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Parallel studies confirm *Francisella halioticida* causes mortality in Yesso scallops *Patinopecten yessoensis*

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ABSTRACT: *Francisella halioticida* is a marine bacterium originally described as the causative agent of mass mortality among giant abalone *Haliotis gigantea.* Recent field studies in Canada and Japan have suggested that this bacterium is also the cause of adductor muscle lesions and high mortality of Yesso scallops *Patinopecten yessoensis*, although a causal relationship has not been established. In the present study, the pathogenicity of *F. halioticida* in Yesso scallops was assessed in both Canada and Japan using bacteria isolated from diseased Yesso scallops in each respective country. Independent laboratory experiments revealed that scallops challenged with *F. halioticida* via bath exposure resulted in high mortality and histological lesions characterized by massive haemocyte infiltration. The presence of *F. halioticida* was confirmed using PCR, and *F. halioticida* was re-isolated from a portion of dead and surviving specimens. These results fulfill Koch's classic criteria for establishing disease causation and provide conclusive evidence that *F. halioticida* causes adductor muscle lesions and high mortality in Yesso scallops.

KEY WORDS: Disease · Bacteria · Bivalve · *Francisella halioticida* · *Patinopecten yessoensis* · Experimental challenge

1. INTRODUCTION

The Yesso scallop *Patinopecten yessoensis* is an economically important species in Northeast Asian countries including China, the Republic of Korea, the Russian Federation and Japan, where it accounted for approximately 20% of the total annual aquaculture production in 2016 (FAO 2018). The Yesso scallop has also been introduced for culture in France, Morocco and Canada, although production in these countries is still relatively low.

A disease state in Yesso scallops characterized by the occurrence of adductor muscle lesions, poor growth and high mortality has previously been reported from Canada, Japan and China. A variety of different microorganisms have been implicated as a possible causative agent for this condition including *Vibrio* spp. (Egusa 1974, Iida et al. 1980, Liu et al. 2013) and *Pseudomonas* sp. (Iida et al. 1980). It has also been speculated that an unidentified bacterial and/or viral pathogen may be the cause (Bower et al. 1992, Kosaka & Yoshimizu 1999, Getchell et al. 2016).

Recently, Meyer et al. (2017) reported that the intracellular bacterium *Francisella halioticida*, originally described as the cause of mass mortality among farmed abalone *Haliotis gigantea* in Japan (Kamaishi et al. 2010, Brevik et al. 2011), was present in diseased Yesso scallop specimens in Canada and hypothesized this bacterium to be the cause of both the lesions and associated mortality. Subsequently, Kawahara et al.

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(2018) found that *F. halioticida* was the most dominant bacterial species present in the lesions of similarly diseased scallops in Japan and succeeded to isolate this bacterium from the lesions. Nevertheless, although these studies by Meyer et al. (2017) and Kawahara et al. (2018) suggested that *F. halioticida* was the most probable cause of lesions and mortalities among cultured Yesso scallops in both Canada and Japan, neither study fulfilled the basic tenets required to definitively establish disease causation.

In the present study, we set out to determine the pathogenic potential of *F. halioticida* using Koch's basic principles for establishing disease aetiology (i.e. passage of disease through purified transfer and recovery of a pathogen) in Yesso scallops using isolates of *F. halioticida* obtained from diseased scallops in both Canada and Japan.

2. MATERIALS AND METHODS

2.1. Experimental challenge conducted in Canada

2.1.1. Bacterial isolation and culture

Francisella halioticida were isolated from an adductor muscle lesion in a Yesso scallop collected from Quadra Island, British Columbia, Canada, on October 23, 2017, using similar methods to those described by Kamaishi et al. (2010) and Kawahara et al. (2018) with a slight modification. Specifically, the isolation medium modified Eugon agar (MEA) (Kamaishi et al. 2010) was supplemented with ampicillin (50 µg ml^{-1}), polymyxin B (100 U ml^{-1}) and erythromycin (10 µg ml^{-1}) to inhibit growth of non-target bacterial species, according to Kawahara et al. (2018). Streaked MEA plates were initially incubated at 15°C for 15 d, and following subculture, the bacteria were incubated at 20°C for 10 to 15 d. For preparation of the inoculum and bath exposure aliquots, the bacteria were cultured in modified Eugon broth (MEB) at 20°C for 48 h with agitation, pelleted by centrifugation at 3800 × *g* for 15 min at 20°C, re-suspended with vortex mixing and washed 3 times in sterile seawater (SSW).

2.1.2. Scallop source

Yesso scallops (1.5 yr old with a mean shell height of 67.1 ± 6.4 mm, n = 100) were collected from an experimental aquaculture site in Departure Bay, British Columbia. This location was chosen because natural infections with *F. halioticida* were anticipated to be low (<1% in 250 scallops sampled over the preceding 6 mo period). Scallops were transported to the nearby Pacific Biological Station where they were divided into 5 groups of 20 and held within plastic mesh baskets suspended in 50 l rectangular tanks supplied with 2 to 3 l min^{-1} of sand-filtered and UV-irradiated seawater. Scallops were allowed to acclimatize to laboratory conditions for 6 d prior to challenge, and water temperature was maintained at 15 ± 1°C for the duration of the study.

2.1.3. Experimental challenge

Challenge trials consisted of the following 5 treatment groups: (1) Low-dose intramuscular injection — 20 scallops were each injected in the adductor muscle with 2.5 × 10^3 CFU of *F. halioticida* in 0.1 ml of SSW. (2) High-dose intramuscular injection — 20 scallops were each injected in the adductor muscle with 2.5 × 10^6 CFU of *F. halioticida* in 0.1 ml of SSW. (3) Injection controls — 20 scallops were each injected in the adductor muscle with 0.1 ml of SSW. All injections were delivered using a 25 gauge stainless steel needle and 1 ml syringe. (4) Bath exposure — 20 scallops were immersed in 5 l of seawater containing 1.0 × 10^7 CFU ml^{-1} of *F. halioticida* and 2.5 ml of Instant Algae (Shellfish Diet 1800®, Reed Mariculture) for 5 h at 15°C. The Instant Algae was added to stimulate filter feeding and increase exposure to the bacteria. (5) Bath exposure control — 20 scallops were immersed in 5 l of seawater containing 2.5 ml of Instant Algae for 5 h at 15°C. Both the bath control and bath exposure treatments were conducted inside 25 l plastic coolers.

Following exposure, scallops were returned to their 50 l tanks, monitored daily for mortalities and fed 3 times per week with Instant Algae re-suspended in seawater. Moribund or dead scallops were removed from the tanks and shucked, and the soft tissues were examined for the presence of macroscopic lesions. Tissue samples (gill, gonad, digestive gland and adductor muscle) were preserved in 95% ethanol for diagnostic testing from all freshly dead specimens. When lesions were observed, these were excised and preserved separately. An additional sample of each respective tissue (and lesions when present) was preserved in Davidson's solution (Howard et al. 2004) for histological examination. At 43 d post challenge (dpc) the experiment was terminated, and all surviving scallops were sampled as above. In addition, from the bath exposure group, re-isolation of the bacteria on MEA media was

attempted from lesions observed in the adductor muscle in 1 moribund scallop at 37 dpc and 2 surviving scallops at 43 dpc.

2.1.4. Detection of *Francisella halioticida* by PCR

DNA extraction was performed using a DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. The extracted DNA concentration and purity of each sample were measured using a Nanodrop (ND-1000) spectrophotometer (Thermo Fisher Scientific). PCR assays were conducted using previously published primer pairs designed to amplify the DNA-directed RNA polymerase beta subunit (*rpoB*) gene: primers Fh-rpoB/F and Fh-rpoB/R (Brevik et al. 2011). Reactions (25 µl total volume) consisted of 1 µl of undiluted template and were performed with final reagent concentrations as follows: 1× HotStar *Taq* DNA polymerase buffer, 5× Q-Solution, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µM forward and reverse primer and 0.05 U μl^{-1} HotStar *Taq* DNA polymerase (Qiagen). Cycling conditions consisted of an initial denaturation of 5 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at 52°C and 30 s at 72°C; and a final extension period of 10 min at 72°C. PCR products were visualized on 1.5% agarose gels stained with SYBR Safe (Thermo Fisher Scientific).

2.1.5. Histology

Tissue samples were processed using routine techniques for paraffin embedding (Howard et al. 2004). Then, 5 µm thick tissue sections were stained with Harris' modified hematoxylin and eosin (H&E), and the slides were examined via light microscopy at 100 to 1000× magnification.

2.2. Experimental challenges conducted in Japan

2.2.1. Bacterial isolation and culture

F. halioticida UTH170823, originally isolated from an adductor muscle lesion of a Yesso scallop grown in southern Hokkaido on August 23, 2017 (Kawahara et al. 2018), was used in both experiments described in Sections 2.2.3 and 2.2.4. In preparation for experimental challenge, the bacteria were cultured in MEB at 20°C for 72 h in a shaker at 190 rpm, pelleted by centrifugation at 2330 × *g* for 10 min, re-suspended and washed 3 times in SSW.

2.2.2. Scallop source

Yesso scallops (1 yr old with a mean shell length of 40.9 ± 4.8 mm, n = 210) were collected from eastern Hokkaido where presumptive *F. halioticida* associated disease has not previously been reported. At the University of Tokyo laboratory, all scallops were initially held in a 1000 l circulation tank in aerated seawater at 15 ± 1°C and fed daily with the diatom-*Chaetoceros calcitrans* (Sunculture, Marinetech). Prior to the challenge, 30 scallops were sampled and screened for the presence of *F. halioticida* using the PCR assay described in Section 2.2.5.

2.2.3. Challenge Expt 1

Two groups of 30 scallops were transferred to experimental tanks (60 × 30 × 36 cm) supplied with 6.7 l min^{-1} of seawater (15°C) for an acclimatization period of 1 wk. During the exposure period, scallops were placed inside plastic tanks (37 × 22 × 24 cm). One group was immersed in 10 l of aerated seawater containing 1.9×10^6 CFU ml^{-1} of *F. halioticida* for 5 h at 15°C. In addition, 5 ml of *C. calcitrans* was added to the suspension to stimulate filter feeding and increase exposure to the bacteria. The second group was treated in the same manner but without the addition of bacteria to act as an experimental control. Following exposure, scallops were returned to the experimental tanks, and one-third of the seawater was exchanged on a weekly basis. Scallops were monitored daily, and mortalities were immediately removed. Re-isolation of *F. halioticida* was attempted from adductor muscle tissue or abscess lesions when present as described by Kawahara et al. (2018). Resulting colonies were confirmed to be *F. halioticida* by the PCR assay described in Section 2.2.5. A sample of adductor muscle was preserved in 70% ethanol for PCR analysis, and an additional sample was preserved in Davidson's solution for histological examination. At 43 dpc the experiment was terminated, and bacterial re-isolation, as well as PCR screening and histopathology sampling, was performed on all surviving scallops.

2.2.4. Challenge Expt 2

A second trial was performed using the same procedures as described in Section 2.2.3, except that 60 scallops were used in each treatment group and the concentration of the bacterial suspension was

increased to 2.5×10^7 CFU ml^{-1} of *F. halioticida*. In addition, 10 live scallops were sacrificed and sampled from both the challenged and control groups at 21 dpc.

2.2.5. Detection of *Francisella halioticida* by PCR

DNA was extracted from tissue samples using a DNeasy mini kit (Qiagen) according to the manufacturer's instructions. DNA was extracted from bacteria grown on MEA by suspending a single colony in 300 µl of sterile distilled water and heating for 10 min at 95°C. Suspensions were then centrifuged at 16 200 × *g* for 1 min, and the supernatant was used as the template.

Detection of *F. halioticida* was conducted using the species-specific primer pair Megai-60 and Megai-480r, developed by Kamaishi et al. (2010). The final volume of the PCR reaction was 20 µl, which contained 1× TaKaRa Ex *Taq* buffer (TaKaRa Bio), 0.3 µM dNTPs, 0.3 µM of each PCR primer, 0.1 µl of TaKaRa Ex *Taq* Hot Start polymerase (TaKaRa Bio) and 1.0 µl of DNA as the template. The thermal cycling conditions consisted of an initial denaturation at 94°C for 4 min; 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s; and a final extension at 72°C for 7 min. PCR products were visualized on 1% agarose gels stained with SYBR Safe (Thermo Fisher Scientific).

2.2.6. Histology

Samples were preserved and processed using the same methods as described for the Canada experiment (Section 2.1.5); however, sections were stained with Giemsa as opposed to H&E.

2.3. Statistical analysis

Kaplan-Meier survival curves generated from all 3 experiments were compared separately by Mantel-Cox logrank tests and adjusted for multiple comparisons using the Bonferroni method.

3. RESULTS

3.1. Challenge experiment in Canada

Most scallops challenged by intramuscular injection with *Francisella halioticida* showed signs of morbidity within 24 to 48 h, including retraction of the mantle margin and slow response to stimuli. In both the high- and low-dose treatment groups, 100% of the scallops died within 4 to 9 dpc, respectively, and no macroscopic lesions were observed; no scallops died in the control group (Table 1, Fig. 1A). Because of the extensive and rapid mortality rate of the *F. halioticida* challenged scallops following injection, samples were not collected for histopathology or PCR screening, nor was re-isolation of *F. halioticida* on MEA media attempted since most individuals were already partially decomposed by the time of collection.

In the bath exposure group, morbidity was first observed at 9 dpc, which corresponded with the first occurrence of mortality. Mortality continued throughout the study, reaching a cumulative 60% by 43 dpc, with most mortality events (n = 9) occurring between 9 and 15 dpc (Table 1, Fig. 1A). Macroscopic lesions similar to those reported among cultured Yesso scallops as shown in Kawahara et al. (2018) were observed in the adductor muscle of 1 moribund scallop at 37 dpc and 2 surviving scallops at 43 dpc. Bacteria were re-isolated from these 3 specimens on MEA media and confirmed to be *F. halioticida* by PCR. The presence of *F. halioticida* DNA was detected by PCR in 90% of the dead scallops and 38% of the survivors (Table 1). There were no mortalities among the bath control group and no macroscopic lesions observed in any of the bath control survivors, nor was *F. halioticida* DNA identified in any bath control tissue samples (Table 1, Fig. 1A). Since

Table 1. Summary of *Francisella halioticida* 42 d challenge experiment conducted in Canada. Numbers in parentheses indicate no. of positive/no. tested. NE: not examined; NA: not applicable

Treatment	Cumulative mortality (%)	Macroscopic lesions	PCR-positive for *F. halioticida*		Microscopic lesions	
			Dead	Survivors	Dead	Survivors
Low-dose injection	100	0% (0/20)	NE	NA	NE	NA
High-dose injection	100	0% (0/20)	NE	NA	NE	NA
Injection controls	0	0% (0/20)	NA	NE	NA	NE
Bath exposure	60	15% (3/20)	90% (9/10)	38% (3/8)	90% (9/10)	38% (3/8)
Bath controls	0	0% (0/20)	NA	0% (0/20)	NA	0% (0/20)

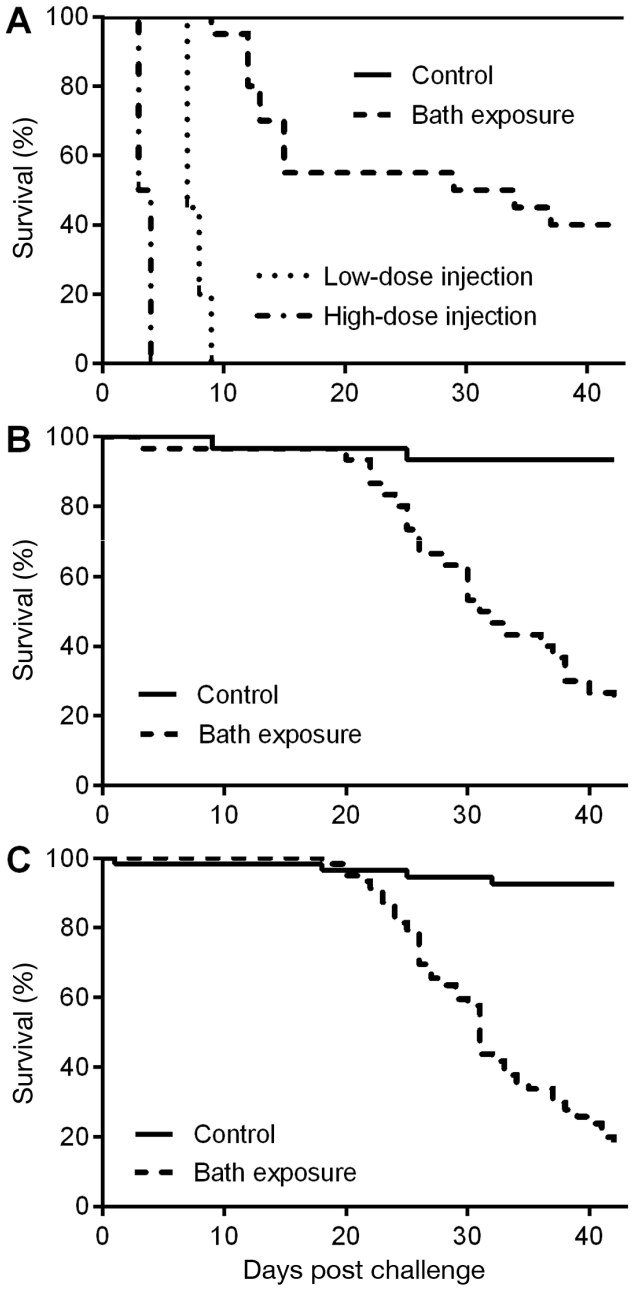

Fig. 1. Kaplan-Meier survival curves for *Francisella halioticida* challenge experiments conducted in (A) Canada and (B,C) Japan. Significant ($p < 0.01$) reduced survival was observed in all *F. halioticida* exposed groups relative to experimental controls in all 3 trials

no diagnostic testing was conducted on the inoculation exposure groups, it was decided that preserving and testing tissue samples from the inoculation control group was unnecessary, especially given that no lesions were observed in either of the control groups. Mantel-Cox logrank comparison of survival curves identified significantly lower ($p < 0.01$) probabilities for survival in all *F. halioticida* challenged groups compared to their respective controls (Fig. 1A). Similarly, histological examination revealed the presence of pathology typical for infection with *F. halioticida* (Meyer et al. 2017, Kawahara et al. 2018) in 90% of the sampled dead scallops and 38% of the survivors, while no significant lesions were observed in any of the specimens from the corresponding control groups (Table 1).

3.2. Challenge experiments in Japan

3.2.1. Prescreening of scallops

Although no macroscopic lesions were observed in the 30 scallops examined prior to the experiments, 20% tested PCR positive for *F. halioticida*.

3.2.2. Challenge Expt 1

Consistent mortality in the challenged scallops began at 20 dpc, with cumulative mortality reaching 77% by 43 dpc. Control scallops experienced 7% cumulative mortality, and Mantel-Cox logrank comparison of survival curves indicated a significantly lower ($p < 0.01$) probability for survival in the *F. halioticida* bath challenged group relative to the control group (Table 2, Fig. 1B). One surviving scallop from the challenge group had a macroscopic lesion in the adductor muscle, from which *F. halioticida* was re-isolated by culture and the species confirmed by PCR; no macroscopic lesions were observed in the control scallops.

In the challenge group, the presence of *F. halioticida* DNA was detected by PCR in 100% of the dead and 29% of the surviving scallops. The presence of *F. halioticida* DNA was also detected by PCR in 39% of the control survivors but was not detected from either of 2 dead control scallops. By culture methods, *F. halioticida* was re-isolated from 52% of the dead and 14% of the surviving scallops from the challenge group but was not isolated from any of the control specimens. Similarly, microscopic lesions like those shown in Fig. 2A were detected in 48% of the dead and 14% of the surviving scallops from the challenge group (Table 2). Additionally, bacteria-like particles similar to those reported by Meyer et al. (2017) were observed in the haemocytes (Fig. 2A), while neither microscopic lesions nor bacteria-like particles were observed in any of the control specimens (Table 2, Fig. 2B).

Table 2. Summary of *Francisella halioticida* 43 d challenge experiments conducted in Japan. Numbers in parentheses indicate no. of positive/no. tested. dpc: days post challenge; NA: not applicable

Treatment	Cumulative mortality (%)	Macroscopic lesions	PCR-positive for *F. halioticida*			Culture-positive for *F. halioticida*			Microscopic lesions		
			Dead	Sampled 21 dpc	Survivors	Dead	Sampled 21 dpc	Survivors	Dead	Sampled 21 dpc	Survivors
Trial 1											
Bath exposure	77	3% (1/30)	100% (23/23)	NA	29% (2/7)	52% (12/23)	NA	14% (1/7)	48% (11/23)	NA	14% (1/7)
Controls	7	0% (0/30)	0% (0/2)	NA	39% (11/28)	0% (0/2)	NA	0% (0/28)	0% (0/2)	NA	0% (0/28)
Trial 2											
Bath exposure	86	2% (1/59)	100% (42/42)	70% (7/10)	29% (2/7)	38% (16/42)	50% (5/10)	14% (1/7)	24% (10/42)	20% (2/10)	14% (1/7)
Controls	7	0% (0/60)	50% (2/4)	0% (0/10)	11% (5/46)	0% (0/4)	0% (0/10)	0% (0/46)	0% (0/4)	0% (0/10)	0% (0/46)

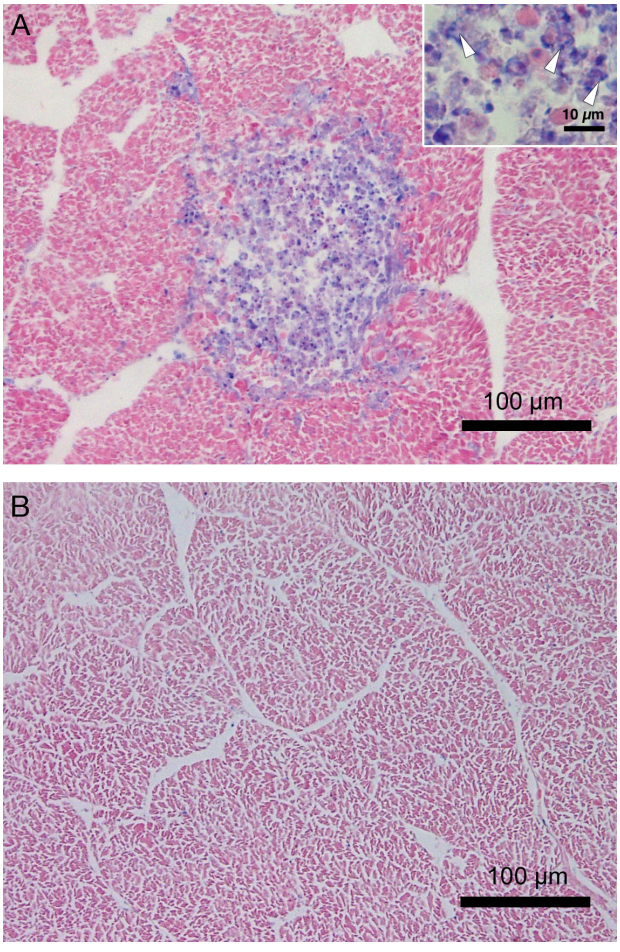

Fig. 2. Histological tissue sections of *Patinopectin yessoensis* stained with Giemsa from a bath exposure challenge conducted in Japan. (A) Typical lesion characterized by focal haemocyte infiltration in the adductor muscle of a challenged scallop. Inset: bacteria-like particles (arrowheads) observed in the host haemocytes. (B) Normal adductor muscle tissue from a control scallop

3.2.3. Challenge Expt 2

Consistent mortality in the challenged scallops began at 18 dpc, with cumulative mortality reaching 86% by 43 dpc. Control scallops experienced 7% cumulative mortality, and again Mantel-Cox logrank comparison of survival curves indicated a significantly lower ($p < 0.01$) probability for survival during the 43 d challenge in the *F. halioticida* bath challenged group relative to the control group (Table 2, Fig. 1C).

In the challenge group, the presence of *F. halioticida* DNA was detected by PCR in 100% of the dead scallops, 70% of the scallops sampled at 21 dpc and 29% of the surviving scallops (Table 2). In the control group, the presence of *F. halioticida* DNA was detected by PCR in 50% of the dead scallops and 11% of the survivors; however, the bacterium was not detected in any of the scallops sampled at 21 dpc (Table 2). Using culture methods, *F. halioticida* was re-isolated from 38% of the dead scallops, 50% of the scallops sampled at 21 dpc and 14% of the surviving scallops from the challenge group but was not isolated from any of the control specimens (Table 2). Microscopic lesions were detected in 24% of the dead scallops, 20% of the scallops sampled at 21 dpc and 14% of the surviving scallops from the challenge group but were not detected in any of the control specimens (Table 2). A macroscopic lesion was observed in 1 of the challenged individuals; however, *F. halioticida* was not detected in the lesion by either PCR or bacterial culture (Table 2).

4. DISCUSSION

In the present study, independent experimental challenges were conducted to evaluate the patho-

genicity of *Francisella halioticida* to Yesso scallops in both Canada and Japan. In Canada, Yesso scallops were challenged via both injection and bath exposure, while in Japan scallops were challenged in 2 separate bath exposure experiments. In all instances, challenged scallops showed significantly lower survival relative to controls, indicating that both immersion and injection with *F. halioticida* can cause high mortality among Yesso scallops.

Injection challenges using 2 different concentrations of *F. halioticida* caused rapid morbidity in most scallops (24 to 48 h), and all scallops from both injection groups died within 4 and 9 d, respectively. Although no macroscopic lesions were observed, we speculate that this was due to the artificially high concentrations of bacteria administered combined with the unnatural route of exposure, which produced overwhelming infections that the scallops were not able to contain or mount a host response against within this short period of time.

In all 3 bath challenge experiments, which were used to simulate a more natural method of exposure, the onset of mortality was delayed and occurred more gradually when compared to the injection challenge method. Detection of *F. halioticida* by PCR and re-isolation of the bacterium by culture was consistently proportionally higher among the dead and 21 dpc sampled scallops than for surviving scallops and thus supports the causal relationship of *F. halioticida* with mortality in these trials. Further, although the prevalence of macroscopic lesions in all 3 experiments was relatively low, the presence of *F. halioticida* in 4 of the 5 macroscopic adductor muscle lesions as determined by both PCR and culture confirms an association between this bacterium and clinical presentation of adductor muscle disease. Microscopic lesions consisting of intense haemocyte infiltration were also observed in 14 to 90% of the bath challenged scallops (depending on sampling time point), whereas no microscopic lesions were observed in any of the corresponding control groups; this is additional confirmation for the association between *F. halioticida* and adductor muscle disease.

In Japan, *F. halioticida* was detected by PCR in 20% of the scallops tested during the pre-screening event and 11 to 39% of the control groups, and the fact that *F. halioticida* could not be isolated by culture and no histopathology was observed in these specimens would suggest that persistent low-level infections were maintained in this population. If this is the case, then the mortality and lesions observed following the bath challenges would indicate that infection with relatively large quantities of bacteria is likely necessary to initiate a disease state in Yesso scallops. Thus, in considering the epidemiology of *F. halioticida* in natural environments, further research into identifying factors which might promote elevated levels of the bacteria and/or compromise scallop physical and immunological defenses (e.g. changes in water temperature) may prove critical. It is also possible that scallops used in Japan were infected with a less-pathogenic strain of *F. halioticida*, and isolation and characterization of the bacterium in the original location of those scallops would be required to confirm this hypothesis.

The results from this study provide experimental evidence that fulfills Koch's postulates for disease causation by *F. halioticida* in Yesso scallops. This study also provides a clear link between *F. halioticida* infection and mortality of Yesso scallops, identifying that *F. halioticida* is not only pathogenic but in some situations can be highly virulent in cultured Yesso scallops in both the eastern and western Pacific. Given that *F. halioticida* was originally described as the cause of mortality among giant abalone, a member of an entirely different class of organisms within the Mollusca phylum than the Yesso scallop (Gastropoda vs. Bivalvia), it is evident that this bacterium has pathogenic potential over a phylogenetically diverse range of mollusc species. This may be an important consideration when implementing future biosecurity measures, exploring disease aetiology and implementing diagnostic screening of other economically important molluscs.

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