difficult to resolve. In this case, we assumed that there were in excess of 500 potential separate sites to sample and that within this range of sites, at least 5% would be infected (if infection did in fact exist). This assumption was based on the belief that some mixing occurs between some of the 500 contiguous populations of wild prawns in some parts of Australia, so that spread between populations was possible. Within WSSV-infected wild populations of crustaceans in Asia, the prevalence of WSSV varies widely. Typical examples include 26% in *P semisulcatus* from the southwest coast of Taiwan, 60% in larvae of *Scylla serrata* from Taiwan, and 67% and 74% in *P monodon* brooders from Taiwan.[7-10] Based on these published works, it was assumed that if WSSV was present in a population, then at least 10% of the population would be infected. It is important to note that when providing evidence to support a case for disease freedom, the null hypothesis being addressed is that disease is present in the population at a given level (the design prevalence). For this survey, the design prevalence was that WSSV is present in at least 10% of susceptible animals in at least 5% of sites.

Confidence required

The survey was designed to provide a 95% confidence of detecting at least one infected crustacean population within Australia. We also wanted to minimise the chance of wrongly concluding that WSSV might be present. False positive reactions can occur with any diagnostic test, and these present a particular problem since each reactor must be investigated to determine whether the result is a true positive. Accordingly, a protocol for investigating positive reactions was developed for this survey.

Testing regimen

The quality of the DNA extracted from all collected samples was assessed using the decapod PCR test. All samples were then tested for WSSV, in one of four separate regional laboratories, using the OIE-recommended PCR test. Any samples that gave a preliminary positive result in testing were retested at the AFDL using both the OIE-recommended PCR test and a real time PCR. The survey was designed to allow any samples that tested positive for WSSV by PCR in both a regional laboratory and the AFDL to be further assessed by bioassay at the AFDL. The aim of this approach was to minimise false positive reactions.

Test sensitivity and specificity

None of the PCR tests for WSSV that were used in this survey have been validated with field samples to determine the true specificity and sensitivity of the technique. However, validated PCR tests routinely have specificities and sensitivities greater than 95% and usually in excess of 98%.[11,12] Although the specificity for the PCR test used was not known, the complete test regimen of retesting preliminary positive samples and the subsequent use of bioassay if required, was assumed to have an overall specificity of 100%. Based on the known sensitivity of other validated PCR tests, the sensitivity of the PCR testing regimen was assumed to be 95%. This overall sensitivity of the test regimen was composed of the combined sensitivities of the PCR tests used and the sensitivity of the bioassay test.

Number of samples required

The number of sites to sample and the number of crustaceans examined per site depend on a range of factors including the expected prevalence of disease (at the site and within a site), the desired confidence level, the sensitivity and specificity of the tests used[13] and the total number of sites. The data used to determine minimum sample size are shown in the presurvey column of Table 1. Figures were calculated in Freecalc using the method described by Cameron and Baldock.[14] The number of independent, noninteracting crustacean populations within Australia is not known. However, considering that there are a number of WSSV susceptible species of crustaceans and a range of habitats along the Australian coast, it was determined for the purposes of this survey that there would be in excess of 500 potential sites to sample. Whether these sites and populations are truly independent is unknown. Given the values outlined for test specificity, sensitivity and confidence level, a two-stage survey was designed. As discussed by Garner,[13] there is some flexibility in selecting the number of sites and the number of crustaceans per site, to sample in order to satisfy the desired confidence level. Taking both convenience and cost into account, it was decided that in this survey, 30 individuals of the one species would be collected at each survey site, and this was used to calculate the minimum number of sites that should be sampled (60). Based on published reports that WSSV is found in a wide range of crustaceans, target species for the survey were the predominant crustacean species in each area.[14,15]

Table 1. Values used for calculation of sample size.

Level	Parameters	
	Pre-survey	Post-survey
Site level		
Site sensitivity	0.95	0.93
Site specificity	1.00	1.00
Number of sites	More than 500	More than 500
Minimum expected site prevalence	5%	5%
Type 1 error (α)	0.05	0.04
Type 2 error (β)	0	0
Confidence	0.95	0.96
Power	1.00	1.00
Number of sites to test	60	64
Crustacean level		
Sensitivity	0.95	.85
Specificity	1.00	1.00
Population crustaceans at a site	More than 500	More than 500
Minimum expected site prevalence	10%	10%
Type 1 error (α)	0.05	0.07
Type 2 error (β)	0	0
Confidence	0.95	0.93
Power	1.00	1.00
Number of crustaceans to test	30	30
Total number to test	1800	1920

Collection of samples

All penaeid prawn samples collected were juveniles or sub-adults, as recommended by the OIE.[1] Crabs were collected using standard baited pots and prawns were collected by beam trawl. Some samples were purchased from licenced professional fishermen. It was recognised that these are not random samples. However, WSSV in wild penaeid prawn and crab populations is subclinical and would not affect the likelihood of being caught and sampled

by the methods described. In farmed and research situations, sample collection was by cast net or dip net. This method is not affected by inappetence and no clinical disease was apparent at the time of sampling. Any other biases in catch techniques were not believed by the authors to significantly affect the likelihood of detecting WSSV infected animals if they were present. Animals were euthanased and gill tissue (2 to 3 mm^3) or pleopod immediately placed into preservation medium (ethanol:glycerol:water 70:20:10) and stored at ambient temperature. Duplicate samples were collected from each specimen; one sample was transported to the testing laboratory and the second retained in the original sample collection facility in case further testing was required. When preliminary positive results were obtained with the first sample, the duplicate sample was forwarded to the AFDL for repeat testing. An unfixed tissue sample from each animal was also stored frozen as a source of material for any potential bioassay.

Extraction of total nucleic acid

Nucleic acid was extracted using either of two procedures, CTAB extraction[17] or the use of a commercial kit.[5] These procedures were shown to yield approximately equivalent amounts of nucleic acid, as judged by equivalent sensitivity in subsequent PCR tests (McColl and Hodgson, unpublished).

Decapod PCR

The quality of the TNA extracted from each sample was assessed using either of two PCR tests that amplified the same region of decapod 18S-ribosomal DNA. In one test (Hodgson, unpublished), decapod Master Mix, which contains PRIMER SET DP3-2 (see Table 2), was overlaid with oil and 1/10th volume of test TNA sample or control sample was added to the tube and mixed by centrifugation immediately before adding to a thermal cycler preheated to 80°C. PCR amplification was 1 x (94°C for 2 min) then 60 x (96°C for 20 s, 55°C for 30 s, 62°C for 20 s, 70°C for 90 s) and finally 1 x (70°C for 5 min, 30°C for 10 min). After thermal cycling, 10 µL of the PCR mix was removed and examined by agarose gel electrophoresis for the large decapod fragment of 830 bp (equivalent to the fragment produced by the OIE test) and for the nested decapod fragment of 240 bp. The second PCR test that was used was that recommended by the OIE.[15]

Standard WSSV nested-PCR assay

TNA extracts were analysed by the OIE standard PCR for WSSV.[1]

Real-time PCR

The real-time PCR was set up in a 96-well plate format, with water being used as a negative control, and TNA from the gills of a prawn that was experimentally-infected with WSSV being the positive control. TNA from each sample was extracted as described previously, and all controls and samples were examined in triplicate. Amplification and analysis of samples was done with the AB 7700 (PE Applied Biosystems) using standard procedures.[18] The sequence of the primers and probe used in the real time PCR test are given in Table 2.

Table 2. Sequence of primers used in PCRs for WSV.

Primer set	Target	Code	Sequence (5'-3')
Real Time Forward			CCGACGCCAAGGGAACT
Real Time Reverse			TTCAGATTCGTTACCGTTTCCA
Real Time Probe			CGCTTCAGCCATGCCAGCCG
DP3-2	Decapod	(20s2)	CTGCCTTATCA(G/A)CTTTCGAT(G/T)GTAGG
	Decapod	(20a2)	ACTTCCCCCGGAACCCAAAGACT
	Decapod	(20s9)	GGGGGCATTCGTATTGCGA

Figure 1 – Location of sampling sites (n=64) for wild and farmed crustaceans within Australia.

Results

At least 30 specimens were collected from each of 47 'wild' sites, 12 commercial crustacean farms and 5 research facilities throughout Australia (Figure 1) resulting in a total of 3051 crustaceans from 64 sites being examined in the survey. At many sites, many more than the required 30 samples were collected (up to 250), so that the level of statistical confidence at these sites exceeded that estimated by the survey design. However, after the survey was completed, concern was expressed that the estimate of sensitivity for the overall test regimen may have been optimistic. A confidence estimate was calculated using a figure of 85% for the sensitivity of the overall test regimen with 30 samples being collected at each site. The postsurvey column in Table 1 shows that with a lower overall sensitivity at the crustacean level and the same design prevalence figures, the survey achieved a 96% level of confidence by having samples collected at 64 sites.

PCR results

Of the 17 sites sampled for farmed crustaceans, 16 were negative following initial testing, that is, WSSV was not detected in these samples. However, two of seven pools of sand crabs (*Portunus pelagicus)* from one site gave preliminary positive results, and duplicate samples were forwarded to the AFDL for repeat testing using both the OIE recommended PCR test and the real time PCR test.

Of the 47 wild population sites sampled, 45 were negative for WSSV, and preliminary positive results were obtained from prawns from two sites. All positive samples were only positive after the second step of the nested-PCR test.

Duplicate tissues from all samples returning preliminary positive results were retested at the AFDL, and were found to be negative by both the OIE PCR and real time PCR. A bioassay was therefore not necessary for any sample.

Discussion

This study was designed to detect the presence of WSSV in Australian crustaceans based on the assumptions outlined. Crustacea susceptible to WSSV (crab, prawn and crayfish populations) were sampled around the entire Australian coastline. The parameters used in the survey design were based on published data related to the epidemiology of WSSV. There is no reason to believe that WSSV would behave differently in Australian waters.

After testing of samples from 3051 crustaceans collected from 64 locations throughout Australia, WSSV was not confirmed in any sample, thus if WSSV were present in Australian crustaceans, based on the assumptions outlined, it would be present at less than 5% site prevalence and less than 10% within site prevalence.

The design and subsequent results of the survey raised several issues relevant to the conduct of such surveys. The large number of samples required the participation of several laboratories. Although the CSIRO-LPL took a central role in distributing protocols and providing advice on methodology, variations in test procedures occurred. This issue was addressed by the conduct of an inter-laboratory proficiency test in which identical coded samples prepared by the AFDL were distributed to participating laboratories. Five of the six participating laboratories (including the AFDL) correctly identified all six samples, and the remaining laboratory correctly identified five of the six samples. Importantly, there were no false positive results generated by participating laboratories.

The occurrence of several false positive results in the survey was not unexpected. The testing regimen allowed for such results and required that each positive preliminary test was retested in a second laboratory and, if positive in that second laboratory, a bioassay would be conducted to provide a definitive result. Although the specificity of the PCR tests was not known, it was assumed that these tests would have specificities similar to those of known validated PCR tests. If the PCR tests had a specificity of 95 to 98%, then 2 to 5% of the test results should be expected to be false positive results. An alternative explanation for the intermittent 'preliminary positive' results is contamination of samples. DNA-based diagnostic techniques are hampered by potential problems including a high susceptibility to contamination.[19] The survey protocol required duplicate tissue samples to be prepared from each specimen but only one sample dispatched to the diagnostic laboratory. Although several initial samples tested positive, subsequent testing of the duplicate tissue sample at the AFDL did not confirm the presence of WSSV. If the preliminary positive results were actual positive results then the duplicate tissue samples should also have returned a positive test. The OIE's *Manual for Standards for Diagnostic Tests and Vaccines*[20] reports that "some investigators have found that the use of nested PCR increased the rate of false-positive results". Further, the *Manual* reports that "several multi-centre studies have shown.…that with known negative samples, several false positives are frequently obtained, indicating the continuing presence of contamination problems". It is for these reasons that PCR when used alone must be interpreted with caution. Care must be taken to ensure that modifications designed to increase the sensitivity of the test do not decrease the specificity of the same test.

In addition, future tests must also cope with sampling error that can arise when animals have a subclinical infection and very low levels of target WSSV occur in an individual. Since only a very small proportion of tissue from each individual is ultimately used in the PCR test and the OIE test has a detection limit of about 20 WSSV genomes, this small sample may not contain sufficient WSSV to be detected.[10]

At the conclusion of the survey, no confirmed positive results had been found. The results of this survey, combined with the:

- Absence of WSSV in the survey of farmed crustaceans conducted in 2000,[5]
- Absence of WSSV in a survey for WSSV published by the Northern Territory,[21]
- Absence of reports of clinical white spot disease in Australia, and
- Historical absence of WSSV from Australian crustacean populations

allow us to conclude that WSSV is not present in Australian waters.

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CORRESPONDENCE

Developments on Animal Welfare in Egypt

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Through the courtesy of your journal may I please update my professional colleagues on recent animal welfare developments in Egypt?

For several years Meat and Livestock Australia, (MLA) LiveCorp and Australian exporting companies have been working alongside livestock importers and the Government Organization of Veterinary Services of Egypt to enhance the standards of animal husbandry and welfare in that country. Over recent years livestock imports into Egypt have fluctuated (none the last few months due to the falling Egyptian Pound and high exchange rates) but Australia continues to work at this relationship. As the partnership and mutual confidence have developed so the scope of the program has matured.

I do not propose to list deficiencies, nor do I intend to deny their existence and claim perfection in everything Egyptian. Suffice it to write that Egypt, like every other country around the world including Australia, has some practices within its livestock industry identified as requiring modification. The partnership cited earlier is working towards achieving this goal. All partners agree that there is a plethora of inter-related social, cultural and financial issues affecting the rate of progress in a country of 71 million people, a capital with 20 million (the entire population of Australia crammed into one urban mass) and suffering from major problems of poverty and unemployment (GDP per capita = US $956).

The philosophy adopted by MLA and LiveCorp is that no 'quick-fix' solution exists and that the best mechanism for progress is commercial collaboration for technology transfer – '*Traid*'. In this, partners work together for dissemination of benefits over time rather than a unilateral declaration of moral superiority and subsequent non-communication.

Within this approach a refurbishment program in Cairo's Basateen Abattoir has been developed over several years to establish a benchmark for standards and practices within this abattoir, Cairo and the rest of Egypt. During the past 9-months work has progressed to the point where the following modifications have now been completed in one slaughter-hall: improved livestock receival platforms and quarantine station; raised standards of stockyard cleanliness; introduced animal handling training program; modified livestock delivery to the slaughter-hall; installation of a slaughter cradle; over-haul of cleaning procedures inside the slaughter hall; new flooring with epoxy coating; re-installed reticulated, hot water wash-facilities and sterilisers; rewiring of electrical systems, refurbished chillers.

With respect to modifying behaviour patterns, an Australian slaughterman travelled to Cairo in late February to share expertise by working alongside Egyptian colleagues on the production line. A training program in welfare and hygiene is now underway, being conducted by Egyptians who have already received support and guidance through 'train-the-trainer' mechanisms.

In addition, I am especially delighted to report that work has now started in a second hall and on other areas within Basateen, funded entirely by the Egyptian authorities. This also encompasses significant renovations to drainage, waste disposal, toilet facilities, and other structures within the abattoir complex. In my opinion this progress results from the support and encouragement given by the Australian Live Export Industry.

I would ask all my professional colleagues to recognise the contribution Australian exporters and producers, LiveCorp, MLA, the federal government (through His Excellency Robert Newton, Ambassador in Cairo) and, not least, my veterinary friends and predecessors in the Middle East - Tony Brightling, John Lightfoot and Neil Buchanan – for the foresight to assist Egypt (along with other countries in the Middle East) in progressing towards improved animal husbandry and welfare.

Despite ill-informed attacks from some quarters, MLA and LiveCorp remain committed to this program and welcome assistance from anyone interested in making genuine progress towards improved animal welfare on a global scale.