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Bundesforschungsinstitut für Tiergesundheit
Federal Research Institute for Animal Health

DTU

# 22nd Annual Workshop

# of the National Reference Laboratory for Fish Diseases

## May 30th – 31st 2018

DTU, Lyngby, Denmark

(presentation May 31st 11:20 – 11:40)

# New research on KHV and KHVD

Dr.  Dr. habil. Sven M. Bergmann

OIE Reference Laboratory for KHVD
German NRL for KHVD

Certified Veterinary Specialist (consultant) for Aquaculture and for Fisheries

# Research update on:

1. KHV diagnostics
2. Treatment against KHVD
3. Confirmation of KHV

# 1. KHV diagnostics

CiD (EU) 2015/1554 (Sept. 11th 2015)
valid from April 1st 2017

Manual of Diagnostic Tests for Aquatic Animals 2017, ch 2.3.7.

**EU**

**only to molecular assays**

**OIE recommendation**

**1. KHV qPCR**
Gilad et al. 2004
(Bergmann et al. 2010)
- pooling
  - health monitoring max. 2 fish

**2. KHV conventional PCR**
Bercovier et al. 2005
(CEFAS / FLI + nested+ seq.)
Engelsma et al. 2013 (+ nested + seq.)

**1. KHV conventional PCR**
Bercovier et al. 2005
Yuasa et al. 2005
(both + seq.)

**2. KHV qPCR for surv. only**
Gilad et al. 2004
- pooling
  - KHVD max. 2 fish
  - health monitoring max. 5 fish

# Pathogenesis and sample collection

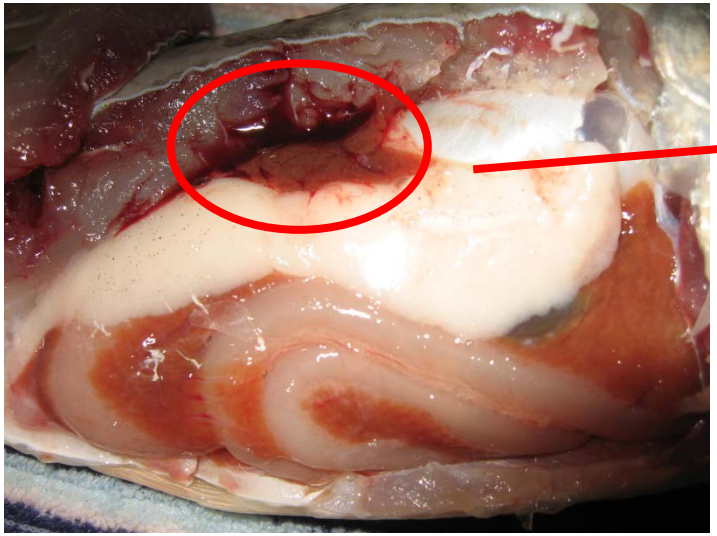
**Early pathogenesis**: 0 – 3 dpi

**to 1st dpi:**
- KHV enters through gill and gut tissues but not via skin (no mRNA)
- after 2 hpi in gill and gut cells (weak mRNA)
- after 4 hpi in leucocytes (weak mRNA – first capsid assembly)
- after 6 hpi in all inner organs (weak mRNA)

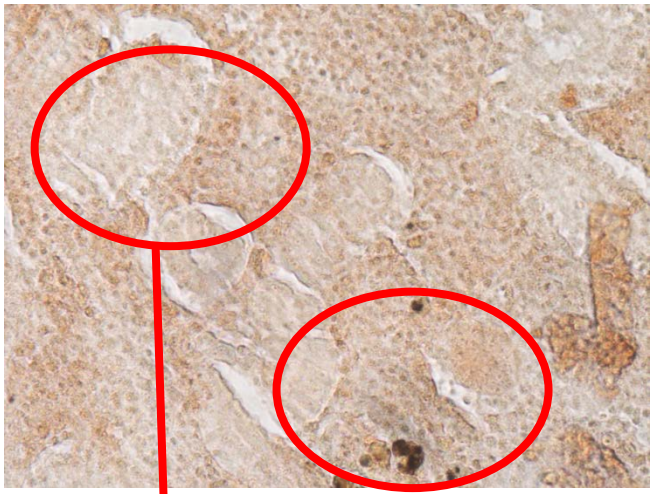
**from 1st dpi** – explosive replication in kidney and liver
- spread by leucocytes
- released via gut

**from 3rd dpi**
- occurrence in skin, gut and gills again in leucocytes but also epithelial cells (gills, perhaps gut)
- virus release via gut and gill

# carp kidney samples and KHV



ISH carp kidney



25 mg

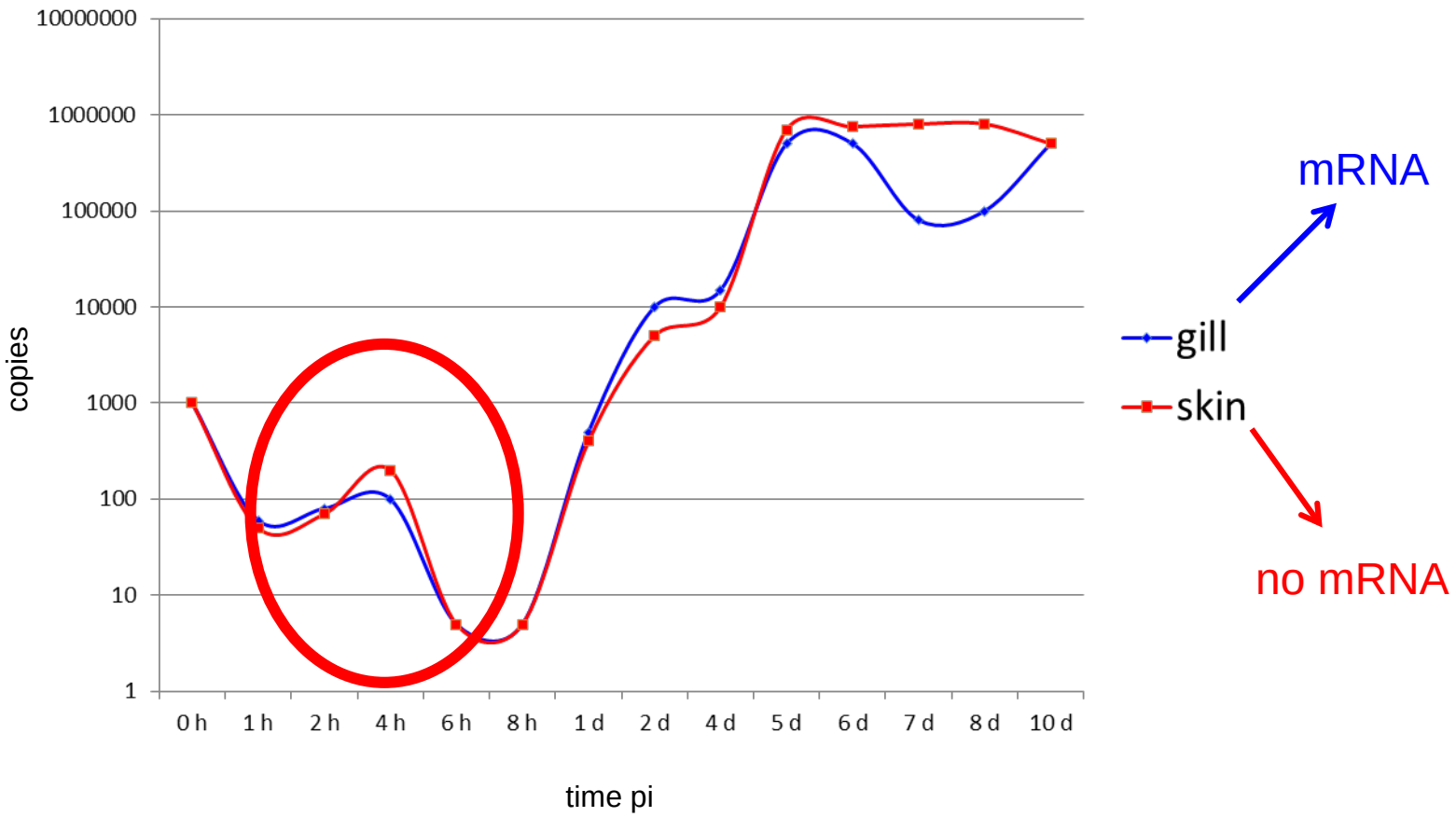
PCR -

PCR +

low sensitivity of virus detection methods due to low concentrations

**+ sensitivity of the assay**
(enzyme etc.)

# **„outside“ tissues** (peracute KHVD)



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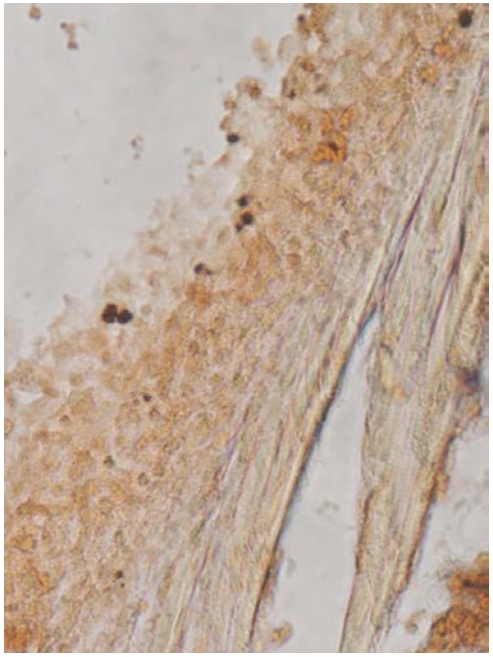
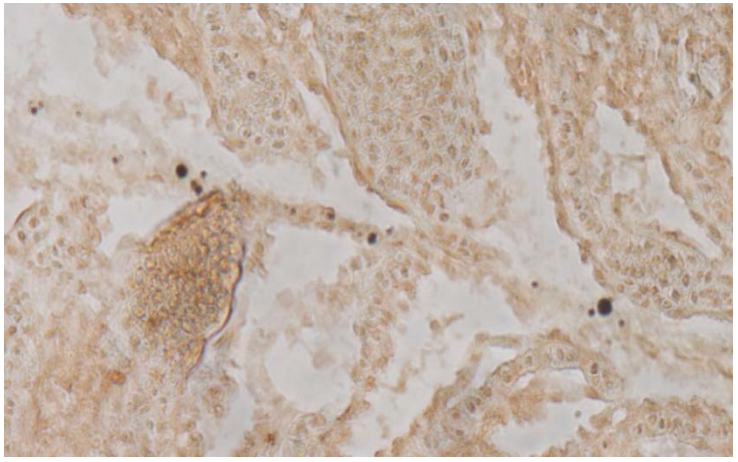
# tissues 1 - 2 h pi

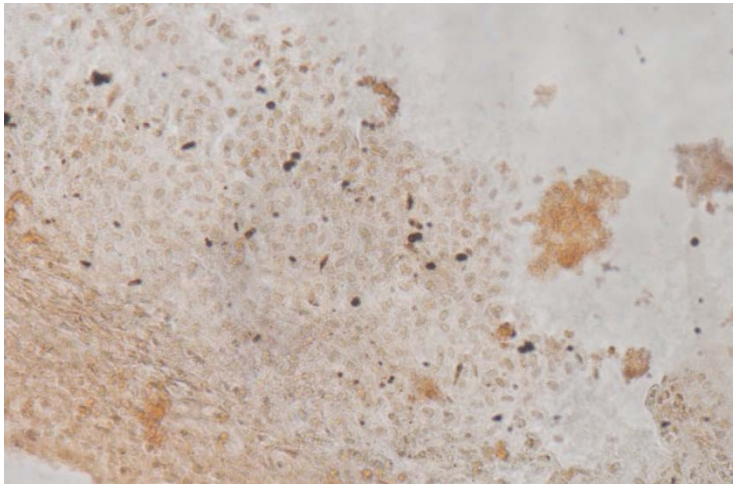
gill



attached from outside

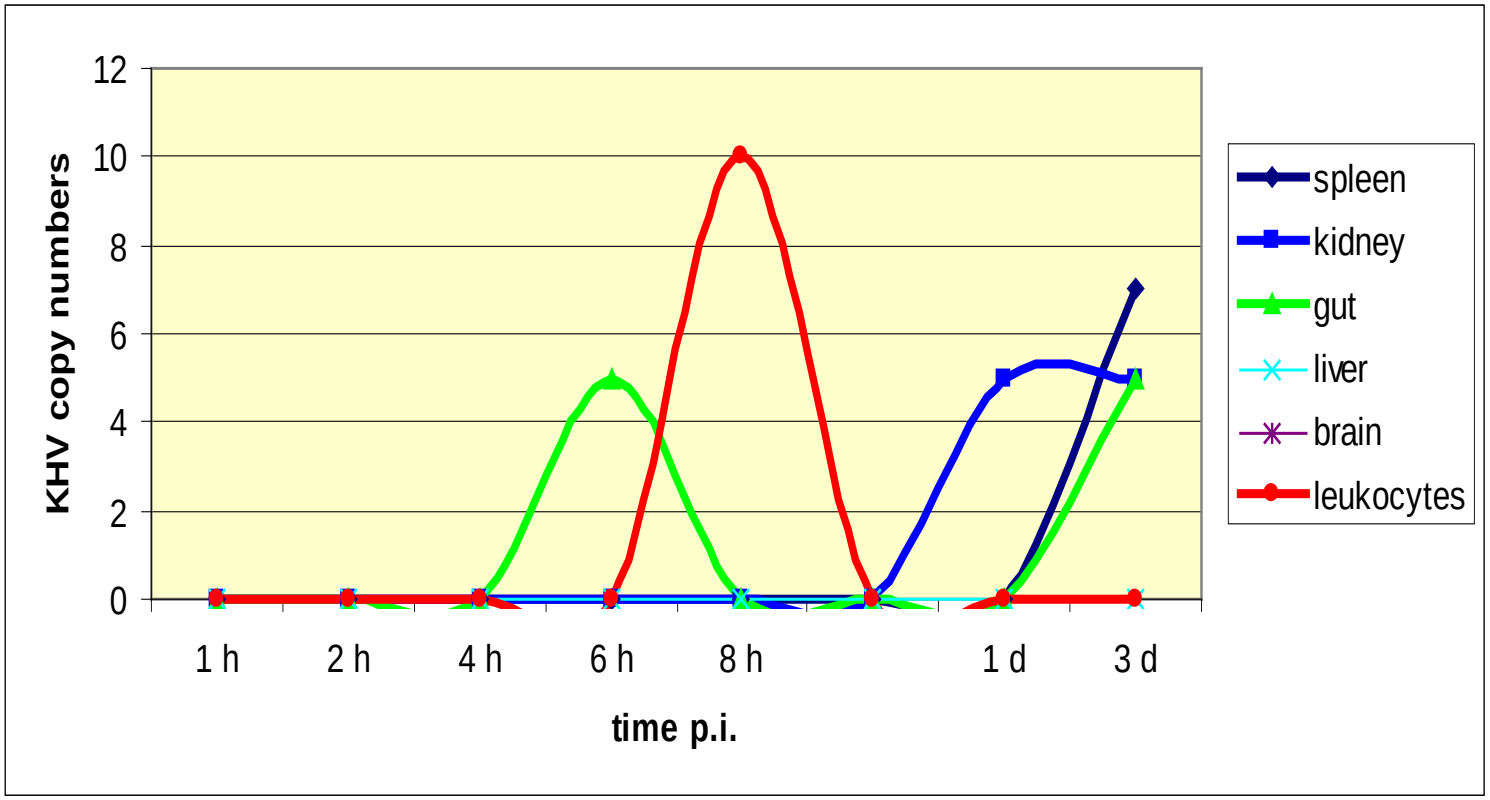
DNA bearing cells in the mucus



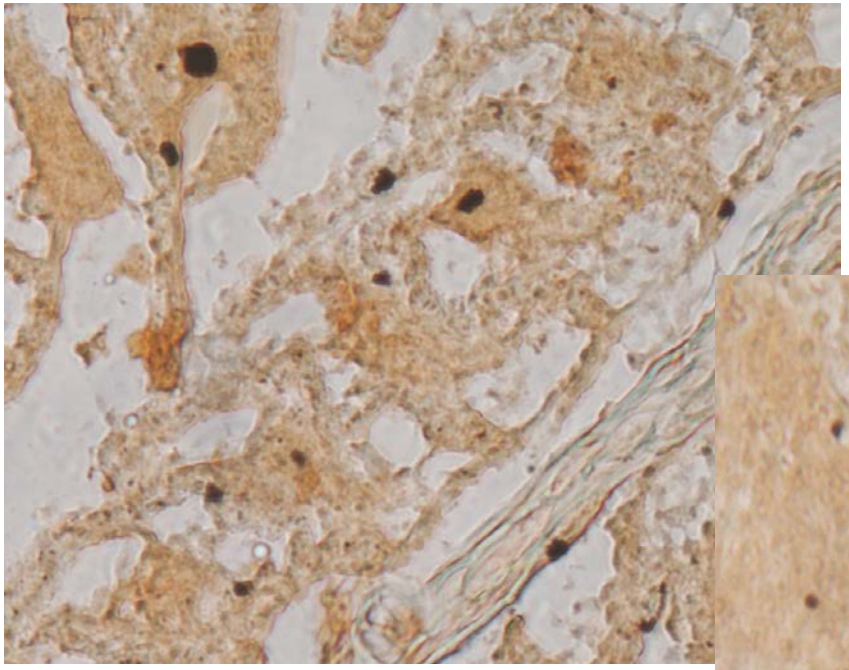


skin
in the mucus only

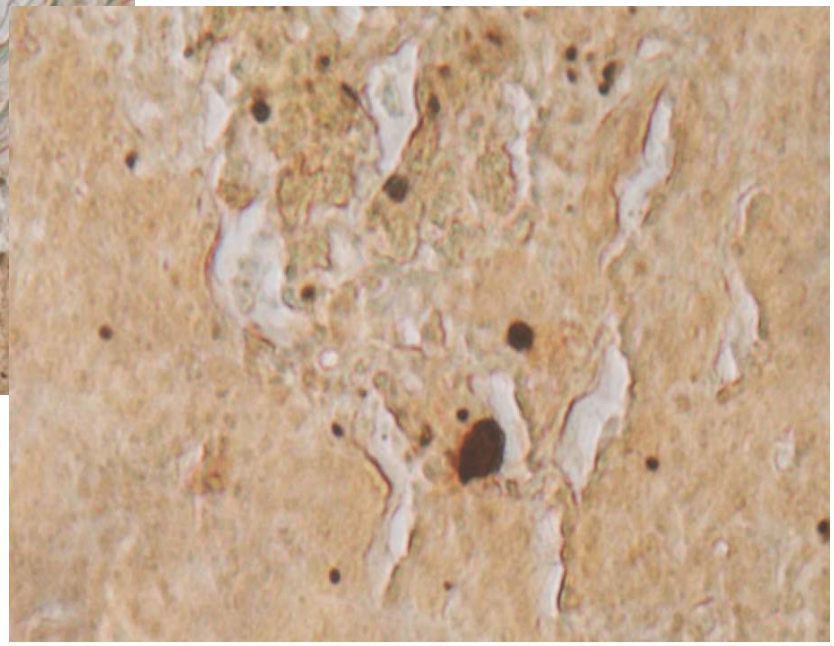
# „inside“ tissues (peracute KHVD)



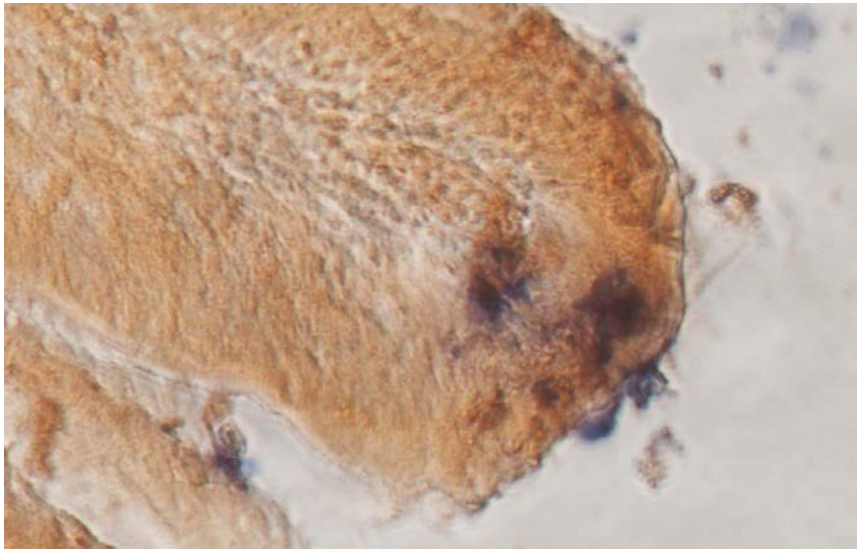
gill

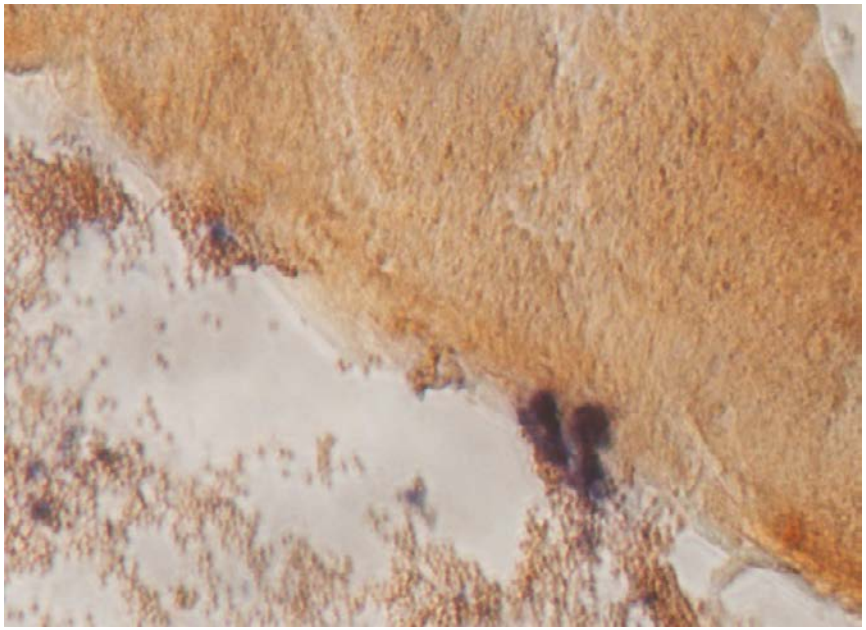


spleen, blood vessels



# gut tissue 4 h pi

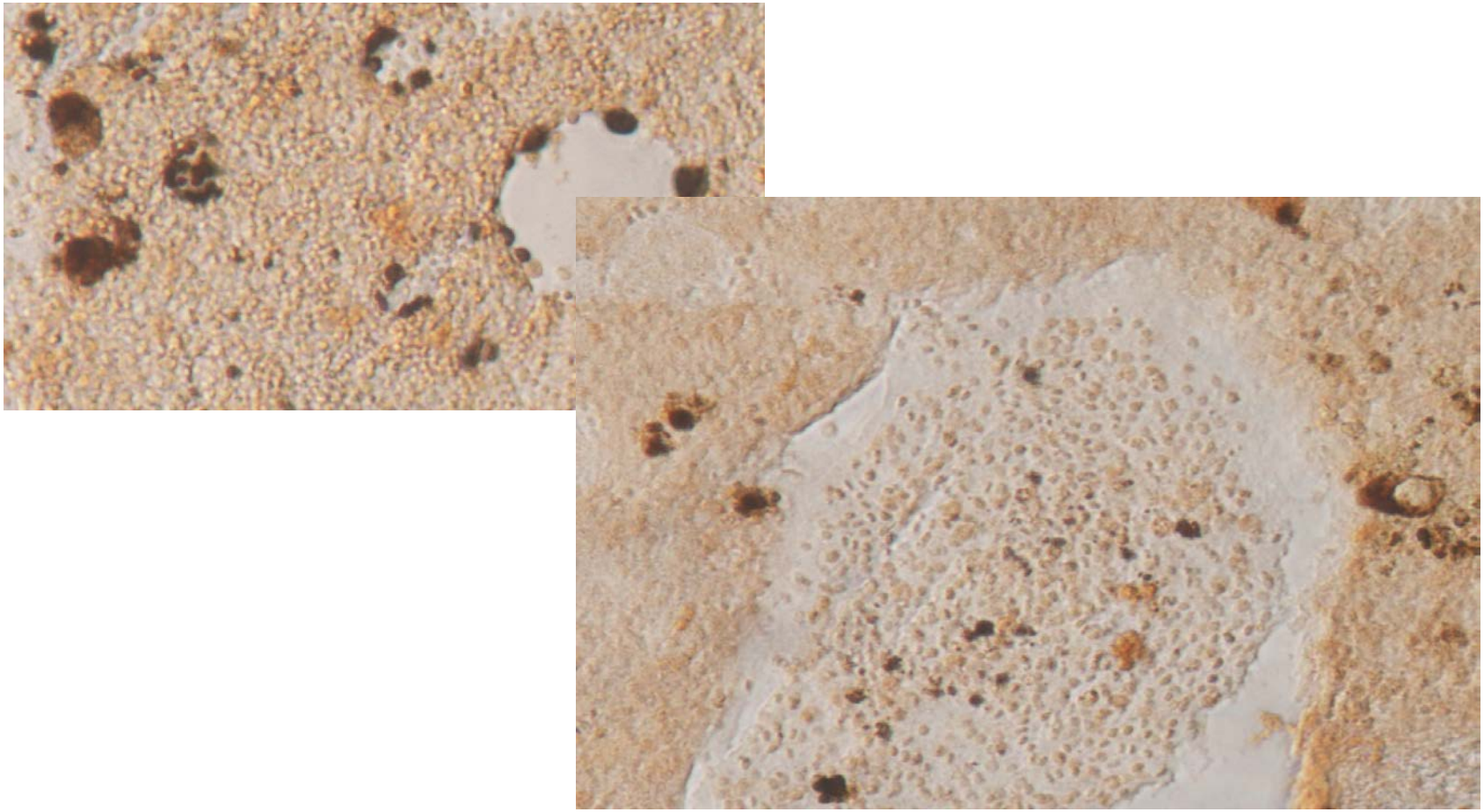




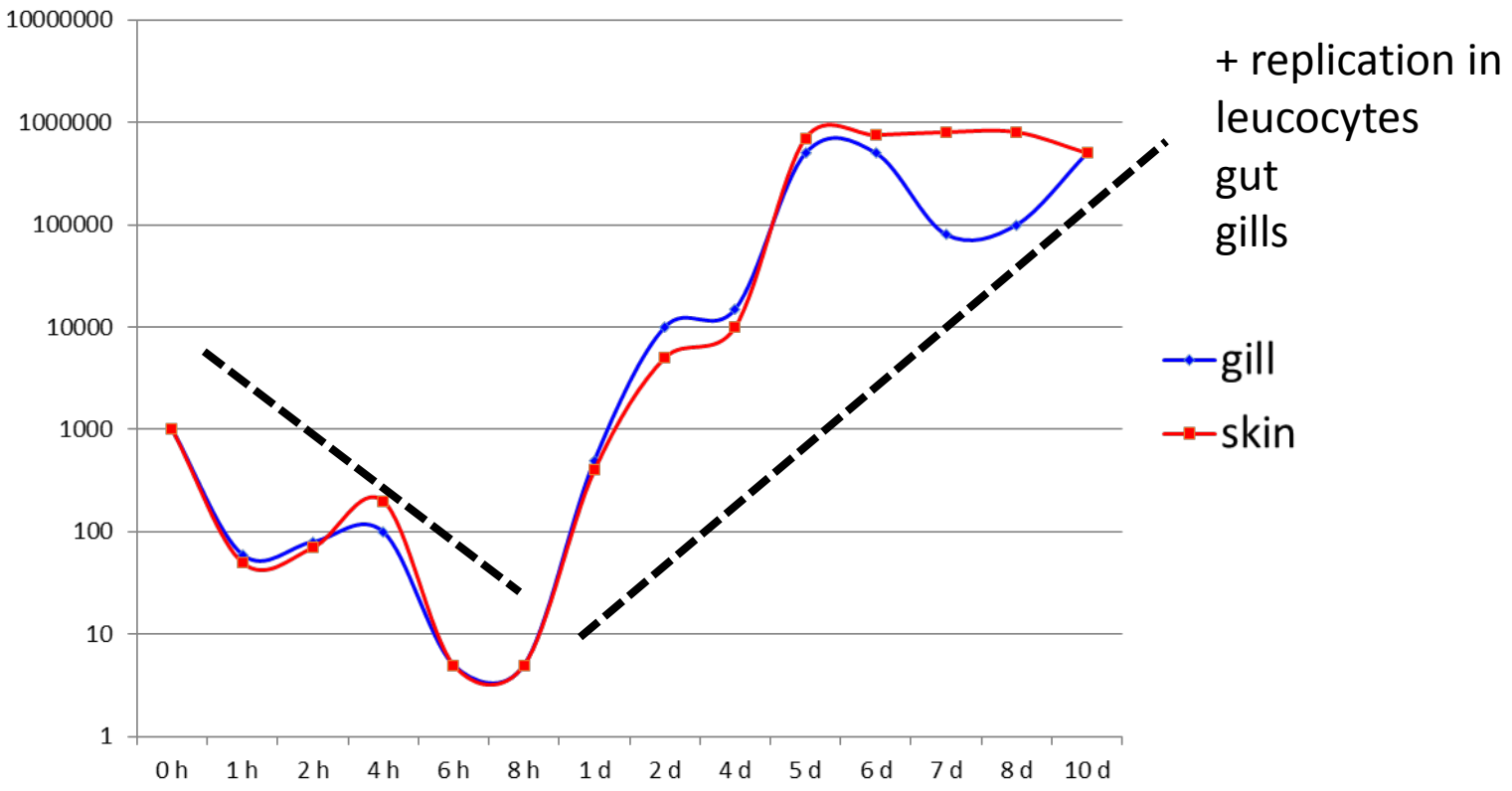
**KHV released at the first time!**

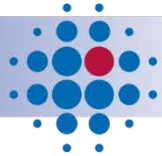
# tissues 4 - 8 h pi

kidney (vessel associated)



# „outside“ tissues





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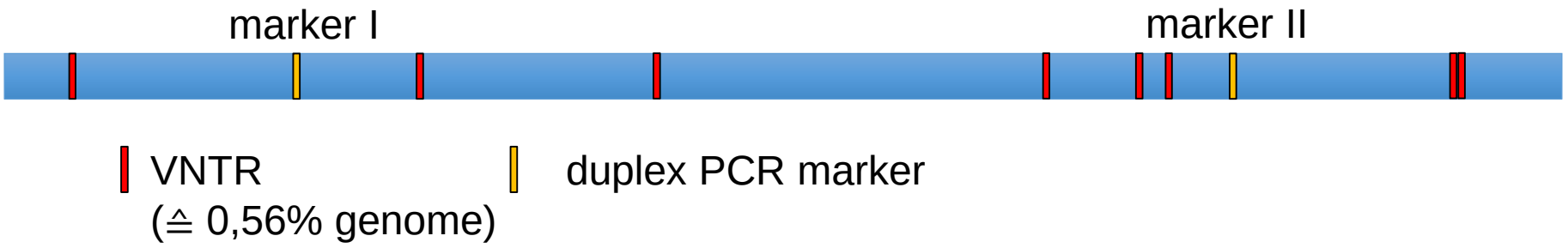
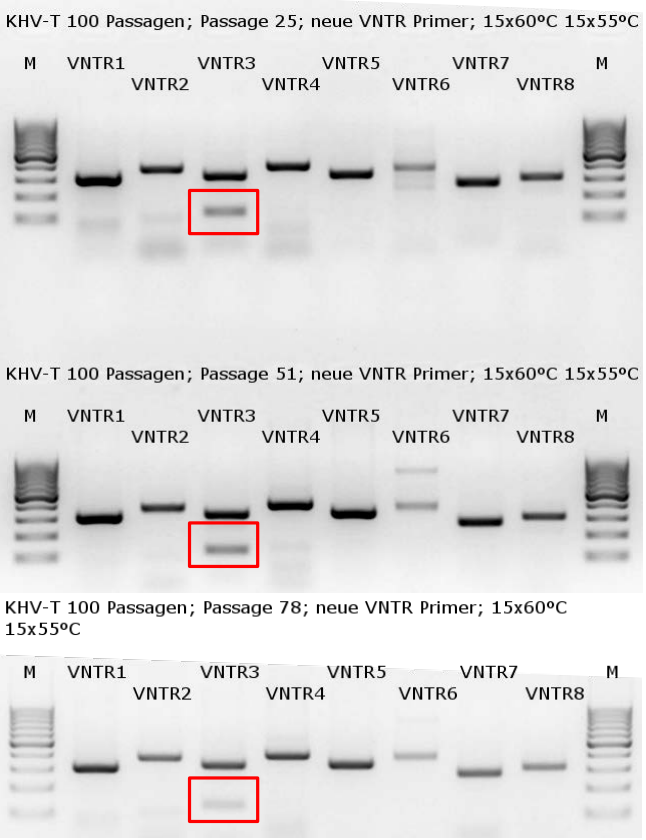
# KHV divertisity

# KHV diversity / variation

- necessary to detect different types auf KHV for vaccine development
- molecular tracing for KHV all over the world



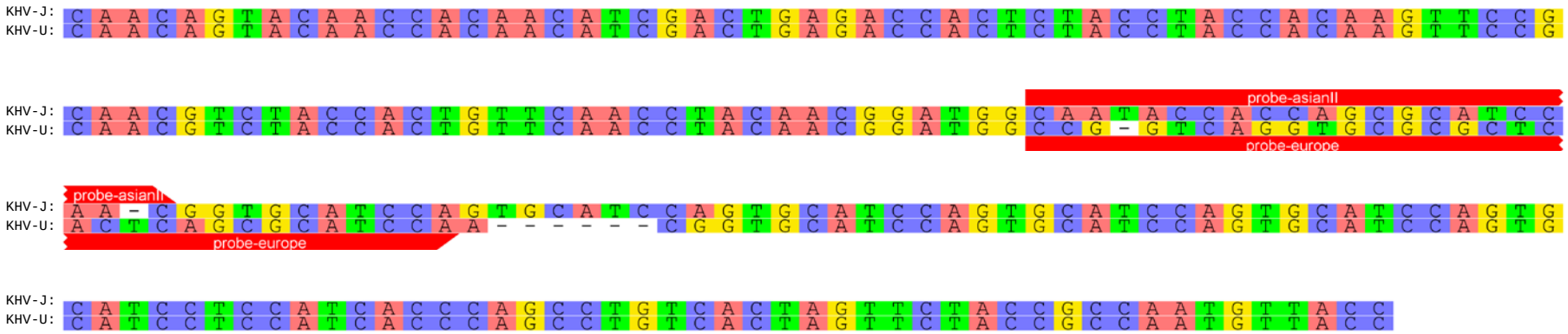
# VNTR PCRs in praxi



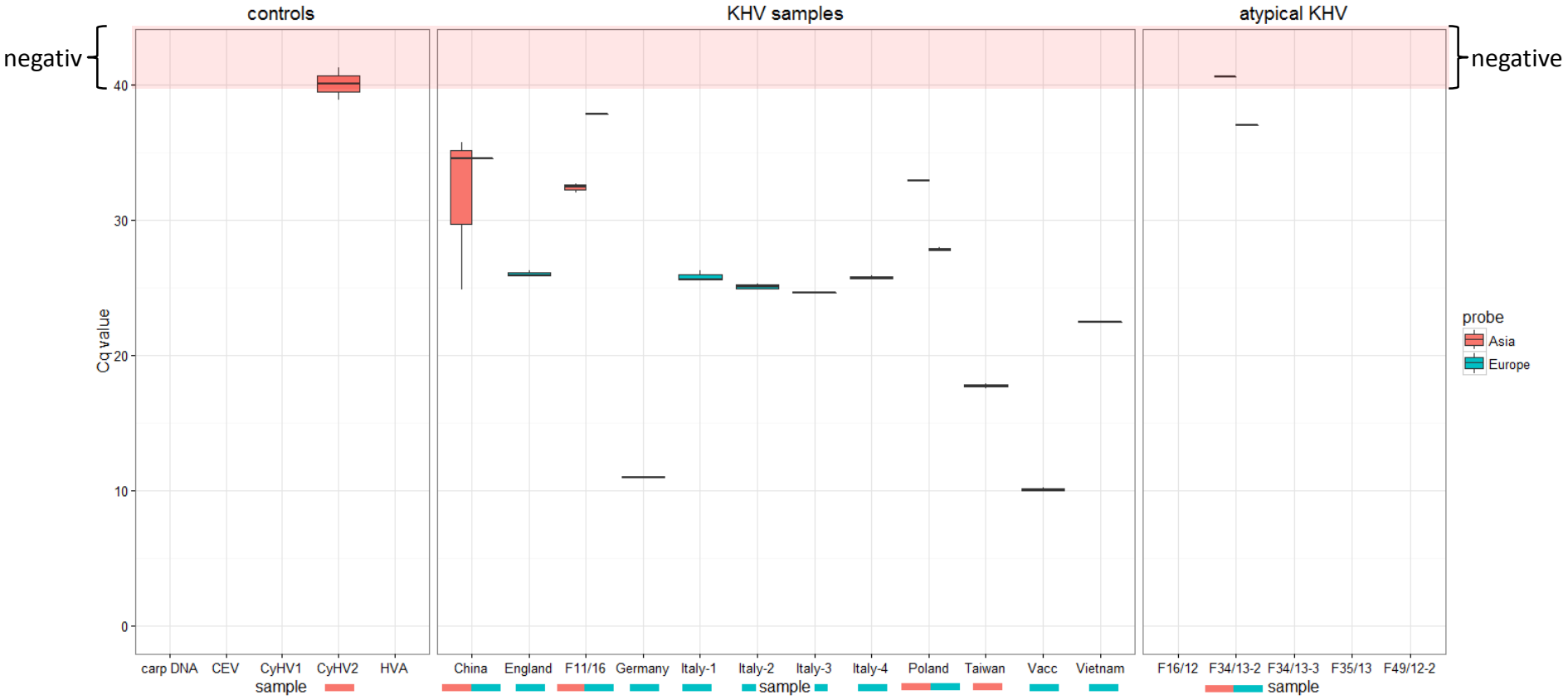

1. new primers
2. diff. annealing temp.
3. differences to CyHV-1 /2 in VNTR3

# MLVA applied to KHV passages (100) onto CCB at 20°C

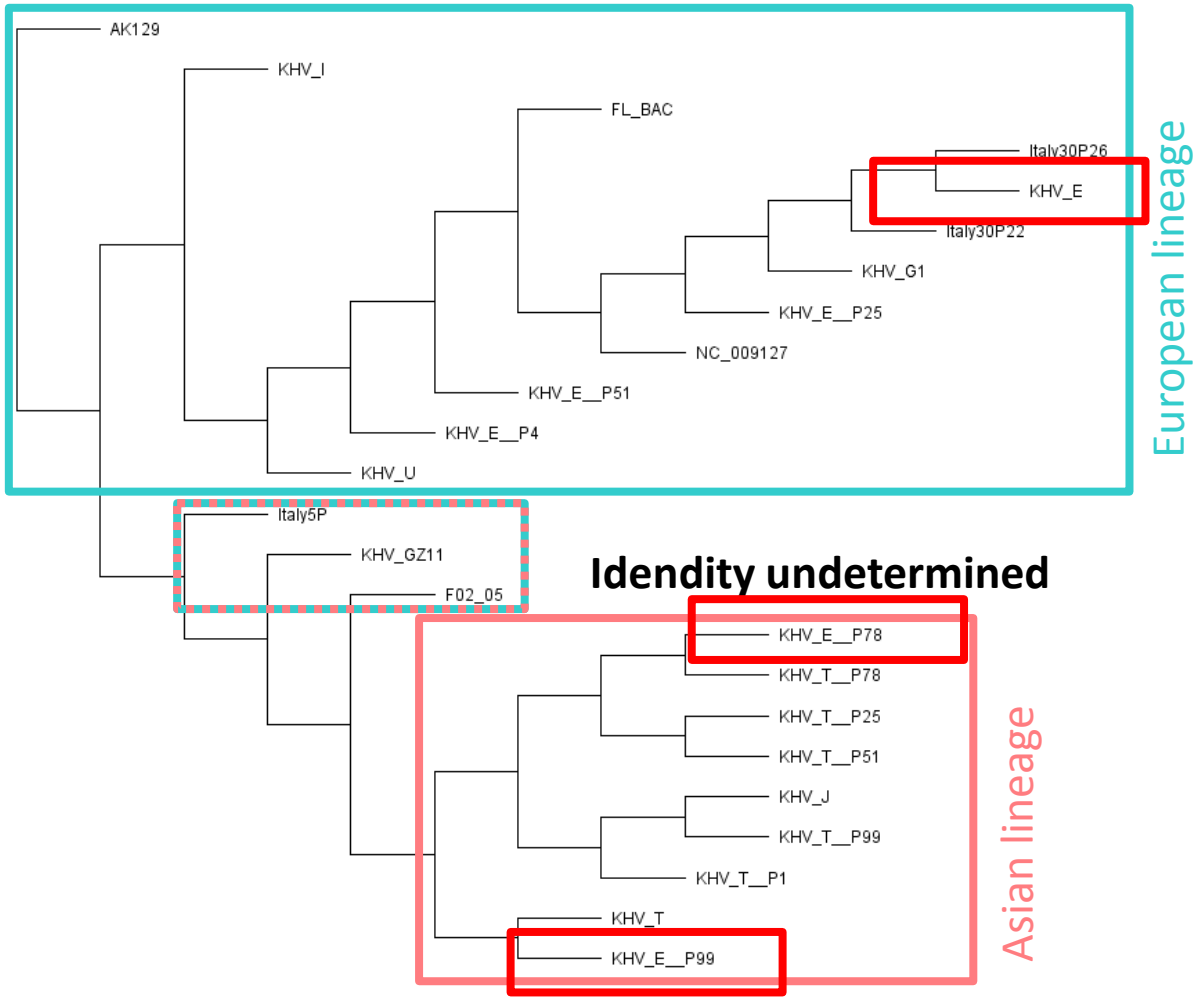
# Development on a new TaqMan qPCR based on VNTR3



# Some results with the new VNTR3 qPCRs

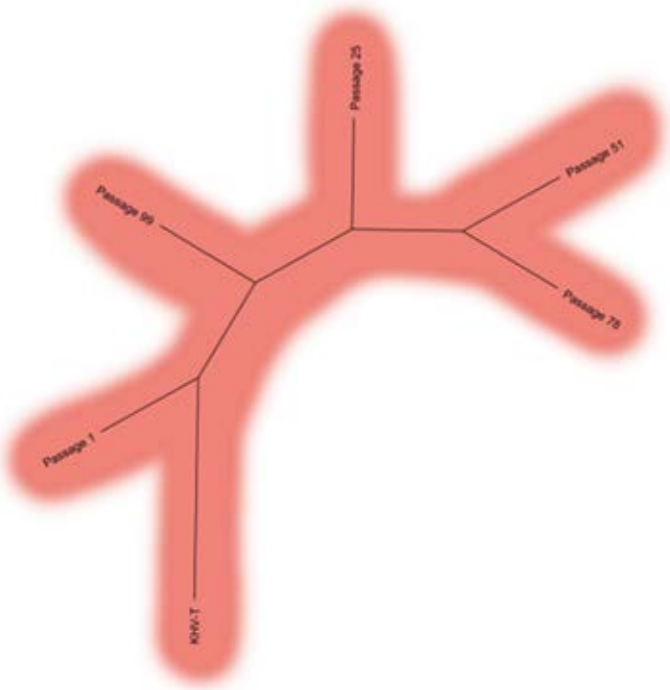


# Sequence analysis using 8 VNTRs

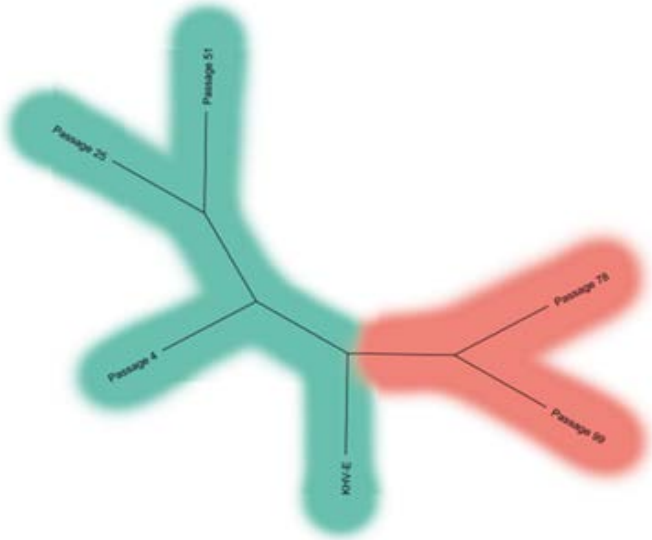


# Variable numbers of tandem repeats qPCR (VNTR-qPCR) with sequence analysis of 8 VNTRs (Klafack et al. 2017)

KHV-T




KHV-E




**Asian lineage**

**European lineage - Asian lineage**

# Recommendation for the diagnostics

1. Use of at least **two molecular assays** (diagnostics + monitoring)
   - qPCR + VNTR3 qPCR
   - qPCR + TK (+ nested + sequnece analysis)
   - qPCR + PAN (+ nested + sequnece analysis)

2. **Sequence analysis** of the fragments

3. **Sample collection** (from 5 dpi)
   - Organs - gill swabs or gill bioptate (non-lethal)
   - leucocytes (non-lethal)
   - gill and kidney (lethal)

4. **Pooling**
   - early infection – single samples (gills)
   - from day 5 pi – two fish (gills)
   - latent infection – stress induction (gills)
     (24 – 48 h) (30 -60% pos.)

# 2. Treatment against KHVD

## 2.1. Hygenic measuremnets

- „clean“ parents generation (cat. I)
- always clean hatchery
- disinfection (biosecurity)

## 2.2. Immunoprophylaxis (immunization)

2.2.1. inactivated vaccine from (a high virulent) KHV
2.2.2. attenuated live vaccine
2.2.3. genteically engeneered vaccine
2.2.4. immunostimmulation with virus eradication (alternative)

# 2.2.1. Inactivated vaccine (heat, BEI)

**KHV-I (10 $^{4}$ $TCID_{50}$/ml)**


**KHV-T (10 $^{6}$ $TCID_{50}$/ml)**


**CCB cells**

**20°C**

**26°C**

**field test**
**10.000 carp ip**

**67.8 % PSR**

**78.8 % PSR**

# 2.2.2. attenuated live vaccine

KV 3 (Israel, KoVax Ltd.)
Cavoy (Canada, Novartis)

**Germany FLI**

**AK 129** (KHV-I)

- passages onto CCB cells
- 129 – 140 x at 28°C

- $10^{2.85}$ $TCID_{50}$ / 10 µl

**results:** survival rate pch (before 92% survival after ip)

| | |
|---|---|
| ip | = 100 % |
| immersion | = 65 % |
| orally | = 43 % |

**Att Vacc G** (KHV-T)

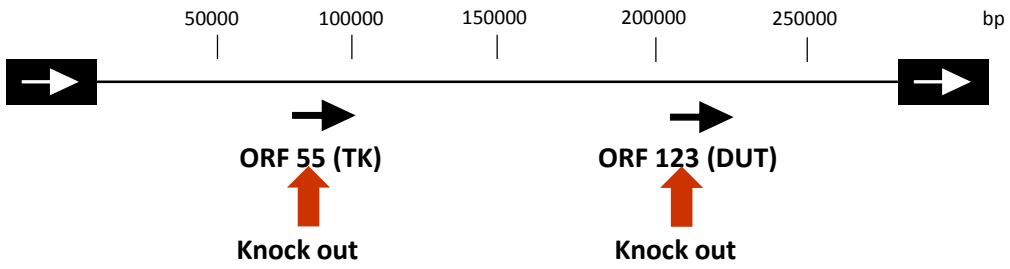
- passages onto CCB cells
- 0 – 100 x at 20°C

- $10^{4-6}$ $TCID_{50}$ / ml

**results:** survival rate pch (P78, P 51, P 99)

| | |
|---|---|
| P 78= 100% | 80% |
| P 51= 10% | - |
| P 99= 20% | - |

# 2.2.3. Geneticly engeneered vaccine (3 variants)

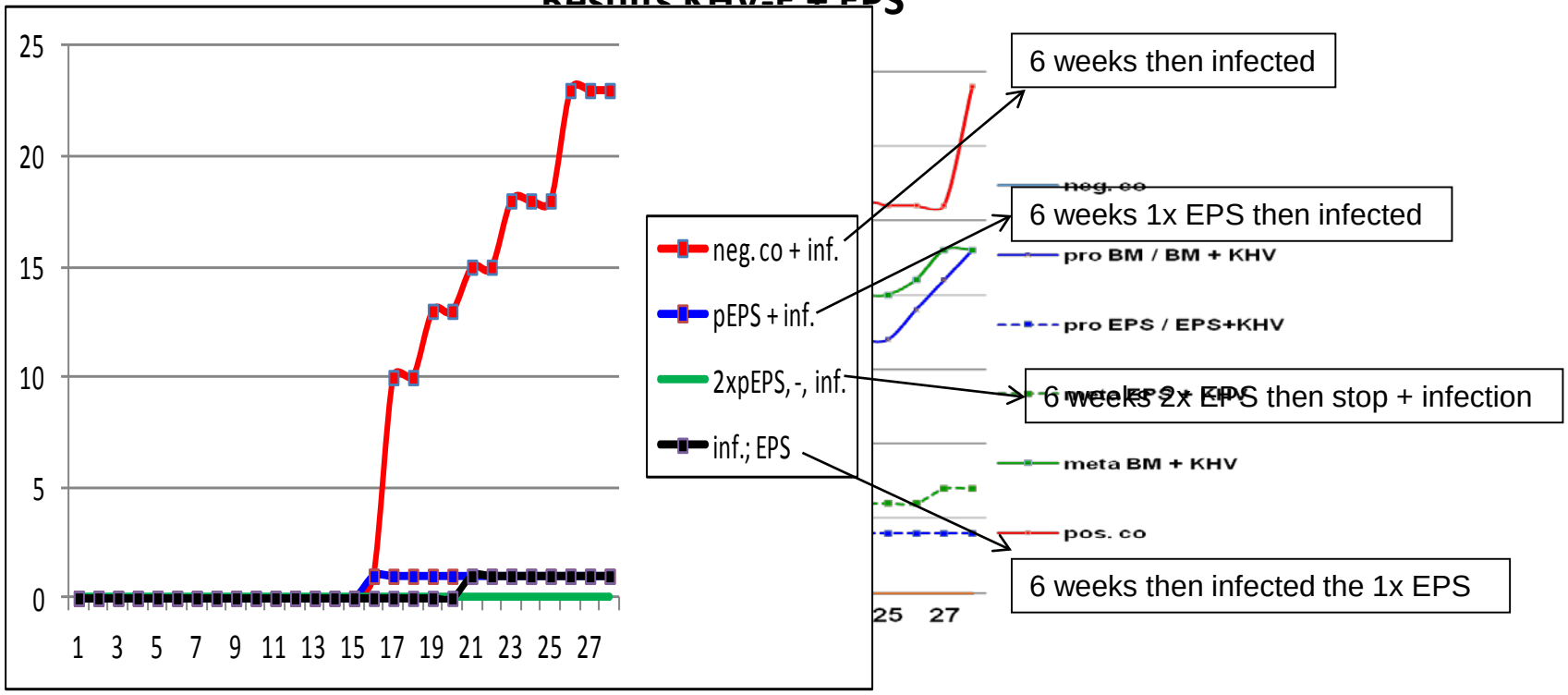



| | immersion vaccination | immersion challenge |
|---|---|---|
| del TK | 92% | 100% |
| del DUT | 92% | 100% |
| del TK/DUT | 95% | 100% |
| rev TK | 78% | 100% |
| rev DUT | 45% | 100% |
| KHV-T | 38% | ´ 90% |

# 2.2.4. immunostimmulation with virus eradication

## Exopolysaccharide (EPS) from marine alagae

### Results KHV-T + EPS



# Conclusion for combat

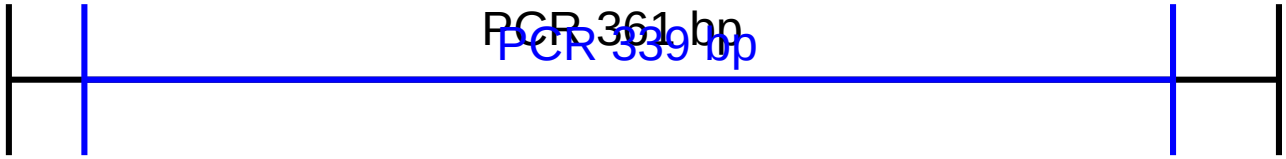
Vaccination and alternatives are possible and can be used very sucessfully.

# 3. Confirmation of KHV

**PCR and nested PCR (Engeslma et a. 2013)**

# PCR according Engelsma et al. 2013

(PAN-CyHV PCR, ORF 79, viral DNA polymerase)




**Recognizing :**

CyHV-1 (capo), CyHV-2 (GHV), CyHV-3 (KHV) and AngHV-1 (HVA)

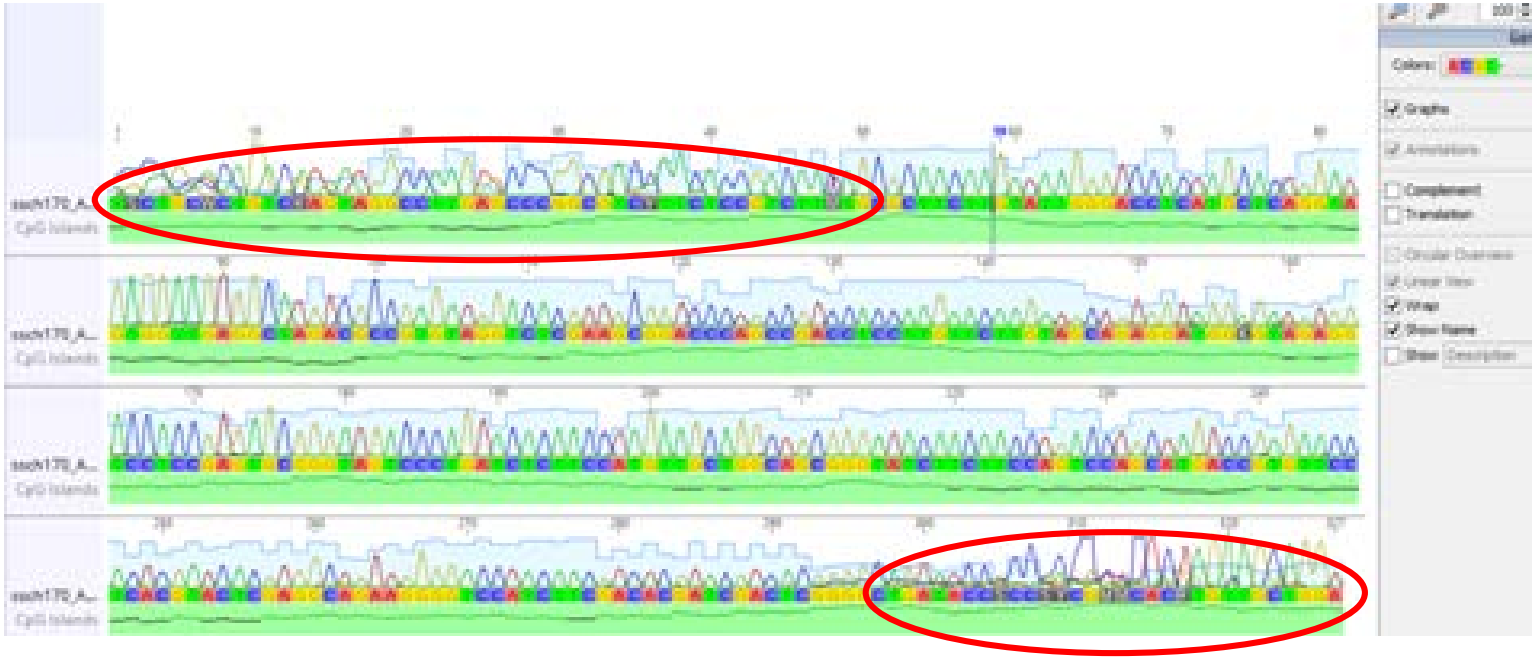
**Sequence analysis of fragments:**

1. Which one? PCR or nested PCR or both?
2. Which part of the ORF 79?

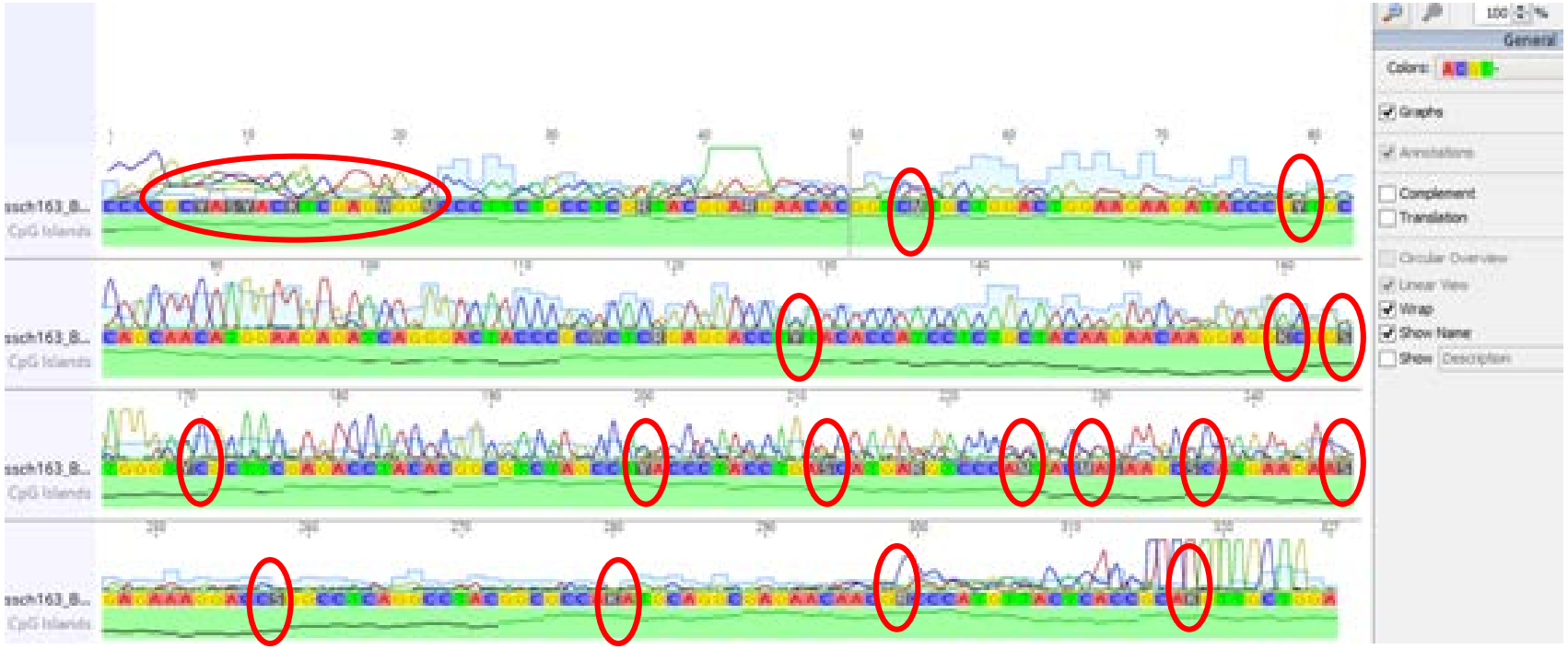
**Recent legislation:**

98% similarity of consensus sequences (3) = KHV

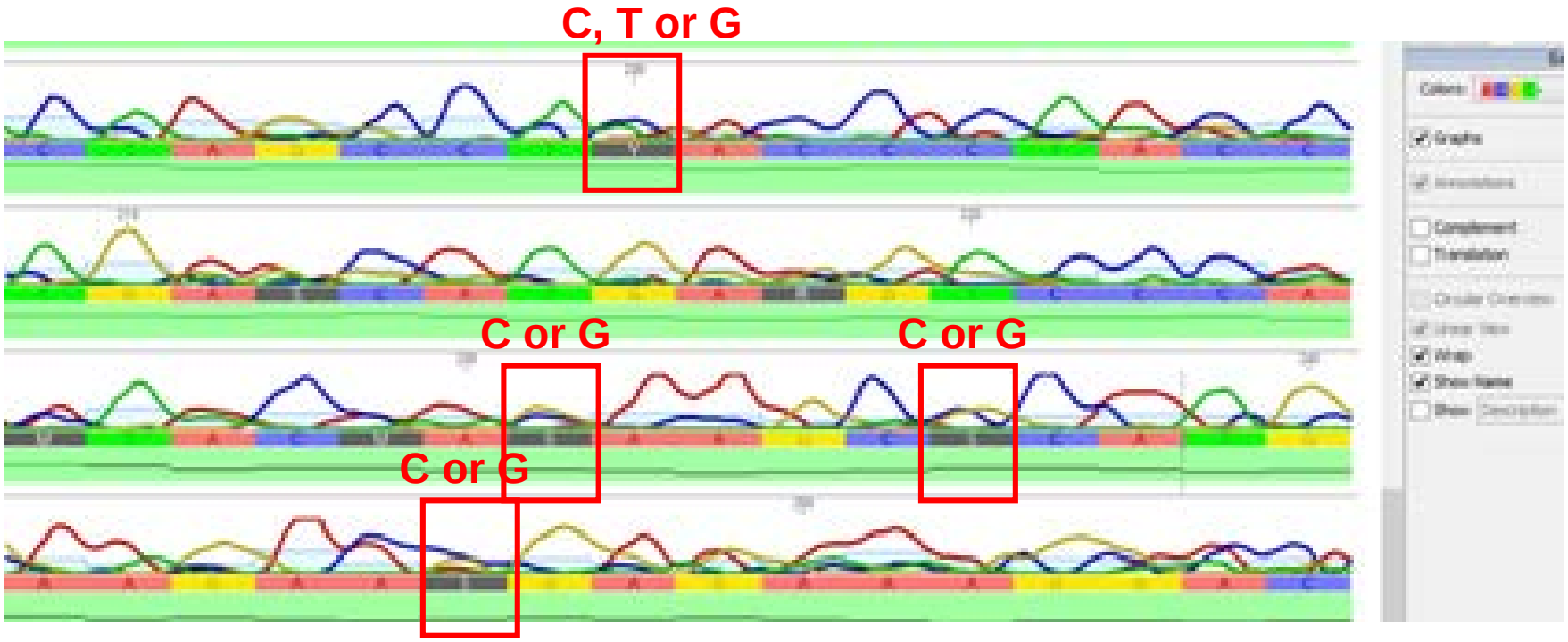
> 98% = no KHV (atypical reacting KHV)



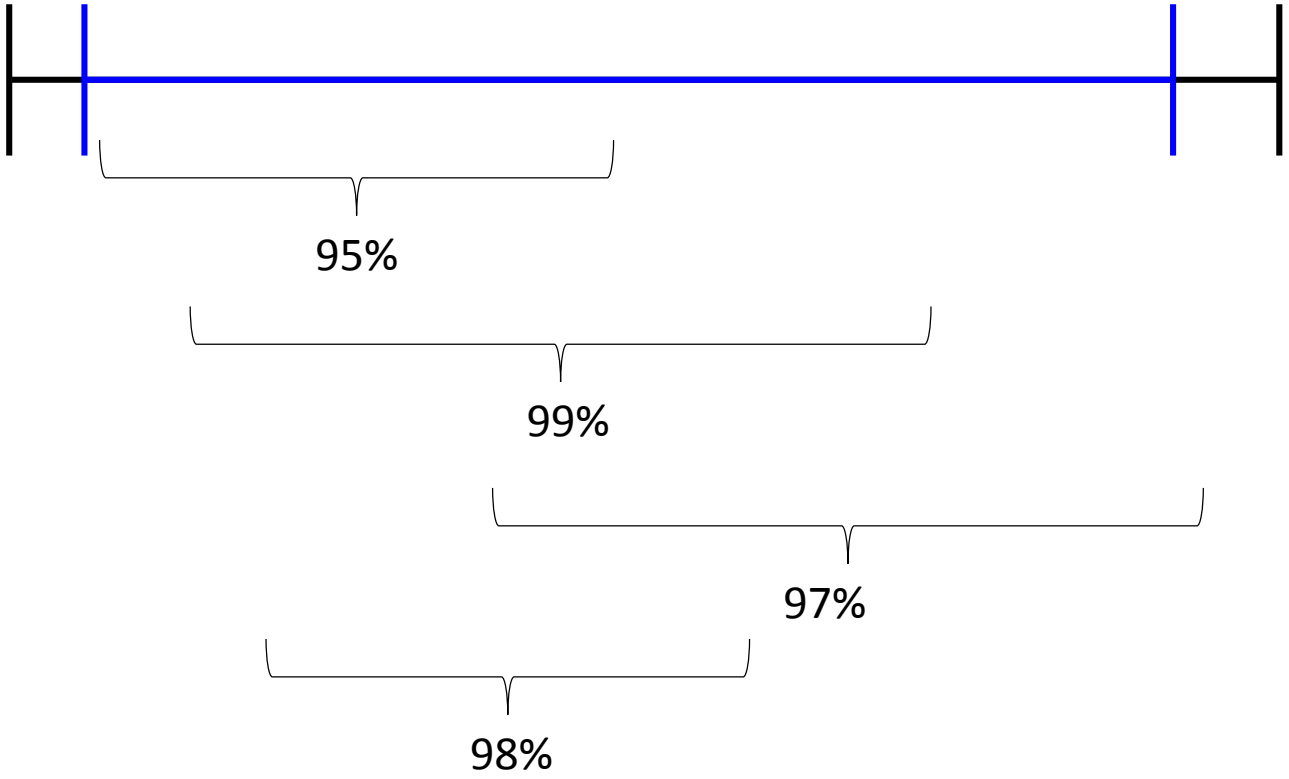
**„normal“ nested PCR fragment**




**„difficult“ nested PCR fragment**




- KHV variants mix
- NGS confirmed by JC Avarre and Bergmann.




What is right and what is wrong?

# Investigation on atypical reacting and normal reacting KHV
(PAN CyHV PCR, % similarity)

| | | 340-280 bp | 150-160 bp | atyp. | Tk/n | ca | GHV | HVA |
|---|---|---|---|---|---|---|---|---|
| atyp. | 2 | 96 | 100 | 97 | -/+ | 86 | 81 | 79 |
| | 4 | 95 | 99 | 96 | -/+ | 86 | 80 | 78 |
| | 5* | 96 | 99 | 97 | -/- | 85 | 78 | 72 |
| | 7* | 97,7 | 99 | 96 | -/- | 82 | 77 | 71 |
| typ. | 8 | 98 | 100 | 98 | +/+ | 84 | 78 | 74 |
| | 10 | 100 | 100 | 97 | +/+ | 83 | 78 | 73 |

* isolates onto CCB cells but from the organs

# Investigation KHV isolates at different temperatures

(PAN CyHV PCR, % similarity from 10th passage)

| | 15°C | 20°C | 25°C |
|---|---|---|---|
| KHV-T | 100 | 100 | 100 |
| KHV-E | 96 | 100 | 97 |

# Conclusion for the confirmative PCR

1. value 98% similarities is very, very artificial

2. if necessary 95% would fit to KHV variants (with mortality!)
   95 -100% = KHV
   >90 % = different herpesviruses

3. other and /or confirmative qPCRs? (qPCR VNTR3 or lin. qPCR?)
   (specific to KHV not to other aquatic herpesviruses)

# Thank you very, very much!

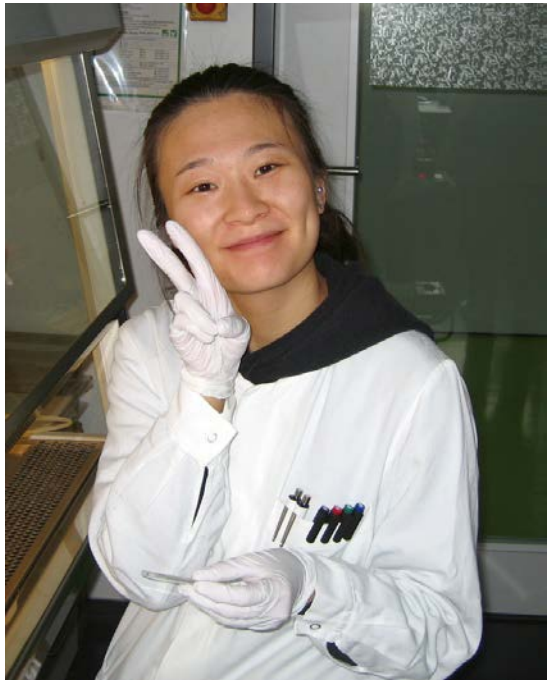
Lars Schröder

Working group Dr. Walter Fuchs

Prof. Dr. Dr. h.c. Thomas C. Mettenleiter

**JC Avarre**

# Thank you very, very much!

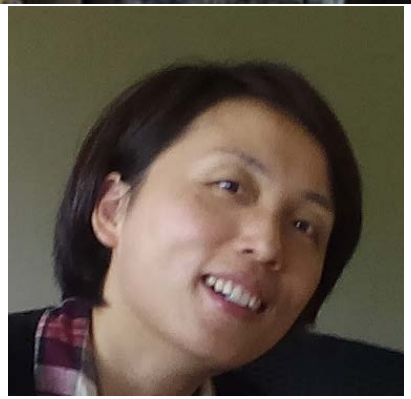


**Yeonhwa Jin**

**Sandro Klafack**

**Kyo Hyun Kim**

**Irina Werner**



**Prof. Wang Qing**
**Dr. Wang Yingying**
**Dr. Zeng Weiwei**
**Li Yingying**
**Prof. Kielpinska Natalia**


**Thank you for your attention!**