Vol. 13: 133–138, 1992 | DISEASES OF AQUATIC ORGANISMS Dis. aquat. Org. | Published July 23

Evaluation of an indirect fluorescent antibody technique for detection of *Aerococcus viridans* (var.) *homari*, pathogen of homarid lobsters

L. J. Marks[1], James E. Stewart[1], Tore Håstein[2]

[1] Department of Fisheries and Oceans, Biological Sciences Branch, Bedford Institute of Oceanography, PO Box 1006, Dartmouth, Nova Scotia, Canada B2Y 4A2
[2] The National Veterinary Institute, PO Box 8156, Dept. 0033, Oslo 1, Norway

ABSTRACT: Application of an indirect fluorescent antibody technique (IFAT) significantly shortened the time required for detection and identification of the lobster pathogen *Aerococcus viridans* (var.) *homari*, from culture media or directly from lobster hemolymph. The normal 4 to 7 d for confirmed diagnoses using traditional bacteriological procedures was reduced to 2 h for detection of heavy infections, or 48 to 50 h when amplification of numbers was required. Of the bacteria checked, only *Staphylococcus aureus* cross-reacted; this was overcome by treatment of fixed slides with papain prior to IFA staining. The validity of the method was confirmed in comparisons between the traditional procedures and IFAT using samples from 1090 lobsters which had shown presumptive signs of infection.

INTRODUCTION

Gaffkemia, the fatal disease of lobsters (genus *Homarus*), caused by the bacterium *Aerococcus viridans* (var.) *homari*, is responsible, periodically, for heavy mortalities among captive homarid lobsters (Snieszko & Taylor 1947, Stewart et al. 1975, Håstein et al. 1977, Stewart 1980, Gjerde 1984). Detection of the organism in freshly captured lobsters has indicated a wide and periodic distribution of infections among lobsters in the wild (Stewart et al. 1966, Audouin & Leglise 1971, Stewart 1978) where, presumably, it causes many deaths. Systemic in nature (Stewart et al. 1969a, b), the disease progresses rapidly with no resistance offered by the host to virulent strains of the pathogen (Cornick & Stewart 1968). An early and reliable diagnosis is of utmost importance to permit measures to be taken to stop the spread of the disease. The presumptive phenylethylalcohol (PEA) broth test and subsequent confirmatory bacteriological procedures devised initially for diagnosis (Stewart et al. 1966) require between 4 and 7 d for a complete analysis, although results can be available sooner with cases involving heavy infections. The substitution of a more rapid method for reliably detecting infections was needed. Accordingly, we developed an indirect fluorescent antibody technique (IFAT); this paper describes its evaluation and application, plus validation through field comparisons involving samples from 1090 lobsters showing presumptive signs of infection.

MATERIALS AND METHODS

Antibody. Antiserum, specific for *Aerococcus viridans* (var.) *homari*, was produced in rabbits, according to the method of Saxegard & Håstein (1978) using National Veterinary Institute (NVI) *A. viridans* (var.) *homari* strains 1030 and 1032. For use, it was diluted 1:70 with phosphate buffered saline (PBS) pH 7.2 (Difco Laboratories).

Indirect fluorescent antibody staining (IFA). The IFA staining followed essentially the procedure of Bullock & Stuckey (1975). The procedure was applied to bacteria grown at 28 °C in Trypticase Soy (TS) broth or TS agar (BBL), phenylethylalcohol (PEA) broth (Stewart et al. 1966) and on smears made from hemolymph, taken from both infected and control lobsters. In the procedure finally adopted, slides, after acetone fixation of 7 to 10 min, were treated with papain (BDH Incorporated) prepared according to the method of Komninos & Tompkins (1963); the activation

0177-5103/92/0013/0133/$ 03.00

step was shown to be unnecessary. Papain was applied to smears and the slides were incubated at 37 °C for 10 min in a moist chamber then washed in PBS pH 7.2 for 10 min. The slides were then blotted and incubated at room temperature in the moist chamber for 30 min with rabbit serum specific for *Aerococcus viridans* (var.) *homari*. After washing the slides with PBS, the process was repeated with fluorescein-labelled goat anti-rabbit globulin (GIBCO BRL Canadian Life Technologies Incorporated or Sigma Chemical Company Limited) (1:50) mixed with Rhodamine counterstain (Difco Laboratories) (1:50) to reduce background fluorescence. After a final wash with PBS, the slides were air-dried and coverslips were applied using mounting fluid at pH 9 (Difco Laboratories).

Slides were examined with a Zeiss Photomicroscope equipped with 6.3 and 16× planoachromatic objectives for scanning and 63× planoapochromatic objectives for intensive examination employing bright field and ultraviolet (UV) epifluorescence.

Lobsters (*Homarus americanus*). Lobsters, 500 to 700 g in weight, were captured locally, the claws were immobilized with rubber bands, and the animals then were acclimated in an open, aerated seawater system to 15 °C for 1 wk prior to use in experimental work. All hemolymph sampling from, and injections into, the lobsters was carried out aseptically via the ventral abdominal sinus. The site was swabbed before and after with 70 % ethanol.

Bacteriology. For experimental infection of lobsters, Rabin's strain of *Aerococcus viridans* (var.) *homari* (virulence maintained by continuous passage in lobsters) was grown in TS broth for 24 h at 28 °C. Cells were washed in sterile 3 % saline and standardized at 420 nm on a Spectronic 20 colorimeter to give ca 1×10^7 colony-forming units (cfu) ml^{-1}. Doses used were 1 ml of the bacterial suspension per kg of body weight. Control lobsters were injected with the same volume of 3 % sterile saline. Viable bacterial numbers (cfu), in suspensions and in samples of lobster hemolymph, were determined by the plate count method of Miles & Misra (1938), using TS agar at 28 °C. All dilutions were made in sterile 3 % saline.

For surveys, the presumptive phenylethylalcohol (PEA) broth test (Stewart et al. 1966) consisted of removing 1 ml of hemolymph aseptically from an adult lobster (500 to 700 g) and transferring 0.5 ml to each of two 4.5 ml tubes of PEA broth. Tubes showing acid production after incubation at 28 °C indicated a presumptive positive test; tubes should be incubated for a minimum of 48 h (preferably 72 h) before being declared negative.

The micrococci, many of the rod-shaped bacteria, and *Aerococcus viridans* (var.) *homari*, tested for cross-reactivity, were isolated previously from lobster hemolymph during past surveys of lobsters and from lobster intestinal tracts (Stewart & Zwicker 1972). All other bacteria were obtained from the American Type Culture Collection (ATCC) and although not normally found associated with the lobster were used to expand the range of the test.

Experimental. (1) The following diagnostic methods were compared using lobsters sampled every 4 h during the first 28 h subsequent to injection with 1×10^7 *Aerococcus viridans* (var.) *homari* cfu kg^{-1} body weight. The growth of the pathogen in the hemolymph was followed by the plate count method of Miles & Misra (1938):

(a) Direct microscopic examination of lobster hemolymph for the presence of Gram positive, tetrad-forming cocci (Stewart et al. 1966).

(b) Examination and culture of presumptive positive PEA broths (those showing acid production after incubation at 28° C) for the presence of catalase negative, beta-hemolytic colonies of Gram positive cocci, occurring frequently in tetrads (Stewart et al. 1966), i.e. the confirmatory bacteriological procedure.

(c) Direct microscopic examination of IFA stained preparations of lobster hemolymph for the presence of fluorescing cocci.

(d) Examination of positive PEA broths by IFAT for the presence of fluorescing cocci.

(2) The effect of papain treatment on IFAT smears made from presumptive positive PEA broths was compared with parallel untreated smears for any improvement in sensitivity.

(3) Presumptive positive PEA broth cultures (1090), collected in the course of several field surveys, were examined with the IFAT and the results were compared with those obtained by the traditional confirmatory bacteriological procedures (Stewart et al. 1966) by the Fish Health Unit of the Halifax Fisheries Research Laboratory, Department of Fisheries and Oceans, during the course of their diagnostic work.

RESULTS AND DISCUSSION

The evaluation of IFA staining of *Aerococcus viridans* (var.) *homari* showed it to be highly specific and suitable for rapid and accurate identification of the bacterium; it also substantially shortened the time required to survey large numbers of lobsters for the presence of the pathogen. The IFA-stained *A. viridans* (var.) *homari* cells are readily recognizable in hemolymph smears (Fig. 1), visible even at the comparatively low magnification obtained using a 6.3× objective. The zone of fluorescence appears to correspond with the size of the bacterial capsule which

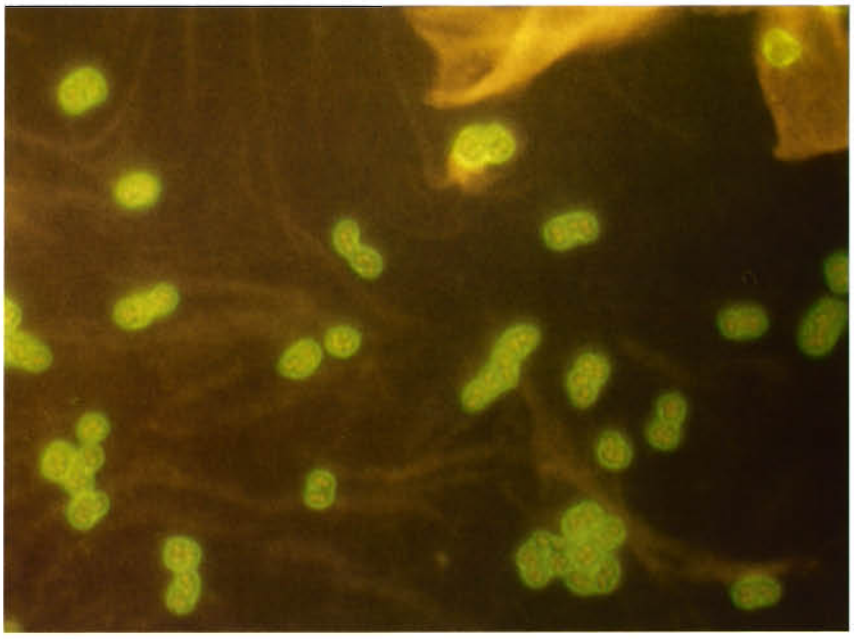

Fig. 1 *Aerococcus viridans* (var.) *homari* in lobster (*Homarus americanus*) hemolymph smears illustrating the tetrads surrounded by the fluorescein labelled specific goat anti-rabbit antibody. Indirect fluorescent antibody technique with rhodamine counterstain. ×2500

varies with growth conditions and apparently virulence. The capsule is widest in cells grown in lobster hemolymph and narrow for those grown on laboratory media (Hitchner & Snieszko 1947) and virtually nonexistent among avirulent strains (Stewart 1984).

The IFAT gave positive results for all strains previously confirmed as *A. viridans* (var.) *homari* and shown to be virulent (Table 1). It reacted with *Staphylococcus aureus*, a cross-reaction commonly recorded with immune (Komninos & Tompkins, 1963) and nonimmune rabbit sera (Bergman et al. 1966). The cross-reaction was eliminated by treating hemolymph smears with papain prior to IFA staining (Komninos & Tompkins 1963), which also enhanced the brightness and clarity of *A. viridans* (var.) *homari* and markedly improved detection. For example, when slides were prepared from 14 presumptively positive PEA broth cultures of hemolymph, only 10 (71 %) were IFAT positive for *A. viridans* (var.) *homari*; however, when parallel slides from the same cultures were treated with papain prior to IFA staining, all 14 clearly showed the presence of the pathogen.

Of the avirulent strains of *Aerococcus viridans* (var.) *homari*, strain ATCC 10400 was IFAT positive exhibiting a narrow band of fluorescence; strain 37R had a very narrow and incomplete band of fluorescence with many cells in the preparation failing to stain. Strains 88A and 88B did not stain. These results are consistent with the findings of Wiik et al. (1986) in which DNA hybridizations indicated that strains 88A and 37R, although conforming to the cultural criteria, may not actually be *A. viridans* (var.) *homari*.

The typical growth curve (Fig. 2) for *Aerococcus viridans* (var.) *homari* in lobsters (Stewart & Arie 1973) illustrates the problem faced in attempting early diagnosis of the infection and conducting extensive surveys among freshly captured individuals. In lobster hemolymph, the pathogen 'fades' to nominal levels during the early stages of infection through aggregation and retention of phagocytosed cells in the heart and hepatopancreas (Johnson et al. 1981). This period of fading, much prolonged at low temperatures, is followed by a logarithmic increase which stabilizes at levels between 1×10^8 and 1×10^9 bacterial cfu ml^{-1} of hemolymph. If diagnosis is attempted using only Gram stained hemolymph smears when bacterial levels are significantly less than 1×10^5 cfu ml^{-1}, the success rate is negligible (10 % or less) (Table 2). Application of IFAT to these hemolymph smears was a substantial improvement over the use of the Gram stain alone (Table 2); however, for practical purposes, unless the bacterial levels in the hemolymph equalled or exceeded 1×10^5 ml^{-1}, IFAT would not ensure detection of all infections.

To guarantee that gaffkemia is detected in lobsters with very light infections, it is necessary to use procedures to amplify the number of *Aerococcus viridans* (var.) *homari* present in the hemolymph. This will be achieved if hemolymph samples are transferred immediately, upon removal from the lobster, to PEA broth and incubated at 28° C. Repeated trials with suspensions of virulent *A. viridans* (var.) *homari* showed that growth in standard PEA broths, inoculated with between 1 and 5 cells, reached and exceeded 1×10^5 cfu ml^{-1} within 48 h. Application of IFAT or the traditional confirmatory procedures at this stage will

Table 1 Examinations for cross-reactivity[a]

Bacterium		IFAT reaction
Micrococcus sedentarius (ATCC 14392)		–
Micrococcus luteus		–
Micrococcus conglomeratus		–
Micrococcus sp.		–
Streptococcus pyogenes (ATCC 19615)		–
Staphylococcus epidermidis (ATCC 122280)		–
Staphylococcus aureus (ATCC 25923)		+
Staphylococcus aureus (ATCC 25923): papain treated		–
Staphylococcus aureus (ATCC 12598)		+
Staphylococcus aureus (ATCC 12598): papain treated		–
Brevibacterium sp.		–
Pseudomonas perolens		–
Bacillus subtilis		–
Flavobacterium sp.		–
Klebsiella pneumoniae (ATCC 13883)		–
Aerococcus viridans (var.) *homari*		
Virulent strains:	RABIN	+
	NVI 1030	+
	NVI 1032	+
	St. Andrew 8	+
	St. Andrew 14	+
	St. Andrew 15	+
	St. Andrew 18	+
Avirulent strains:	ATCC 10400	+
	37R	+/–
	88A	–
	88B	–

[a] Examinations carried out, at minimum, in duplicate (50 fields slide^{-1})

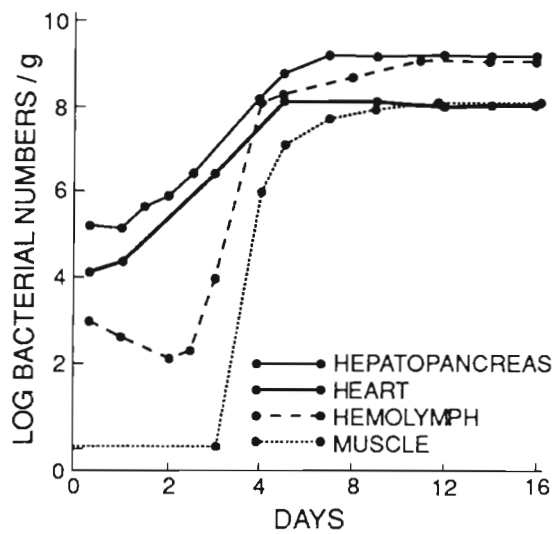

Fig. 2. *Aerococcus viridans* (var.) *homari*. Typical growth curve in live lobsters (*Homarus americanus*) held at 15 °C. Growth of the pathogen is strictly temperature-dependent; thus, at lower temperatures the pathogen's 'hemolymph fading' period during early infection is much prolonged. (Redrawn from Stewart & Arie 1973)

give a 100 % rate of success. The IFAT, however, will provide a confirmatory diagnosis in 2 h, in contrast to the 4 to 7 d required for traditional procedures.

Of the presumptively positive PEA broths, representing 1090 lobsters at various commercial holding facilities, the same 720 were shown to be positive for *Aerococcus viridans* (var.) *homari* via both the IFAT and confirmatory culture techniques. Five percent of IFA stained slides were found to exhibit some degree of stray fluorescence. This associated or stray fluorescence consisted of narrow, usually incomplete, zones and was not equally distributed over all areas of the slide. In only one case did the PEA survey reveal a cross-reacting bacterium: a micrococcus-like sp. with all cells encircled by a narrow zone of fluorescence. In no case was the morphology of these fluorescing bacteria, or the appearance of the IFA staining, consistent with that of *A. viridans* (var.) *homari*; and thus the organisms could not be confused with the pathogen. The associated (stray) fluorescence, and that of the cross-reacting micrococcus-like sp., was lost upon transfer of the bacteria to fresh medium.

Thus, IFAT can be applied directly to the hemolymph

Table 2. Comparison of *Aerococcus viridans* (var.) *homari* detection methods at 4 different infection levels. Values are percent detection

Detection method	Numbers of *A. viridans* cfu ml^{-1} lobster hemolymph			
	10^2	10^3	10^4	10^5
(a) Direct microscopic examination for the presence of Gram positive, tetrad-forming cocci in hemolymph smears	0 (14)[a]	3 (36)	10 (40)	90 (10)
(b) Presence of catalase negative, beta-hemolytic colonies of Gram positive, tetrad-forming bacteria in PEA broth (presumptive positive followed by confirmatory culture procedures)	100 (14)	100 (16)	100 (14)	
(c) Direct microscopic examination for the presence of fluorescing cocci in hemolymph smears stained with IFA	29 (14)	72 (36)	76 (40)	100 (10)
(d) Presence of fluorescing cocci (IFAT) in presumptive positive PEA broth (after 0.5 ml hemolymph containing above numbers was inoculated and then incubated at 28 °C for 48 h)	100 (14)	100 (16)	100 (14)	

[a] Numbers in parentheses indicate the number of slides examined at each infection level (100 fields $slide^{-1}$). *A. viridans* (var.) *homari* levels ml^{-1} hemolymph were obtained using 35 lobsters

smears of heavily infected lobsters in acute situations to give a confirmed positive diagnosis within 2 h. For survey purposes, amplification of pathogen numbers by 48 h growth in PEA broth, or equivalent, is essential. Amplification, followed by substitution of IFAT for the bacteriological confirmatory procedures, will reduce the overall survey time and work to about one-third of that required for the full culture methodology, and still give accurate and reliable results. The Department of Fisheries and Oceans is now employing the technique routinely in the form of a direct fluorescent antibody technique (DFAT) using serum prepared by Dr R. Lallier (Université de Montréal, Montréal, Québec, Canada) from rabbits immunized with Rabin's strain of *Aerococcus viridans* (var.) *homari*.

Acknowledgements. We thank B. M. Zwicker and the Fish Health Unit for collaborating in the evaluation, and J. W. Cornick, B. M. Zwicker, D. J. Scarratt, and J. Jellett for reviewing the manuscript and offering constructive criticism.

LITERATURE CITED

Audoin, J., Leglise, M. (1971). Note préliminaire sur la présence de *Gaffkya homari* dans le sang des homards stockés dans les viviers de la région de Roscoff. Int. Counc. Explor. Sea. Comm. Meet. (Shellfish and Benthos Comm.) K: 26: 1–6 (mimeo)

Bergman, S., Forsgren, A., Swahn, B. (1966). Effects of naturally occurring rabbit antibodies on fluorescent-antibody reactions. J. Bacteriol. 51: 1664–1665

Bullock, G. L., Stuckey, H. M. (1975). Fluorescent antibody identification and detection of the *Corynebacterium* causing kidney disease of salmonids. J. Fish. Res. Bd Can. 32: 2224–2227

Cornick, J. W., Stewart, J. E. (1968). Interaction of the pathogen *Gaffkya homari* with natural defence mechanisms of *Homarus americanus*. J. Fish. Res. Bd Can. 23: 695–709

Gjerde, J. (1984). Occurrence and characterization of *Aerococcus viridans* from lobsters, *Homarus gammarus* L., dying in captivity. J. Fish Dis. 7: 355–362

Håstein, T., Roald, S. O., Kjos-Hansen, B., Slaveland, K. (1977). Occurrence of gaffkemia in lobsters in Norway. Acta vet. scand. 18: 138–139

Hitchner, E. R., Snieszko, S. F. (1947). A study of a microorganism causing a bacterial disease of lobsters. J. Bacteriol. 54: 48

Johnson, P. T., Stewart, J. E., Arie, B. (1981). Histopathology of *Aerococcus viridans* var. *homari* infection (Gaffkemia) in the lobster, *Homarus americanus*, and a comparison with histological reactions to a Gram-negative species, *Pseudomonas perolens*. J. invert. Pathol. 38: 127–148

Komninos, G. N., Tompkins, V. N. (1963). A simple method of eliminating the cross-reaction of Staphylococcus in the fluorescent antibody technic. Am. J. clin. Path. 40: 319–324

Miles, A. A., Misra, S. S. (1938). The estimation of the bactericidal power of the blood. J. Hyg. 38: 732–749

Saxegaard, F., Håstein, T. (1978). Identification of *Aerococcus viridans* by means of co-agglutination. Acta vet. scand. 19: 604–606

Snieszko, S. F., Taylor, C. C. (1947). A bacterial disease of the lobster *Homarus americanus*. Science 105: 500

Stewart, J. E. (1978). The lobster disease Gaffkemia, in relation to fisheries management. Int. Coun. Explor. Sea. Comm. Meet. (Shellfish Comm.) F: 16: 1–9 (mimeo)

Stewart, J. E. (1980). Diseases. In: Cobb, J. S., Phillips, B. F. (eds.) The biology and management of lobsters, Vol. 1. Academic Press, New York, p. 301–342

Stewart, J. E. (1984). Lobster diseases. Helgoländer Meeresunters. 37: 243–254

Stewart, J. E., Arie, B. (1973). Depletion of glycogen and

adenosine triphosphate as major factors in the death of lobsters (*Homarus americanus*) infected with *Gaffkya homari*. Can. J. Microbiol. 19: 1103–1110

Stewart, J. E., Arie, B., Zwicker, B. M., Dingle, J. R. (1969a). Gaffkemia, a bacterial disease of the lobster, *Homarus americanus*: effects of the pathogen, *Gaffkya homari*, on the physiology of the host. Can. J. Microbiol. 15: 925–932

Stewart, J. E., Cornick, J. W., Spears, D. I., McLeese, D. W (1966). Incidence of *Gaffkya homari* in natural lobster (*Homarus americanus*) populations of the Atlantic region of Canada. J. Fish. Res. Bd Can. 23: 1325–1330

Stewart, J. E., Foley, D. M., Ackman, R. G. (1969b). Characteristics of *Gaffkya homari*, the causative agent of the lobster disease Gaffkemia. J. Fish. Res. Bd Can. 26: 1385–1389

Stewart, J. E., Zwicker, B. M. (1972). Natural and induced bactericidal activities in the hemolymph of the lobster, *Homarus americanus*: products of hemolymph – plasma interaction. Can. J. Microbiol. 18: 1499–1509

Stewart, J. E., Zwicker, B. M., Arie, B. (1975). Status report on Gaffkemia in lobsters in Atlantic Canada. Int. Counc. Explor. Sea. Comm. Meet. (Shellfish and Benthos Comm.) K: 20: 1–5 (mimeo)

Wiik, R., Torsvik, V., Egidius, E. (1986). Phenotypic and genotypic comparisons among strains of the lobster pathogen *Aerococcus viridans* and other marine *Aerococcus viridans*-like cocci. Int. J. system Bact. 36: 431–434

Editorial responsibility: Managing Editor

Manuscript first received: February 7, 1992
Revised version accepted: April 13, 1992