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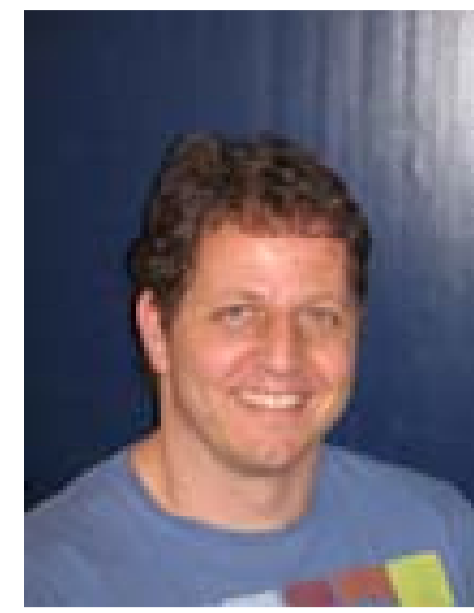
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Validation of a Taqman based real time RT-PCR assay suitable for surveillance and diagnosis of Viral Haemorrhagic Septicaemia Virus worldwide



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Introduction: Viral haemorrhagic septicaemia (VHS) is a serious disease in several fish species. VHS is caused by the rhabdovirus Viral Haemorrhagic Septicaemia Virus (VHSV). To prevent outbreaks it is important to have a fast, sensitive, and specific diagnostic tool to identify infected fish. Traditional diagnosis based on isolation in cell culture followed by identification using e.g. ELISA is sensitive and specific but rather slow. Surveillance for declaring freedom of VHS in fish farms is hitherto based on cell cultivation since no serological- or molecular test had previously been fully validated and documented to have a competitive sensitivity and specificity to the cell culture methods. By switching to RT-PCR for surveillance and diagnosis of VHS the time needed before an answer is available will be considerably shortened and the need for maintaining expensive cell culture facilities reduced. Here we present the validation of a sensitive and specific Taqman based real time RT-PCR that detects all VHSV isolates in a panel of 79 VHSV isolates covering all known genotypes and subtypes. The sensitivity of the real time RT-PCR is comparable to traditional cell based methods and it is possible to obtain a diagnosis within one work day.

Genotype	Number of isolates	Jonstrup	Garver
I	2	21,9	23,2
Ia	18	20,3	21,3
Ib	14	19,7	21,0
Ic	3	19,4	20,3
Id	3	20,8	21,7
Ie	2	21,3	23,1
II	4	21,6	25,1
III	11	18,8	22,7
IVa	16	20,1	19,2
IVb	6	19,1	19,1
Controls	15	No Ct	No test

Figure 3: Analytical specificity test. The average Ct values obtained by using the Jonstrup et al. PCR or the Garver et al. PCR are shown. Each Ct value is based on an average of a number of different VHSV isolates and each of these were performed in triplicates. Also a panel of 15 other fish viruses was used. Unspecific signals were not observed for the Jonstrup et al. PCR indicating an **analytical specificity close to 1**. It was also concluded that our PCR competes with the Garver et al. PCR and especially for genotype II and III it performs significantly better.

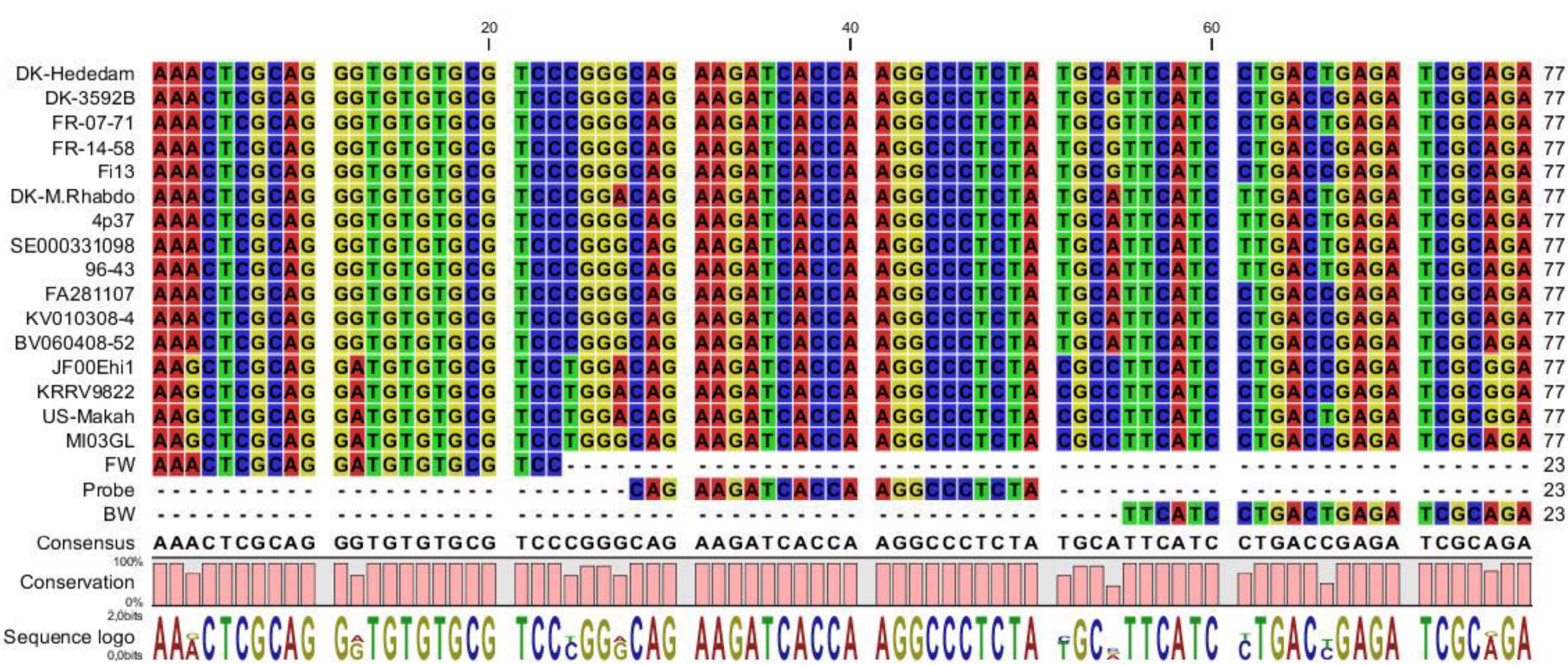


Figure 1: Design of Taqman probe and primers. Alignment of nt 532-608 (according to GenBank accession number Z93412) of the N-gene of different VHSV isolates spanning most genotypes. VHSV sequences were retrieved from www.FishPathogens.eu. The probe was designed to target an area where no variation between isolates were seen while primers were designed so that maximum one mismatch in the FW primer and two in the BW primer was observed.

Diagnostic specificity

Known positive diagnostic samples (16) from recent Danish VHS outbreaks and 43 known negative surveillance samples from Danish VHSV free rainbow trout farms were examined. All positive isolates were detected with Ct values of 15 to 25 and none of the known negative samples gave rise to any false positive reactions. An internal ELF1a control ensured that the lacking signal was not due to degradation of sample. Taken together with the results from the investigations of analytical specificity it was concluded that the diagnostic specificity of this assay is very close to 1.

Diagnostic sensitivity

Rainbow trout (84) from a VHSV infection study were examined using both cell culture and the Jonstrup et al. real time RT-PCR. In 35 fish the cell culture titer was below the detection limit ($<1.9 \times 10^2$ TCID₅₀ ml⁻¹). The remaining 49 fish had titer values evenly distributed from low to high. In 25 of the 35 fish all with titers below detection limit no virus were detected in either test. In 45 fish virus was detected by both methods. 8 fish were positive in PCR but not in cell culture and 6 vice versa. All fish with a titer $\geq 2.7 \times 10^3$ TCID₅₀ ml⁻¹ were detected using both methods. Assuming no false negatives or false positives the diagnostic sensitivity of the real time RT-PCR was determined to 53/59 (0.90), while the diagnostic sensitivity of the cell culture experiment was 51/59 (0.86).

	10 ⁰	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵
BF-2	+++	+++	+++	++		
EPC	+++	+++	+			
Jonstrup et al.	+++	+++	+++	+++	++	+

Figure 2: Analytical sensitivity. 10x dilutions of VHSV isolates were made in triplicate for all 4 genotypes (Ia shown as example here). Each dilution was tested for VHSV by either traditional diagnosis (cultivation on BF-2 or EPC cell lines followed by ELISA) or by the Jonstrup et al. RT-PCR. The number of plusses indicates the number of positive wells found within a specific triplicate diagnosed by a specific method. Sensitivity of the cell culture versus PCR was found to be within the same range for all genotypes (+/- 10²).

Conclusions:

Analytical and diagnostic specificity determined to be very close to 1
- No false positives observed

Diagnostic sensitivity determined to be around 0.90
- When RT-PCR is performed in triplicates
- Cell culture had a sensitivity of around 0.86

Assay also passed tests on (data not shown here):
- Robustness, ruggedness, repeatability, and reproducibility

Performs at the same level or better than other published real-time RT-PCR assays for detection of VHSV

