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Isolation, characterisation and DNA analysis of *Mycoplasma* spp. from moribund prawns *Penaeus monodon* cultured in Australia

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ABSTRACT: For the first time, a total of 14 *Mycoplasma* isolates were cultured from 24 moribund prawns investigated during an outbreak of mid-crop mortality syndrome in northern Queensland, Australia. *Mycoplasma* were isolated from the gill appendages, brains and eyes of the prawns. *Mycoplasma* growth occurred between 20 and 37°C with or without CO_2 in modified Frey's medium containing 0.5 to 3.0% sodium chloride and 20% foetal bovine serum. Optimal growth was observed at 37°C with 5% CO_2. All strains were size filtered and cloned, and their morphology, biochemical and biomolecular characteristics were compared with the characteristics of previously described *Mycoplasma* species. The results showed that these strains belonged to 2 new species, for which the temporary names *Mycoplasma* P1 (*MP1*) and *Mycoplasma* P2 (*MP2*) were designated. Both *Mycoplasma* fermented most of the tested carbohydrates but did not hydrolyse arginine and urea. *MP1* produced films and spots and had high phosphatase activity, but *MP2* did not produce films or spots and had no phosphatase activity. Both species lysed sheep erythrocytes. A genomic library (*Mbo*1 digested) was prepared from *MP1* DNA and cloned into pUC19. Colony hybridisation, using a probe prepared from purified *MP1*, was used to identify colonies of interest. *MP1* DNA fragments were retrieved from recombinant plasmids by digestion with *Eco*RI and *Hind*III. This DNA was used to prepare randomly primed probes for dot blot hybridisation analysis with immobilised DNA from *MP1*, *MP2*, *M. bovis*, *M. dispar*, *M. agalactiae*, *M. bovigenitalium*, *M. ovipneumoniae*, *Mycoplasma* Group 7, *M. arginini* and bacteria belonging to different genera. The probe reacted with genomic DNA from *MP1* only. To further enhance the sensitivity, an *MP1* specific polymerase chain reaction (PCR) assay was designed and produced a 254 bp amplicon which discriminated *MP1* from all other *Mycoplasma* DNA tested. Using the DNA probe and PCR assay, most of the *Mycoplasma* isolated from the diseased prawns could be designated as strain *MP1* (11/14, ~80%).

KEY WORDS: *Penaeus monodon* · *Mycoplasma* · Polymerase chain reaction · DNA probe · Diagnosis bacteria

INTRODUCTION

Mycoplasmas of aquatic animals have not been studied extensively. Nevertheless, *Mycoplasma mobile* has been isolated from diseased fish species showing symptoms of red disease (Kirchhoff et al. 1987), and *M. phocarhinis* and *M. phocacerebrale* have been reported from harbor seals (Giebel et al. 1991) suffering from an acute and fatal disease. In 1994, mid-crop mortality syndrome (MCMS) spread through the *Penaeus monodon* aquaculture industry in northern Queensland, Australia. In a study of filterable agents that might be involved in the mortalities, it was necessary to determine if *Mycoplasma* might have a role in MCMS. Two different *Mycoplasma* were isolated from a number of the diseased prawns from 2 affected farms. We report for the first time the isolation details of these strains, describe the taxonomic evaluation of the 2 strains and compare them with previously described species of *Mollicutes*.

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MATERIALS AND METHODS

Prawns. Isolation of *Mycoplasma* was performed on 24 prawns found moribund or dead at the edges of ponds from 2 aquaculture farms in northern Queensland. Prawns were tested either within 2 h postmortem or after storage at -20°C.

***Mycoplasma* strains, bacteria and growth conditions.** Most of the reference *Mycoplasma* strains were obtained from the National Collection of Type Cultures (Colindale, UK), but some strains were obtained from Oonoonba Veterinary Laboratory of Northern Queensland (Table 1). Other bacterial strains were obtained from our Department. All *Mycoplasma* strains were grown in Frey's medium (Frey et al. 1968) containing 20 or 30% foetal bovine serum (FBS), depending upon the requirement of individual species. Other bacterial strains were grown in brain heart infusion (BHI) medium.

***Mycoplasma* medium.** *Mycoplasma* Frey's medium (Frey et al. 1968) with some modifications was used for isolation of *Mycoplasma* from the diseased prawns. Briefly, mycoplasma broth medium (MBM) was prepared by rehydrating 12.75 g of mycoplasma broth base (Oxoid Laboratories) in 500 ml of dH_2O containing 2.5 ml of 0.4% phenol red and 1.25 ml of 10% thallium acetate. The medium was adjusted to pH 7.8 and autoclaved at 121°C at 15 lbs (ca 6.8 kg) pressure for 15 min. It was dispensed in 80 ml volumes and stored at -20°C. The following filter sterilised solutions were added to each 80 ml of MBM: 20 ml FBS (Commonwealth Serum Laboratory, Melbourne, Australia), 1 ml of freshly prepared yeast extract solution (Herderschee 1963), 1 ml of 0.2% DNA solution (sodium salt isolated from salmon testes) (Sigma Chemical Co., St. Louis); 1 ml of 10% glucose solution, 1 ml of 10 mg ml^{-1} of ampicillin solution.

To prepare *Mycoplasma* agar (MA), technical agar (Oxoid) was added to the MBM to a final concentration of 1% without phenol red and glucose and autoclaved as described above. Once the temperature of the medium had reached about 40°C in a water bath, the following filter sterilised solutions were added to each 80 ml of MA, mixed well and dispensed into 5 ml petri dishes: 20 ml FBS, 1 ml of yeast extract solution, 1 ml of 0.2% salmon testes DNA solution, 1 ml of 10 mg ml^{-1} of ampicillin solution.

For the isolation of arginine-requiring *Mycoplasma* and *Ureaplasma*, modified media were used. To prepare these, 1% arginine hydrochloride or urea was added to MBM and adjusted to pH 7.0. The shift of the phenol red indicator from pink to red indicated growth in this medium.

Culture procedure. Approximately 0.5 g of homogenised organ material (gill appendages, brain and eyes) from individual prawns was suspended in 3 ml of MBM. Once a homogenous suspension had been produced, it was diluted in MBM. Aliquots of 300 µl of suspension were used to prepare serial 10-fold dilutions (up to 10^{-4}) in MBM and modified arginine broth (MAB). To test for any *Mycoplasma* contamination in the FBS and other sources, 1 tube containing MBM was always put up as a negative control. The tubes were incubated at 37°C in rolling drums in which the tubes rotated once per minute. They were examined daily for pH (colour) changes. Whenever the colour of the medium turned to yellow, 300 µl of the culture was transferred to a fresh tube containing 3 ml of fresh MBM. After 6 to 7 d, 50 µl of the contents of each tube with the highest dilution indicating growth was spotted into MA plates. The plates were incubated at 37°C for 14 d in a humidified atmosphere with 5% CO_2. They were examined for colonies using an inverted microscope (Olympus CK2, Japan). Differences in growth of *Mycoplasma* on plates incubated aerobically and in 5% CO_2 were recorded. The cellular morphology of the organisms was assessed by light microscopy after Gram and Giemsa staining.

Table 1. *Mycoplasma* and other bacteria species tested by the *MP1* specific probe and PCR. NC: type strain NCTC, National Collection of Type Culture; Oonoonba: Oonoonba Veterinary Laboratory of Northern Queensland; JCU: Department of Microbiology and Immunology, James Cook University

Species	Strain
Mycoplasma bovis	NC10131
Mycoplasma ovipneumoniae	NC10151
Mycoplasma agalactia	NC10123
Mycoplasma bovigenitalium	NC10122
Mycoplasma bovirhinis	NC10118
Mycoplasma dispar	NC101125
Mycoplasma Group 7	Oonoonba
Mycoplasma bovigenitalium	Oonoonba
Mycoplasma arginini	Oonoonba
Vibrio spp.	JCU
Pseudomonas putida	JCU
Staphylococcus aureus	JCU
Staphylococcus spp.	JCU
Streptococcus uberis	JCU
Streptococcus agalactia	JCU
Corynebacterium bovis	JCU
Klebsiella pneumoniae	JCU
Pseudomonas spp.	JCU
Pasteurella spp.	JCU
Aeromonas spp.	JCU
Brucella abortus	JCU
Yersinia enterocolitica	JCU
Enterobacter cloacae	JCU
Enterobacter facalis	JCU
Serratia marcessans	JCU
Bacillus subtilis	JCU
Escherichia coli	JCU

Staining *Mycoplasma* colonies. To observe *Mycoplasma* colonies and differentiate between *Mycoplasma* and bacterial L-form colonies, isolated *Mycoplasma* colonies were stained with Dienes stain (Timms 1967). A light film of Dienes stain was placed on a microscopic slide and allowed to dry. A block of agar containing *Mycoplasma* colonies was cut from MA and placed colony-side upwards on the dry Dienes stained microscope slide (Carter 1975). The preparation was then examined under the low power of a microscope.

Purification of *Mycoplasma*. Isolated *Mycoplasma* were purified by the single colony technique (Stalheim 1984). A single colony was removed by cutting out a small block of agar using a sterile scalpel. The colony was transferred to a tube containing 3 ml of MBM and incubated for 48 h. The culture was then passed through a 0.45 µm filter, diluted 1/10 and 1/100 in MBM and 50 µl of each dilution was spotted onto MA plates and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 7 d. This purification procedure was repeated 3 times.

Biochemical tests. The tests for breakdown of carbohydrates, arginine and urea, reduction of 2,3,5-triphenyltetrazolium chloride (aerobically and anaerobically), phosphatase activity and film and spot production were performed as described by Aluotto et al. (1970). Because of wide variation in the sensitivity of mycoplasma to methylene blue, this characteristic was investigated by adding 0.002% (w/v) methylene blue to the MA and comparing the growth of inoculated mycoplasma with the growth of mycoplasma cultivated on MA plates without methylene blue (Fallon & Whittlestone 1969). All plates and tests were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 7 d unless otherwise stated.

Sugar fermentation. One percent (w/v) of each sugar (mannitol, dulcitol, xylose, sorbitol, arabinose, mannose, sucrose, sorbose, cellubiose, maltose, glucose) was added to 10 ml of MBM without glucose, then inoculated with 300 µl of culture medium containing the isolate and incubated. Basal medium without sugar was also inoculated as a growth and pH indicator control.

Salt concentration. The salt concentration in 10 ml of MBM was adjusted to 1, 1.5, 2, 2.5, 3, 3.5 and 4% (w/v) by addition of extra sodium chloride to MBM at pH 7.8. Then it was inoculated with 300 µl of culture medium containing the isolated *Mycoplasma* and other *Mycoplasma* species listed in Table 1 and incubated. Normal MBM, containing 0.5% sodium chloride, was also inoculated as a growth and pH indicator control.

Arginine utilisation. One percent (w/v) arginine hydrochloride was added to 10 ml MBM containing 0.4% phenol red and adjusted to pH 7.0. It was then inoculated with 300 µl of culture medium containing the isolated *Mycoplasma*. Basal medium without arginine was also inoculated and incubated as a growth and pH indicator control.

Haemolysis of red blood cells. Haemolytic activity of each isolated *Mycoplasma* was tested on mycoplasma blood agar prepared by incorporating sheep red blood cells into the melted MA at 50°C and at a final concentration of 5% (v/v) red cells.

Sterol requirement. This test was carried out to discriminate the families Mycoplasmataceae (sterol dependent) from Acholeplasmataceae (sterol independent) by cultivation of the *Mycoplasma* isolates in MBM without added serum (i.e. with no sterol).

Digitonin test. This test was carried out to discriminate *Mycoplasma* isolates from *Acholeplasma*, as *Mycoplasma* are more sensitive to lysis and to growth inhibition by digitonin than *Acholeplasma* (Razin & Shafer 1969). The digitonin discs were prepared by dissolving 0.15 g of digitonin (Sigma Chemical Co.) in 10 ml of pure ethanol over a boiling water bath. The 6 mm Whatman filter discs were soaked in 25 µl of digitonin solution and then air dried. An MA plate was flooded with 500 µl of isolated *Mycoplasma* culture and the plate was allowed to dry in a biological laminar flow cabinet. The digitonin disc was then placed on the agar surface and the plate was incubated.

Phosphatase test. One ml of 1% (w/v) sodium phenolphthalein diphosphate solution was added to MBM without phenol red and glucose, inoculated with 300 µl of culture medium containing the isolated *Mycoplasma* and incubated. Uninoculated medium was incubated as a negative control. After 3 to 4 d incubation, 5 N NaOH was added at a final concentration of 5% to all the test tubes. A positive reaction was determined by a pink colour change.

Filtration studies. Filterability of isolated *Mycoplasma* was determined by membrane filtration (Millipore Corp.) at pore diameters of 0.45 and 0.2 µm. An unfiltered suspension was used as a control, and this suspension and each filtrate were diluted in 10-fold steps and spread (100 µl) on MA plates. After incubation for 72 h at 37°C with 5% CO_2, the number of colonies was counted and calculated.

Reversion experiments. The clones were subcultured 6 consecutive times by using liquid or solid growth medium devoid of ampicillin or any other antimicrobial agent in order to determine whether the organisms reverted to bacterial forms. Agar plates and fluid cultures of all passages were examined for alteration in the morphology of clones and cells, respectively. Also, agar culture colonies of each clone were stained with Dienes stain in order to differentiate *Mycoplasma* colonies from bacterial L forms (Timms 1967).

Experimental infection. The pathogenesis of *MP1* was tested in a prawn bioassay. The *MP1* cultures were grown for 48 h at 37°C in 5 ml of MBM. After growing of the *Mycoplasma*, a serial dilution was made in 1 ml MBM and an aliquot spotted on MA. The number of colonies on agar was used to calculate the number of *Mycoplasma* in broth culture. A total of 60 clinically healthy prawns from research breeding facility stocks with no history of untoward mortalities or abnormalities were used in the experimental infections. They were divided into 6 separate groups. Groups 1 and 2 were injected intramuscularly with 50 µl of MBM containing 10^6 colony-forming units (CFU) *Mycoplasma* and Groups 3 and 4 were injected with 50 µl of MBM containing 10^4 CFU *Mycoplasma*. Groups 5 and 6 were control groups that were injected with only 50 µl of sterile MBM. Clinical observations were made on all prawns beginning 1 wk prior to experimental infection.

Genomic DNA extraction. *Mycoplasma* were grown in MBM and DNA was extracted from 200 ml of a late exponential phase culture as previously described (Ghadersohi et al. 1997).

Genomic library construction. For cloning of specific fragments, total DNA from *MP1* was completely digested with *Mbo*1 (Promega) and the fragments generated were cloned into the *Bam*HI site of pUC19 (Sambrook et al. 1989). Fresh, competent *Escherichia coli* JM109 cells were used for transformation with ligation mixtures. Recombinant colonies were grown on 2YT agar containing 150 µg ampicillin, 40 µg X-Gal and 0.5 mM IPTG ml^{-1}. Colony hybridisation with a probe prepared from purified *MP1* DNA was used to identify colonies of interest. Six such colonies were selected for further evaluation. The size of the cloned fragments was determined and fragments were liberated by *Hind*III/*Eco*RI double digestion of the plasmids. The digested DNA was purified and isolated on 2% agarose gels and used to prepare randomly primed probes for dot blot hybridisation.

Dot blot hybridisation. Five hundred ng of chromosomal DNA from different species of *Mycoplasma* or an equivalent amount of other bacterial DNA (Table 1) was spotted onto nitrocellulose filters following the instructions of the manufacturer (Hybond-N+, Amersham) and fixed by baking in an oven at 80°C for 2 h. Radiolabeling of DNA with ^{32}P and hybridisation techniques were as described (Ghadersohi et al. 1997). The membranes were then washed twice in 100 ml of 2 × SSC (3 M NaCl, 0.3 M Na_3citrate) and twice in 100 ml of 0.1% SSC at room temperature (5 min for each wash) with constant agitation. The radiolabelled probes were detected by autoradiography with Fuji X-Ray film for 18 h at –70°C.

Sequence analysis. To determine the DNA homology of isolated *Mycoplasma*, 2 recombinant plasmids (p*MP1*/1-2 and p*MP2*/10-1) with insert sizes of 340 and 1100 base pairs (bp), respectively, were sequenced. The nucleotide sequences of these fragments were determined using an Applied Biosystems Dye Prism kit following the instructions of the manufacturer.

Development of polymerase chain reaction. The probe prepared from the *MP1* DNA fragment retrieved from p*MP1*/1-2 hybridised only with *MP1* chromosomal DNA (Fig. 1). The sequence of this fragment (see Fig. 4) was used to design 2 primers which had no similarity with any sequence contained in GenBank or the EMBL DNA database. These were forward 5'-CTCTG-CACTATCTGATCAGTA-3' and reverse 5'-GAATT-GGGGCTGGTGAAGTAA-3'. They were used to produce a 254 bp amplicon under optimal conditions.

Optimisation of the PCR. To gain maximum sensitivity and specificity, all parameters of the polymerase chain reaction (PCR) were optimised. The amplification was performed in 25 µl reaction mixture containing 2.5 µl 10 × PCR buffer, 10 µM of each primer, 0.1 mM concentration of each deoxynucleotide triphosphate (dNTP), 3 mM $MgCl_2$ and 1 U Taq DNA polymerase (Bresatec, Australia) in 1 µl. One droplet of mineral oil was layered on the reaction mixture to prevent evaporation. The reaction was performed in 38 cycles using the following optimal temperature protocol for the 254 bp *MP1* fragment: initial denaturation for 5 min at 94°C followed by cyclic denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, primer extension at 72°C for 1 min and final extension at 72°C for 7 min.

PCR specificity and sensitivity. Twenty ng of chromosomal DNA from *MP1* strains was used as the template in a *MP1*-primed PCR. One hundred ng of DNA

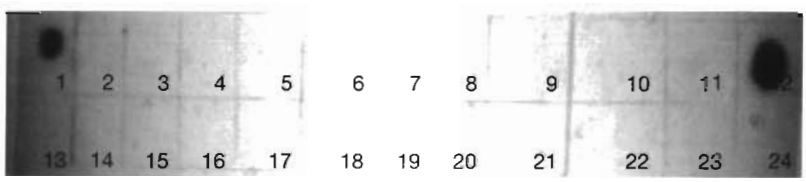

Fig. 1. Autoradiograph of a dot blot hybridisation of various *Mycoplasma* spp. and bacterial DNAs with the radiolabelled probe *MP1*/1-2. 1: *MP1*; 2: *MP2*; 3: *M. bovis*; 4: *M. arginini*; 5: *M. ovipneumoniae*; 6: *M. agalactiae*; 7: *M. bovigenitalium*; 8: *M. bovirhinis*; 9: *M. dispar*; 10: *Mycoplasma* Group 7; 11: *M. bovigenitalium*; 12: positive control recombinant plasmid *MP1*/1-2; 13: *Vibrio* sp.; 14: *Pseudomonas putida*; 15: *Streptococcus uberis*; 16: *Staphylococcus aureus*; 17: *Corynebacterium bovis*; 18: *Klebsiella pneumoniae*; 19: *Aeromonas* sp.; 20: *Escherichia coli*; 21: *Enterobacter cloacae*; 22: *Enterobacter facalis*; 23: *Yersinia enterocolitica*; 24: *Serratia marcescens*

from *MP2*, pUC19 plasmid and other *Mycoplasma* and bacteria (Table 1) was also subjected to *MP1* PCR to determine the specificity of the test. To determine the sensitivity of the test samples of DNA prepared by phenol-chloroform, extraction from a known amount of mycoplasma (CFU) in broth culture was subjected to *MP1* PCR.

Clinical samples. To test the PCR with field material, purified DNA from diseased prawns from aquaculture farms in northern Queensland was subjected to *MP1* PCR. Homogenised organ materials (gill appendages, brain and eyes) from individual prawns were initially suspended in 1 ml NTE buffer (10 mM NaCl, 20 mM Tris hydrochloride pH 7.4, 1 mM EDTA) and incubated overnight at 37°C with 0.5% sodium dodecyl sulphate (SDS) and 100 µg of proteinase K (Promega) ml^{-1}. DNA was extracted with phenol-chloroform as described previously (Ghadersohi et al. 1997). To remove any inhibitors of PCR, the DNA was purified using the Wizard Minipreps DNA purification system (Promega).

RESULTS

Culture of clinical samples

Some of the cultures were overgrown by bacterial contamination with *Corynebacterium* spp. It was found that these bacteria could pass through 0.45 µm membrane filters. *Mycoplasma* were recovered from 14/24 moribund prawns and from none of 20 healthy prawns or from negative control cultures. The *Mycoplasma* isolated decreased the pH of MBM and formed typical 'fried egg' *Mycoplasma* colonies on *Mycoplasma* agar. The colonies rapidly stained with Dienes reagent, confirming that the isolates were true *Mycoplasma* (members of the *Mollicutes*) rather than bacterial L forms (Timms 1967). Liquid cultures stained with Giemsa stain contained characteristic mycoplasmal pleomorphic forms (Klieneberger-Nobel 1962). The isolated *Mycoplasma* were resistant to low pH (4.7) as measured from growth and subculture on MBM and MA for several months.

Biochemical tests

The *MP1* and *MP2* were able to ferment all sugars (*MP2* variably) but they did not utilise arginine and urea (Table 2). They grew at 20 and 37°C, and did not grow in the absence of bovine serum. *MP1* produced films and spots on MA containing 10% (v/v) homogenized egg yolk and it had a strong positive phosphatase reaction, but *MP2* did not produce films and spots and had no phosphatase activity. Both strains lysed sheep erythrocytes and grew with or without CO_2 at 37°C, but growth was faster at 37°C with 5% CO_2. No dye reduction occurred when they were grown in MBM containing 0.025% (w/v) tetrazolium chloride. While the reference *Mycoplasma* from animal origin failed to grow in MBM with increased sodium chloride, the *Mycoplasma* isolated from prawns grew in MBM containing sodium chloride from 0.5 to 3%.

In digitonin tests to discriminate *Mycoplasma* from *Acholeplasma*, the zone of inhibition for the prawn

Table 2. Biochemical characteristics of 3 *Mycoplasma* reference strains and 2 *Mycoplasma* strains isolated from *Penaeus monodon*. N: not tested

Characteristic	*MP1*	*MP2*	*M. mobile*[a]	*M. phocarhinis*[b]	*M. phocacerebrale*[b]
Fermentation of glucose	+	+/–	+	–	–
Fermentation of lactose	+	+	+	N	N
Fermentation of mannitol, dulcitol xylose, sorbitol	+	+	N	N	N
Fermentation of arabinose, mannose, saccharose	+	+	+	N	N
Fermentation of sorbose, cellubiose	+	+	N	N	N
Fermentation of maltose	+	+	+	N	N
Hydrolysis of arginine	–	–	–	–	+
Hydrolysis of urea	–	–	–	–	–
Reduction of 2,3,5-triphenyltetrazolium chloride	–	–	+	+	–
Reduction of methylene blue	–	–	–	–	–
Phosphatase activity	+	–	+/–	+	+
Production of film and spots	+	–	+	+	+
Haemolysis ovine erythrocytes	+	+	+	+	+
Growth at 20°C	+	+	+	N	N
Growth at 30°C	+	+	+	N	N
Growth at 37°C	+	+	–	+	+
Growth in high salt (3%)	+	+	N	N	N

[a]Kirchhoff et al. (1987), [b]Giebel et al. (1991)

Mycoplasma strains was 18 to 30 mm. The zone of inhibition increased with increasing concentration of the stock culture and decreased with increasing incubation time. The isolated *Mycoplasma* were able to pass through membrane filters of 0.45 µm pore size without reduction in number and through filters of 0.2 µm with a slight reduction in number. They did not revert to bacterial forms during 6 consecutive passage in media lacking inhibiting antibiotic substances.

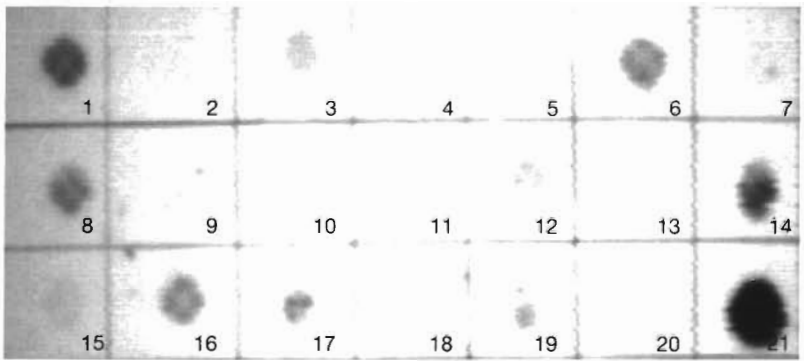

Fig. 2. Autoradiograph of a dot blot hybridisation of DNA of moribund prawns with the probe *MP1*/1-2. 1–19: DNA samples from 19 individual moribund prawns collected from farms; 20: negative control; 21: positive control recombinant plasmid containing *MP1*/1-2

Experimental infections

After challenge with 10^6 CFU *MP1* (Groups 1 and 2), cumulative mortality by Day 17 was 5/10 for Group 2 (i.e. 2 on Day 4, 2 on Day 10 and 1 on Day 17) (Table 3). *Mycoplasma* were isolated from the eye and gill appendages of 3 of the dead prawns, but cultures from the other 2 prawns were overgrown with contaminating bacteria. Also, on Day 14, 2 prawns from control Groups 5 and 6 died but no *Mycoplasma* were found, and cultures were overgrown with contaminating bacteria. On Day 30, 2 prawns from Group 4 died (i.e. challenged with 10^4 CFU *Mycoplasma*). *Mycoplasma* was isolated from only 1 by culture, but *Mycoplasma* DNA was detected in both by PCR (Table 3). There was significantly higher mortality in Group 4 (10^6 CFU challenge) than in both the 10^4 CFU challenge groups (3 and 4) and the control groups (5 and 6) ($\chi^2 = 4.5$, $p < 0.05$, df = 1) (Zar 1974). On Day 60, 5 prawns each from Groups 1 and 2, 4 prawns each from Groups 3 and 4 and 2 prawns each from the control Groups 5 and 6 were sacrificed. *Mycoplasma* were isolated from 3 of the 5 prawns from Group 1 (10^6 CFU) but cultures from the other groups were negative (Table 3).

Table 3. Results from experimental injection of *Penaeus monodon* with *Mycoplasma* P1 (*MP1*). PCR was performed only on prawns that died and were negative on culture

	Group 1 10^6 CFU	Group 2 10^6 CFU	Group 3 10^4 CFU	Group 4 10^4 CFU	Group 5 Control	Group 6 Control
Mortality		5/10		2/10	1/10	1/10
Positive on culture		3/5		1/10	0	0
Positive by PCR		0/2		2/2	0/1	0/1
At termination of experiment (60 d)						
Positive on culture	3/5	0/5	0/4	0/4	0/2	0/2

Identification of a specific probe

The *Mbo*1 digests of *MP1* were constructed into pUC19 plasmid vector. Based on colony hybridisation, 6 white colonies giving high intensity staining with *MP1* whole genomic DNA were selected for detailed study. After double digestion, 8 fragments ranging in size from 0.1 to 2 kb were purified and used to prepare probes for dot blot hybridisation. One fragment of 340 bp called *MP1*/1-2 was found to hybridise with *MP1* only (Fig. 1) and not with DNA from other microorganisms (Table 1). Using fragment *MP1*/1-2 as a probe in dot blot hybridisation tests, most of the isolated prawn *Mycoplasma* (~80%) were identified as strain *MP1* (Fig. 2).

Sensitivity of the probe

To check the sensitivity of the probe *MP1*/1-2, homogenised tissue from a healthy prawn which was negative by dot blot hybridisation (i.e. prawn No. 2, Fig. 2) was seeded with known CFU of *MP1* followed by dot blot hybridisation tests. The results are shown in Fig. 3. The detection limit of this assay was approximately 10^3 CFU. As can be seen in Fig. 3, the size and intensity of the dot blot reaction increased with increasing numbers of *Mycoplasma* in the homogenate.

DNA sequencing and primer determination for PCR

A computer search of GenBank and EMBL databases for sequence simi-

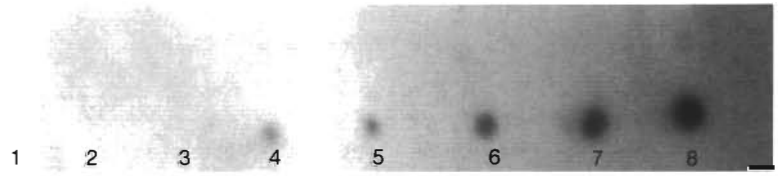

Fig. 3. Sensitivity of the DNA probe *MP1*/1-2 in the dot-blot procedure. Dilutions of a preparation of *Mycoplasma* (*MP1*) with a known titre were added to a crude homogenised healthy prawn sample No. 2 (see Fig. 2), and then extracted DNA from the experimentally seeded samples was subjected to the dot-blot procedure. Samples contained (1) 10, (2) 10^2, (3) 5×10^2, (4) 10^3, (5) 10^4, (6) 10^5, (7) 10^6, (8) 10^7 CFU ml^{-1} of prawn isolated *Mycoplasma*

5'-GGATCTGTTT TCATATCATT AATTAGTTTA GTTCCTTGCA TATTTGCATA TTTATA**CTCT**
GCACTATCTG ATCAGTATGG AGTTGACATT TGAACTAATT GGTTAACTGG TGTAAATGGA
ATTAATACAG CAGCTAGACA AATAGCAATT GTTGCAATTA ACATTGATCC ATTGTAGCAA
TTGTATTAAC ATGCACTCCC ATATCTCTTC ATTTACTCAT TGGTATTTTA TATAAAGGAA
T TGAATAGTA TGTTCCTTTA TTAGTAATTA ACATTAAATG TTGTAAGTTG C**TTACTTCAC**
CAGCCCCAAT TCACATATCA TT TGGTTTTC TTCCAAATAC-3'

Fig. 4. Nucleotide sequence of the 340 bp *MP1* DNA fragment. The sequences corresponding to the primers used for PCR are shown in bold and underlined. These nucleotide sequences have been deposited in the GenBank, EMBL Nucleotide Sequence databases under the accession number AF042859, NCBI REF 374530

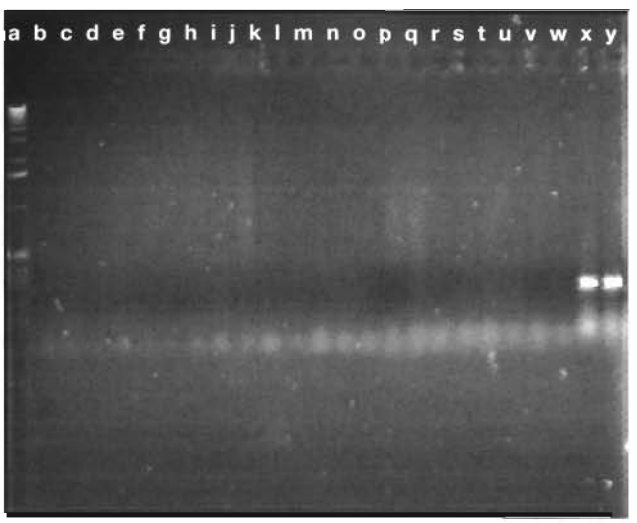

Fig. 5. Photograph of a 1.8% agarose electrophoresis gel showing the PCR amplification reaction products using various genomic DNA samples. Lane a: molecular weight markers (MWM) (BRL 1 kb ladder); Lane b: *Staphylococcus aureus*; Lane c: *Staphylococcus* spp.; Lane d: *Aeromonas* spp.; Lane e: *Streptococcus agalactiae*; Lane f: *Corynebacterium bovis*; Lane g: *Serratia marcescens*; Lane h: *Enterobacter cloacae*; Lane i: *Klebsiella pneumoniae*; Lane j: *Pseudomonas* spp.; Lane k: *Vibrio* spp.; Lane l: *Yersinia enterocolitica*; Lane m: *Mycoplasma bovirhinis*; Lane n: *Mycoplasma* Group 7; Lane o: *Mycoplasma ovipneumoniae*; Lane p: *Mycoplasma arginini*; Lane q: *Mycoplasma dispar*; Lane r: *Mycoplasma agalactiae*; Lane s: *Mycoplasma bovigenitalium*; Lane t: *Mycoplasma bovirhinis*; Lane u: *Mycoplasma bovis* (type strain); Lane v: *MP2*; Lane w: no template; Lane x: isolated *Mycoplasma* (*MP1*); Lane y: recombinant pUC19 (positive control)

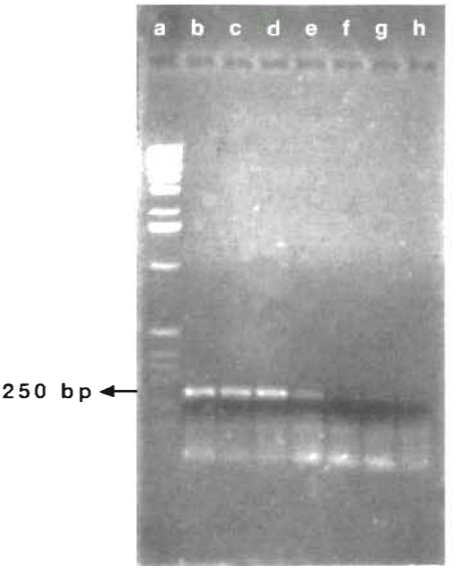

Fig. 6. Photograph of a 1.8% agarose electrophoresis gel showing the PCR amplification reaction products using various dilutions of *MP1* genomic DNA. Lane a: MWM (1 kb ladder); DNA was extracted from: Lane b, 10^7; Lane c, 10^3; Lane d, 500; Lane e, 300; Lane f, 100; Lane g, 10 CFU of *MP1*; Lane h has no template

larity was conducted. Both *Mycoplasma* fragments retrieved from recombinant plasmids gave 70% homology with *Mycoplasma capricolum* DNA for CONTIG MC095 and 61% protein similarity with (Z33071) DNA-gyrase (alpha) of *M. capricolum*. The sequence of the *MP1* insert is shown in Fig. 4.

Sensitivity and specificity of *MP1* PCR

Samples of 20 ng of DNA prepared by phenol-chloroform extraction from prawn isolated *Mycoplasma* were used as templates for *MP1* PCR and the expected 254 bp amplicon was obtained. By contrast, 100 ng DNA templates from *MP2*, 8 other bovine and ovine *Mycoplasma* species and other bacteria (Table 1) gave no amplicoans under the same PCR conditions (Fig. 5). The sensitivity of the *MP1* PCR was for homologous DNA from 300 CFU, as determined by 1.8% agarose gel electrophoresis (Fig. 6). The PCR assay was validated by using DNA from the same diseased prawns that were used in the hybridisation assay (shown in Fig. 2). The PCR assays confirmed the results of the hybridisation assays and a sample from these PCR tests is shown in Fig. 7.

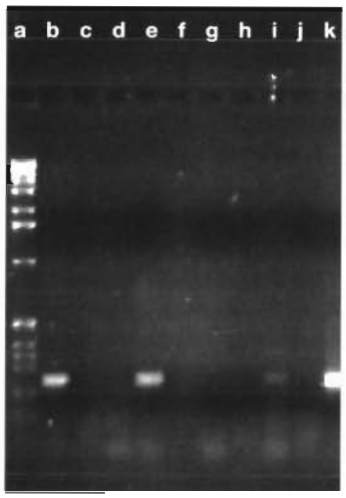

Fig. 7. Agarose electrophoresis gel (1.8 %) of PCR amplification products from DNA samples of individual moribund prawns from aquaculture farms in northern Queensland. A subset of DNA samples (8 of the 19) was drawn from the same lot used in the hybridisation assay for Fig. 2. Lane a: MWM (1 kb ladder); Lane b: prawn 1; Lane c: prawn 2; Lane d: prawn 3; Lane e: prawn 8; Lane f: prawn 10; Lane g: prawn 11; Lane h: prawn 12; Lane i: prawn 13; Lane j: negative control; Lane k: positive control recombinant plasmid

DISCUSSION

The properties of the organisms isolated from diseased prawns fulfilled the essential criteria for Mollicutes as proposed by the International Committee on Systematic Bacteriology Subcommittee on Taxonomy (1995), i.e. typical fried-egg colony form, polymorphic cell form, absence of a cell wall, passage through 0.45 and 0.2 µm filters, lack of reversion to bacteria, resistance to ampicillin. According to digitonin sensitivity and serum requirement, they could be classified in the family Mycoplasmataceae. The inability to hydrolyse urea indicated that the organisms did not belong to the genus *Ureaplasma*. The most exciting property of the isolated *Mycoplasma* strains was their ability to grow in *Mycoplasma* media containing 3.0% sodium chloride and to tolerate low pH (4.7) for a long period. These properties appear to be unique, because other *Mycoplasma* species fail to grow in such media. The *Mycoplasma* isolates grew over a wide range of temperatures.

Some *Mycoplasma* spp., such as *Mycoplasma mobile* isolated from fish, can adhere to glass and plastic surfaces (Kirchhoff et al. 1987) but in this study no such adherence was observed. Furthermore, there was no DNA hybridisation reaction between *MP1* and *Mycoplasma* from other animals. Using a DNA probe and PCR amplification, most of the prawn *Mycoplasma* isolates (~80%) corresponded to strain *MP1*. From the results presented here, it is suggested that the prawn *Mycoplasma* isolates represent 2 new species. This was confirmed by 16S r RNA gene sequencing, the results of which will be presented elsewhere.

The *Mycoplasma* in the experimentally infected prawns did not produce high mortalities. This is not surprising since most *Mycoplasma* diseases are influenced by a variety of host and environmental factors. Moreover, avirulent strains occur naturally and some animals can carry *Mycoplasma* with no signs of disease until they are stressed (Simecka et al. 1992). However, the isolation of pure *Mycoplasma* from the eyes, gill appendages and brain of dead prawns suggests that *MP1* might have some potential to produce disease or predispose the prawns to other viruses and bacteria. Further studies on the route of infection and the loss of virulence during passage in artificial medium (Tully & Razin 1983, Wadler et al. 1990, Miles 1992) are needed.

Current methods used to diagnose *Mycoplasma* infections are inadequate because they lack speed, sensitivity and specificity (Simecka et al. 1992). Since DNA probes and PCR can overcome these problems (Razin 1994), we used these methods to design a specific DNA probe and PCR test to separate the *Mycoplasma* isolates from some previously described *Mycoplasma* species. We obtained a DNA clone (*MP1*/1-2) that was relatively sensitive (i.e. 10^3 CFU ml^{-1}) and highly specific for the detection of *MP1*. We also found a direct relationship between *Mycoplasma* number and intensity of hybridisation dot, so that rough quantitation was possible. Although the sensitivity might be regarded as low, it may be adequate for the detection of active infections even in poorly preserved samples. On the other hand, the use of radionucleotide DNA labeling may be restrictive, since it is not easily adapted to routine laboratory usage. Nevertheless, the probe did not cross react with other microorganisms likely to be found in association with diseased prawns.

The PCR method we developed based on *MP1*/1-2 was highly specific for detection of *Mycoplasma* DNA in prawns since it gave no amplicons even with 100 ng concentrations of DNA (corresponding to 10^7 CFU) from heterologous *Mycoplasma* species (Razin 1994) or from other bacteria. In tests of the PCR assay, no background problems through interference by proteins, fats, haemolymph and ions present in prawn tissue were encountered, and the concurrence of results with those from the hybridisation assay indicated that no false positive or false negative results were obtained. At 300 CFU, it was 10 times more sensitive than the dot blot hybridisation test for *Mycoplasma* DNA detection and it may be more easily applied in shrimp disease diagnostic laboratories. Using the PCR and dot blot

tests, some prawns were shown to be free of *MP1* infection, but the data also indicated infections in prawns from 2 farms 500 km apart. Further investigations should be undertaken on broodstock to determine whether they can be the source of *Mycoplasma* infections. Large-scale screening of prawns is now possible through the highly sensitive and specific PCR.

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