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First detection of *Francisella halioticida* in mussels (*Mytilus* spp.) experiencing mortalities in France

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ABSTRACT

This note describes the first detection of the bacteria *Francisella halioticida* in mussels (*Mytilus* spp.) from locations in Normandy and northern Brittany (France) experiencing high mussel mortalities, while it was not detected in Bay of St Brieuc (N Brittany), which was not affected by abnormal mussel mortality. The distribution of the bacteria in mussels seems to be restricted within inflammatory granulomas as observed in Yesso scallops *Mizuhopecten yessoensis* from Canada and Japan. *Francisella halioticida* has been identified as being involved in mass (>80%) mortality of abalones *Haliotis gigantea* in Japan and high (up to 40%) mortality of Yesso scallops *M. yessoensis* in Canada as well as in lesions reducing marketability of Yesso scallops in Japan. The impact of this bacterium on the health of mussels needs to be investigated in future research, especially since the cause of high mussel mortalities that have been occurring in France for the past few years is still undetermined.

KEY WORDS:

Francisella halioticida, mussels, granulocytomas, polymerase chain reaction, *in situ* hybridisation

1. INTRODUCTION

The gram-negative bacteria *Francisella halioticida* was originally described in farmed abalone (*Haliotis gigantea*) as the cause of mass mortality that occurred in Japan (Kamaishi et al. 2010, Brevik et al. 2011). Subsequently, this bacterium was identified in diseased Yesso scallops, *Mizuhopecten* (=*Patinopecten*) *yessoensis,* in conjunction with mortality events in Canada (Meyer et al. 2017) and Japan (Kawahara et al. 2018). In addition, Kawahara et al. (2019) demonstrated that this bacterium can be highly virulent in Yesso scallops. Thus, *F. halioticida* has shown pathogenic potential in both gastropod and bivalve species of molluscs.

Since 2014, unexplained mass mortalities of mussels (*Mytilus edulis*, *Mytilus galloprovincialis* and their hybrids) have occurred in France from the Atlantic to the English Channel coasts (Béchemin et al. 2014, 2015, Allain & Bernard 2016, Travers et al. 2016, Bernard & Allain 2017), with mortality rates ranging from 40 % to 70 % (Bernard & Allain 2017, Bernard et al. 2018, Charles et al. 2020). Several hypotheses have been proposed to explain these mortalities, including the involvement of pathogenic bacteria belonging to the *Vibrio splendidus* clade (Béchemin et al. 2014, 2015, François et al. 2015, Soletchnik & Robert 2016, Travers et al. 2016), possibility of genomic abnormalities and disseminated neoplasia (Benabdelmouna & Ledu 2016, Benabdelmouna et al. 2018,). Nevertheless, none of these proposals alone could explain such high mortality rates (Charles 2019). High mortality rates were recently observed among mussels in Northern Brittany and although *V. splendidus* strains and disseminated neoplasia were detected, the prevalence in mortality-affected versus non-affected areas did not correlate with mortality and no causative agent was identified (Bernard et al. 2018, Charles et al. 2020).

In a multi-parametric field study to identify potential causes of high mortality of mussels, Charles et al. (2020) reported the occurrence of heavy haemocyte infiltra-

tion and formation of granulomatous lesions in the connective tissue of various organs, however were not able to identify an aetiological agent. Similarities between the histological observations in mussels and the histopathology reported in Yesso scallops infected with *F. halioticida* (Meyer et al. 2017, Kawahara et al. 2018) is what prompted this investigation to test for the presence of *F. halioticida* in French mussel samples from previous studies.

To the best of our knowledge, *F. halioticida* has never been reported in mussels. The current study reveals the presence of *F. halioticida*, identified by polymerase chain reaction (PCR) and by *in situ* hybridisation (ISH), in cultured French mussels experiencing mortalities.

2. MATERIALS & METHODS

The 341 mussels analysed for detection of *Francisella halioticida* in the current study came from two overlapping multi-parametric studies in France: the first was conducted in 2017 with mussels taken from three sites in northern Brittany (Charles et al. 2020), two sites (Brest and Lannion) were affected by high mussel mortality while the third site (St Brieuc) did not experience mortalities. In this first study, mussel seed batches were periodically checked for mortality and sampled (live mussels randomly taken) for diagnosis. The second study was conducted in 2017 and 2018 by analysing mussels for obligatory reporting of increasing mortality (Council Directive 2006/88/EC) in Normandy and Hauts-de-France region (Charles 2019); in this second study, the sampling agents (from Administration and/or the Regional Shellfish Farming Committee) collected mostly moribund mussels and the information on mortality was obtained from Administration reports (Canier et al. 2018, Combette 2018). In the two previous studies, mussels were analysed using histological, bacteriological and molecular diagnostic procedures; and although a number of pathogens and

pathological conditions were detected none of them were believed to be the primary cause of high mussel mortality in northern France (Charles 2019, Charles et al. 2020).

The 17 mussel groups analysed in the current study are listed in Table 1 together with some data derived from the two previous studies, namely the sampling place, the corresponding farming method, the cumulative mortality at sampling and the condition of mussels in the samples (either alive or moribund). It is noteworthy that the mussels collected during the first previous study, designed in advance to monitor mortality, were alive (valves remained closed when out of seawater), while the mussels collected during the second previous study, based on sampling immediately after knowing mortality events, were primarily moribund specimens (valves were gaping and non-responsive) or a mixture of live and moribund. Two molecular diagnostic procedures were used targeting *F. halioticida* DNA, polymerase chain reaction (PCR) and *in situ* hybridisation (ISH). Information on how the mussels had been processed in the previous two studies was reported by Charles (2019) and Charles et al. (2020). Briefly, for histological analysis, an approximately 5 mm thick transverse section of mussel tissue containing mantle lobes, visceral mass (gut, digestive gland) and gills was excised, fixed in Davidson's solution for 48h, kept in 70% ethanol and then dehydrated through an ascending ethanol series and embedded in paraffin wax. From each mussel a pool of minced tissues from foot, adductor muscle, gills, mantle, and digestive gland were preserved in a microcentrifuge tube at -20 °C.

A total of 332 mussels were used for PCR assays, 20 mussels per group except for groups 11 and 15 in which 16 mussels were used. DNA was extracted from preserved tissues of individual sample mussels, using a QIAamp DNA minikit® (Qiagen, Courtaboeuf, France) following the manufacturer's protocol for blood or

body fluids (except elution was performed using 60 µL Qiagen elution buffer AE). Following extraction, the DNA extracts of 5 mussels were pooled, except in groups 11 and 15 in which DNA pools derived from 4 mussels. Thus, 4 DNA pools were produced for each mussel group. PCR assays were conducted using the primer pair Fh-rpoB/F and Fh-rpoB/R (Brevik et al. 2011) designed to amplify the gene corresponding to bacterial DNA-directed RNA polymerase beta subunit (*rpoB*). For the conventional PCR, typical 25 µL reaction mixtures contained 12.5 µL of Premix Ex Taq® 2 X Takara® (Lonza, Verviers, Belgium), 0.5 µL of each primer (20 µM), 9.5 µL of purified water and 2 µL of DNA template (replaced with 2 µL of purified water in the negative control). PCR amplifications were carried out in a T100™ Thermal Cycler (Bio-Rad, France) using the following thermal cycling conditions: 10 s at 95 °C; 30 cycles for 10 s at 95 °C, 30 s at 55 °C, 40 s at 72 °C and a final extension of 3 min at 72 °C. All PCR products were purified and sequenced at LABÉO Frank Duncombe (Caen Cedex, France), and then analysed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).

For ISH assays, only mussels from the first study could be retrospectively analysed; nine archived paraffin blocks of mussels showing different histological characteristics were selected: three healthy mussels (from groups 5, 6 and 7), two mussels with heavy haemocyte infiltration in the visceral mass (from groups 1 and 5), two mussels with granulomas (from group 5), one mussel with massive haemocyte diapedesis through stomach epithelium (from group 4) and one mussel with abundant degenerated digestive gland tubules filled with necrotic cells (from group 6). Samples analysed by ISH corresponded to mussels different from those analysed by PCR. The ISH assays were performed as described by Meyer et al. (2017) except that a 1 % Light Green counterstain was substituted for Bismark Brown to yield better

contrast.

Table 1: Summary of mussel (*Mytilus* spp.) samples collected from France and test results for *Francisella halioticida* detection by conventional Polymerase Chain Reaction (PCR) and *in situ* hybridisation (ISH) assays. Each pool in the PCR assays resulted from pooling DNA from 5 mussels except in mussel groups 11 and 15 in which DNA from 4 mussels was pooled. Individual mussels assayed by PCR were different from those assayed by ISH. *Bouchot*: intertidal mussel stakes; Mix: mixture of alive and moribund mussels; *NA*: missing data; *NP*: not performed.

Group	Site	Region	Sampling date	Farming method	Cumulative mortality (%) at sampling time	Mussels condition	Number of pool of mussels analysed by PCR	PCR-results (positive pools/total pools)	ISH-results (positive mussels/total mussels analysed)
1	Lannion	Brittany	24/02/2017	Longlines	5 [(a) (b)]	Alive	4	0/4	0/1
2	Brest	Brittany	27/02/2017	*Bouchot*	12.5 [(a) (b)]	Alive	4	0/4	*NP*
3	St Brieuc	Brittany	27/02/2017	*Bouchot*	2.6 [(a) (b)]	Alive	4	0/4	*NP*
4	Brest	Brittany	24/04/2017	*Bouchot*	45 [(a) (b)]	Alive	4	0/4	0/1
5	Lannion	Brittany	17/05/2017	Longlines	20 [(a) (b)]	Alive	4	**1/4**	**2/4**
6	St Brieuc	Brittany	28/05/2017	*Bouchot*	4.5 [(a) (b)]	Alive	4	0/4	0/2
7	Brest	Brittany	30/05/2017	*Bouchot*	56.5 [(a) (b)]	Alive	4	**2/4**	0/1
8	Brest	Brittany	20/09/2017	*Bouchot*	66 [(a) (b)]	Alive	4	0/4	*NP*
9	Oye-Plage	Hauts-de-France	20/03/2017	*Bouchot*	25 [(c)]	Moribund	4	**3/4**	*NP*
10	Oye-Plage	Hauts-de-France	24/10/2017	*Bouchot*	10-50 [(c)]	Moribund	4	**4/4**	*NP*
11	Tardinghen	Hauts-de-France	14/03/2018	*Bouchot*	10-50 [(d)]	Moribund	4	**3/4**	*NP*
12	Donville-les-Bains	Normandy	19/06/2018	*Bouchot*	50 [(d)]	Mix	4	**2/4**	*NP*
13	Agon-Coutainville	Normandy	16/07/2018	*Bouchot*	*NA* [(d)]	Mix	4	**2/4**	*NP*
14	Chausey	Normandy	25/09/2018	*Bouchot*	10-50 [(d)]	Mix	4	0/4	*NP*
15	Wimereux	Hauts-de-France	15/10/2018	Wild	*NA* [(d)]	Moribund	4	**4/4**	*NP*
16	Tardinghen	Hauts-de-France	13/10/2018	*Bouchot*	*NA* [(d)]	Alive	4	**2/4**	*NP*
17	Dannes	Hauts-de-France	26/11/2018	*Bouchot*	25 [(d)]	Moribund	4	**4/4**	*NP*

[(a)] Bernard *et al*. (2018); [(b)] Charles *et al*. (2020) ; [(c)] Canier *et al.* (2018); [(d)] Combette (2018)

3. RESULTS

The PCR results as well as the ISH results for detection of *Francisella halioticida* DNA in French *Mytilus* spp., are summarized in Table 1. BLAST results of all the PCR amplified fragments were a 99.9 % match to the reference *rpoB* sequences for *F. halioticida* (Brevik et al. 2011) (GenBank accession numbers JF290381 and JF290374). Regarding the first study conducted in northern Brittany during 2017 (groups 1 to 8), *F. halioticida* DNA was detected by PCR in mussels collected in May at Lannion and Brest which had mortality rates of 20 % and 56.5 % respectively, after 3 months of culture, but it was not detected in samples from St Brieuc, an area that was not affected by abnormal mortality (only 4.5 %). Concerning the groups collected following reports of abnormal mortality in Normandy and Hauts-de-France region in 2017 and 2018 (groups 9 to 17), PCR-positive results were obtained from every group except the one coming from Chausey's Island.

Although ISH assays were not conducted on all groups, positive reactions were observed within granuloma lesions from two specimens (Figure 1). These lesions were characterized by an intense accumulation of granulocytes surrounded by layers of flattened, epithelioid cells (Figure 1A & Figure 2A); and every tested mussel with granulomas showed positive reaction. Other types of pathology, such as heavy haemocyte infiltration and diapedesis, were negative via ISH; furthermore, mussels with no significant histopathology were also negative via ISH. Thus, infection with *F. halioticida* appears to be associated with the host's response and formation of granulomas (Figure 2), which is common in vertebrates infected with other *Francisella* species (Colquhoun & Duodu 2011).

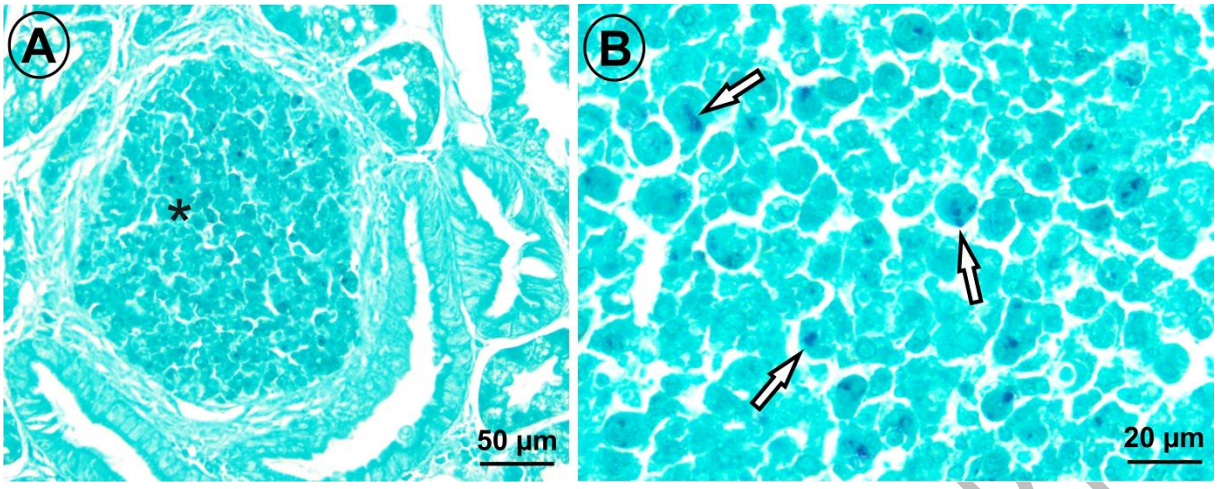

Figure 1. *In situ* hybridisation (ISH) assay showing positive reaction (dark blue deposits). (A) Section through the digestive gland of a mussel showing a granuloma (*) with tiny dark blue deposits within them. (B) Higher magnification of the inside of a granuloma showing dark blue deposits (arrows) indicative of positive ISH reaction within cells.

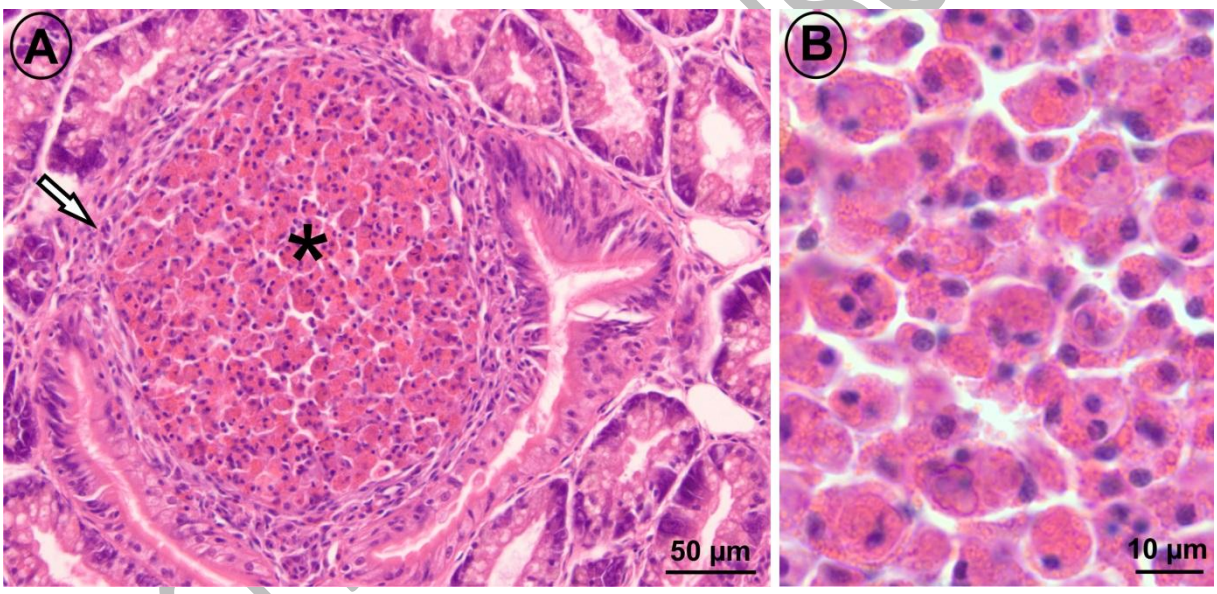

Figure 2. Micrographs of a histological section consecutive to that of the Figure 1, stained with Harris' haematoxylin and eosin. (A) Large granuloma in the connective tissue of the digestive gland consisting of a mass of accumulated granulocytes (*) surrounded by layers of flattened, epithelioid cells (arrow). (B) Higher magnification of the inside of the granuloma showing numerous granulocytes with phagocytosed cells.

4. Discussion

The occurrence of *Francisella halioticida* in French areas affected by high mussel mortality was revealed, whereas it was not detected in two areas, St Brieuc, which was not affected by abnormal mussel mortality, and Chausey's Island, where

mussels are known to suffer from intense predation by ducks, gulls and spider crabs, which were most likely contributing to the mortalities (CRC Normandie-Mer du Nord 2017, pers. com. 2019).. The findings from this study support a hypothesis proposed by Charles et al. (2020) concerning the possibility of missing an uncultivable bacterial pathogen with the classical medium used for bacterial culture. This could also be the case of the network for the monitoring of mollusc pathologies (REPAMO), coordinated by the French institute Ifremer, that monitors reports of mussel mortalities and conducts bacteriology using traditional culture media. Isolation and culture of *Francisella halioticida* requires a special culture medium (*Modified Eugon Agar*) and it can take several weeks for the bacteria to grow (Kamaishi et al. 2010, Kawahara et al. 2018). The limited sample size of this study prevents us from giving conclusive results; nevertheless, given that *F. halioticida* is known to be highly pathogenic in abalone and Yesso scallops, its detection in mussels from Normandy and northern Brittany areas affected by high mussel mortality highlights the need for further research concerning the association between mussel mortalities and infection with *F. halioticida*. Regarding the lack of geographical connection of the French mussel culture areas with the previous locations where *F. halioticida* was found, Canada and Japan, the importation of *M. yessoensis* in the beginning of the year 1987 in a hatchery of Brittany for several aquaculture trials (Buestel et al. 1988) could be considered as a potential hypothetical connection. It would be interesting to retrospectively test for the presence of *F. halioticida* DNA in samples of past mortalities, given that since 2015, several studies highlighted the presence of numerous inflammatory granulomas in French mussels affected by mortalities in the Atlantic coasts without any cause being identified (Robert et al. 2016, Travers et al. 2016, Pépin et al. 2017, 2018, Charles et al. 2020). In fact, some of these authors have even suggested a link

between the presence of the granuloma lesions and mortality. In addition, since 2014, mussels were not the only molluscs in France to be affected by unexplained mortalities: abalone (*Haliotis tuberculata*), cockles (*Cerastoderma edule*), clams (*Ruditapes philippinarum*) and scallops (*Pecten maximus, Chlamys varia*) were also affected (François et al. 2015, Lupo et al. 2016, Osta Amigo et al. 2018).

Considering the contagious and virulent nature of the *Francisella* spp. bacteria, and particularly *F. halioticida* in scallops, the detection of this bacterial species in French mussels opens up a new path to follow for further research concerning the cause of high mussel mortalities that have been occurring in northern France for the past few years. A field approach combined with laboratory challenges assessing the Koch's classic criteria would contribute to clarify the role of *F. halioticida* in mussel high mortality in N France.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. DATA AVAILABILITY STATEMENT

The authors confirm that one part of the data supporting the findings of this study is available within the article and the other part is available on request from the corresponding author.

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