Video Article

Multi-locus Variable-number Tandem-repeat Analysis of the Fish-pathogenic Bacterium *Yersinia ruckeri* by Multiplex PCR and Capillary Electrophoresis

Snorre Gulla[1], Saima Nasrin Mohammad[1], Duncan John Colquhoun[1,2]

[1]Fish Health Research Group, Norwegian Veterinary Institute

[2]University of Bergen

Correspondence to: Snorre Gulla at snorre.gulla@vetinst.no

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Abstract

Yersinia ruckeri is an important pathogen of farmed salmonids worldwide, but simple tools suitable for epizootiological investigations (infection tracing, etc.) of this bacterium have been lacking. A Multi-Locus Variable-number tandem-repeat Analysis (MLVA) assay was therefore developed as an easily accessible and unambiguous tool for high-resolution genotyping of recovered isolates. For the MLVA assay presented here, DNA is extracted from cultured *Y. ruckeri* samples by boiling bacterial cells in water, followed by use of supernatant as template for PCR. Primer-pairs targeting ten Variable-number tandem-repeat (VNTR) loci, interspersed throughout the *Y. ruckeri* genome, are distributed equally amongst two five-plex PCR reactions running under identical cycling conditions. Forward primers are labelled with either of three fluorescent dyes. Following amplicon confirmation by gel electrophoresis, PCR products are diluted and subjected to capillary electrophoresis. From the resulting electropherogram profiles, peaks representing each of the VNTR loci are size-called and employed for calculating VNTR repeat counts in silico. Resulting ten-digit MLVA profiles are then used to generate Minimum spanning trees enabling epizootiological evaluation by cluster analysis. The highly portable output data, in the form of numerical MLVA profiles, can rapidly be compared across labs and placed in a spatiotemporal context. The entire procedure from cultured colony to epizootiological evaluation may be completed for up to 48 *Y. ruckeri* isolates within a single working day.

Video Link

The video component of this article can be found at https://www.jove.com/video/59455/

Introduction

Yersinia ruckeri, a Gram-negative bacterium and member of the Yersiniaceae family, causes yersiniosis in farmed salmonid fish worldwide[1]. It is readily diagnosed from infected fish by cultivation on many types of agar media, but until recently, little was known regarding the population structure and epizootiology of *Y. ruckeri* across the world and in different habitats (host species, etc.). Existing serotyping systems for *Y. ruckeri* are inconsistent, lack mutual compatibility and offer low epidemiological resolution. Some molecular studies on the bacterium have been conducted, employing techniques such as Multilocus sequence typing (MLST), Pulsed-field gel electrophoresis (PFGE) or whole-genome sequence (WGS) analysis[2,3,4,5]. However, MLST does not provide a sufficiently high resolution for routine infection tracing, while PFGE is labor demanding and produces results that are not readily portable across labs. While WGS analysis would provide a near ultimate resolution, the establishment and implementation of such analyses would prerequisite technical- and bioinformatics capabilities that yet remain restricted to a relatively small number of laboratories.

Multi-locus Variable-number tandem-repeat Analysis (MLVA) represents a simple and easily accessible molecular typing tool, which offers a genetic resolution in some cases almost matching that of WGS analysis[6,7]. The technique is based on repeat number variation in selected variable-number tandem-repeat (VNTR) loci, resulting in output data that is highly transportable, making comparison of profiled isolates towards online databases and across labs straightforward. Although MLST remains the gold standard for epidemiological typing of many bacterial pathogens, an increasing number of studies identify a significantly higher discriminatory power of MLVA[8,9,10]. Several protocols have also been published targeting fish-pathogenic bacteria, such as *Francisella noatunensis*, *Edwardsiella piscicida* and *Renibacterium salmoninarum*[11,12,13].

The ten-loci MLVA protocol presented here, which recently formed the basis for an extensive *Y. ruckeri* population study[14], involves extraction of DNA from agar-cultivated colonies, multiplex PCR and capillary electrophoresis (CE), followed by downstream in silico applications. For each examined isolate, two multiplex PCRs, both containing five fluorescently labelled primer pairs (6FAM, NED or VIC) each targeting individual VNTR regions, are run in parallel under identical conditions. Following verification of PCR amplicons by gel electrophoresis (GE), PCR products are diluted prior to CE analysis, and peaks representing the respective VNTR loci are size-called from the resulting electropherogram files. Together with locus-specific formulas accounting for minor, sequence-specific discrepancies in CE migratory patterns, VNTR CE size calls

are then employed for calculating VNTR repeat counts which are concatenated into ten-digit MLVA profiles. These are used as input for epizootiological evaluations (e.g., by cluster analysis in Minimum spanning tree (MST) diagrams).

Protocol

CAUTION: For the entirety of the protocol, it is advisable to conduct all wet-lab procedures sterilely by use of lab coats, disposable gloves and sterile reagents and equipment. It is also advisable to prepare PCR reactions in a separate room (pre-PCR) not used for PCR amplification and/or handling of PCR products (post-PCR). Store all reagents as recommended by the manufacturer. See **Table of Materials** for further details on reagents, equipment and software used.

1. Bacterial Cultivation and Extraction of Genomic DNA

1. Sow out *Y. ruckeri* pure cultures on any suitable agar type (the authors used 5% bovine blood agar) and incubate at 22 °C for 1-2 days, or 15 °C for 3-4 days.
2. From each agar plate, pick a single representative colony with an inoculation loop and transfer to 1.5 mL centrifuge tubes containing 50 µL of ultrapurified water. Suspend, vortex briefly, and incubate for 7 min on a heating block at 100 °C.
3. Centrifuge at 16 000 x *g* for 3 min and use a pipette to carefully transfer the supernatant into an empty 1.5 mL centrifuge tube. Proceed to next step using the supernatant as template DNA or store at -20 °C until such time.

2. Multiplex PCR setup and Cycling Conditions

NOTE: Each multiplex PCR reaction (two per *Y. ruckeri* isolate) should contain 12.5 µL of 2x Multiplex PCR Plus master mix, 0.1 to 0.2 µM of each appropriate primer pair (**Table 1**) and 3 µL of template DNA, adjusted to a final reaction volume of 25 µL by addition of RNase-free water. Aim to keep light exposure of the fluorescently labelled forward-primers at a minimum (e.g., by wrapping their storage tubes in aluminum foil).

1. For each of the two multiplex PCR assays (**Table 1**), prepare master mixes as described above (without template DNA) according to the number of samples plus one positive and one negative control. Additionally, allow 10% surplus volume. Vortex the prepared master mixes gently at low speed.
2. Distribute 22 µL of each master mix separately into individual wells on either PCR strips or plates, as appropriate for the number of samples, and add 3 µL of template to each well (for positive and negative controls, respectively, use DNA from a verified *Y. ruckeri* strain and ultrapurified water). Seal and centrifuge briefly.
3. Run all samples on a PCR thermal cycler with the following programme: (i) 5 min at 95 °C (ii) 30 cycles of 0.5 min at 95 °C, 1.5 min at 60 °C, and 1 min at 72 °C, and (iii) 60 min at 68 °C, followed by cooling to 4 °C indefinitely. The program will complete in less than 3 h.

3. PCR Amplicon Confirmation by Gel Electrophoresis

1. According to the manufacturer's recommendations, prepare a volume of 1.5% (w/v) agarose gel in 1x tris-borate-EDTA (TBE) buffer appropriate for the number of PCR reactions to be tested. Prior to casting, add 5 µL of fluorescent nucleic acid dye per 50 µL of gel solution and mix. Use trays and combs as appropriate for casting, leaving an appropriate number of wells free for DNA reference ladders.
2. After setting, submerge the gel in 1x TBE-buffer in a GE system. Mix 5 µL of PCR product together with 2 µL of loading dye and transfer to gel wells. Add 5 µL of DNA ladder in empty wells for reference.
3. Run the gel at 110 V per 15 cm for approximately 1 h and use a UV-based gel imaging/visualisation system to verify the presence of multiple (up to five) bands representing PCR amplicons (see example in **Figure 1**). Discard the gel. Proceed to the next step or store remaining PCR products at 4 °C until further processing.

4. Capillary Electrophoresis Setup and Run Conditions

1. Following confirmation of PCR amplicons, dilute PCR products 1:10 (v/v) in purified water. Seal, mix and centrifuge briefly.
2. Working in a fume cupboard, prepare a volume of master mix consisting of 9 µL of formamide and 0.5 µL of size standard per PCR product (allow 10% surplus volume). Vortex briefly and distribute 9.5 µL into wells on a plate appropriate for the available CE system, before adding 0.5 µL of diluted PCR product. Seal, mix and centrifuge briefly.
   CAUTION: Handle with care. Mixing formamide with water generates formic acid, which is toxic.
3. Using a PCR thermal cycler, denature the samples at 95 °C for 3 min before cooling to 4 °C indefinitely. Centrifuge briefly and load the plate onto a calibrated CE system according to the manufacturer's instructions.
4. Run fragment analysis CE using reagents as appropriate for the apparatus of choice and the following settings: 60 °C; 5 s injections at 1.6 kV (32 V per cm); 32 min run time at 15 kV (300 V per cm). CE fragment analysis of 24 wells on a 24-capillary (50 cm) will typically take approximately 50 min.

5. VNTR Size Calling, Repeat Count Calculation and MLVA Profiling

NOTE: Step 5.1 describes *Y. ruckeri* VNTR CE size calling from electropherogram files, using the specific software listed in **Table of Materials**. Consult the software manual for additional details and troubleshooting. For use of other software, consult appropriate manuals.

1. **Import CE result files (two per *Y. ruckeri* isolate). Set Analysis Method to Microsatellite Default and select the appropriate product choice under Size Standard, prior to pressing the Analyze button. Verify the correct identification of size standard fragments through the Size Match Editor and rectify any visibly erroneous allocations.**

1. Having selected the sample(s) to be read, hit the **Display Plots** button and press **Ctrl+A** to enable view of the **Sizing Table**. While in the top panel, hold down **Ctrl** while clicking on the five peaks representing the VNTR amplicons (use zooming tool as needed).
   NOTE: For each of the multiplex PCR products, the electropherogram will show five peaks distributed amongst the three dyes employed (see 5' dye labelling of forward primers in **Table 1** and the two examples in **Figure 2**).
2. Press **Ctrl+G** to filter the **Sizing Table**, showing only characteristics of the five highlighted peaks, and record CE size calls for each VNTR locus (with reference to **Table 1**) for downstream application.

2. In order to account for biased amplicon mobility patterns during CE, calculate accurate VNTR repeat counts according to the formula provided below, employing VNTR CE size calls together with locus-specific variables (see **Table 1**). For efficiency, it is advisable to automate this process (e.g., by using a spreadsheet template).

$$VNTR\ repeat\ count = \frac{VNTR\ CE\ size\ call \times s + i - VNTR\ flank\ size}{VNTR\ repeat\ size}$$

3. Round calculated VNTR repeat counts off to the nearest integer and concatenate into ten-digit strings, each representing the MLVA profile of a single *Y. ruckeri* isolate.

6. Minimum Spanning Tree Cluster Analysis of MLVA Data

NOTE: Step 6 describes the creation of MST diagrams from *Y. ruckeri* MLVA data, using the specific software listed in **Table of Materials**. Consult the software manual for additional details and troubleshooting. For use of other software, consult appropriate manuals.

1. Create a new database and opt to activate the MLVA plugin.
2. Import *Y. ruckeri* MLVA profiles and metadata by selecting **Character type data** followed by **Import fields and characters** (further sub-selection depending on storage format). When prompted, specify import rules according to the content of the import file: In the **Destination type** column, classify VNTR repeat counts as **Character value: VNTR**, and the miscellaneous metadata as **Entry information: Entry info field**.
   NOTE: For comparison and context, it is also possible to import the entire dataset published (open access) together with the original paper employing the present MLVA protocol[14]. MLVA profiles and metadata on the diverse collection of *Y. ruckeri* isolates (n = 484) scrutinised in that study is available from its supplemental material (Tables S1 and S2) through the following link: https://aem.asm.org/content/84/16/e00730-18/figures-only#fig-data-additional-files
3. In the **Experiment type** panel, open the **VNTR** entry and set minimum and maximum values for each VNTR locus to **0** and **100**, respectively. Under **General settings**, set the number of decimal digits to **0** and select **Numbers** under **Data type**. Check to consider absent values as zero.
4. **Select imported samples destined for MST cluster analysis and click the Create new comparison button (in Comparison panel).**
   1. If desired for the visual presentation of the MST, allocate the samples to colored groups (e.g., according to a particular metadata trait) by employing the various options available in the **Groups** panel.
      NOTE: Groups can also be created/altered retrospectively, subsequent to the following steps.
   2. Select **Advanced cluster analysis...** and **MST for categorical data** to generate an MST diagram based on the chosen samples.
   3. Further modify the visual presentation of the MST as preferred (e.g., by adding partitioning parameters, node/branch labelling, crosslinks, legends, etc). See example in **Figure 3**.
      NOTE: A cluster (clonal complex) partitioning threshold of ≤4/10 non-identical VNTR loci, in addition to hiding of branch connections representing >5/10 non-identical VNTR loci, has previously been employed for MST cluster analysis based on MLVA data generated using this protocol[14]. Provided the aforementioned dataset of 484 *Y. ruckeri* MLVA profiles was imported, those samples can also be included for MST cluster analysis (as described above) to provide a global and historical context. This will e.g. facilitate identification of any samples affiliated with previously described clonal complexes, as well as those representing yet undescribed lineages. Depending on available metadata, the resulting MST diagram can be scrutinised in different ways, e.g. to discover eventual clustering patterns linked to particular traits (geography, host, time etc.).
   4. If needed, export the finalized MST in a desired format using the **Export image** selection.

Representative Results

Following multiplex PCR as described here, a typical GE image verifying the presence of multiple amplicons from each PCR reaction is shown in **Figure 1**. Downstream CE fragment analysis performed on verified PCR products will, for each *Y. ruckeri* isolate examined, result in two electropherogram files used for size calling of the respective VNTR loci (**Figure 2**). From analysis of 484 diverse *Y. ruckeri* isolates, no overlap in amplicon size range was observed between VNTR loci labelled with the same dye in the same multiplex reaction (**Table 1**)[14]. Each of the electrophoretic peaks can, therefore, be unambiguously identified by color.

Following import of MLVA profiles and relevant metadata into the preferred software, MST diagrams can be constructed as described for scrutiny of any epidemiological patterns of interest in the material. Consult appropriate manuals for additional options available in the respective software. As an example, **Figure 3** shows comparison by MST of MLVA profiles for *Y. ruckeri* isolates recovered from fish associated with five different salmon farms in Norway.

The consistent repeat sizes of the ten VNTR loci, as well as their in vitro and in vivo stability, have previously been verified in the original study based upon this protocol[14]. Briefly, this was done using Sanger sequencing (repeat size), and by MLVA typing of multiple isolates following serial passages (in vitro) and from within individual disease outbreaks (in vivo). Moreover, the environmental stability of the loci over time was examined by typing multiple 'house strain' isolates recovered over several years from persistently infected freshwater production sites for Atlantic salmon.

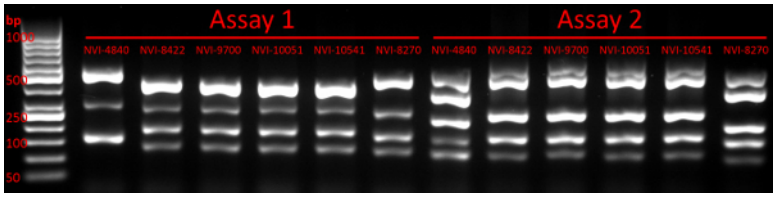

Figure 1: Gel electrophoresis verifying the presence of multiple PCR products. The image confirms the presence of multiple PCR amplicons in all 12 lanes containing samples, with the first lane representing the DNA ladder used. The sizes of selected ladder fragments have been indicated, as have the PCR assay and strain (see Table S1 in Gulla et al. 2018[14]) affiliation of each lane. Please click here to view a larger version of this figure.

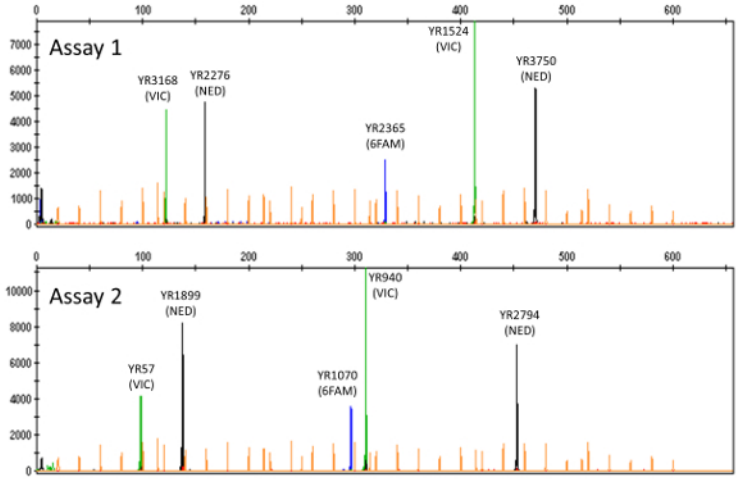

Figure 2: Electropherograms showing peaks corresponding to VNTR amplicons. Names of the different VNTR loci are indicated, with dye labels (VIC = green; NED = black; 6FAM = blue) in parentheses. The two electropherograms (PCR assay 1 top; PCR assay 2 bottom) originate from typing of a single *Y. ruckeri* isolate. Orange peaks (dye LIZ) represent the size standard employed. Please click here to view a larger version of this figure.

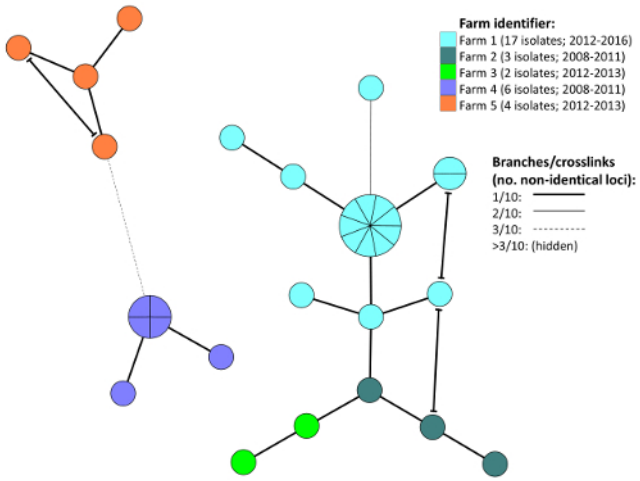

Figure 3: Example Minimum spanning tree for epidemiological evaluation. The diagram is based on MLVA profiles from *Y. ruckeri* isolates recovered from Atlantic salmon in five different Norwegian farms (1-5; see legend) experiencing recurrent yerisniosis outbreaks. A clear clustering tendency linked to farm origin can be observed. Crosslinks show all possible connections involving ≤1/10 non-identical VNTR loci (see legend). Please click here to view a larger version of this figure.

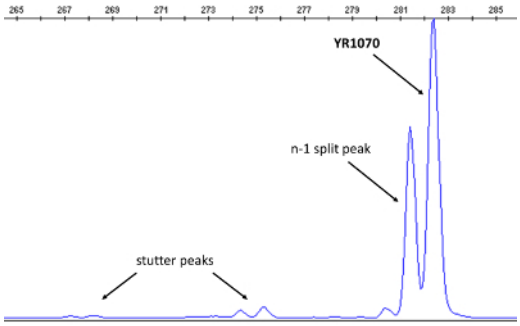

Figure 4: Electropherogram visualizing stutter and split peaks. In this case, both occur simultaneously, which is not always the case. The longer and taller peak, representing the YR1070 VNTR locus, can be readily distinguished. The display is magnified and shows only blue dye peaks. Please click here to view a larger version of this figure.

VNTR locus	Primer sequence (5'-3')		Multiplex PCR		Variables for VNTR repeat count calculation				PCR fragment size range (bp)
	Forward (with 5' dye labelling indicated)	Reverse	Assay	Primer conc. (µM)	VNTR flank size (bp)	VNTR repeat size (bp)	slope (*s*)	intercept (*i*)	
YR2365	6FAM-CCTCGGAAACATAACTTATCGGAC	CCTCTGAAAGAGTACATCTCAGCAT	1	0.2	146	7	1.023	-0.994	195-475
YR3168	VIC-ATCACGAATAAACTCTTGGGTGGA	CCTACCGCATATTCCTGGCTAAAT	1	0.1	95*	7	0.979	3.957	101-319
YR1524	VIC-TAATCCAGGCAGAATGGCAAAAAC	AAAATGTCTGTGATGGACAGTTGC	1	0.1	375	8	0.991	6.346	391-519
YR2276	NED-GTACGGATTGACTTGCATCCAAAA	GATAAATTAATCGGCCACAAGTGA	1	0.1	103	5	1.008	3.224	123-243
YR3750	NED-GAGACAAAGGATGCAGAGTACTGG	CTGATGCAATAATGACAAAGCCCA	1	0.2	315	8	0.990	1.728	339-635
YR1070	6FAM-GGTTATGTATTTTCAACAACCGCGA	TCCAACTCACCAATAACCCATCAA	2	0.2	171*	7	0.990	3.714	198-437
YR57	VIC-CTGAGCTTGTAGTGGTGTACTGAT	CAGCAATGATTTGAGCTGTAGCAA	2	0.1	82	6	1.002	1.602	94-160
YR940	VIC-ACCACAGCATAGTGTTATCCCAAA	TAAACTCAACTTGATCTGTGCCCT	2	0.2	305	8	0.968	13.151	313-585
YR1899	NED-ATCCCAAAACTATCCGGTGACAAT	CACCAAGGTAACCCTAGGCTAATA	2	0.2	87	9	0.989	4.968	105-222
YR2794	NED-TTGGAGCATGAAATGAGTTTTCCG	AACTCTTTGCCGTATTCGGTTTTC	2	0.1	419	6	1.007	-0.252	443-509

*: A single base pair deletion observed in the left flank of some isolates. This does not influence the final VNTR repeat count.

Table 1: VNTR locus characteristics. Relevant characteristics of the ten *Y. ruckeri* VNTR regions targeted in the present MLVA protocol.

Discussion

Both multiplex PCRs presented here have appeared relatively robust in the face of poor template DNA quality, but lack of PCR amplification was nevertheless occasionally observed when using templates with extremely high DNA concentrations. These issues were readily resolved by diluting the templates prior to PCR. Other methods for DNA extraction than the one employed here may also be used (e.g., commercial kits).

Although five amplicons are expected from each multiplex PCR reaction, five visually distinguishable bands should not always be expected from GE, as some (differently labelled) VNTR loci within the same reaction have overlapping size ranges. The final PCR extension time of 60 min may be shortened if required, but will likely result in the increased occurrence of split peaks in subsequent CE electropherograms (see below). Notably, as the purpose of the GE step is purely for qualitative verification of PCR amplicons, the run time, voltage and/or gel recipe may be adjusted as preferred. If particularly weak bands are observed by GE, it may be advisable to reduce the dilution factor of those samples prior to CE.

While the CE protocol described here was run on a specific commercial capillary electrophoresis apparatus (see **Table of Materials**), different CE systems may have different sample requirements, which may in turn prompt some modifications to the protocol. Refer to the manual of the respective CE system manufacturer for instructions on appropriate reagents/equipment, calibration etc. for fragment analysis. There is also a possibility that the biased amplicon mobility patterns observed during CE may differ, relatively, across CE systems and/or machines, as has previously been documented for other MLVA protocols[15,16]. If occurring to an extent where final (rounded) VNTR repeat counts become affected, this means the locus-specific variables *s* and *i* (**Table 1**), used to determine VNTR repeat counts, must be re-calibrated. This involves linear regression on plots comparing accurate sequence sizes versus CE size calls, as described by Gulla et al. 2018[14].

Split peaks and stutter peaks, both well-known artefacts in CE based MLVA typing[17], may be observed in electropherograms during size calling (**Figure 4**). While stutter peaks should be disregarded, the longer peak should consistently be selected for downstream applications in the case of split peaks separated by a single base pair. Moreover, absent peaks indicating lack of particular VNTR loci are rare, but may occur, in which case a repeat count of '0' should be assigned. If the starting culture from which DNA is extracted is not pure (i.e., contains more than one *Y. ruckeri* sub-type), multiple tall peaks corresponding to different alleles of the same locus/loci may be observed following CE. Secondary cultivations must then be performed from single colonies prior to new DNA extraction for re-typing.

As stated in the protocol, template DNA for PCR should by default be extracted from pure cultures of *Y. ruckeri*. In a few cases, however, egg-fluid samples testing positive for *Y. ruckeri* by qPCR (Ct-values < 27) were successfully MLVA typed directly, without prior culturing, using an increased amount of genomic DNA (extracted with commercial kit) as template. Although this approach has not been extensively tested nor verified, it does indicate the potential of this MLVA assay for examination of complex biological matrices containing DNA from a range of different organisms in addition to *Y. ruckeri*.

The entire MLVA typing procedure presented here, from DNA extraction to epizootiological evaluation, may be completed in a single working day. However, the number of samples examined is in a sublinear relationship with the time required for DNA extraction, PCR and CE, and the method is therefore much more time efficient when running multiple samples simultaneously. This is nevertheless the case for most lab-based methods,

and as a tool for epidemiological subtyping of *Y. ruckeri*, the combination of high resolution, simplicity and portability makes this MLVA assay superior to previously published protocols[4,5]. It has also been used to verify the limited epidemiological relevance of *Y. ruckeri* serotyping[14].

Through a comprehensive MLVA based population study involving 484 *Y. ruckeri* isolates recovered from a range of spatiotemporal origins and habitats (host fish, environment, etc.), our understanding regarding the epizootiology and population structure of this important fish pathogen was substantially increased[14]. MLVA typing enabled the tracing of clones disseminated anthropogenically over decades, presumably through transport of fish, as well as identification of locally confined strains. Moreover, while some clonal complexes of the bacterium could clearly be associated with disease in particular fish hosts (rainbow trout and Atlantic salmon, respectively), others were only recovered from environmental sources and/or clinically unaffected fish specimens. The applicability of the method is thus not only limited to infection tracing, as it may also provide information of potential relevance e.g. for vaccine development, risk assessment, and maintenance of national biosecurity. It is currently in active use at the Norwegian Veterinary Institute as a tool for investigating *Y. ruckeri* diagnoses in Norwegian aquaculture.

Disclosures

The authors have nothing to disclose.

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