Vol. 82: 105–109, 2008
doi: 10.3354/dao01972

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published November 20

Real-time PCR detection of *Moritella viscosa*, the likely causal agent of winter-ulcer in Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: We report the development of a real-time PCR protocol for specific detection of *Moritella viscosa*. This bacterium is considered to be the main aetiological agent in development of winter-ulcer, a disease severely affecting salmonid aquaculture in Norway. From a newly elaborated draft version of the genome of *M. viscosa*, the *tonB* gene sequence was selected as a suitable basis for the development of the real-time PCR assay. The real-time PCR demonstrated the presence of *M. viscosa* DNA sequences in 88.1% of samples collected from 35 outbreaks of winter-ulcer in Norwegian fish farms. In contrast, standard culturing on blood agar identified *M. viscosa* in only 39.7% of fish. While the culturing method revealed a similar prevalence (26 to 27%) of *M. viscosa* in kidney and ulcer samples, substantially more ulcer (81.5%) than kidney (49.7%) samples were shown positive by real-time PCR.

KEY WORDS: *Moritella viscosa* · Winter-ulcer · Aquaculture · Salmonids · Norway · Real-time PCR

INTRODUCTION

Moritella viscosa (ex. *Vibrio viscosus*; Benediktsdóttir et al. 2000, Lunder et al. 2000) is a psychrotropic Gram-negative, motile bacterium belonging to the family *Alteromonadales* (NCBI Taxonomy Homepage www.ncbi.nlm.nih.gov/Taxonomy/). In Norway, the bacterium is routinely isolated from farmed Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* suffering from 'winter-ulcer', and the bacterium is believed to be the dominating aetiological agent for this disease (Lunder et al. 1995, 2000). In addition to salmonids, *M. viscosa* has been isolated from Atlantic cod *Gadus morhua* (Colquhoun et al. 2004) and wild-caught plaice *Pleuronectes platessa* kept in captivity (Lunder et al. 2000). In experimental challenges, turbot *Scopthtalmus maximus* and to a lesser degree Atlantic halibut *Hippoglossus hippoglossus* were susceptible to *M. viscosa* infection (Bjornsdottir et al. 2004, Gudmundsdottir et al. 2006). Winter-ulcer is associated with low water temperatures (<8°C) and primarily manifests as external skin and muscle lesions of varying severity. However, gill pallor, fin rot and severe internal pathology may also be observed. Commercial vaccines against *M. viscosa* were first introduced in 1993, and an estimated 90 to 95% of all fish transferred to sea are now vaccinated with vaccines containing an *M. viscosa* component. However, despite extensive vaccination, mortalities may be high, and when combined with a reduced market quality of the surviving fish, the disease is a major cause of economic losses in Norwegian aquaculture (The health situation in farmed fish in Norway 2007, www.vetinst.no/eng/Research/Publications/Fish-Health-Report/Farmed-Fish-Health-Report-2007).

Currently, diagnostic demonstration of *Moritella viscosa* relies on culture on agar supplemented with 2% NaCl, a procedure that is often considered unreliable.

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Due to relatively slow growth, *M. viscosa* may be outcompeted by other bacterial species, leading to a possible underestimation of the prevalence of *M. viscosa* (Benediktsdottir et al. 1998, Bjornsdottir et al. 2004).

Here we report the development of a real-time PCR protocol specific for *Moritella viscosa*. The real-time PCR was used to assess the prevalence of *M. viscosa* in farmed Atlantic salmon and rainbow trout sampled from 35 outbreaks of winter-ulcer along the Norwegian coast during the period 2005 to 2007.

MATERIALS AND METHODS

PCR design. *Moritella viscosa* type strain (NCIMB 13584) was cultured in modified Guz medium (2% w/v yeast extract, 4.2 mM $MgSO_4 \times 7H_2O$, 11.5 mM K_2HPO_4, 0.34 M NaCl, pH 7.3) at 7°C for 48 h. The bacterium was pelleted by standard centrifugation, and total DNA was isolated from the pellet using a DNeasy kit (69504, Qiagen) according to the manufacturer's instructions. The concentration of isolated DNA was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The DNA was shipped on dry ice to 454 Life Sciences, where the *M. viscosa* genome was sequenced and assembled into contigs. Large contigs were annotated using the internet based BAsys automatic annotation service (Van Domselaar et al. 2005). Genes identified by BAsys annotation were manually inspected and verified by standard sequencing and Blast analysis (Altschul et al. 1997; www.ncbi.nlm.nih.gov/BLAST/). Sequencing was performed using a BigDye terminator v3.1 cycle sequencing kit (4337457, Applied Biosystems) and ABI Prism 3130XL-Avant Genetic Analyzer (Applied Biosystems) using 30 cycles of 96°C for 10 s, 50°C for 15 s and 60°C for 4 min, respectively. The *tonB* gene (GenBank EU332345) was the selected target for design of an *M. viscosa*-specific PCR protocol as described below.

DNA was isolated as described above from a number of *Moritella viscosa* isolates and from the very closely related yet non-pathogenic *M. marina* (NCIMB 1144). The *tonB* gene sequence was then sequenced from each *M. viscosa* isolate and *M. marina* using the primers tonB-5′F and tonB-3′R (Table 1). The *M. viscosa* isolates originated from clinical winter ulcer outbreaks along the Norwegian coast and from Atlantic salmon, rainbow trout and Atlantic cod. The *tonB* sequences were aligned using ClustalW (EMBL-EBI; www.ebi.ac.uk/Tools/clustalw/index.html), and the produced alignment was employed to identify sequence stretches that were conserved between all examined *M. viscosa* isolates and different in *M. marina*. Accordingly, primers (Mv-tonB-L1 and Mv-tonB-R1) and a double-dye probe (Mv-tonB-Pr) were designed to be specific for *M. viscosa* (Table 1), generating a PCR amplicon of 133 bp.

Real-time PCR was performed using the Stratagene Mx3005P kit and the Platinum Quantitative PCR SuperMix-UDG kit (11730-025, Invitrogen) according to the manufacturers' instructions. The reactions were performed in a total volume of 15.0 µl, including 4.0 µl of sample. An optimised PCR reaction mixture was found to contain 50% v/v Platinum Quantitative PCR SuperMix-UDG, 6 mM $MgCl_2$, 0.33 µM of each primer, 0.5 µM of double-dye probe and 50 nM ROX. Thermocycling was conducted as follows: 50°C for 2 min, 95°C for 2 min, followed by 40 repetitions of a 2 step temperature cycle (95°C, 15 s; 60°C, 30 s). Water was used as a negative control.

Specificity and sensitivity testing. To verify that positive PCR products would not arise from amplification of DNA/RNA from known pathogens, a broad array of bacterial fish pathogens was tested (Appendix 1). DNA was isolated as previously described and analysed by real-time PCR.

The assay sensitivity, defined as the smallest detectable concentration of *Moritella viscosa* DNA, was evaluated both for isolated *M. viscosa* DNA and for tissue samples spiked with isolated *M. viscosa* DNA. For isolated *M. viscosa* type strain DNA, a series of 2-fold dilutions was prepared, and real-time PCR was subsequently performed in the presence of 1.048 µg to 2.56×10^{-9} µg DNA per reaction. Each dilution was analysed in 3 parallels. The lower detection limit was defined as the lowest amount of *M. viscosa* DNA that was detected in all 3 parallel reactions. For tissues spiked with isolated *M. viscosa* DNA, aliquots of 100 µl of head kidney or muscle tissue lysate were spiked with decreasing amounts (2-fold dilution) of isolated *M. viscosa* DNA (i.e. from 4.17×10^{-1} µg to 3.1×10^{-6} µg). Total DNA was then isolated from the

Table 1. Primers and probes. All primers and probes were supplied by TAG Copenhagen A/S

Name	Function	5′-3′ Sequence
tonB-5′F	Sequencing primer	TGGATGGTGGATAACGGC
tonB-3′R	Sequencing primer	CTTTGGCTGGTACTTCCA
Mv-tonB-L1	PCR primer	CGTTGCGAATGCAGAGGT
Mv-tonB-R1	PCR primer	AGGCATTGCTTGCTGGTTA
Mv-tonB-Pr	Double-dye probe[a]	TGCAGGCAAGCCAACTTCGACA

[a]5′-bound reporter dye: FAM. 3′-bound quencher: Black Hole Quencher 1

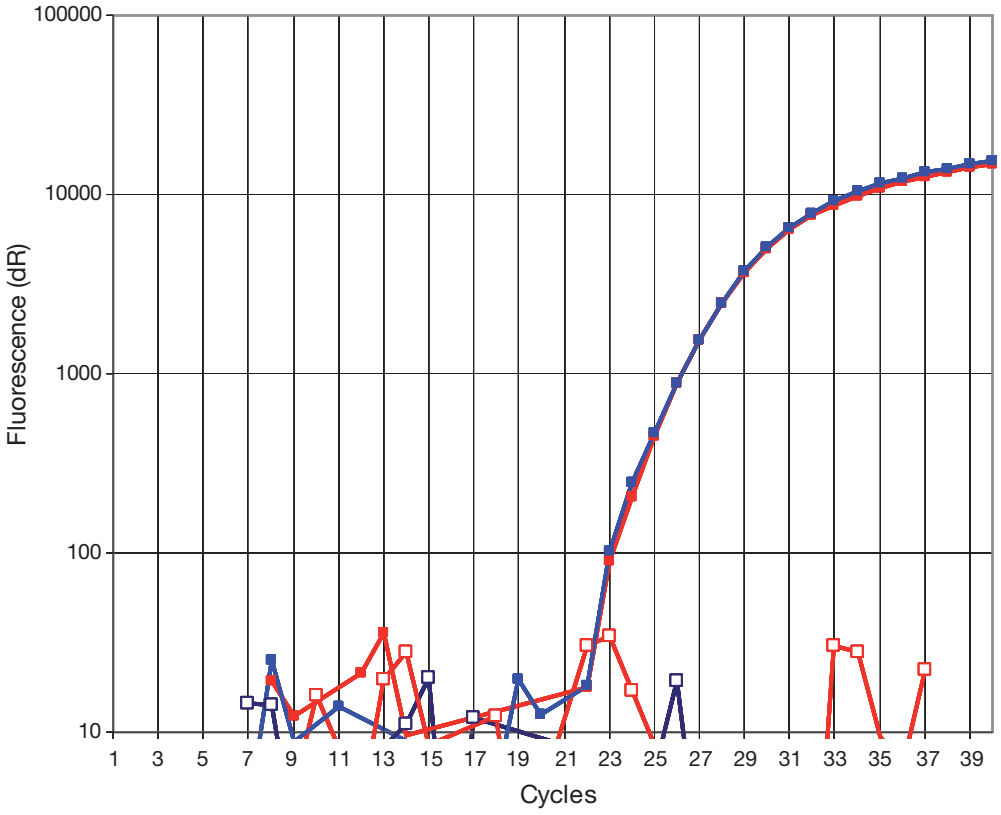

Fig. 1. *Moritella viscosa* and *M. marina*. Amplification plot from real-time PCR testing. Real-time PCR of DNA isolated from the *M. viscosa* type strain (n = 2) resulted in significant amplification and fluorescence (■, ■). No amplification products or fluorescence could be detected when *M. marina* DNA (n = 2) was PCR tested (□, □)

spiked tissue aliquots by the NucliSens easyMAG platform as described below in 'Sample preparation for PCR'. From head kidney lysate aliquots, each originating from 1.8 mg of tissue, a mean of 4.9 µg total DNA was isolated (range 4.6 to 5.3 µg), and from muscle lysate aliquots (6.5 mg tissue) a mean of 1.8 µg total DNA was isolated (range 1.6 to 2.0 µg). For both kidney and muscle, the amount of isolated total DNA was independent of the amount of *M. viscosa* DNA spiked to the aliquots before DNA isolation.

Sample collection. Samples were collected during winter and spring of 2006 and 2007, from 35 outbreaks of clinical winter-ulcer in farmed Atlantic salmon and rainbow trout along the Norwegian coast. The severity of ulcers/abscesses varied considerably between outbreaks and individual fish, from small wounds with almost intact muscle texture to very large wounds with completely necrotised muscle tissue. From each site, 5 freshly dead or moribund fish were sampled and sent on ice to the laboratory by overnight transport. Bacteriological samples from kidney and surface ulcers or abscesses were sown onto heart infusion agar (Difco) containing 2% NaCl and 5% bovine blood (BA), incubated at 15°C and initially read after 48 h (Benediktsdottir et al. 2000, Lunder et al. 2000). Incubation and observation was extended for a further 5 d.

Sample preparation for PCR. Tissue samples from kidney and surface ulcers or abscesses were dissected and the tissues preserved in RNAlater according to the manufacturer's instructions. Tissue slices corresponding to a volume of approximately 0.03 cm^3 were homogenised in 1 ml of lysis buffer. Subsequently, DNA was isolated from 200 µl of homogenate using the NucliSens easyMAG platform for total nucleic acid extraction (bioMérieux) by the off-board protocol according to the manufacturer's instructions. Nucleic acids were eluted in 20 µl of elution buffer.

RESULTS AND DISCUSSION

Sensitivity. Regarding pure *Moritella viscosa* DNA, the real-time PCR showed a lower detection limit of approximately 6.09×10^{-14} g of total *M. viscosa* DNA, equivalent to approximately 10 *M. viscosa* genomes. Although smaller quantities could be detected, these detections were inconsistent. Regarding *M. viscosa* DNA isolated from spiked tissue samples, the real-time

Table 2. *Moritella viscosa.* Comparison of results (no. of positive/no. of negative) from real-time PCR versus culture-based detection from kidney and ulcer tissue sampled from fish with clinical winter-ulcer. Pos: positive; neg: negative

Sample	PCR	Culturing		Tissue total
		Pos.	Neg.	
Kidney	Pos.	**31**	**44**	75
	Neg.	**10**	**66**	76
Tissue total		41	110	151
Ulcer	Pos.	**36**	**87**	123
	Neg.	**3**	**25**	28
Tissue total		39	112	151

Table 3. *Moritella viscosa.* Comparison of detections (no. of positive/no. of negative) in kidney and ulcers/abscesses analysed by either real-time PCR or culture on blood agar supplemented with 2% NaCl. Pos: positive; neg: negative

Analysis/ Total	Ulcer	Kidney		Total
		Pos.	Neg.	
PCR	Pos.	**66**	**57**	123
	Neg.	**9**	**19**	28
Total		75	76	151
Culturing	Pos.	**20**	**19**	39
	Neg.	**21**	**91**	112
Total		41	110	151

PCR showed a lower detection limit of approximately 128 *M. viscosa* genomes. Because of greater variability in the results from spiked tissue samples, arising from variation in the performance of DNA isolation, a higher number of real-time PCR parallels (n = 8) were used for this analysis.

Specificity. When tested against DNA isolated from a selection of bacterial species (Appendix 1), the real-time PCR showed no positive reactions, as shown for *Moritella marina* (Fig. 1). Blast analysis against expressed sequence tag (EST) and nucleotide databases revealed no sequences that could be amplified and recognised by the *M. viscosa*-specific primers and probe.

Frequency of *Moritella viscosa* in farmed salmonids experiencing winter-ulcers. Real-time PCR and culturing were used to evaluate the frequency of *Moritella viscosa* in winter ulcer diseased salmonids, collected from fish farms along the Norwegian coast (Tables 2 & 3). Only fish from which real-time PCR and culturing data that were obtained from both kidney and ulcers were included in the final data set. In important contrast to the culturing method, the real-time PCR may detect *M. viscosa* irrespective of whether the bacterial DNA originates from live or dead bacteria or bacterial remnants including vaccine components. However, DNA outside protective environments, such as the inside of live bacteria or in oily vaccines, will most likely decay rapidly in the fish host (Grove et al. 2003). Further, in fish receiving vaccine by intraperitoneal injection, *M. viscosa* antigens of vaccine origin should be expected to be retained long-term in tissue of (central) lymphoid organs (Lamers & De Haas 1985, Brattgjerd & Evensen 1996) and in the peritoneum (Cox & Coulter 1997) and not in peripheral non-lymphoid tissues such as skin and superficial muscle. Hence detections of *M. viscosa* DNA in ulcers most likely indicate the presence of *M. viscosa* of non-vaccine origin.

The real-time PCR data identified the presence of *Moritella viscosa* in kidney and/or ulcer of 88.1% (133/151) of examined fish. In contrast, culturing demonstrated the presence of *M. viscosa* in only 39.7% (60/151) of fish examined. Considering the generally high sensitivity of PCR, these differences in detection frequency were in accordance with expectations. Using real-time PCR, we found that 81.5% (123/151) of the ulcer samples were positive for *M. viscosa*, whereas only 49.7% (75/151) of the kidney samples were positive. In contrast, agar culture revealed slightly more positives (27.2%; 41/151) from kidney than ulcer (25.8%; 39/151) samples. The observed differences in tissue distribution between the 2 techniques may reflect inhibition of *M. viscosa* during culture by other members of the mixed bacterial flora (most commonly *Vibrio wodanis*), which are often found in ulcers open to the surrounding environment. This is supported by PCR detection of *M. viscosa* in 5 of 6 fish from which *V. wodanis* was grown in apparently pure culture using standard bacteriological techniques (data not shown). The high frequency of *M. viscosa* in winter-ulcer-diseased fish observed in the present study supports the hypothesis that *M. viscosa* is a primary pathogenic cause of the disease. Currently, knowledge relating to the pathogenesis of *M. viscosa* in relation to winter-ulcer is limited. The real-time PCR developed in our study should be a useful tool in elucidation of the disease process as well as a welcome addition to histology, immunohistochemical techniques and culture, for diagnosis of *M. viscosa* as a cause of winter-ulcer.

Acknowledgements. The skilled technical assistance of Randi Faller is greatly acknowledged. The work was supported by The Research Council of Norway, Grant 174241.

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Appendix 1. Bacteria tested for cross-reactivity by real-time PCR. None were detected by real-time PCR. All bacterial strains were from the collection at the National Veterinary Institute, Norway

Atypical *Aeromonas salmonicida*	*Piscirickettsia salmonis*
Aeromonas salmonicida salmonicida	*Rhodococcus erythropolis*
Bacillus cereus	*Staphylococcus aureus*
Brochothrix thermosphacta	*Streptococcus agalactiae*
Carnobacterium piscicola	*Tenacibaculum maritimus*
Francisella philomiragia noatunensis	*Vibrio anguillarum O1*
Escherichia coli	*Vibrio salmonicida*
Moritella marina	*Vibrio splendidus*
Mycobacterium marinum	*Vibrio wodanis*
Pasteurella skyensis	*Yersinia ruckeri*
Pseudomonas fluorescens	

Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: January 14, 2008; Accepted: July 31, 2008
Proofs received from author(s): October 27, 2008