# Francisella halioticida

Research article

# Francisella halioticida, Identified as the Most Probable Cause of Adductor Muscle Lesions in Yesso scallops Patinopecten yessoensis Cultured in Southern Hokkaido, Japan

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**ABSTRACT**—The occurrence of orange/pinkish colored lesions in the adductor muscle of Yesso scallops Patinopecten yessoensis has been known for many years in Japan; however, determination of the causative agent has not been adequately investigated. Histological examination of affected scallops in southern Hokkaido typically revealed intense host responses: hemocyte infiltration, an abundance of necrotic hemocytes, lysis of muscle fibers and in some instances melanin deposits when the lesions occurred adjacent to the shell. Microbiota analysis showed that Francisella halioticida was dominant in the lesions, and in situ hybridization using F. halioticida specific probes also confirmed the presence of this bacterium within the lesions. A F. halioticida specific PCR assay detected this bacterium in the majority of scallop lesions tested. Subsequently, three bacterial isolates were obtained from scallop lesions on modified Eugon agar supplemented with antibiotics, and these bacterial isolates were found to be F. halioticida by 16S rRNA and rpoB gene sequences. These results suggest that infection with F. halioticida is the most likely cause of the adductor muscle lesions observed in Yesso scallops. Field surveys conducted in 2017 of scallops cultured in southern Hokkaido showed that the presence of adductor muscle lesions putatively caused by F. halioticida was significantly related to mortalities and poor growth of scallops.

**Key words:** Francisella halioticida, lesion, adductor muscle, Patinopecten yessoensis, Yesso scallop, etiology, aquaculture, epidemiology

The occurrence of orange/pinkish abscess lesions in the adductor muscle of Yesso scallops Patinopecten yessoensis from northern Japan has been known for many years, resulting in reduced marketability. Egusa (1974) detected Vibrio sp. and Iida et al. (1980a) isolated group F Vibrio, V. alginolyticus, V. anguillarum as well as Pseudomonas sp. from these lesions. Through various experimental challenges, Iida et al. (1980b) hypothesized that environmental bacteria, including Vibrio spp. and Pseudomonas sp., invaded via injuries and caused the lesions in the adductor muscle. On the other hand, Kosaka and Yoshimizu (1999) demonstrated that similar adductor muscle lesions could be reproduced via inoculating healthy scallops with a 0.22 μm filtrate prepared from lesion homogenates, thus suggesting the involvement of a viral pathogen.

A similar disease involving adductor muscle lesions, poor growth and mortalities was reported during the late 1980s and 1990s, among cultured Yesso scallops from western Canada and at the time intracellular bacterium or viral infection was also speculated to be the cause (Bower et al., 1992; Getchell et al., 2016). Recently, a study by Meyer et al. (2017) detected infection with Francisella halioticida in scallop lesions by PCR and in situ hybridization and concluded that F. halioticida was the most probable etiogical agent.

Given that the observations from the current study shared many similarities with the cases reported from western Canada, we decided to test adductor muscle lesions from Yesso scallops cultured in Japan for the presence of F. halioticida which was originally described as the cause of mass mortalities among cultured giant abalone Haliotis gigantea in Japan (Kamaishi et al., 2010; Brevik et al., 2011).

In the present study, histopathology and molecular assays are used to assess the involvement of F. halioticida in the adductor muscle lesions of Japanese scallops. Additionally, the relationships among lesions, growth and mortality of cultured scallops are also exam-

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ined in southern Hokkaido, Japan.

## Materials and Methods

*Histology*

Scallops having abscess lesions in the adductor muscle were collected from anonymous scallop farms in southern Hokkaido from July 2016 to August 2017 (*n* = 78). Visible lesions were excised and preserved in Davidson's fixative for 2–7 days, and processed using routine histology techniques for paraffin embedding. Tissue sections were cut at 5 µm and 3 µm thickness and stained with Mayer's Hematoxylin and Eosin or May Grünwald Giemsa stain, respectively.

*Microbiome analysis in the lesions*

Abscess lesions (*n* = 7) and tissues of adductor muscle without lesions (*n* = 2) were collected in September 2016, November 2016, January 2017 and April 2017, and preserved in 70% ethanol. DNA was extracted using a DNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, and the samples were shipped to an external sequencing facility, Takara Bio, for microbiota analysis as described in Takagi *et al.* (2018).

Briefly, after further purification of DNA using AMPureXP (Bechman Coulter), DNA was quantified by Quant-iT dsDNA HS Assay Kit (Invitrogen), and DNA libraries were constructed by two step PCRs using a 16S (V3-V4) Metagenomic Library Construction Kit for NGS (Takara Bio) to amplify the V3-V4 region of the 16S rRNA gene, and Nextera XT Index Kit (Illumina) to add the index barcode sequences for Illumina sequencer. The constructed libraries were subjected to the sequencing of paired-end 250 bases using the MiSeq Reagent Kit v3 on the MiSeq (Illumina).

Processing of sequence data, including chimera check, operational taxonomic unit (OTU) definition, and taxonomy assignment was performed using QIIME ver. 1.8, CD-HIT-OUT ver. 0.0.1 and RDP classifier ver. 2.2 with GreenGenes database.

The relative abundance of each bacterial species/ group was defined as the total number of reads divided by those in the entire bacteria community in samples.

*In situ hybridization (ISH)*

To confirm infection with *F. halioticida*, ISH was conducted on adductor muscle and digestive gland tissue with visible lesions collected in November 2016, following the procedures published by Kamaishi *et al.* (2010). Briefly, 5 µm tissue sections were deparaffinized and rehydrated in serial dilutions of ethanol and distilled water. After treatment with 0.24 mg/mL of Proteinase K (Takara Bio) in PBS supplemented with 0.1% Tween 20 at 37°C for 15 min, hybridization solution containing the oligonucleotide probes, Megai-110r, Megai-230r and Megai-870r (Kamaishi *et al.*, 2010),

labelled with DIG Oligonucleotide Tailing Kit 2nd Generation (Sigma-Aldrich), was placed on the sections. The sections were heated at 100°C for 3 min, immediately cooled on ice and incubated at 42°C overnight. Hybridized products were visualized using DIG Nucleic Acid Detection Kit (Sigma-Aldrich) according to the manufacturer's protocol.

*Detection of* Francisella halioticida *by PCR*

To assess the involvement of *F. halioticida* with adductor muscle lesions, a total of 76 adductor muscle tissue samples (29 from scallops with lesions and 47 from healthy scallops) were excised and preserved in 70% ethanol. DNA extraction was conducted as described above, and the PCR assay for detection of *F. halioticida* was conducted using the specific PCR primers, Megai-60 and Megai-480r, developed by Kamaishi *et al.* (2010). The final volume of the PCR reaction was 20 µL containing 1 × Takara Ex Taq Buffer (Takara Bio), 0.2 mM dNTP, 20 pmol of each PCR primer, 0.1 µL of Takara Ex Taq Hot Start version (Takara Bio) and 1.0 µL of the extracted template DNA. The thermal cycler program consisted of an initial denaturation at 94°C for 4 min, 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 60 s, and a final extension at 72°C for 7 min. The positive control of *F. halioticida* DNA was extracted from a Shimane isolate of giant abalone *Haliotis gigantea* kindly provided from Dr. Matsuyama, National Research Institute of Aquaculture, Japan. Visualization of the PCR products was conducted as described above. The odds ratio of *F. halioticida* detection to the adductor muscle lesions was calculated, and 95% confidential interval of the odds ratio was statistically calculated according to Szumilas (2010).

*Isolation of* Francisella halioticida

Abscess lesions of adductor muscle were collected in July 13 and August 23, 2017, and spread onto a modified Eugon agar (MEA) prepared according to Kamaishi *et al.* (2010). In order to selectively isolate *Francisella* sp., ampicillin (50 µg/mL) and polymyxin B (100 units/mL) were added to the agar medium (Soto *et al.*, 2009). Plates were incubated at 15°C for 12–17 days, and resulting colonies were then subcultured on MEA without antibiotics and incubated at 20°C for 8 days. Subcultured bacterial colonies were smeared on a glass slide, and stained with Gram stain kit (Muto Pure Chemicals) for microscopic observation.

For molecular identification of the three isolates obtained, one colony from each isolate was aseptically picked up from subculture MEA plates and used for DNA extraction using a DNeasy Mini Kit (QIAGEN). PCR was conducted using the previously published primer pairs, 27F-1492R (Frank *et al.*, 2008), and Fh-rpoB/F-Fh-rpoB/R (Brevik *et al.*, 2011), designed to amplify the bacterial 16S rRNA genes and DNA-directed

RNA polymerase beta subunit (*rpoB*) of *F. halioticida*, respectively.   The final volume of both PCR reactions was 20 $\mu$L containing 1 × Takara Ex Taq Buffer (Takara Bio), 0.2 mM dNTP, 0.1 $\mu$L of Takara Ex Taq Hot Start version (Takara Bio) and 1.0 $\mu$L of the extracted template DNA.   Amounts of PCR primers were 12 pmol for 27F-1492R, and 20 pmol for Fh-rpoB/F-Fh-rpoB/R. The thermal cycler program for 16S rRNA gene amplification consisted of an initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1.5 min, and a final extension at 72°C for 7 min, and that for *rpoB* gene consisted of an initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, 52°C for 45 s and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were visualized on a 1.5% agarose gel stained with SYBR Safe (Invitrogen), and the bands of approximately 1,400 bp for 16S rRNA and 1,000 bp for *rpoB* gene were extracted using FastGene Gel/PCR Extraction Kit (Nippon Genetics).   The extracted PCR products were sent to an external sequencing facility (Eurofins Genomics) and sequenced using the primers used for each PCR.   The obtained sequences were compared to BLAST database provided in NCBI.

*Field survey*

From January to March 2017, Yesso scallops (1-yr old) were collected from 11 different scallop farms located in southern Hokkaido.   At these sites, scallops are cultured using the vertical ear-hanging method, in which approximately 250 scallops are attached to each rope.   A total of 44 ropes were examined (two to six ropes from each site).   After counting all of the attached shells the survival rate was calculated for each rope. Then, all surviving scallops were shucked and examined macroscopically for the presence of the adductor muscle lesions.   Lesion samples collected from the field survey were preserved in 70% ethanol and subsequently tested for *F. halioticida* using the PCR assay described above. The correlation between survival rate and prevalence of the adductor muscle lesion was statistically analyzed using Spearman's rank correlation test ($\alpha = 0.05$).

The relationship between scallop growth and the presence of the lesions was examined for 24 ropes selected from six farms.   After the shell length of all surviving individuals was measured, scallops were then shucked and examined macroscopically for the presence of the adductor muscle lesions.   The mean shell length of healthy and diseased scallops in each farm was statistically analyzed using Student *t*-test ($\alpha = 0.05$).

## Results

*Histology*

The size, shape and color of the lesions observed in the adductor muscle were variable (Fig. 1).   Typically, the lesions ranged from 5–10 mm in diameter and were
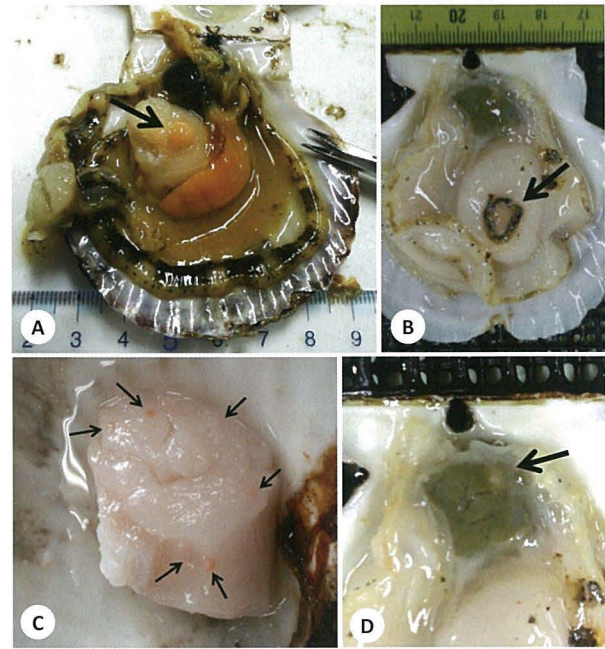


**Fig. 1.**   Yesso scallop *Patinopecten yessoensis* with abscess lesions.   **A**, A large orange lesion in the adductor muscle of scallop (arrow).   The lesion was filled with creamy viscous liquid.   **B**, The lesion adjacent to the shells was brown colored around the peripheral areas (arrows).   **C**, Multiple small orange lesions in the adductor muscle (arrows).   **D**, Yellowish lesion in the digestive gland (arrow).

filled with orange to pink colored pus (Fig. 1A).   Some lesions that occurred adjacent to the shell were brown colored around the peripheral areas, and easily detached from the adductor muscles (Fig. 1B).   In some instances, multiple small lesions, ranging from 1–2 mm in diameter, were observed (Fig. 1C), and on rare occasions, lesions were also present in the digestive gland (Fig. 1D).

The histopathology associated with adductor muscle lesions was characterized by severe hemocyte infiltration, accumulation of necrotic cells and degradation of the peripheral muscle fibers (Fig. 2A and B). Hemocytes engulfing necrotic cells (presumably hemocytes) were commonly observed in the central part of the lesion (Fig. 2C).   On rare occasions severe hemocyte infiltration and accumulation of necrotic cells were observed in the digestive gland, and surrounding connective tissue and the digestive diverticula appeared to be disintegrated and necrotic (Fig. 2D).   In lesions occurring adjacent to the shell, large deposits of brown-yellow particles were observed (Fig. 2E), which stained a green color with May-Grünwald Giemsa stain (Fig. 2F), indicating the accumulation of melanin particles.   Under this melanized surface, epithelial-like cells were observed.

During this study, no protozoan parasites or bacterial infections were detected via histological examination
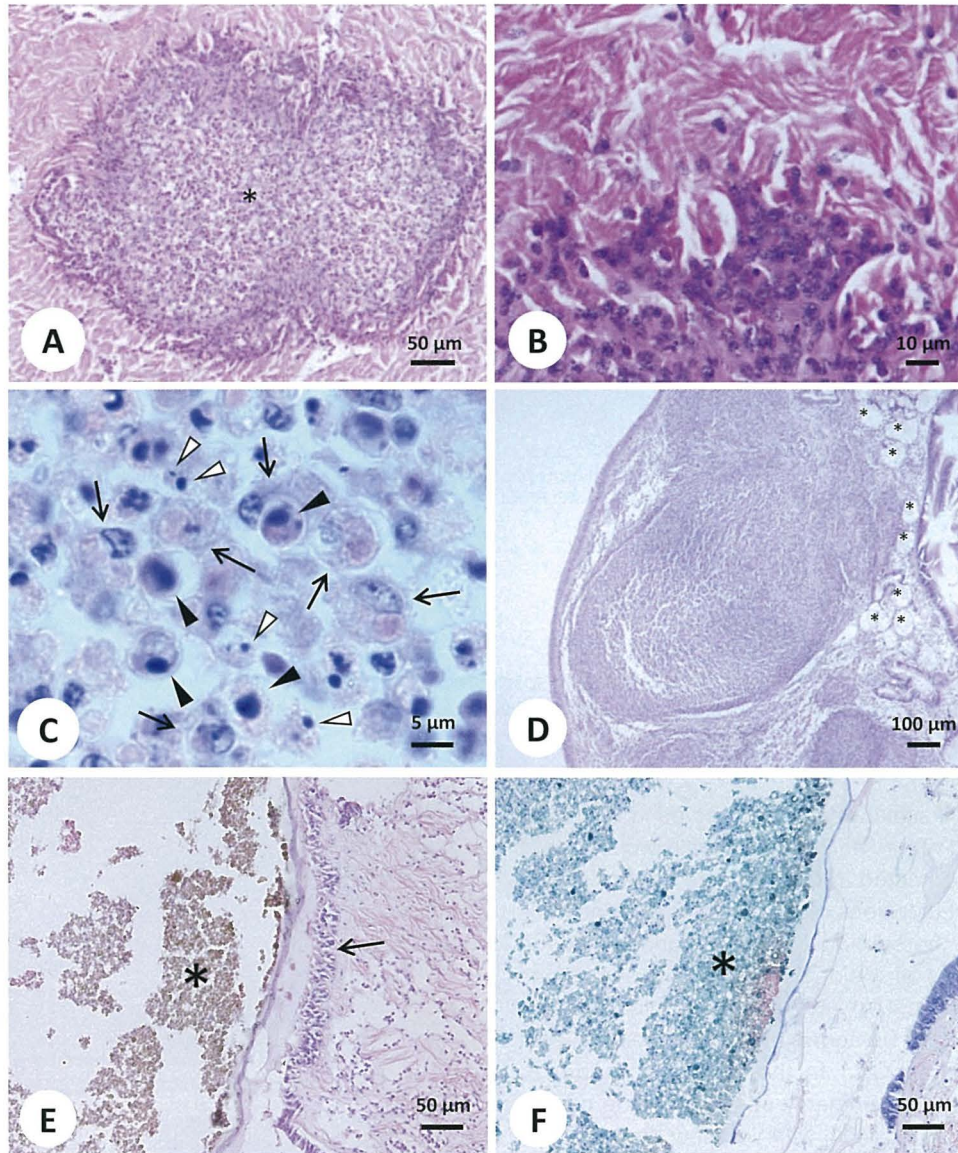
**Fig. 2.**  Histological observations of lesions in the adductor muscle and the digestive gland.   **A**, A lesion (*) in the adductor muscle stained with H&E.   **B**, Infiltration of hemocytes and degradation of muscle fibers were observed at the periphery of the lesion in the adductor muscle.   H&E stain.   **C**, Inner part of the lesion in the adductor muscle.   Hemocytes engulfing necrotic cells (arrows), small nuclei (presumably fragmented nuclei or bacterial cells) (white arrow heads) and necrotic cells with dense chromatin (black arrow heads) were observed.   May-Grünwald Giemsa stain.   **D**, A lesion in the digestive gland.   H&E stain.   Asterisks represents the necrotic digestive tubules.   **E**, An adductor muscle lesion adjacent to the shell.   Brown particles (asterisk) were deposited around the lesion, and the epithelium layer (arrow) was newly formed.   H&E stain.   **F**, An adductor muscle lesion adjacent to the shell.   Deposited particles (asterisk) stained green with May-Grünwald Giemsa stain.

that would explain the cause of the lesions.   In addition, no histopathology was observed in the gonad, mantle or gill tissues.

*Microbiome analysis in the lesions*

Illumina MiSeq sequencing platform yielded 3,028,728 reads over all nine samples for microbiota analysis in the lesion (86,234–1,199,650 reads per individual), and in total 61 operational taxonomic units (OTUs) were detected from the samples.   *Francisella halioticida* was the most abundant bacterial DNA

sequence detected in six out of seven samples with lesions (Fig. 3).   Although *Moritella* spp. was the most abundant bacteria detected in sample No. 6, *F. halioticida* was still present in this sample; albeit at a very low level (0.65%) and therefore not visible on the graph (Fig. 3).   In the adductor muscle samples without lesions (Fig. 3, No. 8 and 9) the most abundant sequence detected was that of an unidentified bacterium associated with *Thyasira* cf. *gouldi*, while the sequence for *F. halioticida* was not detected.
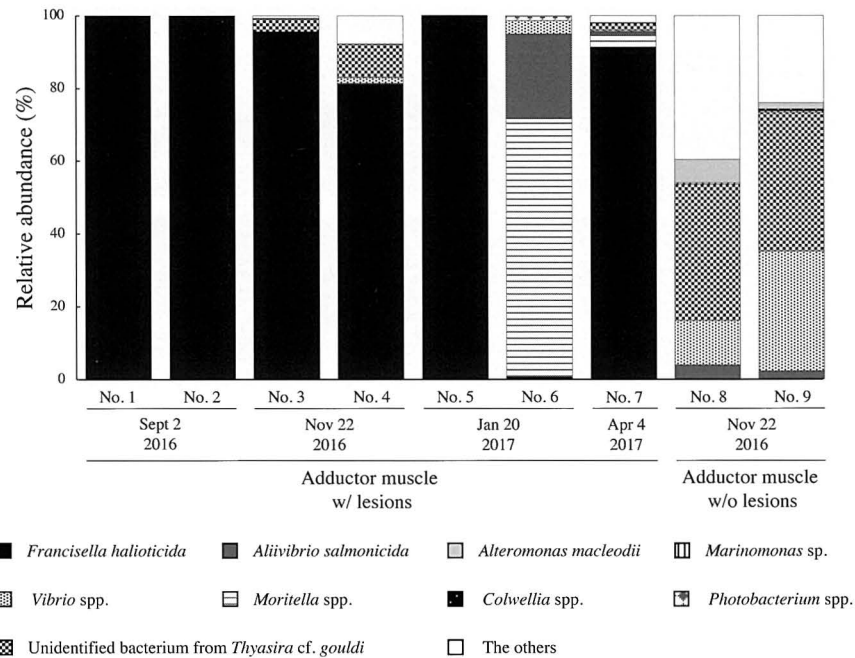
**Fig. 3.** Quantitative profiles of 16S rRNA genes of bacteria species in the adductor muscle with and without lesions of Yesso scallop. The relative abundances of selected representative species/groups were only shown.

## In situ *hybridization*

Positive ISH signal was only observed in the tissue sections treated with *F. halioticida* specific probes, while no signal was detected in the negative control slides. The central part of lesions containing abundant necrotic cells produced the strongest positive signal, while the peripheral areas of lesions typically yielded a less intense signal (Fig. 4A). *F. halioticida* cells were frequently observed to be aggregated within necrotic host cells (Fig. 4B and C). In the digestive gland, *F. halioticida* cells were primarily observed in the central part of the lesion (Fig. 4D), with very few detected outside the lesions. *F. halioticida* was also detected in the epithelial-like membrane surrounding the adductor muscle of only one individual (Fig. 4E), however there was no apparent histopathology in this section stained with H&E (data not shown).

### Detection of *F.* halioticida *by PCR*

PCR products amplified from scallop lesions were confirmed to be *F. halioticida* DNA by direct sequencing and BLAST search, verifying that the PCR assay developed by Kamaishi *et al.* (2010) was a good diagnostic test for detecting *F. halioticida* in Yesso scallops.

Using the PCR assay developed by Kamaishi *et al.* (2010), 27 of the 29 scallops with visible lesions tested positive for the presence of *F. halioticida* DNA, while only two of the 29 with lesions tested negative (Table 1). Conversely, 33 of the 47 scallops without lesions tested negative while fourteen of the 47 tested positive for the presence of *F. halioticida* DNA (Table 1). The odds ratio of *F. halioticida* detection to the adductor muscle
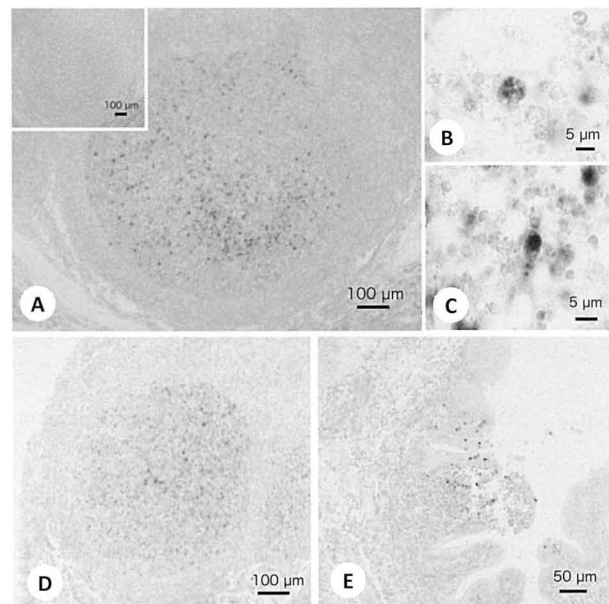


**Fig. 4.** Detection of *Francisella halioticida* by *in situ* hybridization using specific probes. **A**, Positive signals were detected in the lesion of the adductor muscle, while no signal in the control (Inset). **B**, Tiny cells with positive signal were observed in host cell. **C**, Aggregated cells with intense positive signal observed in host cells. **D**, Positive signal observed in the lesion in the digestive gland. **E**, Positive signals observed in the epithelial layers of the adductor muscle.

lesions was 31.82 with a 95% confidential interval of 6.64–152.42 indicating a significant association ($p < 0.05$) between the presence of *F. halioticida* and the occurrence of adductor muscle lesions.

this study tested positive for *F. halioticida* using the specific PCR developed by Kamaishi *et al.* (2010). Additionally, the ISH assay using the probes published by Kamaishi *et al.* (2010) provided further evidence confirming the presence of *F. halioticida* within the lesions of adductor muscles and digestive gland.

Subsequently, bacterial cells were successfully isolated from the lesions using MEA which was developed for isolation of *F. halioticida* from abalone (Kamaishi *et al.*, 2010). Morphology and Gram-stainability of bacterial cells were accordant with *F. halioticida* and 16S rRNA and *rpoB* genes of these isolates were 100% identical to those of *F. halioticida*, further supporting the hypothesis that *F. halioticida* is the causative agent of the lesions of Yesso scallops as suggested by Meyer *et al.* (2017).

The findings from our current study combined with the previous report of infection with *F. halioticida* from Yesso scallops cultured in western Canada (Meyer *et al.*, 2017) and a DNA sequence identical to *F. halioticida* obtained from lesions in Yesso scallops from China (GenBank Accession No. KC852839); suggest that *F. halioticida* has a wide distribution in north western to eastern Pacific and represents a serious health concern for Yesso scallops.

The current study provides good evidence showing an association between infection with *F. halioticida* and lesions. The microbiota analysis showed that *Moritella* spp. and *Aliivibrio salmonicida* were more abundant than *F. halioticida* in one lesion sample, suggesting that these bacterial species, to a lesser degree might also cause lesions in Yesso scallops.

Although Iida *et al.* (1980b) and Liu *et al.* (2013) proposed involvement of *V. alginolyticus*, *V. anguillarum*, *V. splendidus* and *Pseudomonas* sp. for the lesions of Yesso scallop adductor muscle, our microbiota analyses did not show the association of these bacteria with the lesions. As for viral infection suggested by Kosaka and Yoshimizu (1999), our TEM observations did not confirm any viral particles in the lesions (data not shown). Considering that very similar disease conditions have been reported from the Atlantic sea scallop *Placopecten magellanicus* which were determined to be caused by infection with *Mycobacterium* sp. (Grimm *et al.*, 2016), involvement of a variety of different microorganisms can never be completely ruled out, which no doubt varies between geographic locations.

Data from field surveys conducted in scallop farms located in southern Hokkaido provides evidence showing there is a good correlation between the prevalence of adductor muscle lesions with poor growth and mortalities among Yesso scallops. A previous study by Iida *et al.* (1980a) concluded that the impact of adductor muscle lesions on the scallop industry was limited to the loss of marketability of diseased scallops. However, in this study poor growth and mortalities of Yesso scallops

were recorded from populations with high prevalence of the adductor muscle lesions, suggesting that this disease can have a serious negative impact on overall scallop production. Given that the adductor muscle functions as the means for closing the shell, as well as storage of energy for various physiological activities (Barber and Blake, 1981), it is not surprising that the presence of lesions would have a serious negative impact on scallop physiology and cause mortality.

In conclusion, the present study indicates that *F. halioticida* is considered to be the most probable causative agent of adductor muscle lesions in scallops and that this disease is associated with mortalities and poor growth of Yesso scallops in Japan. Experimental transmission of *F. halioticida* is still required to fulfill Koch's postulates for this disease. However, future experimental challenges will require a source for *F. halioticida*-free scallops, which highlights the need for further epidemiology surveys concerning the distribution of *F. halioticida* infection over a wide range of scallop production areas. Moreover, since *F. halioticida* was originally isolated from giant abalone, biological characterization and comparison of isolates of *F. halioticida* from both scallop and abalone will be required to establish preventive measures of abalone and scallop francisellosis.

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# ホタテガイ閉殻筋の膿瘍形成要因と考えられる
## *Francisella halioticida* の感染

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良永知義・伊藤直樹

　商品価値の喪失をもたらすことが問題視されていたホタテガイ閉殻筋に見られる膿瘍の病原体調査を実施した。その結果，*Francisella halioticida* が膿瘍部に優占していることが明らかとなり，改変ユーゴン培地を用いることで膿瘍部より本菌が分離された。さらに，特異 PCR 法より膿瘍部の多くから本菌の遺伝子が検出されたことから，*F. halioticida* 感染のホタテガイ閉殻筋における膿瘍形成への関与が強く疑われた。2017年に北海道南部海域で実施した調査では，生残率および殻長は閉殻筋の膿瘍発生率と強い負の相関を示したことから，*F. halioticida* の感染によって生ずると考えられる閉殻筋膿瘍はホタテガイの生産量にも影響することが疑われる。今後は病原性を確認するため，分離菌株を用いた感染実験が必要となる。