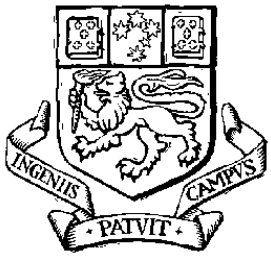
Pathogenic *Vibrio parahaemolyticus* in Australian oysters

Dr T. Lewis, Dr M. Brown, Mr G. Abell, Prof. T. McMeekin and Dr J. Sumner



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2002/409 Pathogenic *Vibrio parahaemolyticus* in Australian oysters

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OBJECTIVES

1. Obtain total *V. parahaemolyticus* counts from oysters from NSW, SA and Tasmania.
2. Determine prevalence of pathogenic *V. parahaemolyticus* amongst these isolates.
3. Compile and analyse data for potential inclusion in the FAO-WHO global risk assessment of *Vibrio parahaemolyticus* in oysters.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

The present work, while establishing for the first time the presence of pathogenic strains of *V. parahaemolyticus* in Australian oysters, also records their presence at levels far below those associated with food poisoning outbreaks. The findings were of immense value in the FAO-WHO Expert Consultation held in August 2002 in Bangkok where the USA modellers published predicted illness from *V. parahaemolyticus* in oysters in Canada, USA, Japan, NZ and Australia. For Australia, modelled estimates exceeded 200 cases/annum, including 120 cases each January. Clearly, this estimate is exaggerated, as health authorities indicate no recent illnesses from oyster consumption linked with *V. parahaemolyticus*, even though *V. parahaemolyticus*-related illnesses from other sources have been reported.

The data from the present study were presented to the FAO-WHO Expert Consultation modellers and will be used for final predictions. It is probable that predicted illnesses will be much reduced, reflecting Australian epidemiology for this pathogen:product pairing. The FAO-WHO will report the final outcomes to the Codex Committee on Food Hygiene in early-2003.

The new Australian data provided by this project, and to be incorporated in the final global risk assessment, will ensure that predicted illness from Australian oysters will be based on correct information, rather than assumptions based on USA data.

This project was designed to produce a "snapshot" of the prevalence of the bacterium *Vibrio parahaemolyticus* in Australian oysters during March and April of 2002.

V. parahaemolyticus occurs in two main forms: pathogenic and non-pathogenic. In the past 3 years there have been several large outbreaks of food poisoning in North America from oysters contaminated with the pathogenic type of *V. parahaemolyticus*.

This project examined samples of oysters from NSW, Tasmania and South Australia for the total and pathogenic *V. parahaemolyticus*. Single samples of 10-12 oysters were collected from selected sites in each state. Bacteria from these oysters were isolated and examined using DNA probes designed to detect either total or pathogenic *V. parahaemolyticus* isolates.

Total *V. parahaemolyticus* was found in 80% of NSW oysters, 60% of Tasmanian and 20% of SA oyster samples, with levels ranging to 2000/g. Pathogenic strains were found in 20% of NSW and Tasmanian oysters and in 10% of SA oysters with maximum levels being 350/g in NSW samples, 300/g in Tasmanian and 200/g in SA oysters. The present study is the first to confirm the presence of pathogenic *V. parahaemolyticus* strains in Australian oysters.

The prevalence and levels of both total and pathogenic *V. parahaemolyticus* established in the present study were much lower than those recorded in the USA - where the organism can be present in 100% of oysters at harvest levels up to 10,000/g. After harvest, total numbers approach 1 million/g. In the present study all oysters were ready-to-eat and the highest levels were 2000/g.

The present project indicates the effectiveness of tailoring data gathering towards satisfying the requirements of risk assessment.

KEYWORDS: *Vibrio parahaemolyticus*, oysters, pathogenicity, risk assessment.

BACKGROUND

During the past 2 decades there have been several major food poisoning incidents involving oysters from New South Wales in which more than 3600 people contracted viral disease. The most recent incident - in which Wallis Lake oysters caused Hepatitis A in more than 400 consumers, one of whom died - also attracted protracted litigation against both government and industry bodies.

During 2001, two processes were begun which have relevance for the Australian oyster industry both domestically and in the export sector. Firstly, the Codex Committee on Food Hygiene (CCFH) commissioned FAO and WHO to set up a team to undertake a risk assessment (RA) on "*Vibrio*'s in seafoods". The drafting group proposed a global risk assessment of *V. parahaemolyticus* in oysters–using the risk model formulated during the USFDA assessment–to accommodate data from the oyster industries of Canada, New Zealand, Australia and Japan.

Secondly, the United States Food and Drug Administration released its risk assessment for *V. parahaemolyticus*, together with mitigation strategies for the oyster industry to reduce the possibility of illness.

A major mitigation was rapid chilling of oysters immediately after harvest. At face value, the burden of this mitigation strategy would be especially onerous for Sydney Rock oysters which, under current regulation, may be held at up to 25°C for up to 72 hours.

During the 1970s and 80s a great deal of work was done on controlling pathogens (especially faecal pathogens) in NSW oysters. *V. parahaemolyticus* was also studied by a number of workers who found, in summary, relatively low levels (<1,000/g). Perhaps not surprisingly, given the low levels of the organism, there have been no reported food poisonings in NSW oysters from *V. parahaemolyticus*.

In the past 3 years there have been several large outbreaks of food poisoning in North America from oysters contaminated with a pathogenic strain of *Vibrio parahaemolyticus* (O3:K6). Interestingly, there have been no reports of pathogenic *V. parahaemolyticus* in Australian oysters.

A major consequence of these outbreaks was the commissioning of a risk assessment by the US Food and Drug Administration. A sensitivity analysis indicated that *V. parahaemolyticus* levels in oysters at harvest is the most important factor in determining the risk of illness in the absence of subsequent post harvest mitigations such as mild heating or frozen storage. The RA established water temperature as the major influence on *V. parahaemolyticus* in harvested oysters, and included rapid chilling of oysters immediately following harvest as a mitigation step to prevent food poisoning from consumption of raw oysters. This strategy was implemented by the Canadian oyster industry on the west coast during the summer of 2000. An alternate mechanism proposed was cessation of oyster harvesting during summer months, a mitigation strategy which is utilised in Japan.

It was noted by the Australian member of the drafting group that the USA model, with its basis on temperature and time, would discriminate against industry practices in Australia, particularly those in NSW where the Sydney rock oyster is often held up to 25°C for up to three days. An initial review of *V. parahaemolyticus* illness from oyster

consumption in Australia indicated no epidemiological linkage, confirming the probability that the Australian industry would fare badly on the basis of the USA model.

During the 1970s and 80s a great deal of work was done on *V. parahaemolyticus* in NSW oysters. In summary, the studies found relatively low levels of *V. parahaemolyticus* (<1,000/g) and no record of pathogenic strains.

In 1999, Seafood Services Australia (SSA) initiated a risk-based approach to assessing and managing hazards. Regarding oysters, two risk assessments were undertaken, one on viruses and one on *Vibrio*s. Focusing on the latter hazards, the risks associated with the three main species: *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* were assessed. It was noted that *V. parahaemolyticus* had caused major outbreaks in USA from consumption of oysters due to a "new" pathogenic strain, O3:K6. For Australia, no work on pathogenic *V. parahaemolyticus* in oysters could be found, which introduced an uncertainty to the assessment. The present project investigated this area of uncertainty.

NEED

In July, 2002 the FAO/WHO risk assessment team released a draft global risk assessment of *V. parahaemolyticus* in oysters. The assessment used the USA risk model with data from the oyster industries of Canada, New Zealand and Japan. Because no Australian data were available on levels of pathogenic *V. parahaemolyticus,* the modellers made assumptions based on USA data.

Among the strategies to mitigate *V. parahaemolyticus* risk is chilling oysters as early as possible after harvest. In the case of the Canadian industry in British Columbia, the strategy has been used successfully in the summers of 2000 and 2001. This strategy is directly opposed to current summer handling practices by the NSW industry, which allow product to remain as warm as 25°C for up to three days.

This preliminary project was designed to provide data of the occurrence and prevalence of pathogenic and non-pathogenic *V. parahaemolyticus* in Australian oysters. This knowledge will be invaluable to the NSW industry in particular - in case the FAO/WHO assessment recommends chilling of oysters as a pre-requisite for market access.

If this work was not done and Codex Committee on Food Hygiene recommended a mitigation strategy of rapid icing, the local industry has a number of choices:

1. Ignore the CCFH recommendation on the grounds that we don't export large volumes of oysters. The problem with this approach is that Codex has just as much application and force for domestic production as it does in the export arena.
2. Undertake a study to try to show that there is no problem with *V. parahaemolyticus* from Australian product. This would probably be a larger study than the present one and would be mounted to attempt to gain exemption for Australia. It's always difficult to unwind global hygiene edicts.
3. The present project, for a relatively modest investment, effectively places Australian data into a global risk assessment. As such the data will have great force in the modelling phase.

OBJECTIVES

1. Obtain total *V. parahaemolyticus* counts from oysters from NSW, SA and Tasmania.
2. Determine prevalence of pathogenic *V. parahaemolyticus* amongst these isolates.
3. Compile and analyse data for potential inclusion in the FAO-WHO global risk assessment of *Vibrio parahaemolyticus* in oysters.

METHODS

Oyster samples were obtained from NSW (Sydney rock oyster, *Crassostrea commercialis*), SA and Tasmania (Pacific oyster, *Crassostrea gigas*).

In NSW 20 samples, each of 10 oysters were obtained from a major processor in the Wallis Lake region. The oysters came from seven growers in the Wallis Lake area, had been harvested and depurated in the week prior to purchase (April 6, 2002). Oysters were transported live to the University of Tasmania's Centre for Food Safety and Quality microbiology laboratory in Hobart, where they were stored overnight at 4°C and processed for bacteriological analysis.

In SA, oyster samples, each comprising 12 oysters were obtained from Smokey Bay, Coffin Bay, Coobowie and Denial Bay. Oysters were shipped, on ice, on 3 separate days during the week 11-15 March 2002, and received at UTas within 2-4 days. Samples were processed immediately upon receipt.

In Tasmania, oyster samples, each comprising 10 oysters, were obtained from leases at Little Norfolk Bay, Dunalley, Triabunna, Pittwater, Barilla Bay, Pipeclay Lagoon and Little Swanport. These were collected by on 08 April 2002, stored overnight at 4°C, and processed on 09 April 2002.

Live (i.e. not gaping) oysters were processed according to the method detailed in Appendix 3, of which salient details are presented here. Oyster shells were cleaned using a stiff brush under running tap water. Meat was removed from the shells and rinsed in cool (~4°C) sterile (autoclaved) seawater. Washed meat from 10-12 oysters from a single sample were then weighed into a Stomacher bag (Colworth Stomacher 400, AJ Saward, UK), along with an equal weight of sterile phosphate buffered saline and "stomached" for 2 minutes. Serial dilutions were made from the meat-water homogenate, and 0.1 mL aliquots inoculated (spread plate technique) onto duplicate Petri dishes containing sterile T1N3 growth medium (Appendix 3). Samples were incubated at 25°C for 5-7 days, then stored at 4°C prior to probing for total and pathogenic *V. parahaemolyticus*.

Total and pathogenic *V. parahaemolyticus* isolates were enumerated using the protocol developed by Cook *et al* (2000) in which colonies from the T1N3 plates described above were blotted directly on to two filter papers (87mm #541, Whatman, USA). Each filter paper was then probed with either of two specific DNA gene probes (TLH-L AP-probe and TDH-M2 AP-probe: DNA Technology A/S, Denmark) to detect and enumerate total (tlh gene) and pathogenic (tdh gene) *V. parahaemolyticus* isolates. Positive reactions to each probe were evaluated after comparisons with the intensity of probe reactions with each of the control colonies (*V. vulnificus* tlh^{-}, tdh^{-}; *V.*

parahaemolyticus tlh$^+$, tdh$^-$; *V. parahaemolyticus* tlh$^+$, tdh$^+$). Control colonies were included for each probing reaction, to accommodate differences in staining intensity between runs.

The numbers of total and pathogenic *V. parahaemolyticus* cells in original samples were calculated from numbers of positive colonies for each probe-sample combination.

RESULTS AND DISCUSSION

Using the methodology described above, total and pathogenic *V. parahaemolyticus* colonies were counted and recorded as count/g oyster meat. Colonies with positive reactions to the two probes appeared as shown in Figure 1 and Figure 2.

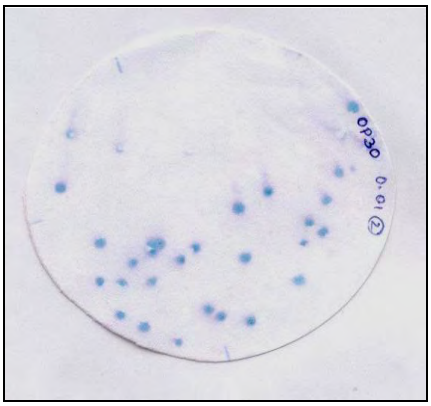

Figure 1: Filter showing total *V. parahaemolyticus* colonies, visualised using the tlh probe

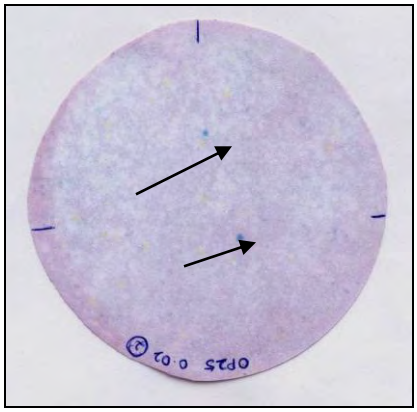

Figure 2: Filter showing pathogenic *V. parahaemolyticus* colonies (arrowed), visualised using the tdh probe

In Table 1 are presented the prevalence and levels of total and pathogenic *V. parahaemolyticus* in oysters from NSW, SA and Tasmania. It can be seen that *V. parahaemolyticus* was isolated from 16/20 (80%) of oysters from NSW, 6/10 (60%) from Tasmania and 2/10 (20%) from SA.

Table 1: Prevalence of total and pathogenic *V. parahaemolyticus* in Australian oysters

	Total *V. parahaemolyticus*			Pathogenic *V. parahaemolyticus*		
	Positive/total	Mean log/g (SD)	Max count/g	Positive/total	Mean log/g (SD)	Max count/g
NSW	16/20	2.4 (0.5)	900	4/20	2.0 (0.4)	350
Tasmania	6/10	2.5 (0.5)	2000	2/10	2.4 (0.1)	300
SA	2/10	3.0 (0.4)	2000	1/10	2.3	200

The prevalence of *V. parahaemolyticus* in the present survey (80%) is similar to that established in previous studies on Georges River oysters over the period 1978-85. Desmarchelier (1978) surveyed *V. parahaemolyticus* in oysters from 8 sites. In Survey 1, 41/60 samples were positive and in Survey 2 128/633 samples were positive for *V. parahaemolyticus.* Davey *et al*. (1982) detected *V. parahaemolyticus* in all four subsamples of undepurated oysters tested and Eyles *et al*. (1985) found 19/21 oyster meat samples were positive. In late summer in 1989 and 1990, 8/13 and 31/44 samples of oyster meat were positive in a survey undertaken by the NSW Department of Health (Table 2).

Table 2: Prevalence of *V. parahaemolyticus* in Sydney rock oysters (NSW Dept Health)

Year	Month	Number Positive/total
1989		
	April	8/13
	May	1/2
	June	3/4
	July	0/1
	Aug	4/5
	Sept	Not done
	Oct	3/3
	Nov	5/9
	Dec	11/12
1990	Jan	10/11
	Feb	42/62
	Mar	12/19
	April	31/44
	May	12/19

The log count (2.0-3.0) found in the present survey is somewhat higher than the levels established in studies on Georges River oysters in the period 1978-1985, which may reflect the difference between classical microbiological techniques and DNA probe technology. Desmarchelier (1978) found numbers generally <10/g while Davey *et al*. (1982) detected *V. parahaemolyticus* in three subsamples at 4-6/g and in one subsample at 0.8/g. Eyles *et al*. (1985) found a geometric mean MPN 7.3/g and range 0.3-50/g.

Desmarchelier (1978) noted a relationship between water temperature and level of *V. parahaemolyticus* (Table 3) and the present study was based on taking oyster samples from waters which were at, or close to, their annual maximum.

Table 3: Numbers of *V. parahaemolyticus* in Sydney rock oysters (Desmarchelier, 1978)

Water temp (°C)	Log *V. parahaemolyticus* /100g
<16	Not detected
16-20	< 1
21-24	1-2
25	3

For NSW oysters, Wallis Lake was chosen as the sampling site because it has warm (>20°C) waters and represents a large proportion of Sydney rock oyster production. In Table 4 are summarised temperature and salinity data for the summer of 2002 (supplied by SafeFood NSW), which include the harvest period at end March/early April of oysters used in this study. It is clear that oysters used for the present study were harvested at water temperatures associated with elevated levels of *V. parahaemolyticus.*

Table 4: Water temperatures at Wallis Lake growing area in Summer, 2002

		Temp			Salinity			Samples (#)
Year	Month	Mean	Max	Min	Mean	Max	Min	
2002	January	24.8	26.9	23.3	34.5	36.5	32.8	35
	February	23.9	25.1	20.9	28.7	34.6	9.9	49
	March	25.2	26.7	24.2	33.5	34.7	30.9	28
	April	21.3	23.9	18.3	18.3	29.3	29.3	35

For SA samples water temperatures for March 2002 were 18.5-20.8°C (Ken Lee, SASQAP, pers comm), 3-4 °C colder than the previous year. At Tasmanian leases from which samples were obtained, mean monthly temperatures were generally between 13.5 and 16.7°C (Ray Brown, TasSQAP, pers comm).

Previous reports have not mentioned the presence of pathogenic strains of *V. parahaemolyticus* in Australian waters or in marine products (see review by Desmarchelier, 1997 on pathogenic *Vibrios*). In 1990 and 1992, there were two outbreaks of gastroenteritis, in which one person died, caused by *V. parahaemolyticus* in chilled, cooked shrimps imported from Indonesia (Kraa, 1995). However, it is likely that the lack of an epidemiological linkage between the organism and Australian seafoods has made the search for pathogenic strains of low priority.

In the present study, pathogenic *V. parahaemolyticus* were isolated from oysters from all three states. Between 10% and 20% of samples had pathogenic strains (tdh^+). In NSW, 4/20 samples from the Wallis Lake growing area were positive; in SA one sample from Denial Bay was positive and in Tasmania, samples from Dunalley and Little Norfolk Bay contained pathogenic strains. Pathogenic *V. parahaemolyticus* ranged between 50/g (the limit of detection) and 350/g.

The prevalence and levels of both total and pathogenic *V. parahaemolyticus* established in the present study were lower than those recorded in the USA where the organism is present in 100% of oysters at harvest levels up to 10,000/g. After harvest, total numbers

approach 1 million/g. In the present study all oysters were ready-to-eat and the highest levels were 2000/g.

Pathogenic strains are estimated to be in the range 10,000-100,000/g in USA oysters compared with a maximum of 350/g recorded for Australian oysters. Culture techniques may influence levels of the organism. In USA, oysters are harvested directly from the sea floor, whereas in Australia they are taken from racks well off the bottom. Since *V. parahaemolyticus* is associated with substrates this may account for the lower levels in Australia. As well, the practice of managing harvesting according to rainfall events, although intended for control of faecal pathogens, also has importance for controlling *V. parahaemolyticus* by not harvesting at salinities <23 ppt. Finally, the Sydney rock oyster is a particularly hardy species which can remain alive out of water for at least 2 weeks at ambient temperature. It may be that this species, because it is alive, also can eliminate *V. parahaemolyticus* from its tissues and shell liquor.

The present study, based on only 40 samples of oysters from three states, should not be regarded as definitive in the quantitative sense; the latter can come only from a longitudinal study over an annual cycle. However, pathogenic strains have been isolated, a finding that has qualitative importance, especially for those areas where water temperatures are high for several consecutive months.

The present study was formulated to provide key data for a global risk assessment commissioned by the Codex Committee on Food Hygiene (CCFH). The risk assessment model used for the global assessment is based on the USA model and relies heavily on temperatures and times. Not surprisingly, when these data are incorporated, the model predicts a significant quantum of annual illness from consumption of raw Sydney rock oysters. The risk estimates (Table 5) predict more than 220 annual illnesses from Wallis Lake oysters, with 120 each January alone, when consumption is high.

Table 5: FAO-WHO draft predicted illnesses from *V. parahaemolyticus* in various countries according to season (note hemisphere difference for New Zealand and Australia)

					USA			
	Japan	Australia	NZ	Canada	Gulf	Mid-Atlantic	North-Atlantic	Pacific NW
Jan-Mar	4	157	13	0	39	0	0	0
Ap-June	1	28	17	1	1559	12	14	1
June-Sept	0	10	0	7	3808	21	42	10
Oct-Dec	1635	33	5	0	329	23	23	0

In contrast to predicted illness, enquiries to the Queensland and NSW Department of Health indicate no illnesses from oyster consumption linked with *V. parahaemolyticus* (John Bates and Craig Dalton, personal communications). Importantly, given two key events in NSW, the Wallis Lake incident of 1997 and the *V. parahaemolyticus* gastroenteritis involving imported shrimp, health authorities are attuned to illnesses involving both *V. parahaemolyticus* and oysters and systems exist for screening patients for this organism.

The USA modelling team will incorporate data from the present study into the final model and include salinity effects as manifested in management of rainfall events.

BENEFITS

The present study will provide several benefits to Australian researchers and producers:

The new Australian data provided by this project, and to be incorporated in the final global risk assessment, will ensure that predicted illness from Australian oysters will be based on correct information, rather than assumptions based on USA data.

From an industry viewpoint, the findings provide an opportunity to re-evaluate the need for a more thorough risk profile/assessment of pathogenic *Vibrio*'s in Australian oysters

From the scientific viewpoint, the presence of pathogenic strains of *V. parahaemolyticus* has been demonstrated in Australian oysters. In addition, linkages have been made with laboratories in USA, Denmark, India and Japan which are using gene technology to isolate and enumerate pathogenic *V. parahaemolyticus.*

FURTHER DEVELOPMENT

This is the first report of the isolation of pathogenic *V. parahaemolyticus* strains from oysters grown in Australia. Industry should consider this result carefully, and evaluate the need for a further, longitudinal study of the prevalence and distribution of pathogenic *Vibrio*'s (*V. parahaemolyticus* and *V. vulnificus*) over an entire growing season. Such data will be necessary to properly evaluate the risk of these organisms to consumers, and to determine if and how individual producers and/or the industry as a whole should manage this risk.

There will be further use of the data as the WHO-FAO drafting team completes its study.

CONCLUSIONS

This project has successfully met its objectives, *viz*:

- Obtain total *V. parahaemolyticus* counts from oysters from NSW, SA and Tasmania.
- Determine prevalence of pathogenic *V. parahaemolyticus* amongst these isolates.
- Compile and analyse data for potential inclusion in the FAO-WHO global risk assessment of *Vibrio parahaemolyticus* in oysters.

A "snapshot" of the *V. parahaemolyticus* status of cultured oysters in three Australian states during late summer of 2002 was obtained. *V. parahaemolyticus* was found in 80% of NSW oysters, 60% of Tasmanian and 20% of SA oyster samples, with levels ranging to 2000/g. Pathogenic strains were found in 20% of NSW and Tasmanian oysters and in 10% of SA oysters with maximum levels being 350/g in NSW samples, 300/g in Tasmanian and 200/g in SA oysters. The prevalence and levels of both total and pathogenic *V. parahaemolyticus* established in the present study are much lower than those recorded in the USA where the organism is present in 100% of oysters at harvest levels up to 10,000/g.

This substantive data is now available for use by the following sectors:

- **The Australian oyster industry:**
  - To assess the implications of the first robust survey of the distribution of pathogenic *V. parahaemolyticus* in Australian oysters.
  - To address the predicted Codex mitigation strategy of rapid icing (c.f the current Australian system of holding Sydney rock oysters at up to 25°C for up to 72 hours).
- **Australian regulatory authorities:**
  - To allow the SSA risk assessment on *Vibrio*'s in Australian seafood to be strengthened.
- **FAO/WHO Codex Risk Assessment of *Vibrio*'s in seafood:**
  - To allow the FAO/WHO global risk assessment of *V. parahaemolyticus* in oysters to be compiled with the inclusion of Australian data.

While establishing the presence of pathogenic strains of *V. parahaemolyticus* in Australian oysters, this data also records their presence at levels far below those associated with food poisoning outbreaks. These findings already have been of immense value in the FAO-WHO Expert Consultation held in August 2002 in Bangkok, where the USA modellers published predicted illness from *V. parahaemolyticus* in oysters in Canada, USA, Japan, NZ and Australia. For Australia, modelled estimates exceeded 200 cases/annum, including 120 cases each January.
The data from the present study were presented to the modellers and will be used for final predictions. It is probable that predicted illnesses will be much reduced, reflecting Australian epidemiology for this pathogen:product pairing. The FAO-WHO will report the final outcomes to the Codex Committee on Food Hygiene in early-2003.

The success of the present project indicates the effectiveness of tailoring data gathering towards satisfying the requirements of risk assessment.

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APPENDIX 1: INTELLECTUAL PROPERTY

It is not anticipated that any intellectual property leading to project income will be generated from this project.

APPENDIX 2: STAFF

Abel, Mr Guy	University of Tasmania
Brown, Dr Mark	University of Tasmania
Lewis, Dr Tom	Australian Food Safety Centre
McMeekin, Prof Tom	Australian Food Safety Centre
Sumner, Dr John	M&S Food Consultants Pty Ltd

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- Ken Lee (PIRSA, South Australia) and Ray Brown (Dept of Health and Human Services, Tasmania) for supply of oysters and associated data
- Andrew Derwent, SafeFood NSW for water temperature and salinity data for Wallis Lake and for various discussions
- Damian Ogburn (NSW Fisheries) for historical temperature and salinity data
- Angelo De Paola, USDA, Dauphin Island, USA for supply of *Vibrio* control cultures and for advice and discussions on probing techniques
- Idya Karunasagar, University of Mangalore, India for advice and discussions on probing techniques

APPENDIX 3: ENUMERATION OF *VIBRIO PARAHAEMOLYTICUS*

DIRECT PLATING PROCEDURE FOR THE ENUMERATION OF TOTAL AND PATHOGENIC *Vibrio parahaemolyticus* IN OYSTER MEATS

Protocol developed by
David W. Cook, Angelo DePaola, and Susan A. McCarthy
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Dauphin Island, AL 36528-0158

INTRODUCTION

At the 1999 Interstate Shellfish Sanitation Conference, Issue 98-107 was adopted as an Interim Guidance Document to assist states in taking a proactive approach to *Vibrio parahaemolyticus* in oysters. Methodology to support this issue is based on direct plating of homogenized oyster tissue onto a nutrient medium and performing colony lifts to transfer the colonies to a filter that can be tested by DNA gene probes to detect total (*tlh* gene) and pathogenic (*tdh* gene) *V. parahaemolyticus*. The methods described herein are designed to enumerate *V. parahaemolyticus* in oysters at harvest. However, in the hands of a trained analyst, these procedures may be applicable for research on a variety of seafoods.

The following analytical procedures are based on a combination of procedures from the following sources:

1. Direct plating procedure:

   DePaola, A. et al. 1997. Evaluation of an alkaline phosphatase-labeled DNA probe of enumeration of *Vibrio vulnificus* in Gulf Coast oysters. Journal of Microbiological Methods 29:115-120.

2. Identification of total *V. parahaemolyticus* by DNA probe:

   McCarthy, SA., et al. 1999. Evaluation of alkaline phosphatase- and digoxigenin-labelled probes for the detection of thermolabile hemolysin *(tlh*) gene of *V. parahaemolyticus*. Letters in Applied Microbiology, 28:66-70

3. Detection of pathogenic *V. parahaemolyticus*:

   McCarthy, S.A. et al. 1999 Comparison of PCR and DNA hybridization methods for detection of the *tdh* gene in *V. parahaemolyticus*. Abstracts of the 99th General Meeting of the American Society for Microbiology, p. 512.

A) EQUIPMENT AND MATERIALS

1. Autoclave for sterilizing media
2. Incubator, 35ºC ±1ºC
3. Balance, Top loading, 0.01 g sensitivity
4. Water bath, shaking, heating to 42ºC and 54ºC±0.1ºC
5. Orbital shaker, small, for use at room temperature
6. Pipetter, single channel, variable volume, 2-20 µl
7. Pipetter, single channel, variable volume, 20-200 µl
8. Tips for pipetter
9. Microwave oven, 1000 watt
10. Blender and blender cups
11. Brushes for scrubbing shellfish
12. Shucking knives
13. Blender and sterile blender jars
14. Sterile culture dishes, 100x15 mm, glass or plastic
15. Tweezers for handling filters
16. Sterile, bent glass or plastic spreader rods
17. Whatman #541 filters, 85 mm (filters this size may be hand cut from larger diameter filters or ordered as a special cut from the manufacturer)
18. Fine tip waterproof marker (black)
19. Culture dish lids, glass, 100x15mm
20. "Washing" container, wide mouth jar, plastic with straight side, 500 ml, screw cap (Nalgene 2117-0500)
21. Plastic Whirl-Pak® bags (4.5"x9") (NASCO B00736WA)
22. Culture dishes, glass, 100x20 mm
23. Control cultures : *tdh+ V. parahaemolyticus*
    tdh- V. parahaemolyticus
    Vibrio vulnificus

B) MEDIA AND REAGENTS

1. Alkaline Peptone Water (APW) or
2. Phosphate Buffered Saline (PBS)
3. T1N3 Agar Plates
4. TCBS Agar Plates
5. T1N1 Agar Slants
6. Lysis Solution (0.5M NaOH 1.5M NaCl)
7. Ammonium acetate buffer (2M)
8. Standard Saline Citrate (SSC) Solution (20x)
9. 5x SSC
10. 3x SSC
11. 1x SSC
12. Stock Proteinase K (ProK) Solution

13. Hybridization Buffer
14. 1xSSC/SDS solution
15. 3xSSC/SDS
16. NBT/BCIP solution
17. Alkaline phosphatase labeled *tlh* gene probe
18. Alkaline phosphatase labeled *tdh* gene probe

C) COLLECTION OF SHELLFISH FOR *VIBRIO PARAHAEMOLYTICUS* ANALYSIS

1. Plan collection of shellfish so that analysis will be initiated in the same day as harvest. If this can not be done, shellfish must be held at a temperature of less than 10ºC, but not frozen, and analysis initiated within 36 hrs of harvest.

2. Shellfish may be harvested by dredging or tonging. Immediately after harvest, shellfish should be culled and rinsed to remove excess mud. For each sample, place 13 to 15 shellfish in a plastic bag, label bag, and place oysters into an insulated chest that has a false bottom and drain to prevent melt water from accumulating and possibly contaminating shellfish. Cover bag of shellfish with sheet of bubble wrap and place a bag containing about 5 pounds of crushed wet ice on top of bubble wrap. Keep the shellfish in the chest until delivered to the analytical laboratory.

D) SAMPLE PREPARATION AND CULTURE

Note: **T1N3 plates** to be used in this section must be dried in an inverted position with lids partly open in a 35ºC incubator for 30 to 60 min. before use. This will permit the sample to be completely absorbed into the medium and prevent colonies spreading .

1. Verify that the temperature of the shellfish when received at the laboratory is <10ºC by checking the internal temperature of one or more animals. Using a knife, pop the hinge of the animal and insert a temperature probe into the meat. If temperature is >10ºC do not initiate analysis of sample. Investigate reason for high temperature and take corrective action. [Note: If shellfish have been harvested within 3 h. of examination, sufficient cooling time may not have elapsed and shellfish may be >10ºC. Consider such shellfish as acceptable for use.]

2. Wash shellfish with a stiff brush under cold running tap water to remove mud and debris. Place shellfish on absorbent paper to drain.

   Note: The remaining steps in this section must be carried out without delay.

3. Shuck 10 to 14 shellfish (200 to 250 g meat and shell liquor) into a sterile blender jar. Add an equal weight of sterile **phosphate buffered saline (PBS)** or **alkaline peptone water (APW).**

4. Blend for 90 to 120 sec on high speed. This produces a 1:1 shellfish:diluent homogenate. (Figure 1)

5. Prepare a 1:10 shellfish:diluent homogenate by weight (20 g homogenate and 80 g **PBS** or **APW**.)

6. Using a balance having a sensitivity of 0.01 g, weigh 0.2±0.01 g of 1:1 shellfish:diluent homogenate onto the surface of each of four (4) dried **T1N3 plates**. Immediately spread the sample over the surface of the plate with a sterile spreading rod and continue spreading each plate until all liquid is absorbed. All 4 plates may be spread with the same rod. Label these plates as inoculated with 0.1 g shellfish. Colonies developing on one of these plates will be probed for the *tlh* gene. Two plates will be replicate plates to be probed for the *tdh* gene. The forth plate is for archiving.

7. Place 100 µl of the 1:10 shellfish:diluent homogenate onto the surface of two dried **T1N3 plates**. Immediately spread the sample over the surface of each plate with a sterile spreading rod. Label this plate as inoculated with 0.01 g shellfish.

8. Incubate all **T1N3 plates** at 35ºC overnight (16 to18 hr).

E) PREPARATION OF COLONY LIFTS

Note: One of the filters inoculated with the 0.1 g shellfish homogenate and one filter with the 0.01 gram shellfish homogenate will be run using the *tlh* gene probe to determine total *V. parahaemolyticus* counts. Two of the filters (replicates) inoculated with 0.1 g shellfish homogenate will be run using the *tdh* gene probe to determine the pathogenic *V. parahaemolyticus* count. The two remaining filters should be archived and developed later if needed.

1. Mark the sample number, sample volume and test (*tlh* or *tdh*) on the edge of a 85 mm Whatman #541 filter paper disk with a fine point permanent marker. Place the filter, labeled side down, on the surface of the **T1N3 plate** with colonies. Use a spreading rod to press the filter directly against the agar surface to insure colonies are transferred. Filters may be lifted as soon as filter is wet or may remain on the plate for up to 30 min.

2. Place 1 ml of **lysis solution** in the center of an inverted 100x15 mm glass petri dish lid (one for each filter to be lysed). Remove the #541 filter with colonies from the **T1N3** plate and place colony side up over the lysis solution. Position filter to exclude all air bubbles between the filter and the glass. The process is intended to wet the entire filter with the lysis solution. Do not let the lysis solution run over the surface of the filter.

   Note: See **section H** procedure for handling of **T1N3** plates for recovery of ***tdh***+ cultures.

3. Place glass petri dishes (maximum of 6) with filters in microwave and heat on full power (1000 watts or less) for 30 sec/filter. Filters should be completely dry, but not brown. If needed, heating time may be extended.

4. Into a clean plastic "washing" container, place 4 ml of **ammonium acetate buffer** for each filter to be neutralized. Add the filters one at a time insuring that each filter is saturated before adding the next. Let filters remain in buffer for 5 min. at room temperature with swirling on the orbital shaker.

5. Decant the **ammonium acetate buffer** from the "washing" container. Add 10 ml of **lx SSC** solution per filter. Swirl container for 1 to 2 minutes. Decant the liquid and rinse a second time by adding 10 ml of **1x SSC** per filter to the container and swirling for 1 to 2 minutes. Decant solution.

6. Continue with probing of filters or place filters, colony side up, onto absorbent paper and allow to air dry at room temperature. Once dry, filters can be stored indefinitely in plastic bags until ready to probe.

F) PROBING THE COLONY LIFTS

(Use Attachment 1, Quick Reference and Checklist, to insure that all steps are completed.)

Note: Filters being probed for *tdh* and *tlh* genes can be combined in steps 1, 2, 6, 7, and 8. However, care must be taken to insure that the gene probes are not mixed and that the filters are processed with the correct probe.

1. Into the "washing" container, add 10 ml of **lxSSC** and 20 µl **stock ProK** for each filter to be treated. Warm to 42ºC and add filters one at a time to insure that each is saturated with the solution before the next is added. Incubate with shaking (~50 spm) in a water bath at 42ºC for 30 min.

2. Rinse filters 3 times in **lxSSC** (l0 ml/filter) for 10 min at room temperature in the "washing" container with shaking on the orbital shaker at ~125 rpm. Filters can be dried, as above, at this point and stored indefinitely or you can proceed with hybridization.

   Note: In steps 3 through 5 it is critical that the temperature be maintained at a constant 54ºC. Check the temperature of your water bath with a certified thermometer to insure correct temperature.

3. Place 1 to 5 filters marked *tlh* in a plastic (Whirl Pack 4.5"x9") bag marked to receive the *tlh* gene probe and filters marked *tdh* in bag marked to receive the *tdh* gene probe. Add a control strip to each bag (see section G). Add 10 ml of **hybridizing buffer** to each bag. Weight corner of bags so that they remain completely submerged, but are free to move with shaking. Soak filters for 30 min. at 54ºC with shaking (~50 spm).

4. Pour buffer from the bag and add 10 ml of fresh pre-warmed **hybridizing buffer**. Hold the bag so that buffer pools on one side. Add 5 picamoles of **probe** and mix into buffer by massaging the bag gently. Close the bag while excluding air bubbles. Weight the bags so that they remain completely submerged but are free to move with shaking. Incubate 1 hr in the water bath at 54ºC with shaking (~50 spm).

5. Remove filters from bags, placing those probed with *tlh* and t*dh* into separate "washing" containers.
   a. Rinse *tlh* filters 2 times with **1xSSC/SDS** (l0 ml filter) for 10 min in bath at 54ºC with shaking.
   b. Rinse *tdh* filters 2 times with **3xSSC/SDS** (l0 ml filter) for 10 min in bath at 54ºC with shaking.

6. Filters can now be combined into one "washing" container. Rinse 5 times for 5 min. in **1x SSC** (l0ml/filter) at room temperature with shaking on the orbital shaker.

7. Into a glass petri dish (100x20 mm), add 20ml of **NBT/BCIP solution** and add up to 5 filters. Incubate with shaking at room temperature or at 35ºC for faster results. Cover to omit light during incubation. Check development of positive control on the control strip every half hour. Full development is usually complete in 1to 2 hours.

8. Stop the development reaction when control filters are developed by placing filters into a "washing" container with distilled or deionized. water (10 ml/filter). Rinse 3 times for 10 min. each time.

9. Place filters on absorbent paper in the dark to dry. Count and record the number of colony blots that develop a bluish-gray or dark brown color. Colony blots that are colorless, yellow, gray or light brown are negative. Filters should be stored in the dark to prevent color change. Filters may be photocopied or scanned into a computer to produce a permanent record.

10. Express results in colony forming units (CFU) per gram of oysters in sample. Each colony on a 0.1 g filter represents 10 CFU/g and each colony on a 0.01 g filters represents 100 CFU/g

G) CONTROL STRIPS

Control strips are prepared using *V. vulnificus*, a *tdh*- strain of *V. parahaemolyticus*, and a *tdh*+ strain of *V. parahaemolyticus*. These strains are spot inoculated in multiple lines on a T1N3 plate and incubated overnight. Colony lifts are made from the plate and the filters are lysed. The filters are cut into strips so that each strip contains all of the three controls. Control strips can be mass produced, dried and stored for later use. A control strip should be used in each bag of filters being probed. The expected reactions from the controls are as follows.

Culture	*tlh* Probe	*tdh* Probe
V. vulnificus	-	-
tdh- V. parahaemolyticus	+	-
tdh+ V. parahaemolyticus	+	+

H) PROCEDURE FOR ISOLATING *TDH*+ STRAINS FROM T1N3 PLATES INOCULATED DIRECTLY WITH OYSTER HOMOGENATES

The *V. parahaemolyticus* monitoring program uses a direct plating procedure for enumerating total and pathogenic (*tdh*+) *V. parahaemolyticus* in oyster homogenates. Below is a procedure for recovering viable cultures of *tdh*+ *V. parahaemolyticus* in the event of detecting colonies on the Whatman 541 colony lifts that appear to hybridize with the *tdh* probe.

In step E.1, prior to overlaying plates (T1N3 plates with overnight growth), the 541 Whatman filters designated for use with the *tdh* probe should be marked with a short line near the label on the filter and one on the opposite side of the filter in permanent ink.

When the filter is placed on the agar surface for the colony lift, make marks with permanent ink on the petri dish bottom that line up with those on the filter.

After the colonies are lifted, leave plates at room temperature for 4 to 6 h to allow partial regrowth of colonies and then store plates in a sealed plastic bag in a refrigerator.

Note: Attempts to recover colonies should be made as soon as the filters are probed and *tdh*+ colonies are detected, but plates should not be held under refrigeration for more than two weeks.

If filters show *tdh*+ colonies, use the filters to locate the appropriate colony (viable cells) on the corresponding plates. The colonies will probably be obscured and possibly mixed with adjacent colonies as a result of the colony lift procedure but viable cells of the target colony should be present in the general area of the colony's original location on the plate.

Place the filter, colony side up on a table top or preferably a light box. Remove the top from the corresponding plate and place the plate in the inverted position directly over the filter and align the lines on the plate over the lines on the filter.

Make a circle (approx. 0.5 cm diameter) on the plate bottom directly over each *tdh* positive spot on the filter. The position of the circles can be confirmed by placing the filter on top of the inverted plate and holding it up to the light to see if the *tdh* positive spot lies within the circle.

Touch an inoculating loop or needle to the agar surface within the circled area and streak one or more TCBS plates for colony isolation. Incubate overnight at 35ºC. From each TCBS plate , transfer 5 to 10 typical (green) colonies into APW in separate wells of a 96-well plate. Incubate at 35ºC until growth is detected and use a 48 prong replicator to inoculate cultures onto two T1N3 plates. After overnight incubation, perform colony lifts and probe one filter with *tlh* probe and the other with the *tdh* probe.

If any wells produce growth that hybridizes with both *tlh* and *tdh* probes, streak APW onto T1N3 to check for purity. Send purified culture on T1N1 slants to GCSL for confirmation and further characterization.

MEDIA AND REAGENTS

(M & R numbers correspond to BAM numbers.)

Note: Distilled or deionized water (d. water) may be used for all media and reagents.

Phosphate Buffered Saline (PBS) [R 59]

NaCl	7.650 g
Na_2HPO_4, anhydrous	0.724 g
KH_2PO_4	0.210 g
d. water	1000 ml

Dissolve ingredients in d. water. Adjust pH to 7.4 (with 1 N NaOH). Autoclave 15 min at 121ºC.

Store at room temperature. Discard after 90 days.

Alkaline Peptone Water (APW) [M9]

Peptone	10 g
NaCl	10 g
d. water	1000 ml

Dissolve ingredients. Adjust pH with NaOH so that value after sterilization is 8.5+0.2. Dispense in 100 ml amounts in media bottles and autoclave 15 min at 121C. Store under refrigeration. Discard after 90 days.

T1N3 Agar

Tryptone	10 g
NaCl	30 g
Agar	20 g
d. water	1000 ml

Adjust pH to 7.2 before heating. Autoclave, cool and pour into plates. Immediately after plates solidify, package in plastic bags and store under refrigeration. Discard after 90 days.

Lysis solution (0.5M NaOH 1.5M NaCl)

NaOH	20.0 g
NaCl	87.0 g
d. water	1000 ml

Store room temperature. Discard after 90 days.

Ammonium acetate buffer (2M)

Ammonium acetate	154 g
d. water	1000 ml

Store room temperature. Discard after 90 days.

Standard Saline Citrate (SSC) Solution [R77]

20xSSC

NaCl	175.4 g
Sodium Citrate . $2H_20$	88.2 g
d. water	1000 ml

Dissolve in 800 ml d. water and adjust to pH 7 with 10 N NaOH. Bring to volume of l000 ml. Store at room temperature. Discard after 90 days.

5x SSC

20X SSC	25 ml
d. water	75 ml

3x SSC

20X SSC	150 ml
d. water	850 ml

1xSSC

20X SSC	50 ml
d. water	950 ml

Dilute prior to use. Discard lx SSC after 1 day.

Stock Proteinase K (ProK) solution

Add 5ml distilled water to 100 mg bottle of proteinase K (P 6556, Sigma Chemical). The stock ProK solution will contain 20mg/ml. Divide into 200 µl amounts and store frozen at -20C.

Hybridization Buffer

Bovine Serum Albumin (BSA) (Fraction V Powder)	0.5 g
Sodium Dodecyl sulfate (SDS) Sodium Lauryl Sulfate)	1.0 g
Polyvinylpyrrolidone (PVP-360)	0.5 g
5x SSC	100.0 ml

Store at 4ºC for no longer then 1 week. Warm to 54ºC before use.

1XSSC/SDS solution

Sodium Dodecyl Sulfate (SDS)	10.0g
1xSSC	1.0 liter

Store at room temperature. Discard after 90 days.

3SSC/SDS solution

Sodium Dodecyl Sulfate (SDS)	10.0 g
3xSSC	1.0 liter

Store at room temperature. Discard after 90 days.

NBT/BCIP solution

Just before use, dissolve 2 NBT/BCIP ready-to-use tablets (Boehringer Mannheim, Cat. No. 1697471) in 20 ml of d. water in a glass petri dish. Discard after one use.

TLH-L AP-probe

Probe sequence is 5' XAA AGC GGA TTA TGC AGA AGC ACT G 3' where X = alkaline phosphatase conjugated 5' Amine-C6. Probe available from DNA Technology A/S, Forskerparkern/Science Park Aarhs, Gustav Wieds Vej 10 A, DK-8000 Aarhuys C. Denmark; Phone +45 87 32 30 00; Fax +45 87 32 30 11; E-mail Oligo@DNA-technology.dk. Probes vary in strength and a volume equal to 5 picomoles must be calculated for each batch. Store probes under refrigeration. DO NOT FREEZE. Shelf life is unknown, but exceeds one year.

TDH-M2 AP- probe

Probe sequence is 5' XGG TTC TAT TCC AAG TAA AAT GTA TTT G 3' where X = alkaline phosphatase conjugated 5' Amine-C6. Probe available from DNA Technology A/S, Forskerparkern/Science Park Aarhs, Gustav Wieds Vej 10 A, DK-8000 Aarhuys C. Denmark; Phone +45 87 32 30 00; Fax +45 87 32 30 11; E-mail Oligo@DNA-technology.dk. Probes vary in strength and a volume equal to 5 picomoles must be calculated for each batch. Store probes under refrigeration. DO NOT FREEZE. Shelf life is unknown, but exceeds one year.

Calculation of probe amount

Data sheets provided with each batch of probe will show the concentration of the probe in nanomoles (nmol) and the total volume of probe shipped. Enter those values into the following equation:

(µl probe equal to 5 picomoles) = (volume in µl x 5) ÷ (concentration in nmol x 1000)

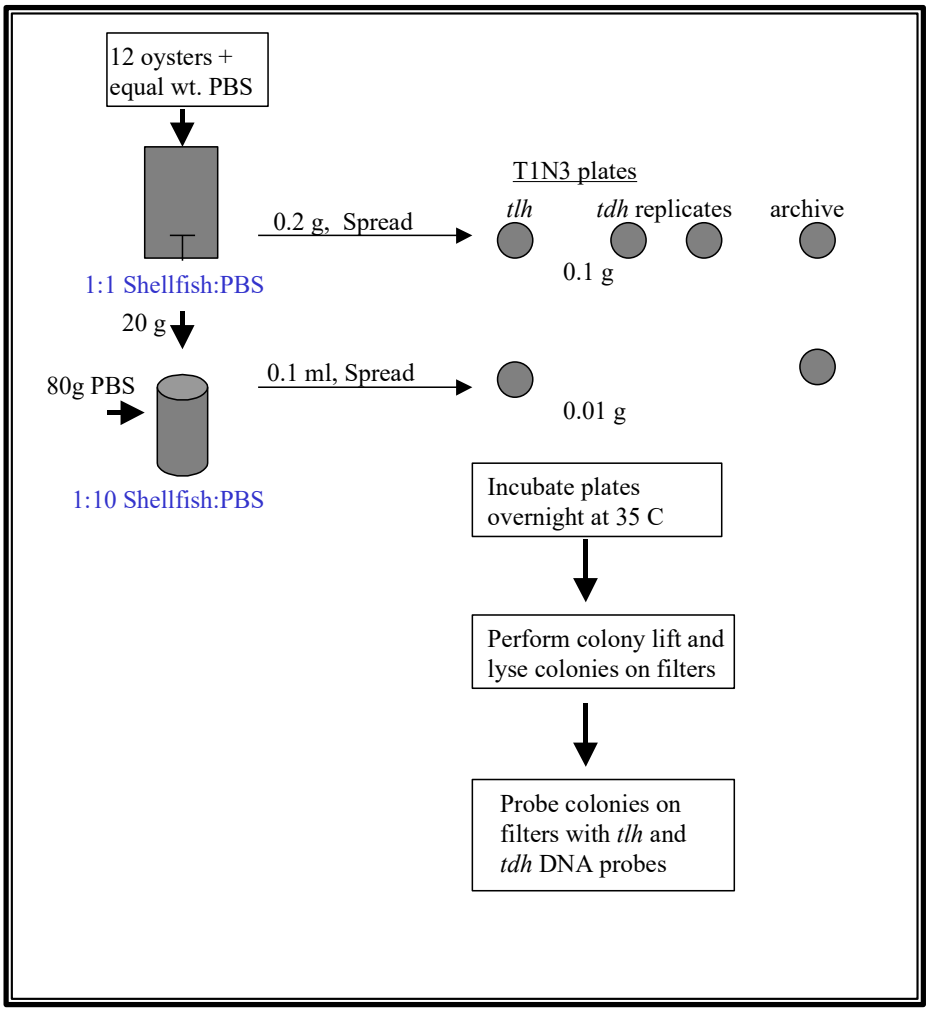
Attachment 1:

Quick Reference and Checklist for AP-DNA Probe Development

Steps in Developing AP-DNA Probe	Temp	Time	Date				
Pro K treatment (10 ml 1xSSC & 20 :l stock Pro K per filter)	42C	30 min					
Rinse 1x SSC buffer (10 ml/filter)	RT	10 min					
Rinse 1x SSC buffer (10 ml/filter)	RT	10 min					
Rinse 1x SSC buffer (10 ml/filter)	RT	10 min					
Hybridizing buffer (10 ml/bag)	54 C	30 min					
Hybridizing buffer (10 ml/bag) & Probe	54 C	60 min					
FOR *tlh* PROBE							
Rinse w 1xSSC/SDS (10 ml/filter)	54 C	10 min					
Rinse w 1xSSC/SDS (10 ml/filter)	54 C	10 min					
FOR *tdh* PROBE							
Rinse w 3xSSC/SDS (10 ml/filter) ,	54 C	10 min					
Rinse w 3xSSC/SDS (10 ml/filter)	54 C	10 min					
Rinse 1x SSC Buffer (10 ml/filter)	RT	5 min					
Rinse 1x SSC Buffer (10 ml/filter)	RT	5 min					
Rinse 1x SSC Buffer (10 ml/filter)	RT	5 min					
Rinse 1x SSC Buffer (10 ml/filter)	RT	5 min					
Rinse 1x SSC Buffer (10 ml/filter)	RT	5 min					
NBT/BCIP (20 ml/5 filters)	RT or 35C	60 to 120 min					
Rinse d. water (10 ml/filter)	RT	10 min					
Rinse d. water (10 ml/filter)	RT	10 min					
Rinse d. water (10 ml/filter)	RT	10 min					
Dry filters							

RT = Room Temperature (~25ºC)
Use √ marks to indicate step has been completed.

Figure 1.


12 oysters + equal wt. PBS
1:1 Shellfish:PBS
20 g
80g PBS
1:10 Shellfish:PBS
0.2 g, Spread
0.1 ml, Spread
T1N3 plates
tlh
tdh replicates
archive
0.1 g
0.01 g
Incubate plates overnight at 35 C
Perform colony lift and lyse colonies on filters
Probe colonies on filters with tlh and tdh DNA probes