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Short Communication

A novel bacterial infection of the edible crab, *Cancer pagurus*

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ABSTRACT

There are few reports of bacterial diseases in crabs. A juvenile edible crab (*Cancer pagurus*) with a rickettsial-like infection was found in the intertidal zone at Freshwater East in South West Wales in July, 2012. Large numbers of bacteria-like particles were found in the haemolymph and within fixed phagocytes of the hepatopancreas. Molecular sequencing and subsequent phylogenetic analysis showed that the infectious agent was a member of the order Rhizobiales and therefore distinct to bacteria classified as rickettsia.

1. Introduction

Crustaceans are subject to a range of diseases caused by bacteria (Wang, 2011). While there are many reports of such diseases in some crustaceans such as shrimp (e.g. Austin, 2010) fewer reports have been published on these conditions in brachyuran crabs (Wang, 2011). Although the haemolymph of crabs often contains small numbers of viable bacteria (e.g. Welsh and Sizemore, 1985) their potential to cause sepsis is unknown (Smith et al., 2014).

Rickettsia and rickettsia-like organisms have been widely reported in a variety of crustaceans (e.g. Johnson, 1984; Edgerton and Prior, 1999; Cordaux et al., 2007; Nunan et al., 2013) and in a small number of brachyuran crabs including *Carcinus aestuarii (mediterraneus)* (Bonami and Pappalardo, 1980) and the European shore crab, *C. maenas* (Eddy et al., 2007). Rickettsia are Gram-positive, obligate, intracellular parasites and their inability to grow in standard bacteriological media has been used as evidence of their rickettsial-like nature but without any reliable approach to identification. Therefore, some of these aetiological agents, originally described as "rickettsia-like", may not belong to the order Rickettsiales (Wang et al., 2004; Wang, 2011). An exception to this is the finding that necrotizing hepatopancreatitis, a serious condition of shrimp, is caused by a newly-described bacterium, *Candidatus* Hepatobacter penaei, a member of the order Rickettsiales (Nunan et al., 2013).

This paper reports on a novel rickettsia-like infection found in a juvenile edible crab, *Cancer pagurus*, recorded as part of a wider disease survey already reported (Thrupp et al., 2015).

2. Materials and methods

The infected edible crab (an intermoult female of 44 mm carapace width) was collected from Freshwater East, South West Wales, U.K. (51°39′0″N, 4°52′0″W; Grid Reference SS016984) in July 2012 as part of a wider survey of disease prevalence (Thrupp et al., 2015). It was transported back to the laboratory in damp seaweed, placed in a sea water aquarium and examined *ca.* 24 h post-collection.

Haemolymph was collected aseptically from a jointed area at the base of a pereiopod and *ca.* 50 µl was examined using phase contrast microscopy. A further 100 µl of haemolymph was added to 100 µl of sterile marine saline (see Smith et al., 2014 for composition) and aliquots spread onto Petri dishes containing either tryptone soya agar (TSA + 2% sodium chloride) or thiosulphate citrate bile sucrose agar (TCBS + 1% sodium chloride) (Becton Dickinson, Oxford, UK). Plates were incubated for 24–48 h at 25 °C prior to examination. A further 50 µl haemolymph sample was placed in *ca.* 2 ml of ice-cold absolute ethanol and stored at −20 °C for later 16S rRNA sequence analysis.

The infected crab was injected with Davidson's seawater fixative and left for *ca.* 1 h. The hepatopancreas, gills and antennal gland were removed and placed in fresh Davidson's seawater fixative. These tissues were left in the fixative for *ca.* 24 h, after which the tissue was removed and placed in 70% ethanol until later dehydration and embedding in paraffin wax, as detailed previously (Smith et al., 2014). Blocks were sectioned at 7 µm and stained

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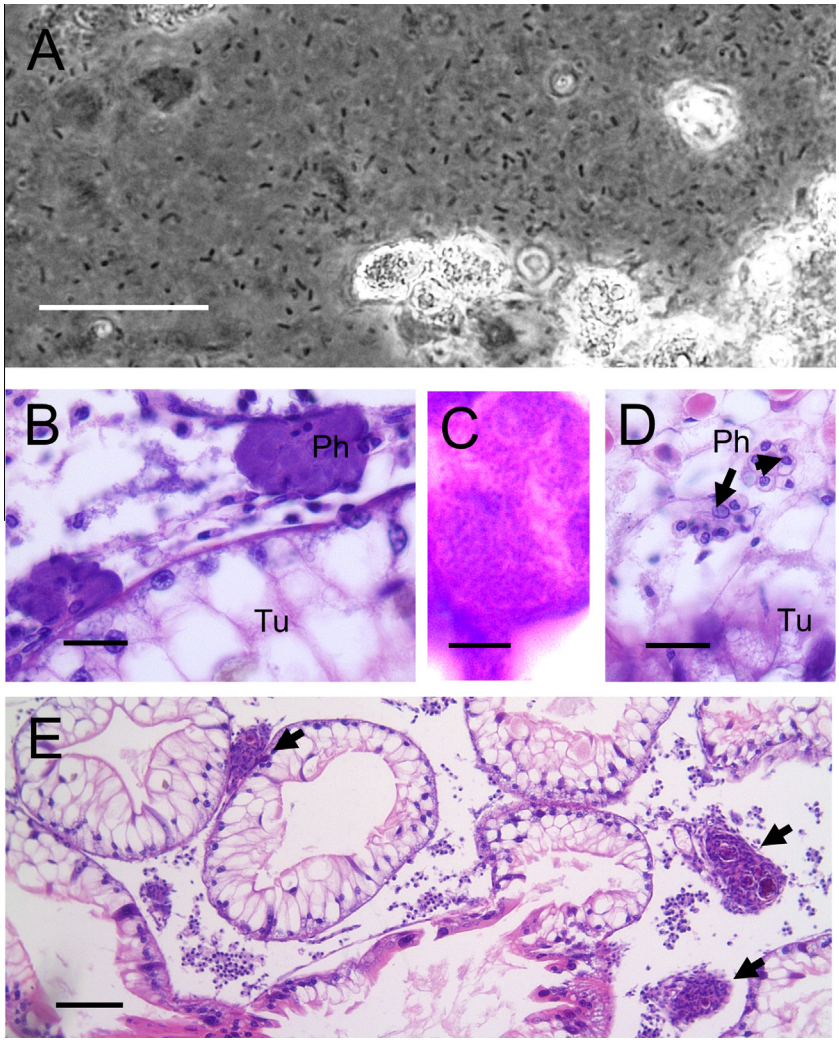

Fig. 1. (A) Phase contrast micrograph showing bacteria-like particles free in the haemolymph of the edible crab collected in July from Freshwater East. (B) Histological appearance of enlarged fixed phagocytes (Ph) in the interstitial space of the hepatopancreas from the affected crab shown in (A). Note hepatopancreatic tubule (Tu). (C) High power micrograph shows the presence of large numbers of bacteria-like particles within these fixed phagocytes. (D) Normal appearance of fixed phagocytes (Ph) in the interstitial space surrounding the tubules (Tu) of the hepatopancreas from an uninfected juvenile edible crab. (E) Low power micrograph of the hepatopancreas of the infected crab showing small numbers of nodules (arrows) in the interstitial spaces between the tubules (Tu). Note lack of damage to tubules. Scale bars = 25 μm (A, B, and D), 5 μm (C), 100 μm (E).

using Cole's haematoxylin and eosin. Sections were examined using an Olympus BX 41 microscope fitted with an Olympus SC30 camera.

To identify the bacteria-like particles observed in the haemolymph, 16S rRNA sequence analysis was undertaken on the infected haemolymph. The sample was centrifuged and the pelleted cells extracted and processed with a Qiagen DNeasy® Blood and Tissue Kit; incorporating an initial lysozyme-based disruption step optimised for Gram-positive bacteria, followed by proteinase K digestion as detailed in Whitten et al. (2014). Bacterial 16S rRNA was amplified by touchdown PCR. Cycling conditions were: 3 min at 94 °C followed by 33 cycles of 30 s at 94 °C, 30 s at 61.4 °C (−0.7 °C/cycle for the first 18 cycles and 49.5 °C thereafter), and 30 s at 72 °C, followed by 5 min at 72 °C. The primers were 968-f: 5′-ACGCGAAGAACCTTAC-3′ and 1401-r: 5′-CGGTGTGTACAA GACCC-3′ (Lindh et al., 2008), but with a modified C-rich GC clamp at the 5′ end of the forward primer to prevent total strand dissociation during ensuing electrophoresis: 5′-CGCCCGCCGCGCCCCGCG CCCGGCCCGCCGCCCCCGCCCC-3′. The resulting amplicons were then profiled using the PCR-temporal temperature gradient gel electrophoresis (TTGE) method to visualise the diversity of bacterial species in the DNA extract. Amplicons from the above PCR reaction were separated by TTGE using the BioRad DCodeTM Universal Mutation Detection System (BioRad Laboratories Inc., Hemel Hempstead, UK) with 7% polyacrylamide gels supplemented with 7.5 mol/L urea and 2% *v/v* glycerol. The optimised running temperature ramped from 55 to 68.6 °C at a rate of 0.8 °C/h at 69 V. The running buffer was 1.25x tris–acetic acid-EDTA buffer. A strong dominant band and a weak minor band was visualised by TTGE, indicating a single dominant bacterial species had infected the haemolymph. This band was excised and the DNA within was amplified by PCR using the above protocol and primers, but lacking the GC clamp. The resulting 403 bp 16S rRNA fragment was sequenced and then screened using the DECIPHER online analysis tool chimeras (http://decipher.cee.wisc.edu/FindChimeras.html; Wright et al., 2012). The terminal portion of the sequence was found to contain potential chimeras, so for the purposes of depositing the sequence with NCBI GenBank it was cropped to a chimera-free 276 bp fragment. Both the longer (403 bp) and cropped (276 bp) sequences were aligned to 16S rRNA sequences obtained from the NCBI GenBank database, using programmes within the Ribosomal Database Project (RDP Release 11; Cole