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NOTE

Immunodetection of specific *Vibrio* bacteria attaching to tissues of the giant tiger prawn *Penaeus monodon*

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ABSTRACT: An *in vitro* study of the attachment of *Vibrio* pathogens to tissues of the giant tiger prawn *Penaeus monodon* (Fabricius, 1798) was carried out using species-specific and genus-specific monoclonal antibodies with indirect FITC-immunofluorescence microscopy. The attachment of *V. alginolyticus* and *V. parahaemolyticus* to different tissues was always stronger than the attachment of *V. anguillarum*, and overall, these data are consistent with epizootiological studies. Strains of other *Vibrio* species used in this study showed no evidence of the attachment.

KEY WORDS: *Vibrio* attachment · Immunofluorescence · Prawn

Infection of marine organisms, and particularly those in mariculture, by *Vibrio* species has a long history dating back to an outbreak of 'red-pest' in eels during 1718 (Bonaveri 1761). Although the current importance of vibrios as fish and shellfish pathogens has been well documented (Austin & Austin 1987, Lightner et al. 1992), there is still much to be done in regard to our knowledge of pathogenicity and epizootiology.

Of the major *Vibrio* pathogens, *V. anguillarum* is well known to be associated with disease problems of salmonids. One recent study has found that particular serotypes of *V. anguillarum*, viz. O1, O2, O4, and O8 in order of significance, were the most common disease isolates (Myhr et al. 1991). *In vitro* attachment of different *V. anguillarum* serotypes to trout tissues was found to correlate with the importance of the serotype in the disease situation (Chen & Hanna 1992). That is, serotype O1 was by far the most common serotype identified from diseased fish and it was also the serotype that showed the strongest attachment to trout tissues.

Evidence from a number of reports indicates that *Vibrio* species are common isolates from diseased penaeid prawns (Anderson et al. 1988, Song et al. 1990, 1993, Lavilla-Pitogo et al. 1992, Lightner et al. 1992, Nash et al. 1992, Owens et al. 1992, Ruangpan & Kitao 1992). Of the *Vibrio* species that have been isolated, the most common were *V. alginolyticus* and *V. parahaemolyticus*. This was emphasised in a review by Lightner (1983) in which the relative frequencies of isolation of *V. alginolyticus* and *V. parahaemolyticus*, amongst all species of vibrios and other bacteria, were 42 and 7%, respectively. As a consequence, we carried out a study to determine which *Vibrio* species attached to penaeid prawn tissues, and the results are presented in this report.

Materials and methods. Adult *Penaeus monodon* (Fabricius, 1798) prawns of 4 to 12 cm were obtained from the Bribie Island Aquaculture Centre, Queensland. Live prawns were air-freighted in sealed plastic bags, containing seawater and oxygen, for frozen sectioning. Others were snap-frozen using dry-ice and then air-freighted in a frozen state. These frozen prawns were stored at –80 °C until required.

Tissue smears were made of muscle, intestine, and gills. Frozen tissues were first rinsed with 0.9% (w/v) NaCl and then soaked for 15 min in 0.9% (w/v) NaCl containing 200 mg ml^{-1} (w/v) streptomycin (Glaxo) before being gently rubbed on clean glass microscope slides. The adhering cells were then fixed in 2% (v/v) formalin.

Cryostat sections of prawn tissues were prepared from freshly killed prawns. Exoskeletons were removed and small pieces of tissues were cut from the tail muscle, intestine, and gills. These tissues were placed in a solution containing equal volumes of 50% (w/v) sucrose in distilled water and Tissue-Tek (Miles), overnight at 4 °C. They were then placed in isopentane for 30 s before transfer to storage at –80 °C. Sections of 10 µm were cut on a Reichelt-Jung microtome and placed on clean glass slides prior to fixing in 2% (v/v) formalin. Adjacent cross sections were double stained with haematoxylin and eosin to identify tissues.

Monoclonal antibodies (mAbs) used in the study (see Table 1) were usually specific to the *Vibrio* spp. listed (Chen et al. 1992, Hanna et al. 1992), thereby providing identification of bacteria that attached to prawn tis-

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sues. However, to study the attachment of *Vibrio* spp. for which there were no specific mAbs available, the mAb F11P411F was used as it was known to react strongly with all species of the genus *Vibrio*, but not with other Gram-negative bacteria.

Indirect immunofluorescence was used to determine which *Vibrio* species attached to cryostat sections and smears of the prawn tissues. Preparations of the sections and tissue smears were washed in 0.05 M phosphate-buffered saline (PBS), pH 7.4, after which 100 µl volumes of *Vibrio* suspensions in PBS, containing 1×10^9 cells ml^{-1} at stationary phase (based on standard curves of OD_{620nm} against viable counts), were added for 45 min at room temperature (RT). Following 3 washes with PBS to remove unattached bacteria, the tissues were incubated for a further 45 min at RT with 100 µl volumes of mAbs that reacted with the bacteria used in the previous step. After 3 washes in PBS, 100 µl volumes of goat anti-mouse FITC-conjugate (Silenus), diluted 1:40 in PBS, were added for 45 min at RT. The preparations were washed another 5 times in PBS and then mounted in PBS containing 90% (v/v) glycerol and 4% (v/v) propyl gallate. The prawn tissues were then examined by epifluorescence microscopy for the attachment of bacteria. With each preparation, attachment was ranked as being very strong (+++), strong (++), weak (+) or none (–), depending on the relative numbers of bacteria observed to be fluorescing.

Results and discussion. Attachment of 11 *Vibrio* species to cryostat sections and tissue smears of *Penaeus monodon* is recorded in Table 1. The attachment of *V. alginolyticus* and *V. parahaemolyticus* to cells of different tissues was invariably strong, whereas attachment of *V. anguillarum* was always weaker. None of the other *Vibrio* species listed showed evidence of attachment. In addition, it was found that fewer bacteria attached to muscle tissue compared with intestine or gill tissues.

Ten *Vibrio anguillarum* serotypes (Sørensen & Larsen 1986) were tested for the ability to attach to prawn tissues. The results showed that only *V. anguillarum* of the O1 serotype attached. This observation was similar to that for trout, although some additional *V. anguillarum* serotypes did attach to trout tissues (Chen & Hanna 1992). Further research is now required to determine whether all *V. anguillarum* isolates from diseased prawns are of the O1 serotype.

The very strong attachment of *Vibrio alginolyticus* to prawn cells is shown in Fig. 1, in which each bacterial cell is identified by immunofluorescence. In particular, the bacteria preferentially attached to cryostat sections and tissue smears of intestine and gills. There was also attachment of bacteria to epidermal cells, exposed through the removal of the exoskeleton. Fewer bacteria attached to muscle and vascular tissues. Controls using non-*Vibrio* species and omitting the primary

Table 1. Attachment of *Vibrio* strains to *Penaeus monodon* tissues detected by indirect FITC-immunofluorescence. Attachment: (+++) very strong; (++) strong; (+) weak; (–) none. ACMM: Australian Collection of Marine Microorganisms; AFHRL: Australian Fish Health Reference Laboratory; ATCC: American Type Culture Collection

Vibrio strains	Identifying mAb	Attachment to cryostat sections			Attachment to tissue smears		
		Gill	Intestine	Muscle	Gill	Intestine	Muscle
V. alginolyticus ACMM 101	F15P12B	+++	++	++	+++	++	++
V. anguillarum AFHRL 1	F13P13F	++	++	+	++	++	+
V. anguillarum O1 ATCC 43305	F13P13F	++	++	+	++	++	+
V. anguillarum O2 ATCC 43306	F11P411F	–	–	–	–	–	–
V. anguillarum O3 ATCC 43307	F11P411F	–	–	–	–	–	–
V. anguillarum O4 ATCC 43308	F11P411F	–	–	–	–	–	–
V. anguillarum O5 ATCC 43309	F11P411F	–	–	–	–	–	–
V. anguillarum O6 ATCC 43310	F11P411F	–	–	–	–	–	–
V. anguillarum O7 ATCC 43311	F11P411F	–	–	–	–	–	–
V. anguillarum O8 ATCC 43312	F11P411F	–	–	–	–	–	–
V. anguillarum O9 ATCC 43313	F11P411F	–	–	–	–	–	–
V. anguillarum O10 ATCC 43314	F11P411F	–	–	–	–	–	–
V. carchariae ATCC 35084	F24P56G	–	–	–	–	–	–
V. cholerae O1 Inaba 569B	F7P25A	–	–	–	–	–	–
V. cholerae non-O1 V3	F27P61H	–	–	–	–	–	–
V. damsela ATCC 33537	F23P11C	–	–	–	–	–	–
V. harveyi ACMM 130	F12P411E	–	–	–	–	–	–
V. ordalii ATCC 33509	F18P66C	–	–	–	–	–	–
V. parahaemolyticus FC1011	F6P55C	+++	+++	+	+++	++	+
V. splendidus I ACMM 140	F11P411F	–	–	–	–	–	–
V. tubiashii ATCC 19109	F11P411F	–	–	–	–	–	–
V. vulnificus (Sweden)	F31P46F	–	–	–	–	–	–

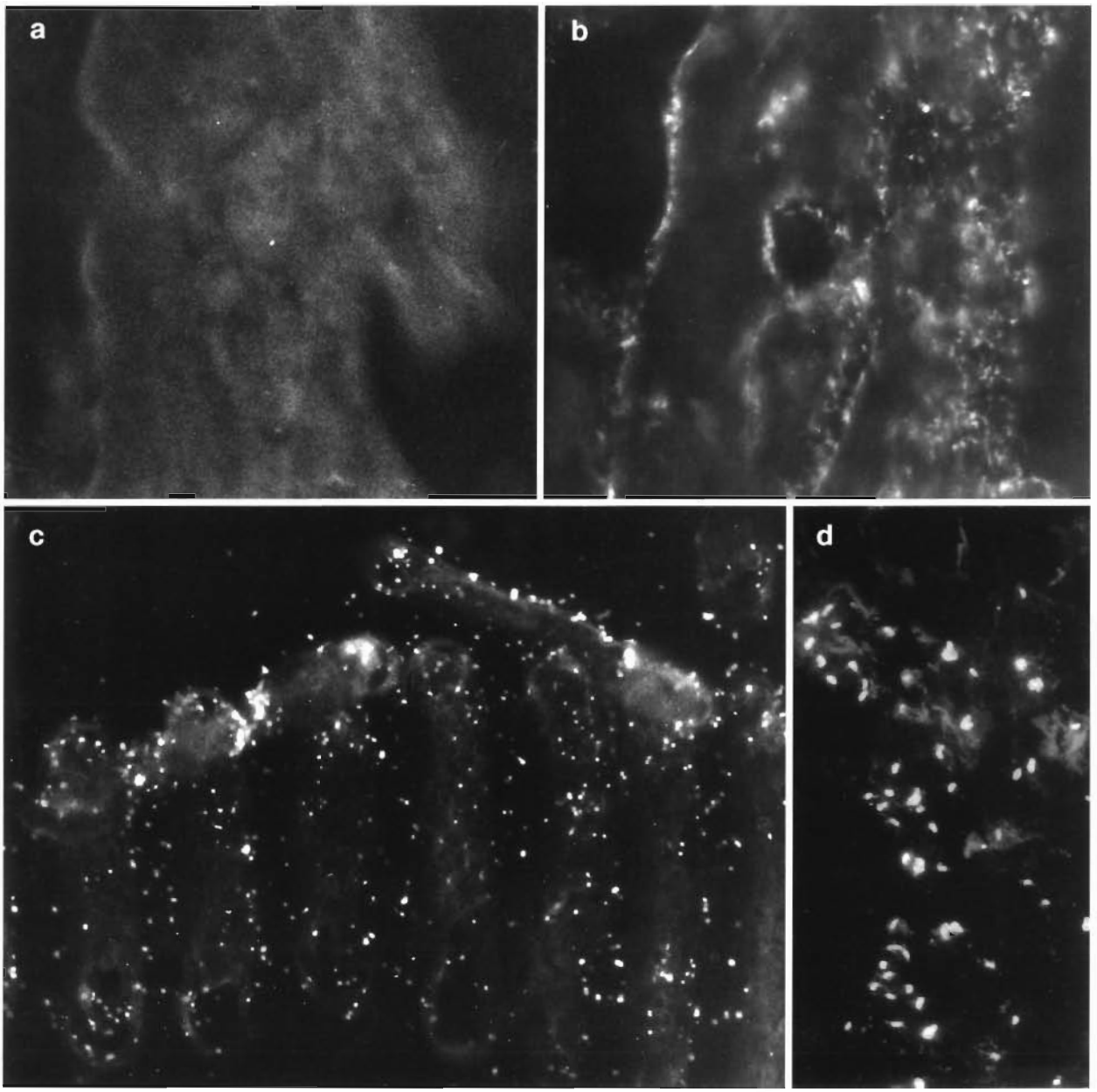

Fig. 1. Attachment of *Vibrio alginolyticus* bacteria to giant tiger prawn *Penaeus monodon* tissues. Photomicrographs are indirect FITC-immunofluorescence of tissues using diagnostic monoclonal antibodies as the primary antibodies, viz. F15P12B for *V. alginolyticus* and F7P25A for *V. cholerae*. (a & b) Cryostat sections of intestine showing (a) the non-attachment of *V. cholerae* and (b) the attachment of *V. alginolyticus*; × 300. Isotype and secondary conjugate controls were similar in appearance to (a). (c & d) Attachment of *V. alginolyticus* to (c) a cryostat section of prawn gills, × 300, and (d) a smear of prawn gills, × 1100

mAb step showed no immunofluorescence. This also held true for the *Vibrio* species and serotypes that were reported in Table 1 as being negative in their attachment.

Overall, these data are consistent with the increasing epizootiological evidence indicating that *Vibrio alginolyticus* and *V. parahaemolyticus* are the major problem species of *Vibrio* in penaeid prawns. Each species attached very strongly to prawn cells *in vitro*. As with trout gills (Chen & Hanna 1992), smears of prawn gills appeared to be particularly good test preparations for the rapid immunoidentification of vibrios pathogenic to prawns.

It has been found that bacteria isolated from bacterial septicemias of penaeid prawns are usually vibrios, and, of these, the most commonly identified species are *Vibrio alginolyticus* and *V. parahaemolyticus* (Anderson et al. 1988, Lightner et al. 1992). A recent case

involving major losses in an Australian prawn hatchery tends to confirm this. In that case our mAbs were used to rapidly identify *V. alginolyticus* as the cause of the problem. The source of the pathogen was a contaminated microalgal culture fed to the prawn larvae.

Another important isolate of diseased penaeid prawns has been reported to be *Vibrio anguillarum* (Lightner 1983). The current study showed that cells of this species were not as strong in attaching to prawn cells as *V. alginolyticus* and *V. parahaemolyticus*, thereby supporting the epizootiological data. However, there were species of *Vibrio* that did not attach in this study and it is possible that additional strains of the same species may have the ability to attach. For example, *V. harveyi* ACMM 130 did not attach, but it is reported that some *V. harveyi* and *V. vulnificus* strains have caused problems in prawn culture in Australia and S.E. Asia (Song et al. 1990, Owens et al. 1992, I. G. Anderson pers. comm.).

The attachment of *Vibrio cholerae* or *V. parahaemolyticus* is an important first step in infection of humans (Yamamoto et al. 1988, Yamamoto & Yokota 1989), and it is likely to be similar in *Vibrio* pathogenesis of aquatic organisms. For example, *V. parahaemolyticus* has been shown to attach *in vitro* onto chitin and copepods (Kaneko & Colwell 1975) and our recent research showed that *V. anguillarum* serotype O1, the main pathogenic serotype, strongly attaches to salmonid tissues (Chen & Hanna 1992). The current research has now shown a strong attachment of *V. alginolyticus* and *V. parahaemolyticus* to various penaeid prawn tissues, and suggests that further studies on the attachment process are in order. In particular, we are investigating the association between surface components of pathogenic *Vibrio* strains and the receptor sites on host cells.

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