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Detection of '*Candidatus* Xenohaliotis californiensis' (Rickettsiales-like prokaryote) inclusions in tissue squashes of abalone (*Haliotis* spp.) gastrointestinal epithelium using a nucleic acid fluorochrome

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ABSTRACT: Rickettsiales-like prokaryotes appear to be etiologic agents of a number of newly described diseases of fish and shellfish. '*Candidatus* Xenohaliotis californiensis' is a Rickettsiales-like prokaryote responsible for withering syndrome, a fatal disease of wild and farmed Eastern Pacific abalone, *Haliotis* spp. The bacterium proliferates in gastrointestinal epithelial cells, forming large intracytoplasmic inclusions. We describe a method of rapidly detecting and assessing the intensity of '*Candidatus* Xenohaliotis californiensis' infections in abalone gastrointestinal tissue using the nucleic acid-specific fluorochrome Hoechst 33258. In excised tissue pieces dried onto slides, rehydrated in the Hoechst stain and viewed with ultraviolet light, the large bacterial inclusions were strongly fluorescent and could be easily distinguished from smaller host cell nuclei. This provided a rapid, inexpensive alternative to paraffin section microscopy or molecular techniques, allowing detection of the pathogen within minutes of tissue excision. Comparison of the fluorochrome method with conventional histological analysis for the ability to detect inclusions in 109 samples was 90% accurate, with discrepancies due to false negative diagnosis of low-level infections. An alternative nucleic acid-specific fluorochrome, propidium iodide, showed a staining pattern identical to that of Hoechst 33258. These methods should prove useful for the rapid detection of inclusion-forming Rickettsiales-like prokaryotes in tissues from many host species.

KEY WORDS: '*Candidatus* Xenohaliotis californiensis' · Nucleic acid · Rickettsiales · Abalone

INTRODUCTION

Rickettsiales and Chlamydiales bacteria are small, Gram-negative obligate intracellular pathogens of many taxa, including mammals, fish, crustacea and molluscs (Krieg & Holt 1984, Fryer & Lannan 1994). Some species replicate freely in the cytoplasm of host cells while others are confined to distinct phagosomal or phagolysosomal vacuoles, forming large, dense inclusions packed with individual bacteria (Krieg & Holt 1984). The latter group includes '*Candidatus* Xenohaliotis californiensis' (RLP) (Friedman et al. 2000), a Rickettsiales pathogen of several species of eastern Pacific abalone, *Haliotis* spp. The prefix '*Candidatus*' in the taxon indicates a provisional status (Murray & Stackebrandt 1995) because the species was described largely on morphology, life history and DNA sequence-

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based data and lacks extensive biochemical and serological characterization. This bacterium has recently been identified as the etiologic agent of withering syndrome, an infectious disease that has caused mass mortality of wild and cultured abalone since the mid-1980s (Gardner et al. 1995, Friedman et al. 1997, 2000, Moore et al. 2000). The pathogen forms large (17 × 55 µm) ovoid inclusions in epithelial cells of the posterior portion of the esophagus (postesophagus), crop, digestive gland transport ducts and, less frequently, the intestine (Friedman et al. 2000). These inclusions can be readily observed in hematoxylin and eosin-stained paraffin sections and can be detected by *in situ* hybridization (Antonio et al. 2000) or by amplification of DNA from tissue samples via a polymerase chain reaction (Andree et al. 2000). However, these methods require at least several hours and typically several days to obtain results. A method that can be used to confidently identify a pathogen and estimate infection intensity within minutes is necessary to obtain live, heavily infected tissue for use in pathogen purification protocols, transmission studies and ultrastructural examination. Detection of RLP inclusions in squash preparations of whole tissue using phase contrast microscopy is problematic because of poor contrast between inclusions and host tissue. Additionally, colorimetric-based Rickettsial stains designed for use in smears of infected yolk sac or mammalian cell cultures are not applicable because of strong staining of normal tissue components in the complex epithelia that comprise host abalone target tissue. We report here that rapid detection and estimation of intensity of infections in abalone tissue can be achieved using nucleic acid-specific fluorochromes on tissue squash preparations.

MATERIALS AND METHODS

Black abalone *Haliotis cracherodii* and red abalone *H. rufescens* collected at various locations throughout California in 1997–8 were killed, and portions of the gastrointestinal tract from the posterior portion of the esophagus to the posterior end of the crop (collectively termed postesophagus, Harris et al. 1998) were excised. Samples for histological analysis were fixed in Davidson's solution (Shaw & Battle 1957) and embedded in paraffin, and 5 µm hematoxylin and eosin-stained sections were evaluated for the presence and intensity of RLP inclusions in epithelial cells. The intensity of infection in histological samples was quantified using the scale of Friedman et al. (1997) based on the average number of RLP inclusions per 200× magnification field of view: absent = (0), 1 to 10 = (1), 11 to 100 = (2), or greater than 100 = (3). Additional pieces of this tissue were minced to approximately 2 mm^2 and placed on a microscope slide. The tissue pieces were gently pressed onto the slide using a second slide and dried with low heat from a blow dryer for 20 min. Dried samples were prepared for examination immediately or held indefinitely at 4°C with dessicant. To prepare for examination, the tissue was flooded with a 10 µg ml^{-1} solution of Hoechst 33258 (bisBenzimide, Sigma, St. Louis, MO, USA) in distilled water, covered with a coverslip, incubated in the dark for several minutes and viewed at 100 to 400× with epifluorescent ultraviolet light and filters appropriate for its spectra (356 nm excitation, 465 nm emission, Latt & Stetten 1976). The intensity of RLP infection in Hoechst 33258-stained samples was subjectively rated as absent, low, moderate or high, roughly coinciding with the semi-quantitative numerical scale used for histological sections (above). Ten dried tissue squash samples were stained with an alternative nucleic acid-specific fluorochrome, propidium iodide (10 µg ml^{-1} in distilled water, Sigma), and viewed with ultraviolet light and appropriate filters for its spectra (530 nm excitation, 615 nm emission, Arndt-Jovin & Jovin 1989).

The accuracy, sensitivity and specificity of Hoechst-stained squash preparations were assessed using conventional histological analysis as the reference technique and the methods of Cooper et al. 1982:

Accuracy = [# true (+) + # true (–)] / # examined
Sensitivity = # true (+) / [# true (+) + # false (–)]
Specificity = # true (–) / [# true (–) + # false (+)]

where # true (+) and # true (–) are the number of samples diagnosed as positive and negative by both histological analysis and the fluorochrome method, respectively, # false (–) is the number diagnosed as positive by histological analysis and negative by the fluorochrome method, and # false (+) is the number diagnosed as negative by histological analysis and positive by the fluorochrome method.

RESULTS

Viewed with conventional light microscopy, RLP inclusions were difficult to detect in either fresh or dried and subsequently rehydrated postesophagus tissue (Fig. 1A). In tissues rehydrated in Hoechst 33258 fluorochrome and viewed with epifluorescent ultraviolet light, small nuclei and large inclusions fluoresced bright blue against a black to dull red background (Fig. 1B). Even in severe infections, inclusions were typically present in a patchy distribution with densely infected areas scattered among uninfected tissue. Only

nuclei fluoresced in uninfected portions of tissue (Fig. 1C) while adjacent regions had large, oval staining bodies corresponding in size, shape and location to inclusions (Fig. 1D). The RLPs in hematoxylin and eosin-stained paraffin sections were similar in size and appearance to those observed in Hoechst-stained tissue squashes (Fig. 1E,F respectively). As with histological and molecular methods, no difference was seen in inclusion morphology or tissue distribution between red and black abalone. Scanning the luminal surface of infected tissues provided an interesting alternative view to that of histologic sections, and allowed rapid identification of particular regions of the gut that were most infected. Staining of freshly excised tissue (without drying) resulted in weak superficial staining that gradually increased over time. Dried, RLP-infected tissue squashes that were rehydrated in a propidium iodide solution and viewed with the appropriate filter set also showed specific staining of host cell nuclei and RLP inclusions. Nucleic acid stains emitting at the commonly used wavelength of fluorescein isothiocyanate (518 nm) could not be used because of intense autofluorescence emitted by host tissue.

Detection of RLP inclusions by the fluorochrome method and conventional histological analysis was performed for a set of parallel samples from 109 black and red abalone. Inclusions were detected in 57 samples with the fluorochrome method and in 68 samples with histological techniques (Table 1). Comparison of the fluorochrome with the histological method as a reference technique indicated that there were 57 true positives, 41 true negatives, zero false positives and 11 false negatives. These data were used to calculate values for the following parameters: accuracy: 0.90; sensitivity: 0.84; and specificity: 1.00.

The values for sensitivity and specificity indicate that the lower accuracy of the fluorochrome method was due to false negative rather than false positive diagnoses. In a set of parallel samples from 84 individuals, categorization of infection intensity with the fluorochrome method as absent, low, moderate or high agreed reasonably well with a similar semi-quantitative scale used for histological samples, with better agreement at higher intensity scores (Table 2). False negative diagnoses tended to occur primarily in animals with low-level infections.

Table 1. Comparison of Hoechst 33258-stained tissue squashes with conventional hematoxylin and eosin (H&E)-stained paraffin tissue sections for detection of '*Candidatus* Xenohaliotis californiensis' inclusions in abalone postesophagus tissue (samples from 24 black abalone and 85 red abalone)

	Sample type/stain	
	Paraffin section / H&E	Squash / Hoechst 33258
Positive	68	57
Negative	41	52
Total	109	109

Table 2. Comparison of Hoechst 33258-stained tissue squashes with conventional hematoxylin and eosin-stained paraffin tissue sections for estimation of infection intensity of '*Candidatus* Xenohaliotis californiensis' inclusions in abalone postesophagus tissue (samples from 20 black abalone and 64 red abalone). *Average number of inclusions per 200× field view; **relative abundance

Paraffin section / H&E		Squash / Hoechst 33258	
Category*	# samples	Category**	# samples
0	30	Absent	37
1–10	17	Low	10
11–100	20	Moderate	16
>100	17	High	21
Total	84	Total	84

DISCUSSION

Conventional stains for diagnosis of rickettsial infections are designed for use in smear preparations derived from infected yolk sac material or cultured cells (Gimenez 1964, Presnell & Schreibman 1997). Such stains are not Rickettsiales-specific but rather are nucleic acid stains that have been shown to stain rickettsial particles well and use counterstains that differentiate the bacterium from normal tissue components. In our experience, these preparations do stain RLP inclusions in smear preparations, but strong staining of normal tissue components results in poor distinction between host and pathogen. Anderson & Greiff (1964) reported a method for detection of *Rickettsia mooseri* in infected yolk sac or other tissue by fluorescence following staining with acridine orange, although this method required quenching of tissue autofluorescence with an alcian blue, periodic acid-Schiff stain. A similar method was also recommended for detection of Rickettsiales-like prokaryotes in kidney imprints of salmonids (Lannan & Fryer 1991). All of the above methods involve detection of individual bacterial particles among thinly scattered host cell components and debris. Our method is unique in that superficial staining of whole tissue preparations viewed with epifluorescence allowed for detection of bacterial inclusions *in situ*. The whole tissue squash preparations used in these studies are excessively thick for staining with colorimetric methods.

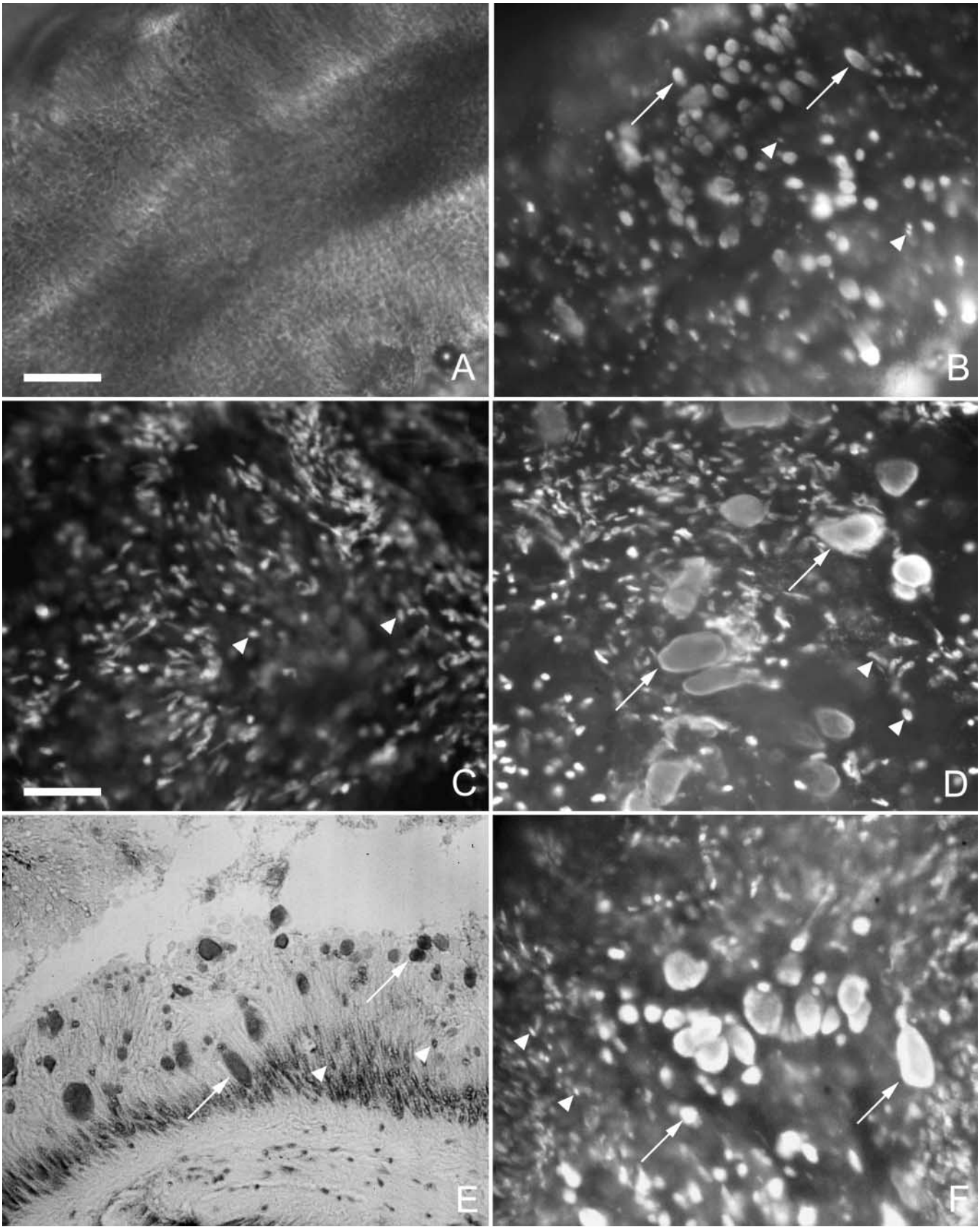
A
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Fig. 1. Detection of '*Candidatus* Xenohaliotis californiensis' inclusions in abalone postesophagus tissue. Arrows point to examples of inclusions and arrowheads point to examples of host cell nuclei. (A) Conventional light microscopic view of dried postesophagus, rehydrated with Hoechst 33258 stain. (B) Same view as (A) but with exposure to ultraviolet light, showing brightly fluorescing inclusions of varying size and uniform small nuclei. (C) Region of uninfected tissue stained with Hoechst 33258 and exposed to ultraviolet light; only fluorescing nuclei are present. (D) Tissue adjacent to that of (C), with a high density of bacterial inclusions. (E) Hematoxylin and eosin-stained postesophagus tissue section showing inclusions in epithelial cells viewed with conventional light microscopy. (F) Hoechst 33258-stained squash tissue preparation from the same animal as (E) viewed with ultraviolet light, showing strongly fluorescing inclusions of similar size and shape. Scale bar in A = 100 µm and is valid for A and B; scale bar in C = 50 µm and is valid for C to F

Hoechst 33258 is poorly permeant (but not completely impermeant; Latt & Stetten 1976) to living cells, and drying or fixation of the tissue before staining greatly enhances the rate of stain uptake. Drying the tissue was chosen over solvent or aldehyde fixation because of the additional benefit of permanently attaching tissue to the slide. Nevertheless, bright fluorescence of inclusions in aldehyde fixed, deparaffinized tissue sections showed that the fluorochrome can bind highly processed DNA (unpubl. obs.). The closely related fluorochrome Hoechst 33342 is more membrane permeable and worked equally well in rehydrated tissue squashes (unpubl. obs.), in agreement with an earlier study that concluded that the 2 dyes can be used interchangeably to label DNA in fixed cells (Arndt-Jovin & Jovin 1989). Propidium iodide is impermeant to living cells, and drying or fixation of the tissue is required for the fluorochrome to cross the cell membrane. In dried tissue squashes the performance of this fluorochrome was similar to that of Hoechst 33258.

All techniques using conventional or nucleic acid stains are non-specific, and species identity should be confirmed by a more informative technique that, in the case of '*Candidatus* Xenohaliotis californiensis', may include histological analysis, *in situ* hybridization or polymerase chain reaction. Well-characterized piscine and mammalian Chlamydiae and Rickettsiae are typically diagnosed by immunologic methods using infected tissue or infected cells grown in culture (Lannan et al. 1991, Lennette et al. 1995). Such methods involve production of antisera or monoclonal antibodies using highly purified source material that, in the absence of susceptible cell lines, must be obtained from infected animals. Studies in our laboratory are underway to purify '*Candidatus* Xenohaliotis californiensis' from host abalone tissue.

The RLP identification tool described herein allows for identification of abalone with advanced RLP infections within minutes of death. Such highly infected material is necessary for efficiency in protocols involving experimental transmission, purification and ultrastructural examination of pathogens. This method should be applicable for the detection of inclusion-forming bacteria from diverse host species and tissue types.

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