

# Application of photostable quantum dots for indirect immunofluorescent detection of specific bacterial serotypes on small marine animals

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## Abstract

An indirect immunofluorescence approach was developed using semiconductor quantum dot nanocrystals to label and detect a specific bacterial serotype of the bacterial human pathogen *Vibrio parahaemolyticus*, attached to small marine animals (i.e. benthic harpacticoid copepods), which are suspected pathogen carriers. This photostable labeling method using nanotechnology will potentially allow specific serotypes of other bacterial pathogens to be detected with high sensitivity in a range of systems, and can be easily applied for sensitive detection to other *Vibrio* species such as *Vibrio cholerae*.

## 1. Introduction

The emerging health concerns (e.g. gastroenteritis) associated with increased incidence of water-borne pathogenic bacteria, such as *Vibrio parahaemolyticus* in coastal regions, reflect existing uncertainties regarding the survival, residence times, associations and movements of pathogens which sometimes occur in relatively low numbers and avoid commonly used detection methods [4, 20]. Copepod exoskeletons and other chitin-bearing particulates (zooplankton molts, fecal pellets, sediments, and organic aggregates) are thought to play a significant role in the survival, fate, and persistence of pathogenic *Vibrios* and other bacteria in coastal environments [2, 18].

Chitin is an *N*-acetylglucosamine (GlcNAc)-based complex polysaccharide, and is the second most abundant polysaccharide (after cellulose) in the biosphere, with annual copepod chitin production alone estimated at several million tons [12]. *Vibrio* growth has been found to be 10 000-fold higher in chitin-saline solutions than in saline solutions alone [8], and the initial *Vibrio* attachment and colonization of copepods and chitinous exuvia is salinity, pH, and temperature dependent [9, 13]. This means that coastal ocean waters, containing copepods and their chitinous exuvia, may be a potentially invisible reservoir for *Vibrios* and other bacteria. Although ecological relationships between human pathogens such as

*V. cholera* and *V. parahaemolyticus*, and planktonic copepods have been reported [5, 8, 9, 16, 17], almost nothing is known about the role of benthic chitin-bearing organisms and other particulates in the culture and transmission of seafood-borne bacterial pathogens.

Quantum dots (QDs) have recently emerged as a significant tool for immunofluorescence detection because of their unique qualities such as a long-term photostability, when compared to more conventional organically based labels [3]. Therefore, QDs provide an excellent tool to localize specific types of microorganisms when used in combination with specific antibodies or DNA probes.

Here we report that the state-of-art approach of confocal scanning laser microscopy (CSLM) in combination with QDs indirect immunofluorescent techniques can be utilized for detecting relatively low abundances of a specific bacterial pathogen on the chitinous exoskeleton of a small marine animal such as a benthic copepod, *Microarthridion littorale*. The approach is easily adapted for the sensitive detection of specific bacteria in other environments, or artificial systems.

## 2. Experimental details

### 2.1. Sample collection

Marine benthic copepods, *M. littorale*, were collected from the *Spartina alterniflora* tidal marsh in the North Inlet off

the coast of South Carolina (latitude 33°20'58" N; longitude 79°11'34" W) during two sampling trips in July 2005 and February 2006. To collect the copepods, mud samples containing copepods were gently removed from the top layer (approximately 2–3 cm) of sediment in proximity to the marsh grasses during low tide. Freshly collected sediment was initially filtered through a 500  $\mu\text{m}$  stainless steel sieve to remove larger debris. Sediment material was retained for further analyses using a 125  $\mu\text{m}$  sieve with a 4:1 ratio of seawater to sediments. The freshly sieved sediment was taken to the laboratory immediately for live copepod separations. Approximately 15–30 individuals of *M. littorale* copepods were gently removed from the sediment within several hours of collection using an Olympus dissecting microscope, fixed in 4% paraformaldehyde and stored at 4 °C.

## 2.2. Detection of *Vibrio parahaemolyticus* using CHROMagar *Vibrio* medium

Initially, ten live (i.e. freshly collected) individuals of *M. littorale* were homogenized using sterile plastic pestles in 1.5 ml microcentrifuge tubes containing 100  $\mu\text{l}$  sterile seawater and streaked on CHROMagar<sup>TM</sup> (CHROMagar, France) *Vibrio* chromogenic medium [7] for isolation and detection of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*. After 24 h of incubation at 37 °C, colony color on the plate was observed. Additionally, an oxidase test was performed on suspected colonies, using tetramethyl-*p*-phenylenediamine at a concentration of 10 mg ml<sup>-1</sup> [7].

Serotyping of the bacterium *V. parahaemolyticus* has been recognized as a very important tool for epidemiological investigations [10]. So far, more than 75 combinations of O and K serotypes have been described. Serotype O4:K12 has been associated with food-borne illness outbreaks in the United States and Mexico [22]. Although, recently, fluorescence *in situ* hybridization (FISH) using DNA probes has become a widely used method for the detection of microorganisms, it was not possible to differentiate the epidemiologically relevant serotypes of *V. parahaemolyticus*. Thus, we employed an indirect immunofluorescence approach using a polyclonal antibody against the *V. parahaemolyticus* serotype O4 in combination with a QD 605 conjugated anti-rabbit secondary antibody.

## 2.3. Scanning electron microscopy

In order to demonstrate bacterial attachment, individual copepods were fixed in 2.5% glutaraldehyde in seawater for 2 h, post-fixed with 1% osmium tetroxide and dehydrated through an ethanol series (50%, 70%, 80%, 90%, 95%, and 100%), then critical-point dried, mounted on aluminum stubs, gold-ion sputter coated and imaged using an environmental scanning electron microscope (FEI Quanta 200 ESEM) at 30 kV. QDs were not applied for the ESEM imaging.

## 2.4. Confocal scanning laser microscopy (CSLM)

Individual copepods, fixed in 4% paraformaldehyde, were washed several times with phosphate buffered saline (PBS)

in a 1.5 ml microcentrifuge tube. Copepods were incubated (25 °C, 2 h) in 3% bovine serum albumin (BSA) in PBS to block any non-specific binding. Anti-*Vibrio parahaemolyticus* serotype O4 polyclonal primary antibody (Denka Inc., Japan) was diluted at a 1:100 ratio using 1% BSA (wt/vol) in PBS. Copepods were incubated with the diluted antibody for 1 h at 25 °C. Pre-immune rabbit serum, instead of primary antibody, was used as a control.

After thoroughly washing the copepods three times with 1% BSA in PBS for 10 min, the copepods were incubated with QD 605 (10 nM) conjugated with anti-rabbit IgG secondary antibody (Invitrogen) diluted with 1% BSA in PBS for 1 h at room temperature. Copepods were again washed three times with 1% BSA in PBS, then rinsed with PBS, and mounted on glass slides with 1 drop of Citifluor mounting media (Ted Pella, Inc.) using a 0.5 mm (depth) Coverwell Adhesive Imaging Chamber (Grace Bio-Labs, Inc.). A Zeiss LSM 510 META confocal scanning laser microscope (CSLM) was used to image QD 605-labeled *V. parahaemolyticus* on the copepods. QD 605 was excited with a blue UV laser (excit/emiss = 405/605 nm). Reflection of the UV laser was used for capturing the overall images of copepod.

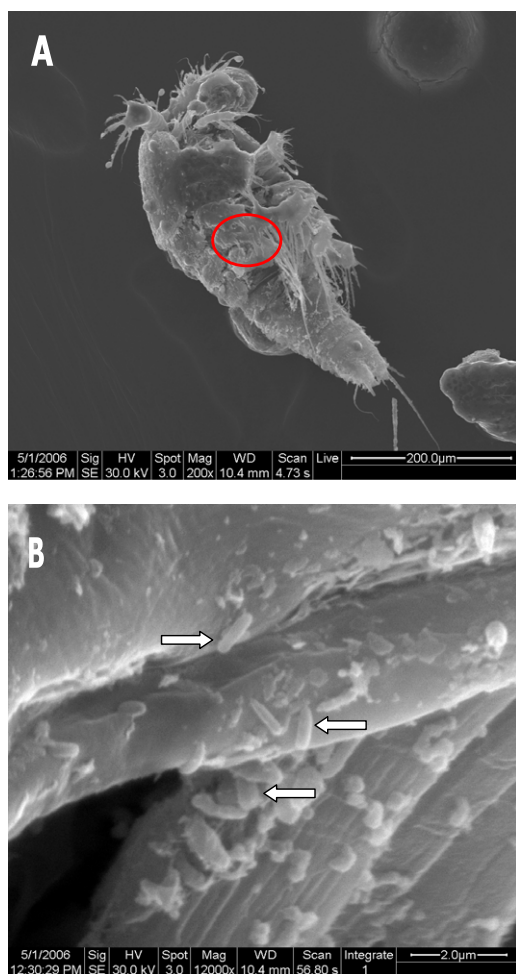
## 3. Results and discussion

The presence of rod-shaped bacteria attached to the exoskeletons of harpacticoid copepods, *M. littorale* (figure 1), indicated that not only planktonic copepods [8, 9] but also benthic copepods may be potential carriers of bacteria, including human pathogens.

The QD-labeled indirect immunofluorescence method was successfully used to localize *V. parahaemolyticus* cells attached to benthic copepods, *M. littorale* (figure 2). Several bacterial colonies isolated from *M. littorale* were confirmed to be *V. parahaemolyticus* by its specific color (mauve) and positive oxidase test (results not shown). Since polyclonal antibodies against 74 serotypes K and 11 serotypes O of *V. parahaemolyticus* are commercially available, the combination of QDs and polyclonal antibodies against different serotypes of *Vibrio parahaemolyticus* in conjunction with CSLM will provide a powerful tool to detect various *V. parahaemolyticus* serotypes on the bodies and molted exuvia of benthic copepods and other small marine animals. Since many antibodies can be raised against various bacterial human pathogens, and are commercially available as serotypes within each species of bacteria, the combination of primary- and secondary-QD antibody conjugates will be a useful approach for the detection of bacterial pathogens and their serotypes.

Some of the benefits over conventional fluorescent labels include: (1) long-term photostability for live-cell imaging; (2) fixability for follow-up immunofluorescence from *in vivo* studies; and (3) brilliant fluorescence for imaging from simple, single-excitation sources, or multicolor analyses [1, 11, 14, 15, 19]. Finally, the long-term photostability of QDs makes them an ideal label for the detection of bacteria using confocal laser microscopy.

The detection of bacteria using a microplate-based QD immunofluorescence assay has been reported by several



**Figure 1.** (A) An environmental scanning electron microscopy (ESEM) image of a harpacticoid copepod *M. littorale*. Note: the imaged copepods do not contain QDs. Scale bar = 200  $\mu\text{m}$ . (B) ESEM image of rod-shaped bacteria attached to the surface of the copepod *M. littorale*. Scale bar = 2.0  $\mu\text{m}$ .

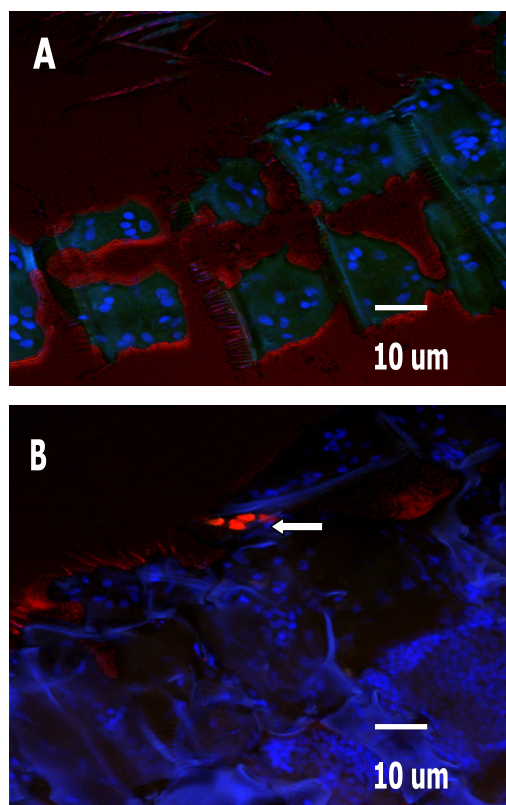
groups [3, 6, 21]. Thus, our approach can be easily applied for the quantification of bacteria using a microplate-based QD immunofluorescence assay.

#### 4. Conclusion

In summary, we successfully demonstrated that the combination of photostable quantum dots and serotype-specific polyclonal antibodies can be used to detect *V. parahaemolyticus* on marine benthic copepods.

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**Figure 2.** (A) CSLM image showing pre-immune serum control with a secondary QD605 anti-rabbit IgG antibody. (B) Anti-*V. parahaemolyticus* primary antibody with a secondary QD605 anti-rabbit IgG antibody was used to detect *V. parahaemolyticus* on the benthic copepod, *M. littorale*. Scale bar = 10  $\mu\text{m}$ .

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