

About me!

- **Veterinarian** (2014, Universidade de Sao Paulo, Brazil)
 - Toroviruses, Noroviruses and Kobuviruses in cattle.



- **Master of Science** (2016, Universidade de Sao Paulo, Brazil)
 - Isolation and characterization of Ranavirus from bullfrogs and fish.
 - **Internship at DTU Vet** – 6 months (2015)



- Experimental infection of Rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar L.*) with Piscine Reovirus and Virus Y. (FAPESP Scholarship abroad: 2015/04027-6)

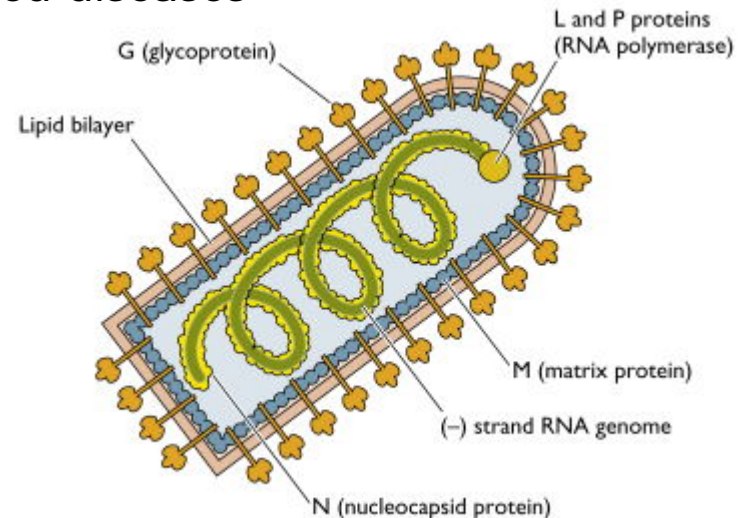
About me!

- PhD at DTU Vet
 - “Identification of virulence markers in two *Novirhabdoviruses* causing serious diseases in fish.”
 - Supervised by Professor Niels Jørgen Olesen and co-supervisors Thomas Bruun Rasmussen (DTU Vet) and Michel Bremont (INRA, France)
 - Started on July 2016;
 - Feb 2017: External research stay at INRA (Jouy en Josas, France)



Background & Introduction

- **Viral Hemorrhagic Septicemia Virus (VHSV)**
- **Infectious Hematopoietic Necrosis Virus (IHNV)**
 - Council Directive 2006/88/ECC; listed diseases
- *Rhabdoviridae*
 - genus *Novirhabdovirus*
- RNAss genome (~ 11000nt)



Background & Introduction

- VHSV is endemic among marine fish species in EU = Reservoir
- Severe disease in RT;
 - Mortality varies



Background & Introduction

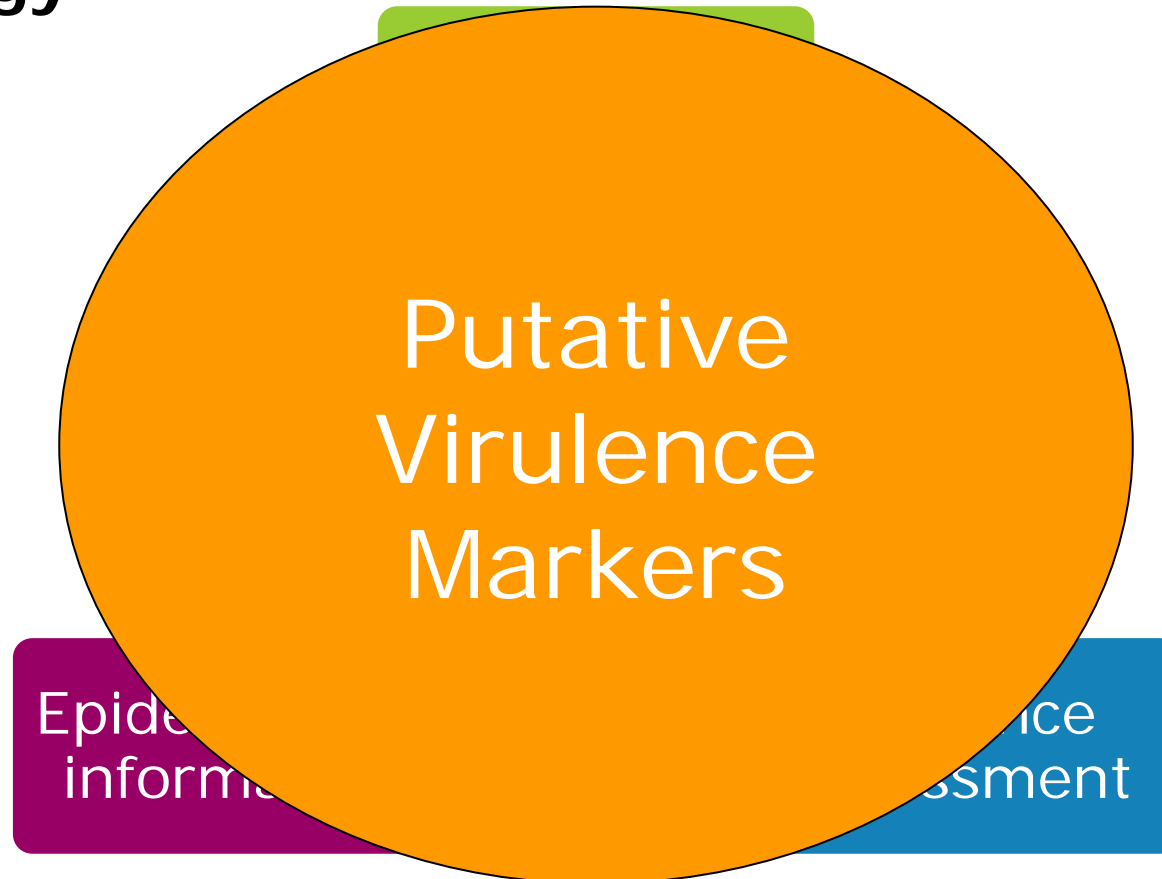
- VHSV : Four genogroups
- IHNV : Five genogroups
 - All based on G and N genes – related to origin;
- VHSV and IHNV can infect a large range of species of fish;
- Marine isolates produce medium to no mortality in RT and same occurs in reverse;
- However, marine isolates are indistinguished from RT isolates through serological methods.

Objectives

1. Identify **virulence markers** and traits of both IHNV and VHSV;
2. Using recombinant virus technology, access and **characterize** the efficacy of the virulence determinants;
3. Develop fast and reliable **diagnostic methods for discriminating** between virulent and non-virulent virus isolates.



Strategy



Recovery of NV Knockout Infectious Hematopoietic Necrosis Virus Expressing Foreign Genes

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Limited Interference at the Early Stage of Infection between Two Recombinant Novirhabdoviruses: Viral Hemorrhagic Septicemia Virus and Infectious Hematopoietic Necrosis Virus^{∇†}

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What have we done so far?



Selection of VHSV isolates

Sample nr	Genotype	Name	Original Passages in cells	Description	Year
2016-13894-01	Id	Fin Ka 423/00	1 pass in BF2	From seafarmed rainbow trout in Finland	2000
2016-13894-02		Fin Ka 423/00	1 pass in BF2	From seafarmed rainbow trout in Finland	2000
2016-13894-03		No-A 163/68		From farmed freshwater rainbow trout in Norway	1968
2016-13894-04	Ie	GE 1.2		From rainbow trout in Georgia	1981
2016-13894-05		Trabzon 207111		From rainbow trout in Turkey	
2016-13894-06		Fikret Koksal 2783	P14 RTG2	From Sea bass in Turkey	
2016-13894-07	Ic	DK 3612	2 pass EPC	From Blåhøj, DK	1986
2016-13894-08		DK 2149	? Pass in BF2	From St Karlskov, DK	1978
2016-13894-09	I	F1-8		Passage 8 of DK isolate for REVA st (medium virulent)	1966
2016-13894-10		F1-253	3 pass BF2	Passage 253 of REVA st for live attenuated vaccine (low virulence)	1966
2016-13894-11		F1-506	7 pass EPC 1 pass BF2	Passage 506 of REVA st for live attenuated vaccine (non-virulent)	1966
2016-13894-12	Ia	DK 3592B		Isolate from Voldbjerg, DK (1989) used as high virulent strain in trials	1986
2016-13894-13		DK 203490	"0" pass org 3	Isolate from Hesselvig, DK (2003). Modern high virulent strain.	2003

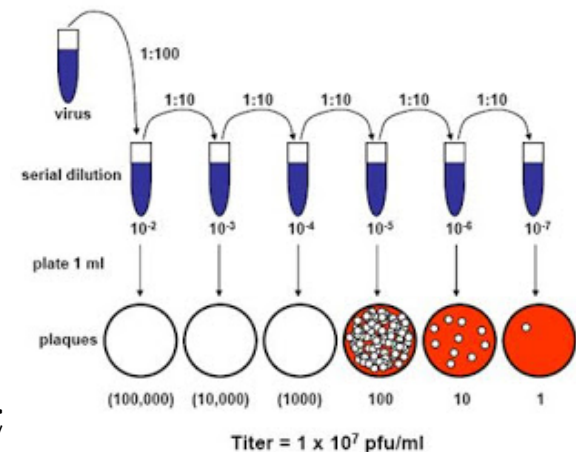
Propagation of selected isolates

- Initial propagation of each isolate in cell culture (EPC and BF-2);

- Plaque cloning of each isolate;

- Collected plaques and propagated in cell culture;

- Titrated and aliquoted for use: both infection trial and whole genome sequencing.



Evaluation of virulence of field isolates

- **Infection trial on DTU Vet experimental facilities**
- 12-09-2016 to 12-10-2016 (31 days)
- 14 groups (13 isolates + Negative Control)
- 3 tanks (replicates) per isolate
- 50 fish (rainbow trout SPF, 1g) per tank
- In total: 2100 RT in 42 tanks



Evaluation of virulence of field isolates

- All fish were subjected to immersion in water containing 10^5 TCID₅₀/mL of each VHSV isolate for 5 hours;
- Monitored daily for clinical signs and mortality;
- Fish that showed acute clinical signs were euthanized and collected-kept under -80C.



Evaluation of virulence of field isolates

- Fish collected during the trial will be tested on cell culture;
- Positive samples on cell culture – ELISA for VHSV;
- Also sent all isolates for whole genome sequencing at ANSES;

Sequence comparison: Ic Genotype

	DK 3612 x DK 2149
Mortality	19% mortality x 66% mortality
N gene	5nt / 3aa
P gene	6nt / 2aa
M gene	1nt
G gene	5nt / 1aa
NV gene	2nt
L gene	18nt / 7aa

In all genes analyzed for Ic isolates, there are only **37 nt differences** and only **13** of these substitutions cause alterations on aa profile (the others are silent).

Sequence comparison: Ie Genotype

	Fikret Koksal x Trabzon 207111
Mortality	79% mortality x 31% mortality
N gene	100% similar
P gene	100% similar
M gene	100% similar
G gene	2nt / 2 aa
NV gene	14 nt / 9 aa
L gene	5nt / 2 aa

In all genes analyzed for Ie isolates, there are only **18 nt differences** and only **13** of these substitutions cause alterations on aa profile (the others are silent).



Sequences comparison

- Look for a motif / combination of nt/aa

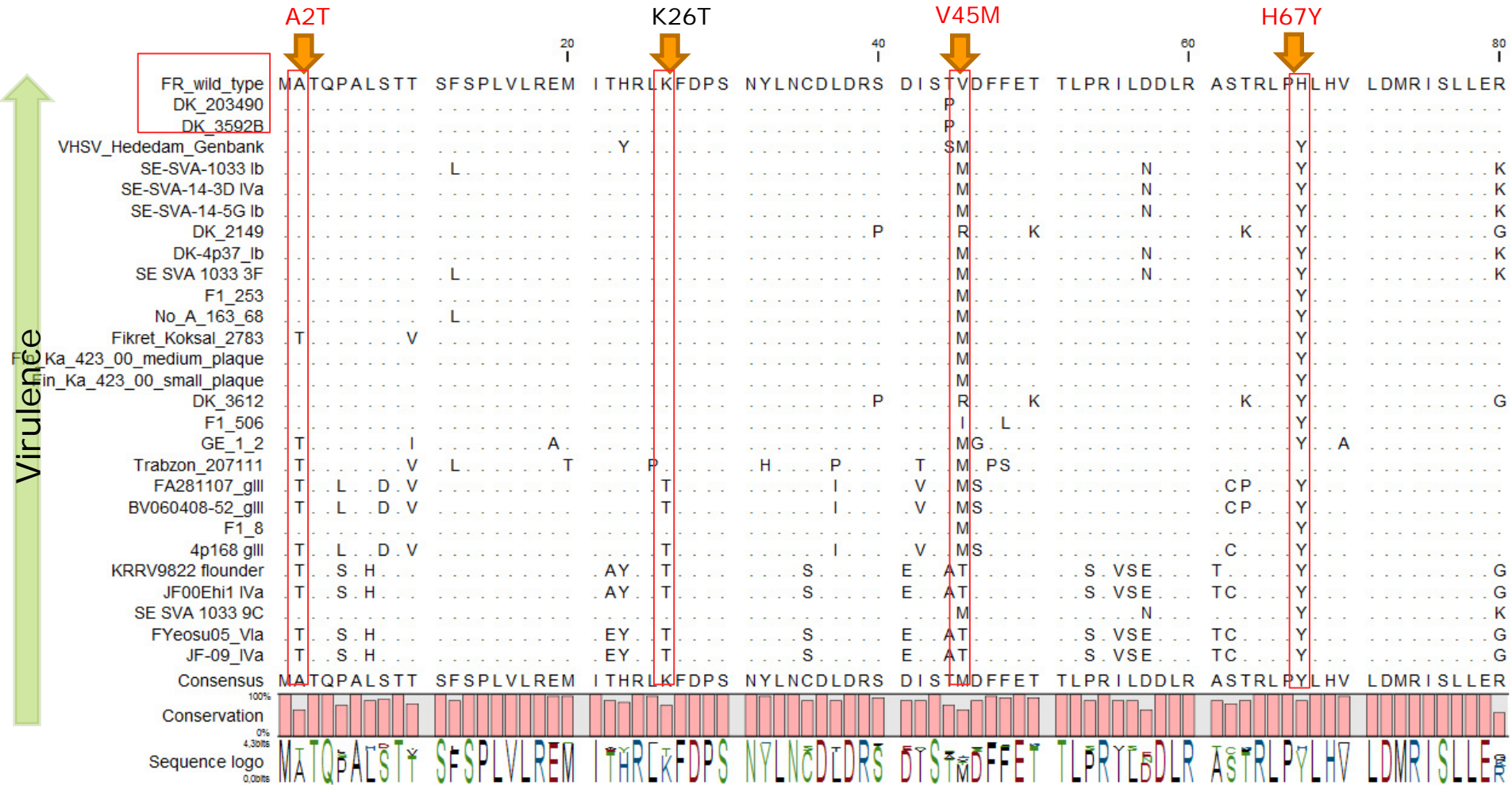
Assessment of the epitope related site of mAb VHS-3.80; ExDGKV (Ito *et al.*, 2012)

Isolates	Variant clone	Accession no.								
DK-3592B (Typical isolate as GIa)	KC778774	1	MEGGIRAAFS	GLNDVRIDPT	GGEGRVLPVPG	DVELIVYVGG	FG	EEDGKV	IV	50
4p37 (Typical isolate as GIb)	FJ460590	1	MEGGIRAAFS	GLNDVRIDPT	GGEGRVLPVPG	EVELIVYVGG	FG	EEDGKV	IV	50
M Rhabdo (GIb)	Z93414	1	MEGGIRAAFS	GLNDVRIDPT	GGEGRVLPVPG	EVELIVYVGG	FG	EEDGKV	IV	50
SE-SVA-14-3D	AB839745	1	MEGGIRAAFS	GLNDVRIDPT	GGEGRVLPVPG	EVELIVYVGG	FG	KEDGKV	IV	50
SE-SVA-14-5G	AB839746	1	MEGGIRAAFS	GLNDVRIDPT	GGEGRVLPVPG	EVELIVYVGG	FG	KEDGKV	IV	50
SE-SVA-1033-3F	AB839748	1	MEGGIRAAFS	GLNDVRIDPT	GGEGRVLPVPG	EVELIVYVGG	FG	EEDR	RKIV	50
SE-SVA-1033-9C	AB839747	1	MEGGIRAAFS	GLNDVRIDPT	GGEGRVLPVPG	EVELIVYVGG	FG	EEDGKV	IV	50
DK-3592B (GIa)	KC778774	51	DALSALGGPQ	TVQALSVLLS	YVLQGNTOED	LET	KCKVLT	MGFKVTQA	VR	100
4p37 (GIb)	FJ460590	51	DALSALGGPQ	TVQALSVLLS	YVLQGNTOED	LGMKCKVLT	MGFKVTQA	AAR		100
M Rhabdo (GIb)	Z93414	51	DALSALGGPQ	TVQALSVLLS	YVLQGNTOED	LGMKCKVLT	MGFKVTQA	AAR		100
SE-SVA-14-3D	AB839745	51	DALSALGGPQ	TVQALSVLLS	YVLQGNTOED	LGMKCKVLT	MGFKVTQA	AAR		100
SE-SVA-14-5G	AB839746	51	DALSALGGPQ	TVQALSVLLS	YVLQGNTOED	LGMKCKVLT	MGFKVTQA	AAR		100
SE-SVA-1033-3F	AB839748	51	DALSALGGPQ	TVQALSVLLS	YVLQGNTOED	LGMKCKVLT	MGFKVTQA	AAR		100
SE-SVA-1033-9C	AB839747	51	DALSALGGPQ	TVQALSVLLS	YVLQGNTOED	LGMKCKVLT	MGFKVTQA	AAR		100

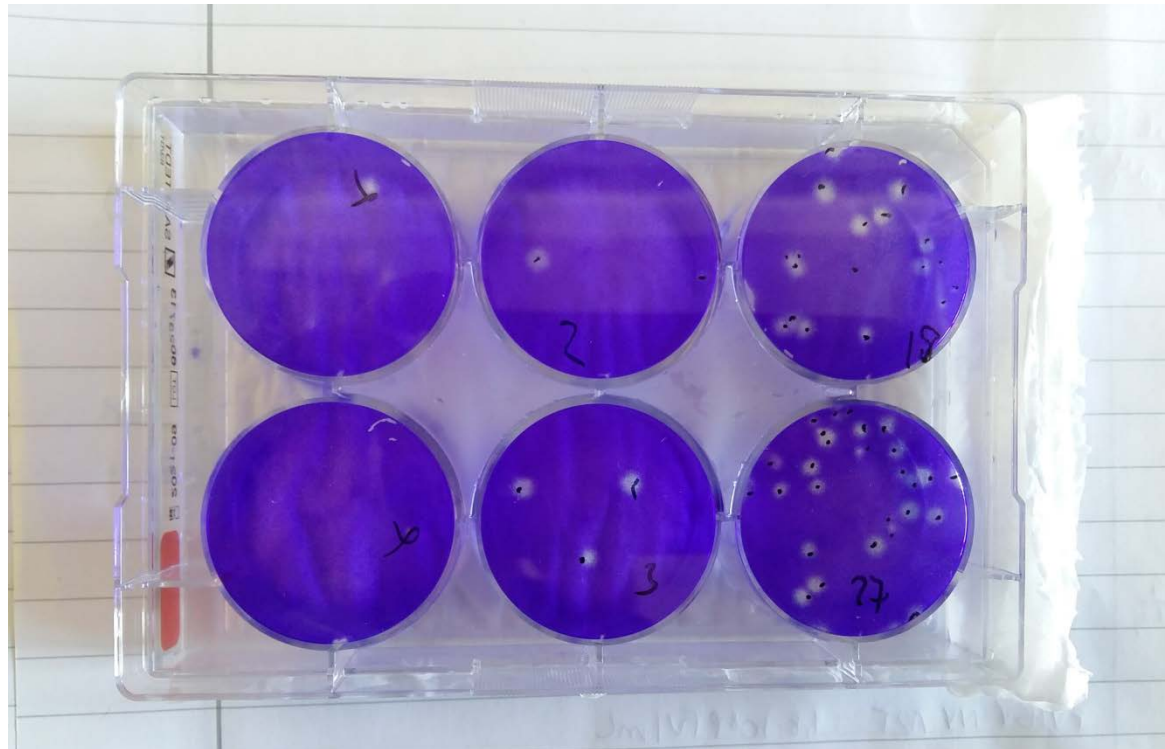
- Virulence determinants might be genotype specific (?)
- Intergenic regions ?

Another approach (INRA)...

NV gene (example)



Reverse Genetics at INRA



Titration by plaque assay

Currently...



More sequences!



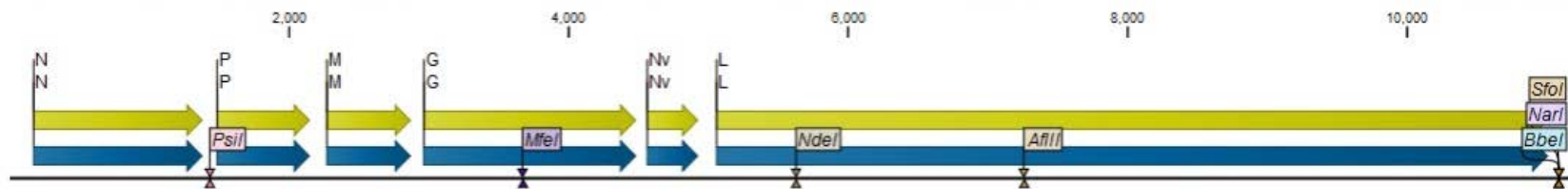
Tube number	Isolate	Genotype	Source isolate
1	FPL2006-005, Goby 1-5	IVb	Groocock et al. (2007)
2	JF-JF00Ehil	IVa	Nishizawa et al. (2002)
3	DK-297 (HEDEDAM)	I	Vestergård Jørgensen (1974)
4	AU-8/95	Ia	University of Veterinary Medicine, Vienna (unpubl.)
5	PL-202473	Ia	National Veterinary Research Institute, Pulawy, Poland (unpubl.)
6	FIN-2ka 66/2000	Id	Einer-Jensen et al. (2004)
7	NO-A163-68 EG46	Id	Håstein, Holt & Krogsrud (1968)
8	IR206239-1	Ie	Ito et al. (2012)
9	FR-L59x	IIIa	Thiery et al. (2002)
10	NO-2007-50-385	IIIb	Dale et al. (2009)
11	CAN-99-019	IVa	Ito et al. (2012)
12	DH2008	IVa	Kim et al. (unpubl.)
13	Blue gill, Budd Lake, MI	IVb	USGS (unpubl.)
14	New Brunswick	IVc	Groocock et al. (2007)
15	DK-3971	Ia	Jonstrup et al. (2009)
16	DK-6137	Ia	Jørgensen et al. (1995)
17	DK-7974	Ia	Jonstrup et al. (2009)
18	DK-9695377	Ia	Einer-Jensen et al. (2004)
19	DK-200051	Ia	Jonstrup et al. (2009)
20	DK-200149	Ia	Jonstrup et al. (2009)
21	DK-1p8	Ib	Mortensen et al. (1999)
22	DK-1p40	Ib	Mortensen et al. (1999)
23	DK-1p116	Ib	Mortensen et al. (1999)
24	DK-5p276	Ib	Skall et al. (2005)
25	DK-4p37	Ib	Mortensen et al. (1999)
26	DK-2835	Ic	Jonstrup et al. (2009)
27	DK-5131	Ic	Jonstrup et al. (2009)
28	DK-5123	Ic	Jonstrup et al. (2009)
29	DK-1p53	II	Mortensen et al. (1999)
30	DK-2p51	IIIa	Snow et al. (1999)
31	UK-H17/5/93	IIIa	Smail (2000)
32	UK-860/94	IIIa	Ross et al. (1994)
33	USA-MAKAH	IVa	Brunson et al. (1989)
34	USA-KHV	IVa	Hopper (1989)
35	USA-Elliott Bay	IVa	USGS (unpubl.)
36	Minter Creek, WA	IVa	USGS (unpubl.)
37	Tokul Creek, WA	IVa	USGS (unpubl.)
38	Port Angels, WA	IVa	USGS (unpubl.)
39	CAN-3624	IVa	Traxler et al. (1995), Pac. Biol. Stat. (unpubl.)
40	Quatsino, BC	IVa	USGS (unpubl.)
41	JP-Obama 25	IVa	Takano et al. (2000)
42	BR01Ehil	IVa	Ito et al. (2010)
43	JF01Oit1	IVa	Ito et al. (2010)
44	JSL02Yam1	IVa	Ito et al. (2010)
45	PM05Ehil	IVa	NRIA FRA (unpubl.)
46	DK-3946	Ia	
47	DK-5151	Ia	
48	USA-BC'93		
49	M. rhabdo		
50	DK-1e62	Ib	
51	MLA98/4CO1		???
52	H19/1/93		
53	H16/7/95		
54	DK-4p59		
55	DK-5p405	Ib	
56	DK-5p26	Ib	
57	DK-5E63	Ib	
58	DK-5e454	Ib	

59	H17/1/95	
60	DK-1p125	Ib
61	DK-5p393	Ib
63	DK-5p508	Ib
64	DK-5p795	
65	DK-5p31	Ib
66	DK-5p263	Ib
67	MLA98/4PT1	
68	DK-5p11	Ib
69	MLA98/4PC1	
70	DK-1p50	Ib
71	DK-1p128	Ib
72	DK-5p457	Ib
73	DK-5p785	
74	DK-4p100	
75	F13.02.97	
76	DK-6047	Ia
77	DK-9695152	Ia
78	DK-3345	Ia
79	DK-5133	Ia
80	DK-5243	Ia
81	DK-6143	Ia
82	DK-6435	
83	DK-7054	Ia
84	DK-7217	Ia
85	DK-7299	
86	DK-7300	Ia
87	DK-7824	Ia
88	DK-7843	Ia
89	DK-9795159	Ia
90	DK-9795265	Ia

Propagation in cell culture
and full genome sequencing

Design a backbone with low virulent isolate

- SE-SVA-1033-9C isolate
- Ib genotype
- Full genome sequenced already
 - Test the hot spots suggested by Ito and the mutations made in INRA



3	Rainbow trout (DTU Vet) [I.p. injection]	SE-SVA-14 wild-type	84 ± 1.7	-	77/81
		SE-SVA-14-3D	31 ± 5.4 [^]	-	34/35
		SE-SVA-14-5G	12 ± 1.9	-	9/10
		SE-SVA-1033 wild-type	61 ± 16.7	-	66/67
		SE-SVA-1033-3F	40 ± 8.7 [^]	-	35/42
		SE-SVA-1033-9C	19 ± 1.2	-	16/20
		Negative control	3 ± 3.6	-	0/3
	[Immersion]	DK-3592B	73 ± 12.7	-	50/50
		SE-SVA-14 wild-type	8 ± 1.7	-	8/8
		SE-SVA-14-3D	4 ± 4.2	-	3/4
SE-SVA-14-5G		2 ± 3.2	-	0/2	
SE-SVA-1033 wild-type		5 ± 4.0	-	4/5	
	SE-SVA-1033-3F	13 ± 12.9	-	7/15	
	SE-SVA-1033-9C	4 ± 2.3	-	1/4	
	Negative control	6 ± 4.4	-	0/6	



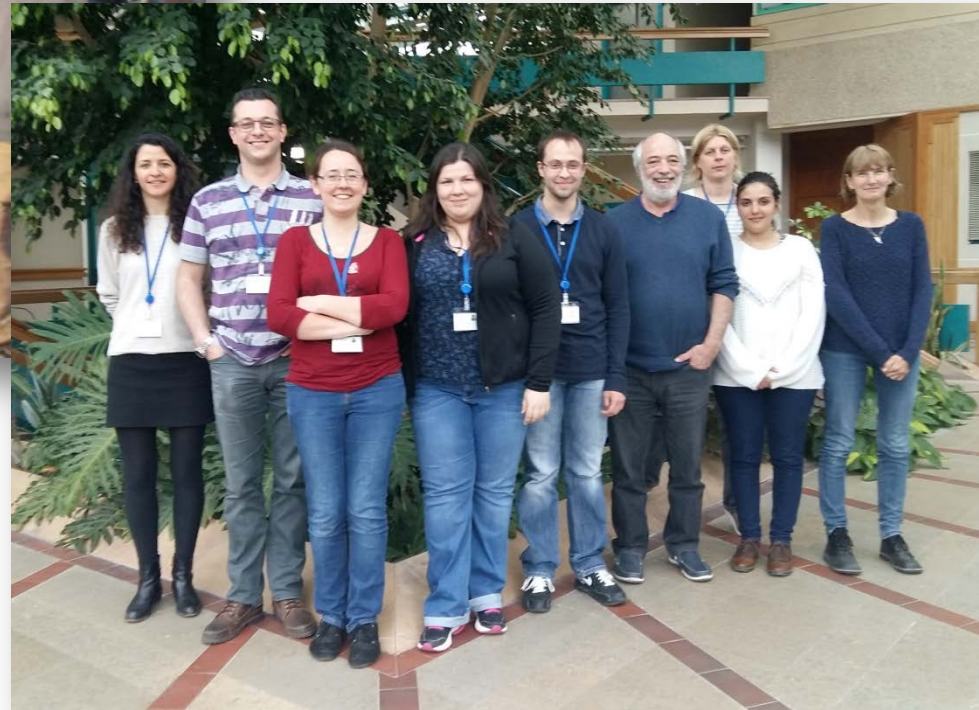
Thanks!



Thanks!



Everybody at the fish diseases group at DTU Vet, DK!



Everybody at the molecular virology of fish diseases group at INRA, France