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Ulcerative enteritis in *Homarus americanus*: Case report and molecular characterization of intestinal aerobic bacteria of apparently healthy lobsters in live storage

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ABSTRACT

An intermoult male American lobster, *Homarus americanus*, with severe intestinal lesions was encountered while collecting samples of aerobic intestinal bacteria from lobsters held in an artificial sea-water recirculation aquarium system. Grossly, the intestine was firm, thickened, and white. Histologic examination revealed a severe, diffuse, ulcerative enteritis which spared the chitin-lined colon, somewhat similar to hemocytic enteritis of shrimp. The bacterial isolates from this lobster were compared to 11 other lobsters lacking gross intestinal lesions. Two organisms, one identified as *Vibrio* sp. and another most similar to an uncultured proteobacterium (98.9%), clustering with *Rhanella* and *Serratia* species using 16S rDNA PCR, were isolated from the intestines of the 11, grossly normal, lobsters and the affected lobster. An additional two intestinal isolates were cultured only from the lobster with ulcerative enteritis. One, a Flavobacterium, similar to *Lutibacter litoralis* (99.3%), possibly represented a previously described commensal of the distal intestine. The second, a *Vibrio* sp., was unique to the affected animal. While the etiology of the ulcerative enteritis remains undetermined, this report represents the first description of gross and histologic findings in *H. americanus* of a condition which has morphologic similarities to hemocytic enteritis of shrimp. An additional observation was a decrease in the number of intestinal isolates recovered from the 11 apparently healthy lobsters compared to that previously reported for recently harvested lobster. More comprehensive studies of the relationship between the health of lobsters, gut microbial flora and the husbandry and environment maintained within holding units are warranted.

1. Introduction

The American lobster (*Homarus americanus*) fishery has historically provided a sustainable economic resource for coastal communities along the north eastern seaboard of the United States of America and the Atlantic provinces of Canada. Annual lobster landings from 1990 to 2004 ranged between 40–50,000 t in Canada and 30–40,000 t in the USA (Canada: Agriculture and Agri-Food Canada (2006). Benchmarking study on Canadian Lobster. http://www.ats.agr.gc.ca/can/4217_e.htm). Post-harvest, lobsters may be held in specialized storage facilities from a few days to months prior to either being sold for processing or shipped live to satisfy the needs of specific markets. Lobster holding facilities vary greatly within the industry but all attempt to maintain product quality for future sales. Empirically, the most efficient holding facilities maintain lobsters at temperatures <2.5 °C which reduces the lobster's metabolic activity and therefore they do not require feeding during holding. Regardless of these endeavors, mortalities approaching 10–15% are anecdotally reported by the industry.

Most losses apparently occur from a combination of handling stress and high density storage. Common diseases observed under holding conditions include: vibriosis, caused by mixed *Vibrio* infections (Brinkley et al., 1976; Tall et al., 2003); gaffkemia, caused by the bacterium *Aerococcus viridans* var. *homari* (Rabin, 1965; Stewart et al., 1969; Battison et al., 2004); bumper car disease, caused by the ciliate *Anophryoides haemophila* (Aiken et al., 1973; Cawthorn et al., 1996; Greenwood et al., 2005); and impoundment shell disease, caused by an assemblage of various chitinolytic bacteria (Hess, 1937; Malloy, 1978).

During collection of aerobic intestinal bacteria from 12 apparently healthy lobsters held in a recirculating artificial seawater system, an animal with marked gross abnormalities of the intestinal tract was encountered. To further investigate the cause of lesions, tissues were collected for histologic examination and the bacterial isolates from the affected lobster and the unaffected animals were compared using a combination of biochemical phenotypic assays and 16S rDNA PCR identification. While no definitive etiology for

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the intestinal lesions was determined, bacteria unique to this lobster were identified and a newly recognized condition in the American lobster is described. In addition, notable variation in the type and numbers of bacteria recovered from the remaining 11 lobsters compared to that in previous reports was noted (Stewart and Zwicker, 1972). The possible effects of long-term, live holding, on microbial diversity and crustacean industries as a whole are also discussed.

2. Materials and methods

2.1. Animals and housing

All protocols used in the study were approved by the Animal Care Committee of the University of Prince Edward Island, Canada in accordance with guidelines of the Canadian Council on Animal Care. All lobsters were obtained from a commercial supplier (Clearwater Fine Foods Inc., Bedford, NS, Canada). Ten lobsters had been held in a flow through system for one week after capture without feeding prior to arrival at the Atlantic Veterinary College Lobster Science Centre (AVCLSC). The two remaining lobsters had been in the holding facilities at the AVCLSC for approximately 15 months.

All lobsters were maintained in individually compartmentalized plastic trays in a recirculating artificial sea-water (Instant Ocean®, Aquarium Systems Inc., Mentor, OH, USA) aquarium system between 1.5 and 2.5 °C with a 300 μ filter. Lighting was set at a 14 h/10 h low light (<3 lux)/dark cycle. Lobsters were not fed during the holding period. Water quality was evaluated weekly to ensure that the biofilter was maintaining the ammonia (0.0–0.3 ppm, measured as total ammonia nitrogen), nitrate (10–40 ppm), nitrite (0.0–0.6 ppm) (FasTest, Aquarium Systems Inc., Mentor, OH, USA), dissolved oxygen (9.7–12.9 mg/l) (YSI 550DO, Yellow Springs Instruments, Mentor, OH, USA), salinity (30 ± 2 ppt) and pH (7.41–8.48) within the listed limits. Dissolved oxygen concentrations and water temperature were also monitored and recorded on a continuous basis (CR7 Measurement and Control System, Campbell Scientific Inc., Logan, UT, USA).

2.2. Necropsy and sample collection

Lobsters were euthanized by potassium chloride injection and examined for gross external and internal lesions (Battison et al., 2000). Appropriate tissues were collected from any lobsters with gross lesions and fixed in a 1% glutaraldehyde: 4% formalin solution, sectioned (5 μm), and processed routinely for hematoxylin and eosin staining (Howard and Smith, 1983). Additionally, Gram, acid-fast (Ziehl Neelsen), and Warthin–Starry silver staining was performed to identify bacteria when required.

2.3. Bacterial isolation, culture conditions and phenotypic characterization

Swabs of the proximal and distal intestine were collected from all lobsters. Swabs were also collected from the hepatopancreas and gill of a single lobster which had gross lesions at necropsy. All swabs were streaked onto sheep blood (5%) agar supplemented with 2% NaCl (BA) and onto TCBS (Thiosulfate–Citrate–Bile Salts) agar and incubated at 28 °C for 72 h. Plates were examined daily and growth recorded. Single, well defined colonies (representing all distinguishable colony morphology types) were re-streaked onto BA and MacConkey's agar at 28 °C. Biochemical profiles of all bacterial isolates were evaluated using the commercial Analytical Profile Index-API 20 NE (bioMériux Canada Inc., Montreal, PQ, Canada). Isolates were also inoculated into 2% skim milk and stored at −80 °C. These samples were later thawed and streaked onto trypticase soy agar (TSA, Oxoid Inc., Nepean, ON, Canada) at 28 °C and used to create stock cultures stored at −80 °C in trypticase soy broth (TSB, Oxoid Inc., Nepean, ON, Canada) supplemented with 15% (v/v) glycerol. Stock cultures were used for DNA extraction studies.

Previously identified bacterial isolates, *Bacillus* sp. 1975 and *Pseudomonas perolens* (Halifax strain) from the intestinal tracts from normal healthy lobsters (Stewart and Zwicker, 1972) were kindly provided as historical lobster intestinal reference isolates by Dr. J.E. Stewart, Department of Fisheries and Ocean, Bedford Institute of Oceanography, Dartmouth, NS, Canada.

2.4. Genomic DNA extraction, polymerase chain reaction and sequencing

Individual colonies for each morphological isolate were picked from TSA plates for inoculation into TSB supplemented with 3% (w/v) NaCl and incubated at 28 °C for 24 h. DNA was extracted using the GenElute™ Bacterial Genomic DNA kit (Sigma–Aldrich Ltd., Oakville, ON, Canada) and assessed in a 0.8% agarose gel containing 0.5 μg ml^{-1} ethidium bromide.

All bacterial isolate 16S rDNA genes were amplified using modified versions of the universal bacterial primers BSF-8/20 (5′-AGAGTTTGATCCTGGCTCAG-3′), positions 8–27 in the *Escherichia coli* 16S rDNA gene and BSR-1541/20 (5′-AAGGAGGTGATCCAGCCGCA-3′), *E. coli* positions 1541–1522 (polylinkers removed from both primers) (Weisburg et al., 1991; Daly et al., 1993; Wuyts et al., 2002). Approximately 1–50 ng of genomic DNA was amplified in a 50 μl reaction using 10 pmol of each primer, BSF-8/20 and BSR-1541/20, 200 μM of each dNTP (A, G, C and T), 1.5 mM $MgCl_2$, 1× PCR buffer (10 mM Tris, pH 8.8, 50 mM KCl and 0.08% Nonidet P40) and 1.25 U of Taq DNA polymerase (Fermentas International Inc., Burlington, ON, Canada). A negative control of molecular biology grade water (Sigma–Aldrich Ltd.) was used in each run. Amplification was performed in a MJ Research PTC-200 thermal cycler (MJ Research Inc., Waltham, MA, USA) under the following conditions: initial denaturation at 94 °C or 2.5 min, followed by 25 cycles of denaturation at 94 °C or 1 min, annealing at 45 °C for 1 min, and extension at 72 °C or 1.5 min. Final extension was at 72 °C for 5 min. Amplified DNA was purified using a GenElute™ PCR clean-up kit (Sigma–Aldrich Ltd.) and separated in a 0.8% agarose gel containing 0.5 μg ml^{-1} of ethidium bromide. Purified PCR amplicons were sequenced at the Guelph Molecular Supercentre (Laboratory Services Division, University of Guelph, ON, Canada) on an ABI Prism 377 sequencer using Big-Dye™ terminators (Applied Biosystems Inc., Foster City, CA, USA). Amplicons were sequenced in both directions using primers from the European Ribosomal RNA Database (http://oberon.fvms.ugent.be:8080/rRNA/index.html). Forward sequencing primers used were BSF-8/20 and BSF-784/16 (5′-AGGATTAGATACCCTG-3′) *E. coli* positions 784–799 and reverse sequencing primers used were BSR-1541/20 and BSR-799/16 (5′-CAGGGTATCTAATCCT-3′) *E. coli* positions 799–784.

Sequences were aligned using the Clustal W application in BioEdit (Hall, 1999). All 16S rDNA sequence electropherograms were carefully examined to ensure that only single peaks and therefore single species DNA was amplified and sequenced (Drancourt et al., 2000). Sequences were further screened for the presence of chimeric sequences using the program Chimera Check (Cole et al., 2005).

2.5. Phylogenetic analysis

The Ribosomal Database Project-II (RDP-II) programs (Cole et al., 2005) were used to find similar sequences and phylogenetic trees were constructed using MEGA version 3.1 (Kumar et al., 2004) using neighbour-joining (Kimura 2-parameter with gaps and missing data handled by complete deletion) and maximum