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Hahella chejuensis is the etiological agent of a novel red egg disease in tilapia (*Oreochromis* spp.) hatcheries in Thailand☆

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ABSTRACT

The present study is the first report of a novel disease called *Hahellosis* or "red egg disease" that has been affecting tilapia (*Oreochromis* spp.) hatcheries in Thailand. *Hahella chejuensis*, a red pigmented Gram negative bacterium, was recovered from samples of the red egg and identified to species level based on 99.5%–99.7% nucleotide homology to 16S rDNA of the type strain *H. chejuensis* KCTC 2396. Experimental infection of eggs indicated that *H. chejuensis* was able to cause red egg disease and also reduce their hatching. PCR protocols were developed for detection of *H. chejuensis* in tilapia samples including eggs and ovaries and testes of tilapia broodstocks. Positive signals were obtained in the ovaries and testes of tilapia broodstocks with *in situ* DNA hybridization using probes specific for *H. chejuensis*, suggesting possible vertical transmission of the red pigmented bacteria from broodstock to eggs.

Statement of relevance: The authors strongly believe that our manuscript would provide significant knowledge to fish aquaculture especially to that of the tilapia (*Oreochromis* spp.) hatcheries.

1. Introduction

Tilapia (*Oreochromis* spp.) is a highly valuable freshwater fish which is commercially farmed in Thailand as well as other Asian countries (Bhujel, 2011; Fitzsimmons et al., 2011). Intensification of tilapia production has seen an increase in the number of infectious disease outbreaks, which are principally caused by bacterial pathogens such as *Streptococcus agalactiae* (Dong et al., 2015c; Kayansamruaj et al., 2014; Suanyuk et al., 2008), *Francisella noatunensis* subsp. *orientalis* (Jeffery et al., 2010; Nguyen et al., in press; Soto et al., 2012), *Aeromonas veronii*, and *Flavobacterium columnare* (Dong et al., 2015a, 2015c; Figueiredo et al., 2005). Most studies generally focused on diseases in grow-out tilapia, but information is lacking regarding disease problems in eggs.

Occurrence of a novel "red egg syndrome" was first observed in red tilapia (*Oreochromis* sp.) in the middle part of Thailand in 2000. Recently, it has affected both red tilapia and Nile tilapia (*Oreochromis niloticus*) hatcheries. The syndrome was characterized by a change in the color of the eggs from normal yellowish to a reddish color during incubation. All egg stages before hatching can be affected in which the eggs turned to red and eventually failed to hatch. According to producers, the problem has been considered as an emerging infectious disease in tilapia eggs, especially during the cold season (<24 °C) in Thailand from early December to mid-February. Cumulative loss of fry production was recorded to be 10% and increased to 50% during very cold weather in Thailand. Affected hatchery owners suggest that up to 50% of eggs in some batches were lost due to "red egg syndrome" during an unusually cold period between December 2013 and March 2014 and that mortality has continued at reduced levels in 2014 and 2015. Since the causative agent is undetermined, this study, therefore, aimed to 1) identify the cause of "red egg syndrome" and to 2) develop DNA-based detection methods for the pathogen.

☆ The GenBank accession numbers for sequences reported in this paper are KT971015–KT971018.

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2. Materials and methods

2.1. Tilapia eggs and broodstocks

Apparently normal (yellow eggs) and abnormal (red eggs) (Fig. 1a–b) were collected from both Nile tilapia (*O. niloticus*) and red tilapia (*Oreochromis* sp.) in two affected hatcheries in Prachinburi province during November, 2014 to February, 2015. Each batch contained 30–50 eggs. The eggs were packed in plastic bags containing rearing water, kept in a cool foam box (8–10 °C) and transported to our laboratory (Centex Shrimp, Mahidol University). The ovary and testis samples were collected from five pairs of male and female Nile tilapia broodstocks. The samples were preserved in absolute alcohol for PCR analysis and 10% buffered formalin for *in situ* hybridization. Extraction of DNA from tilapia eggs and tissues was performed using a conventional phenol–chloroform extraction method. Extracted DNA was adjusted to the concentration of 100 ng μL^{-1}.

2.2. Rapid staining, bacteria isolation, and phenotypic tests

Four to five eggs from normal or red egg samples were separately homogenized in a 1.5 mL microtube using a disposable polypropylene pestle. The lysates were then used for rapid Gram staining and bacterial isolation. Bacterial isolation was performed by streaking the sample directly on tryptic soy agar (TSA, Difco, USA) plates supplemented or without 1.5% NaCl. Streaked plates were incubated at 30 °C for 24 to 48 h. Four red pigmented bacterial isolates obtained from red egg samples (see below) were further cultured for purification and then subjected to Gram staining, conventional biochemical (oxidase and catalase) tests, and 16S rDNA sequence analysis.

2.3. 16S rDNA sequencing and phylogenetic analysis

Genomic DNA of four pure red pigmented bacterial isolates, designated HN01–HN04, was extracted using a simple boiling method as previously described by Dong et al., (2015c). Amplification of a 16S rDNA fragment (~1.5 kb) was carried out using universal primers Unibact-F and Unibact-R (Table 1) (Weisburg et al., 1991). The PCR mixture consisting of a 25 μL volume composed 0.25 μM of each primer, 0.2 mM of dNTPs, 0.25 μM of $MgCl_2$, 1 unit of Platinum Taq polymerase (Invitrogen), 1× reaction buffer, 3 μL of DNA template, and nuclease-free water. The thermocycler conditions were designed as follows: 94 °C for 5 min, 30 cycles of 94 °C for 40 s, annealing at 50 °C for 40 s, and extension at 72 °C for 1 min. Amplified products were visualized using 1.0% agarose gel electrophoresis and subjected to purification using a Favogen Gel/PCR Purification Kit (Taiwan) prior to cloning into pPrime cloning vector (5PRIME). Plasmid DNA purification using a FavoPrep Plasmid Extraction Mini Kit was performed with recombinant clones to confirm the presence of the inserted gene by colony PCR (data not shown). DNA sequencing was carried out by 1st BASE Pte Ltd. (Malaysia) using T7 and SP6 sequencing primers. Assembly of forward and reverse sequences was carried out using ContigExpress software. The homology search of 16S rDNA sequences was performed using Nucleotide BLAST program from National Center for Biotechnology Information (NCBI).

Following multiple alignment (Clustal W method, MEGA 6.0) of 4 sequences of red pigmented bacterial isolates in this study and their closed taxa including *Hahella chejuensis* KCTC 2396 (NR074812), *H. antarctica* IMCC3113 (NR044254), *H. ganghwensis* FR1050 (NR043091), *Endozoicomonas atrinae* (KC878324), *Kistimonas asteriae* KMD 001 (NR116386), and *Zooshikella ganghwensis* JC2044 (NR025668), the neighbor-joining phylogenetic tree was constructed based on 1463 bp of 16S rDNA sequences (positions 29–1491 *Escherichia coli* numbering) using p-distance model with pair wise delete option of MEGA 6.0 software. Bootstrap value was designed as 1000 replicates.

2.4. Experimental challenge

Apparently healthy fertilized Nile tilapia eggs ($n = 400$) were obtained from a hatchery, which had never experienced "red egg syndrome", for the experimental challenge. The eggs were distributed into two groups containing 200 eggs each. Prior to challenge test, a single colony of *H. chejuensis* HN01 was inoculated in 10 mL tryptic soy broth (TSB) supplemented with 1.5% NaCl, incubated at 30 °C with shaking (250 rpm) for 18 h. One milliliter of bacterial culture was then transferred to a flask containing 300 mL of TSB and incubated as described for 10 h. Bacterial cells were collected by centrifugation at 12,000 rpm for 15 min, washed twice with sterile 0.85% NaCl water, and resuspended in 300 mL of de-chlorinated water adjusted to 3 ppt salinity with artificial marine salt. The concentration of bacteria used in the challenge was determined retrospective as 2.7×10^4 CFU mL^{-1} using a conventional plate counting method (Table 2). The eggs were immersed in 300 mL of bacterial suspension (challenge group) or bacteria-free salt water (control group) for 36 h before being transferred to a circulated tank system that contained a small glass tank (1 L) inside a 10 L plastic container. Circulating water was controlled by a pump that allowed the eggs to gently circulate in the small inner tank. Hatching and number of red eggs present were recorded for 3 days. Water temperature during the experiment was maintained at 23 ± 0.5 °C in an air-condition room to mimic cold weather in Thailand. At the end of the experiment, 5 red eggs in the challenge group and 5 eggs in the control group were subjected to *Hahella*-specific PCR assay (see below).

2.5. Development of PCR detection methods

Two primer sets (HF & HR and HCH-F & HCH-R), which specifically targeted to 16S rDNA fragment of *Hahella* genus and a HCH_06026 gene locus (phosphoenolpyruvate synthase/pyruvate phosphate dikinase coding gene located in prodigiosin biosynthesis gene cluster) of *H. chejuensis* species, respectively were designed using Primer3 and Primer Blast (NCBI) (Table 1). PCR reaction mixture included each pair of primers, *H. chejuensis* DNA template, and other reaction components

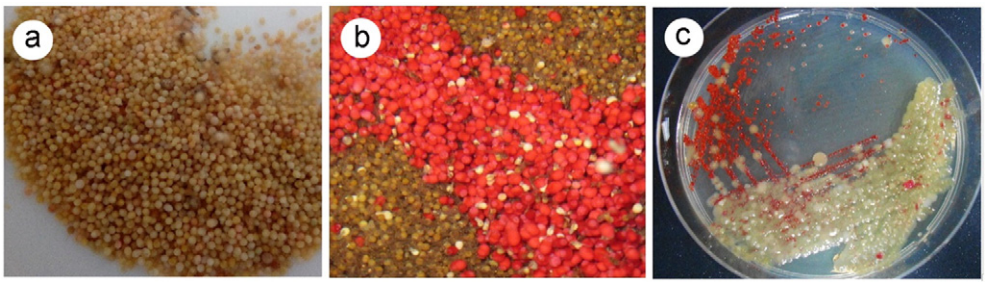

Fig. 1. Tilapia eggs and isolated bacteria. Normal looking tilapia eggs (a), red eggs (b), and bacterial colonies streaked from red eggs grown on TSA plate at 30 °C overnight (c).