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Development of a microplate-based fluorescence immunoassay using quantum dot streptavidin conjugates for enumeration of putative marine bacteria, *Alteromonas* sp., associated with a benthic harpacticoid copepod

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ABSTRACT

Attached bacteria inhabit the surfaces of many marine animals – a process that may play important roles in the survival and transport through aquatic systems. However, efficient detection of these bacteria has been problematic, especially small aquatic animals such as benthic harpacticoid copepod. Quantum dots (QD) have recently emerged as a significant tool in immunofluorescence detection because of their unique properties compared to other fluorescent probes.

In the present study, a polyclonal antibody was raised against the Gram-negative marine bacterium, *Alteromonas* sp. A microplate-based immunofluorescence bioassay using QD streptavidin conjugates was developed for quantifying putative *Alteromonas* sp. cells located on the surfaces of a marine harpacticoid copepod, *Microarthridion littorale*. The number of attached *Alteromonas* sp. was estimated to be $10^2 \pm 8$ CFU using this method. The QD approach, coupled to a microplate assay can potentially provide an efficient and accurate method for rapidly detecting multiple bacteria species attached to small invertebrate animals because of their unique excitation and emission characteristics.

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1. Introduction

Bacterium-benthic copepod interactions may play an important role in marine ecosystems (Nagasawa, 1989; Hansen and Bech, 1996; Carman and Dobbs, 1997; Long and Azam, 2001; Tang, 2005). A marine bacterium, *Alteromonas* sp., located on outer cuticles of copepods, has been reported to play important roles in antagonistic relationships with other bacteria by secreting 2-*n*-pentyl-4-quinolinol (Long et al., 2003). However, many studies have focused on the relationship between planktonic copepods and human pathogens due to its importance of public health (Kaneko and Colwell, 1975; Huq et al., 1983; Heidelberg et al., 2002; Cellini et al., 2004; Huq et al., 2005).

In order to understand the unique interactions of *Alteromonas* sp. and other pathogenic bacteria on harpacticoid copepods, it is important to develop techniques to quantify attached bacteria, such as *Alteromonas* sp., on benthic copepods.

Quantum dot (QD) nanocrystals have several physico-chemical properties that make them ideally-suited for cellular and *in-vivo* labeling, and long-term spectral imaging (Arya et al., 2005; Liu, 2006; Pinaud et al., 2006; Jamieson et al. 2007; Ma et al., 2007). The QD core can be composed

of a few different semiconductor materials, depending on the emission wavelength range that will be targeted. The organic coating adds approximately one additional nanometer in diameter. In our study, cadmium selenide (CdSe) quantum dots, with size range from 3 to 10 nm, were used for detection of visible spectrum emissions using confocal laser scanning microscopy (CLSM). There are several significant differences in the utility of QDs versus the more-commonly used organic dyes. QDs absorb a greater portion of the excitation photons than organic dyes, a process which translates into a brighter signal and ultimately higher sensitivities in detection assays (Arya et al., 2005). Compared to conventional dyes such as FITC and Alexa, QDs display an extremely narrow spectrum of emission, which results in much less overlap between the emissions of two different dyes, especially when the two dyes have similar emission wavelengths (Pinaud et al., 2006). Importantly, QDs are not as susceptible to degradation or photo-bleaching when compared to conventional fluorescent organic dyes (Ma et al., 2007).

Although QDs have been an efficient and sensitive tool for imaging purposes, their application to microplate-based fluorescence immunoassays has not been straightforward since the antibody binding mechanism to QDs is different, when compared to binding using more-conventional fluorescent dyes. In the case of QDs, six to eight antibodies bind to the surface of a QD. When QD secondary antibody conjugates are used to recognize the primary assay in a microplate assay, it is not easy to establish linearity between the number of bacterial cells and fluorescence intensity. Therefore, the combination

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of a dye and a linker, such as biotinylated secondary antibody and a QD streptavidin conjugate, is often needed to correct this problem.

Here we report the development of a microplate-based fluorescence immunoassay using a quantum dot-streptavidin conjugate approach that is used to quantify the bacterium, *Alteromonas* sp. attached to a marine benthic copepod.

2. Materials and methods

2.1. Copepod collection

Marine benthic copepods, *Microarthridion littorale*, were collected from the *Spartina* tidal marsh in the North Inlet (latitude 33°20'58"; longitude 79°11'34") off the coast of South Carolina during two sampling trips in July 2005 and February 2006. Mud samples, containing copepods, were gently collected from the top layer (approx. 2–3 cm) of sediment in proximity to *Spartina alterniflora* grasses during low tide. Freshly-collected sediment was initially filtered through a 500 µm stainless steel sieve, and sediment material was retained using a 125 µm sieve for further analyses in a 4:1 ratio of seawater to sediments. The freshly-sieved sediment was taken to the laboratory immediately for live copepod separations. 15 to 30 *M. littorale* were gently removed from sediment within several hours of collection using Olympus dissecting microscope, fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and stored at 4 °C.

2.2. Isolation of marine bacteria, *Alteromonas* sp

Ten individuals of live *M. littorale* were placed in a 500 µl of sterile sea water in 1.5 ml eppendorf tubes, respectively and were sonicated for 30 s at 30 Hz using Aquasonic Model 150D sonicator (VWR Scientific, West Chester, Pa.) to remove attached bacteria from copepods. Then, an aliquot of 100 µl of seawater, containing bacteria, was plated on Difco Marine Agar 2216 (Becton, Dickinson and Company, Sparks, MD) (Koren and Rosenberg, 2006) and incubated at 30 °C for 24 h. A dominant bacterial colony was isolated and cultured in 10 ml Difco Marine Broth 2216 (Becton, Dickinson and Company, Sparks, MD) in a rotary shaker. Bacterial identification was conducted using 500 bp 16S rDNA sequence at the Microbial Insights, Inc (Rockford, TN). The 16S rDNA sequence of this colony closely matched that of *Alteromonas* sp. (99%). The *Alteromonas* sp. isolate from copepods was used in this study.

2.3. Anti-*Alteromonas* sp. polyclonal IgG antibody production

The environmental isolate, *Alteromonas* sp., was aerobically grown in 100 ml marine broth on a rotary shaker (140 rpm) at 30 °C for 3 days and centrifuged at 10,000 rpm. The collected pellet was suspended in phosphate buffer saline (PBS), pH 7.4 and shipped to Animal Pharm Services (Healdsburg, CA) for polyclonal antibody production. An anti-*Alteromonas* sp. antiserum was raised in a New Zealand white male rabbit according to a standard 45 day protocol by Animal Pharm Services Inc., Healdsburg, CA). Briefly, a New Zealand white male rabbit was initially injected with approximately 1 ml of *Alteromonas* sp. suspension in Freund's complete adjuvant (Chemicon, Temecula, CA). One month later, a second injection was performed in a similar fashion using Freund's incomplete adjuvant (Chemicon, Temecula, CA). Two weeks after the second injection, the rabbit was exsanguinated under anesthesia and sera were separated from blood. Polyclonal IgG antibody purification from sera was performed using a commercially available Protein G agarose affinity column method following the manufacturer's instructions (Invitrogen, Carlsbad, CA).

2.4. Confocal scanning laser microscopy (CSLM)

Marine benthic harpacticoid copepods, *M. littorale*, were fixed in 4% paraformaldehyde immediately after collection. After fixation of at

least 1 h, copepods were washed several times in a 1.5 ml microcentrifuge tube. Copepods were incubated (30 min) in 3% Bovine Serum Albumin (BSA)/PBS blocking solution at room temperature. The copepods were washed with 0.05% Tween/PBS. The anti-*Alteromonas* IgG primary antibody was diluted in 1% BSA/PBS (1.6 ng/100 µl). Copepods were incubated with the diluted antibody at room temperature and washed. Then, Qdot 525 goat F(ab')₂ anti-rabbit IgG conjugate (H+L) (Invitrogen, Carlsbad, CA) diluted in 1% BSA/PBS (10 nM) was incubated for 2 h with the samples at room temperature. Some samples, which received rabbit preimmune serum instead of primary antibody, were used as control. Copepods were then washed and mounted on slides for viewing. The fluorescence of labeled *Alteromonas* sp. on was assessed, and images were captured using a Zeiss LSM 510 META confocal scanning laser microscope (CSLM).

2.5. Enumeration of *Alteromonas* sp. using 96-well microplate-based fluorescence immunoassays with coupled QD streptavidin conjugates

One hundred *M. littorale* were collected and placed in the insert column separated by 65 µm mesh net in a 1.5 ml sterile microcentrifuge tube. The microcentrifuge tube containing the copepods in the separation column was sonicated at 30 Hz for 5 min using Aquasonic Model 150D sonicator, then centrifuged at 3000 rpm. The pelleted cells in the bottom of microcentrifuge tube were fixed in 100 µl of 4% paraformaldehyde in 0.2 M PBS (pH 7.4) for at least 1 h at 4 °C.

Diagram of 96-well microplate-based fluorescence immunoassays with coupled QD streptavidin conjugate was provided in Fig. 1. Serial dilutions of above-fixed bacteria cells were carried out with 0.2 M PBS within the 96-well microplate. Sample cells were allowed to settle and adhere to the microplate wells for at least 2 h. The efficiency of bacterial adherence to the microplate well was determined by plate colony counting of bacteria in well solution at time 0 and time 2 h. All incubations were conducted at room temperature, unless otherwise noted. Then, microplate wells were blocked for non-specific binding with 3% bovine serum albumin (BSA) in PBS solution for 2 h at room temperature. Microplate wells were washed with 0.05% Tween in PBS, then attached bacteria were incubated with anti-*Alteromonas* polyclonal IgG in wells at a dilution of 1:20 in 1% BSA in PBS for 1 h. After washing, secondary biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA), diluted in 1% BSA in PBS (1.5 ng/ml), was added to each well and incubated for 1 h. After washing, the fluorescently-labeled QD 525 streptavidin conjugate (Invitrogen, Carlsbad, CA), diluted in 1% BSA in PBS (10 nM), was added to each well and incubated for 1 h. Finally, microplate wells were washed and fresh PBS was added into each well for fluorescence intensity measurements in the 96-well plate. Fluorescence intensity (excit./emiss. = 340/528 nm) was measured with FLx800 microplate fluorescence reader (Bio-Tek Instruments, Inc.). Wells that received only QD conjugates were used as background fluorescence controls.

Alteromonas sp. cultures were used to establish a standard curve (CFU vs. fluorescence intensity) in a 96 microplate as described below, with each sample point collected in duplicate. Briefly, *Alteromonas* sp. was grown aerobically to stationary phase (i.e. approx. 48 h) in Difco Marine broth 2216 at 37 °C on a rotary shaker (at 140 rpm). Then, 100 µl aliquots of *Alteromonas* sp. cell suspension were placed in duplicate wells of the microplate. Two-fold serial dilutions of *Alteromonas* sp. cells were carried out in the microplates. Then, cells were left to adhere to the bottom of microplate wells for at least 2 h on a shaker at room temperature. After discarding supernatant from each well, microplate-based fluorescence immunoassays using QD streptavidin conjugates and conventional marine agar plate counting were applied to establish the standard curve (fluorescence intensity vs. CFU). To demonstrate the specificity of polyclonal antibody, we conducted the same plate assay on the following other unrelated four bacteria; *Escherichia coli* K12 (ATCC 29425), *Agrobacterium tumefaciens* (ATCC 11158), *Bacillus megaterium* (ATCC 10778), and

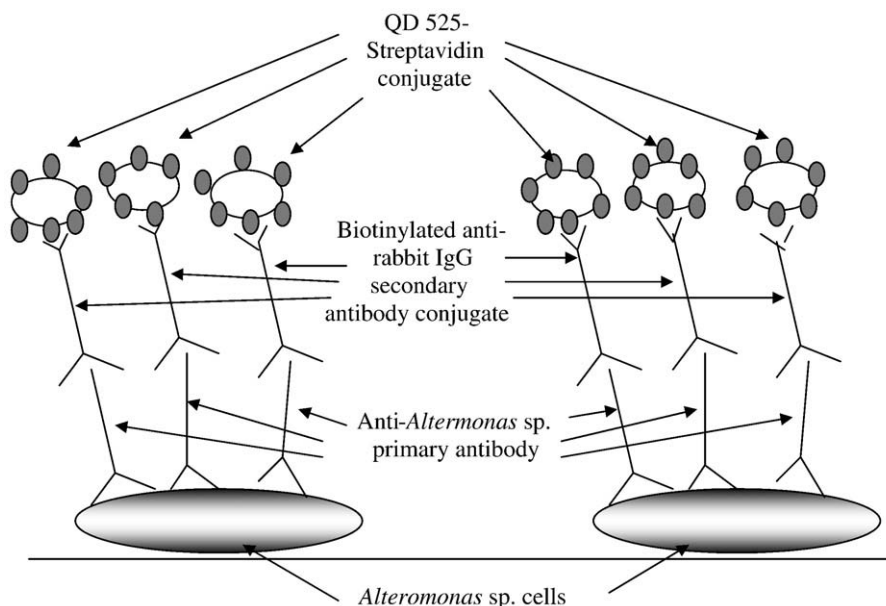


Fig. 1. Diagram of a microplate-based fluorescence immunoassay using quantum dots streptavidin conjugates for enumeration of putative marine bacteria, *Alteromonas* sp.

Staphylococcus epidermis (ATCC 12228). These bacteria strains were obtained from American Type Culture Collection (Manassas, VA). Briefly, these bacteria were grown aerobically to stationary phase (i.e. approx. 48 h) in nutrient broth (Becton, Dickinson and Company, Sparks, MD) at 30 °C on a rotary shaker (at 140 rpm).

2.6. Enumeration of total bacteria associated with copepods using the non-specific nucleic acid fluorescent probe, SYBR green I

Alteromonas sp. cultures and the non-specific nucleic acid fluorescent probe SYBR green I (Invitrogen, Carlsbad, CA) were used to determine numbers of total bacteria in the 96-well plates used for generating the standard curve (CFU vs. fluorescence intensity). Serial dilutions of bacteria cells were carried out using 0.2 M PBS, (pH 7.4) within the 96-well microplate. Sample cells were allowed to settle and adhere to the microplate wells for at least 2 h. All incubations were conducted at room temperature, unless otherwise noted. Approximately 99% of cells were attached to the bottom of each well. After 2 h of incubation, the supernatants from each well were discarded. Then, 100 µl of SYBR green I diluted in PBS (1:10,000) was added directly to each well and stained for 10 min. Attached cells were briefly washed with PBS, and 100 µl of fresh PBS were put in each well for fluorescence intensity measurements. The mean fluorescence intensity of duplicate wells without bacteria cells was used as the control. Based on CFU and fluorescence intensity from each well, the standard curve for counting cells was established.

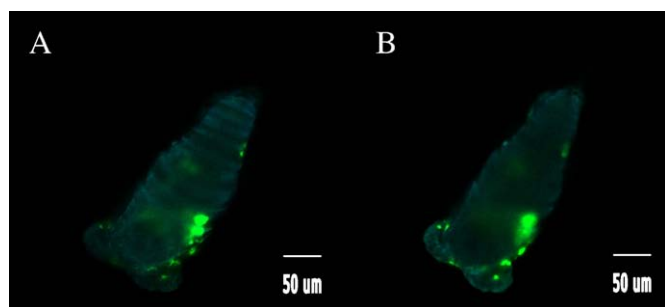


Fig. 2. The copepod *M. littorale* collected in winter(A) and summer (B), and probed for the bacterium *Alteromonas* sp. with Qdot 525-anti-*Alteromonas* sp. conjugates (green fluorescence). Scale bar=50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

In the present study, quantitative measurements of the ecologically important marine bacterium, *Alteromonas* sp. attached to the harpacticoid copepod *M. littorale* were successfully determined using a microplate assay based on quantum dot (QD)-linked immunofluorescence. A secondary Qdot 525-goat anti-rabbit IgG fluorescent label was employed for localization and imaging. The QD labeling clearly showed marine bacteria, *Alteromonas* sp. attached to the exoskeleton of copepods (Fig. 2). We recently reported that the bacterial human pathogen, *Vibrio parahaemolyticus*, was localized on the exoskeleton of a benthic copepod, *M. littorale*, using the same approach (Decho et al. 2008).

Plate counting confirmed that 99% of bacterial cells were adhered to the bottom of microplate wells (data not shown). Fig. 3 shows the total number of attached cells removed by sonication from 100 *M. littorale* copepods. SYBR green I was used to determine the number of total attached cells on the copepods. Following a polynomial transformation of the best fit six-point curve ($y = -6E - 06x^2 + 1.4285x + 139.33$), the adjusted R^2 value equaled 0.9956. The experimental point shown as a red square is the number of cells in 100 µl of the original sample. From

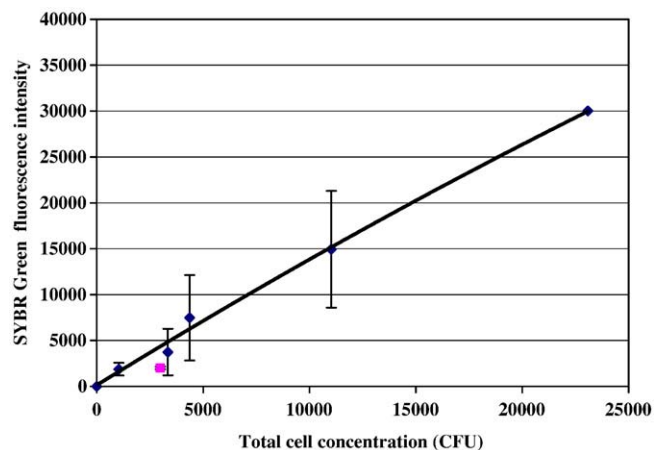


Fig. 3. Total number of bacteria cells attached to cultured copepods, estimated using the *Alteromonas* sp. cell standard curve (SYBR green I). The square indicates the total cell counts (2002.7 ± 143.5 CFU/100 µl; mean \pm SD) from the experimental sample.

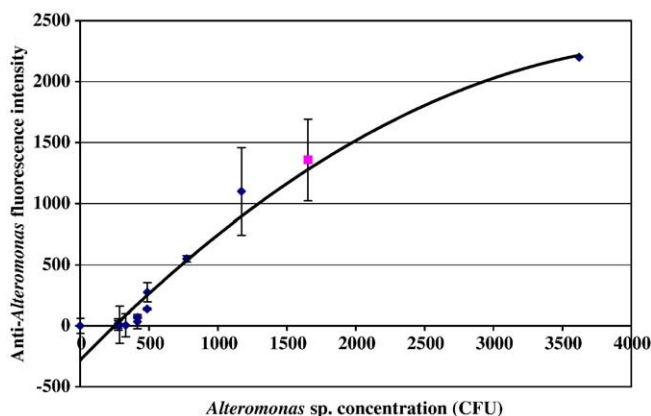


Fig. 4. Twelve-point standard curve for *Alteromonas* sp. labeled with quantum dot 525 (excited at 340 nm). The square indicates the *Alteromonas* cell count (1653 ± 335 cells/100 μ l; mean \pm SD) from the experimental sample.

this point, it was determined that the number of cells attached to 100 *M. littorale* was (*ca.*) 2×10^4 CFU. Therefore, approximately 2×10^2 (CFU) total cells were attached to the surface of a single copepod.

Fig. 4 shows the *Alteromonas* sp. standard curve when QD 525 streptavidin conjugates were labeled as a fluorescent indicator. Anti-polyclonal antibody was shown to be specific to *Alteromonas* sp. cells because we could not establish the standard curve using four bacteria species (data not shown). After a polynomial transformation, yielding a best fit twelve-point curve ($y = -0.0001x^2 + 1.1574x - 282.71$), the adjusted R^2 value equaled 0.9638. The square, superimposed on the curve, is the total number of *Alteromonas* sp. cell per 100 μ l of original sample (Fig. 4). This empirical estimate was used to determine an approximate number of *Alteromonas* sp. cells sonicated from (i.e. previously attached to) 100 copepods. The original sample concentration of *Alteromonas* sp. cells was approximately $10^4 \pm 120$ CFU per one hundred *M. littorale*. Therefore, the number of *Alteromonas* sp. cells attached to a single cultured copepod was calculated to be 10^2 *Alteromonas* sp. cells. This translates into *Alteromonas* sp. representing approximately 53% of the total attached cells on *M. littorale*.

In comparison, total bacteria associated with various sizes of zooplankton were enumerated by Heidelberg et al. (2002). In their study, bacteria were distinguished using EUB 338 and Vvul3 *in situ* hybridization probes. Bacteria abundances were determined using flow cytometry. Abundances associated with large zooplankton (>202 μ m), and a similar size range to the copepod, *M. littorale*, were found to range between 2.8×10^3 to 9.6×10^5 per individual zooplankton. Furthermore, the number of γ -Proteobacteria and *Vibrio-Photobacterium* ranged from 1.0×10^3 to 2.3×10^5 , and 1.6×10^2 to 1.2×10^5 , respectively. The authors enumerated down to the species-specific level of several vibrio species, which ranged from 3.0×1 to 3.3×10^3 per copepod. Therefore, the calculated number of *Alteromonas* sp. of 10^2 CFU per individual *M. littorale* (>202 μ m) observed in the present study were within the range determined by previous studies using other copepods.

The proportion of *Alteromonas* sp.: total bacteria was also computed using bacteria sonicated from (attached to) *M. littorale*. Previous studies (Heidelberg et al., 2002) estimated that 6 to 37% of the total bacteria associated with large zooplankton were of the γ -Proteobacteria subclass, while *Vibrio-Photobacterium* sp. comprised approximately 1 to 26% of the total bacteria associated with the large zooplankton. In the present study, we determined that over half (e.g. 53%) of the total bacteria could be assigned to *Alteromonas* sp.

The detection of bacteria using a microplate-based QD immunofluorescence assay has been reported previously by several groups (Hahn et al. 2005; Yang and Li, 2005). In one study, a QD streptavidin-biotin system was utilized to couple antibodies and QDs (Yang and Li, 2005). In their study, the coupling of QDs and streptavidin was carried

out before the microplate assay and successfully demonstrated the simultaneous detection of two species of food borne pathogenic bacteria, *E. coli* O157:H7 and *Salmonella typhimurium*. It is important to note that conventional fluorescent dye-secondary antibody conjugates are not likely to be replaced by QD – secondary antibody conjugates, and QDs require a streptavidin–biotin system to be used in the microplate quantification assay.

4. Conclusion

In the present study, we successfully developed a method to enumerate the marine bacterium *Alteromonas* sp. attached to a benthic harpacticoid copepod using a microplate-based QD immunofluorescence assay. Microplate-based QD immunofluorescence assays offer the potential for relatively-rapid quantification of bacteria, and by using multiple-fluorescent immunoassays, may be further developed to detect multiple bacteria species simultaneously. Future work utilizing various combinations of QDs and antibodies, and multiplexed QD immunofluorescence assays offer the potential to rapidly detect multiple bacteria species associated with copepods.

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