Workshop on bluetongue virus real time RT-PCR

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Section Epizootic Diseases

Overview

- bluetongue virus
- OIE protocol
- Belgian protocol (sample→result)
- performance characteristics
- results BT crisis

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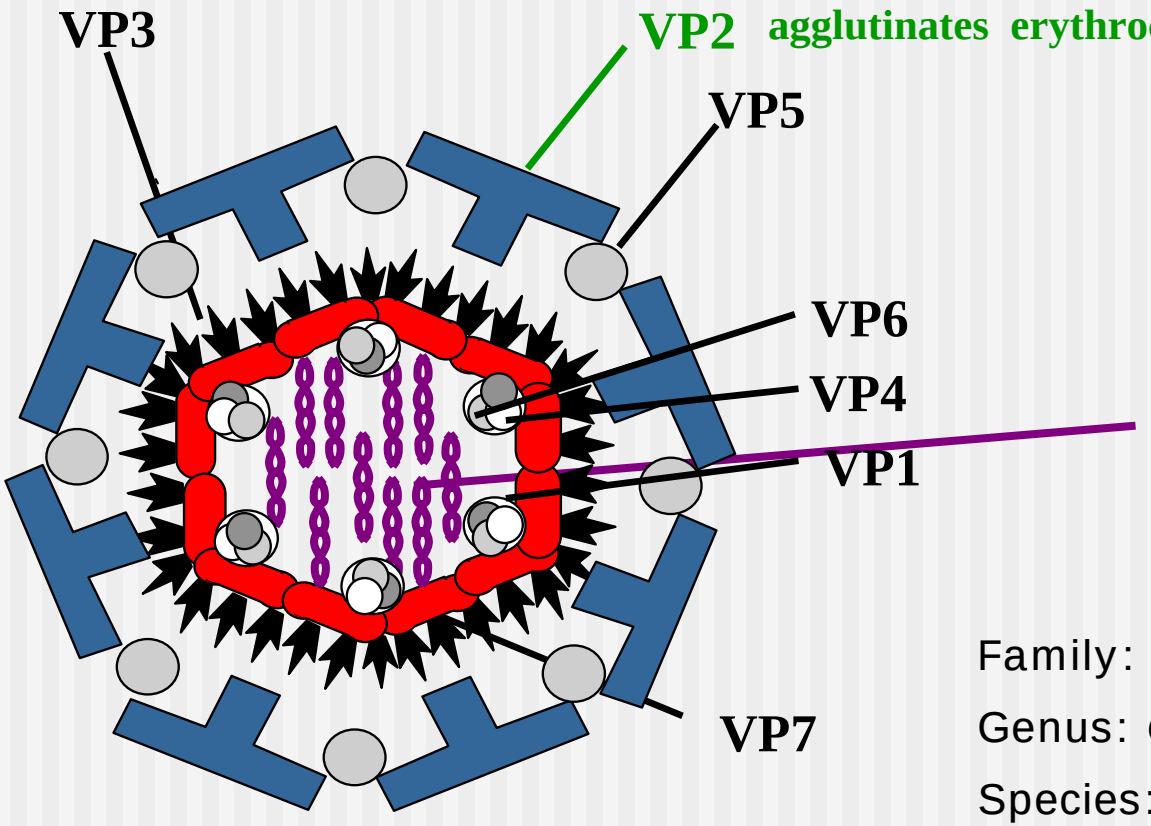
Bluetongue virus



Family: *Reoviridae*

Genus: *Orbivirus*

Species: *Bluetongue virus*

Overview

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OIE RT-PCR protocol

- prescribed by OIE for international trade
- protocol
  - segment 5 (NS1)
  - nested RT-PCR
  - gel-based
  - does not detect Belgian BTV-8

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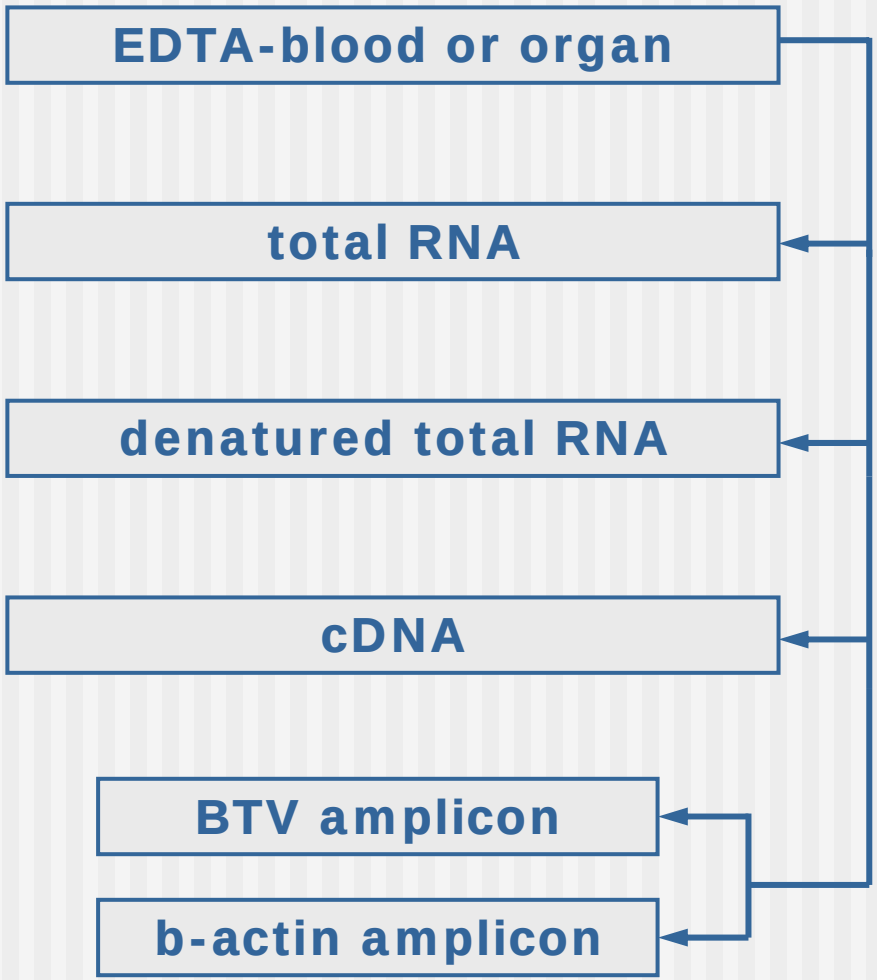
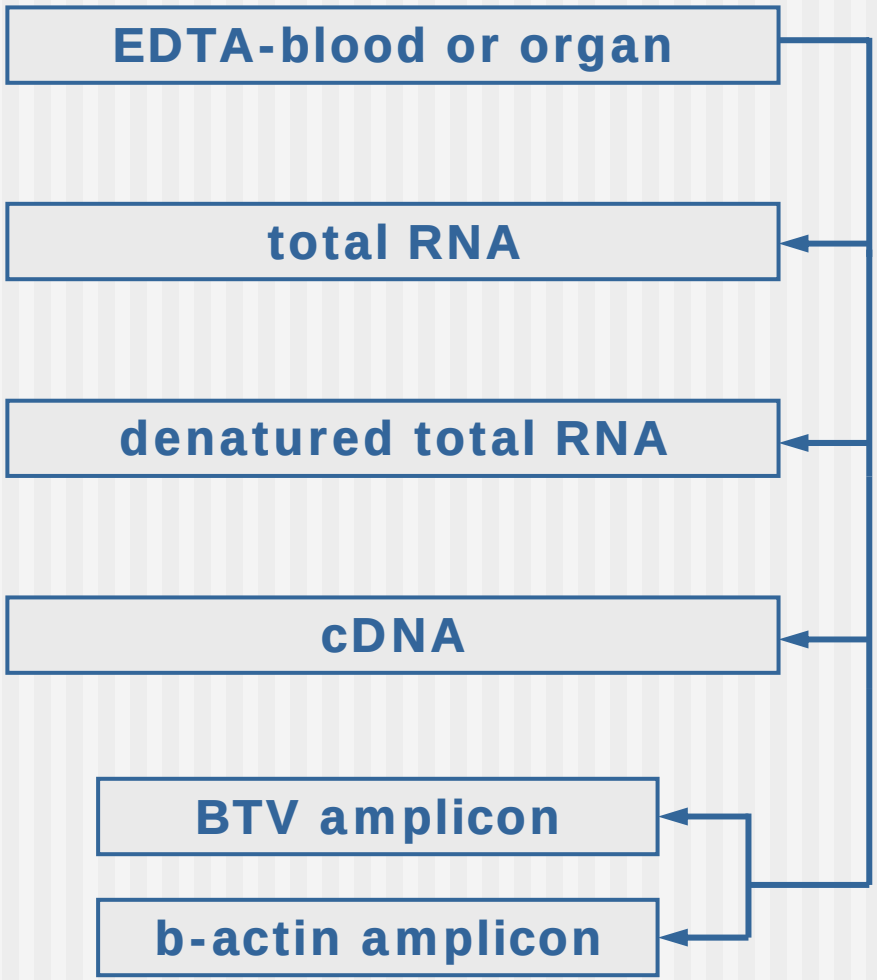
Belgian protocol: overview



1. RNA extraction
2. denaturation
3. reverse transcription (RT)
4. real time PCR (qPCR)

Toussaint et al. 2006

Belgian protocol: overview



1. RNA extraction

2. denaturation

3. reverse transcription (RT)

4. real time PCR (qPCR)

Toussaint et al. 2006

Belgian protocol: sample preparation and extraction (1)

- EDTA-blood sample preparation
  - remove plasma
  - wash with phosphate buffered saline (PBS)
  - haemolyse 2x with DEPC water
  - resuspend in 250µl DEPC water
- trizol-chloroform extraction
  - extract with 750µl trizol and 200µl chloroform
  - precipitate total RNA with isopropanol
  - wash with 70% ethanol
  - resuspend in 30µl DEPC water
- control
  - negative extraction control: water sample

duration: appr. 3 hours 30 min

Belgian protocol: sample preparation and extraction (2)

- organs from autopsies: spleen, lymph node, skin mucosa, etc.

- trizol-chloroform extraction
  - cut piece of 25mg
  - add 250µl DEPC water and 750µl trizol
  - homogenize using Tissuelyser (Qiagen)
  - add 200µl chloroform
  - precipitate total RNA with isopropanol
  - wash with 70% ethanol
  - resuspend in 30µl DEPC water

- control
  - negative extraction control: water sample

duration: appr. 3 hours

Belgian protocol: commercial alternatives?

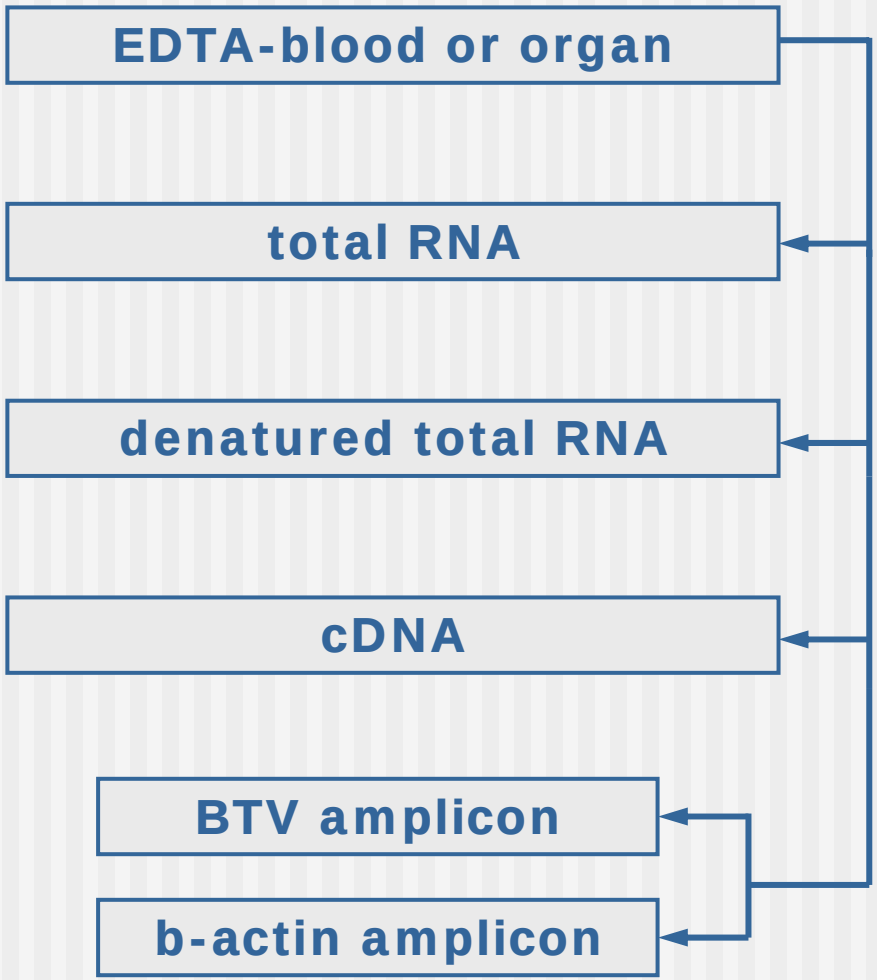
- comparison of commercial RNA extraction kits
  - RNeasy 96 Kit (Qiagen)
  - Nucleospin RNA II kit (Macherey-Nagel)
  - Nucleospin RNA virus kit (Macherey-Nagel)

→ high sensitivity with Nucleospin RNA virus kit

- comparison with conventional trizol extraction

→ highest sensitivity with trizol extraction

Belgian protocol: overview



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Toussaint et al. 2006

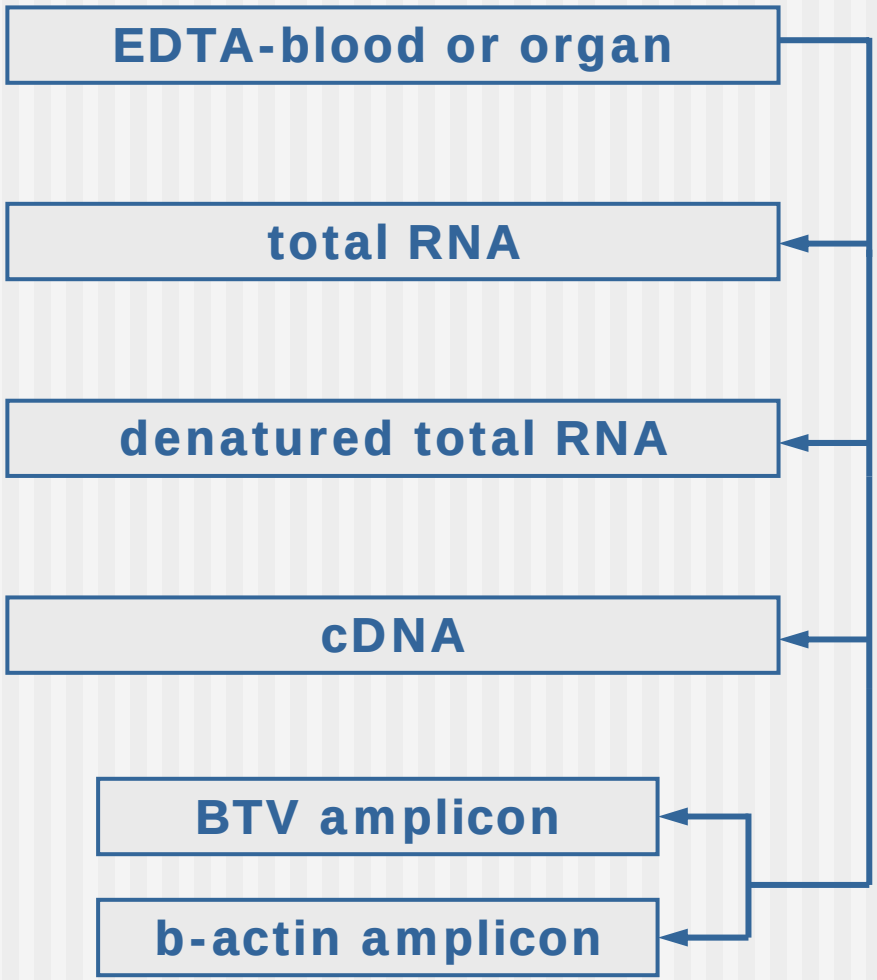
Belgian protocol: denaturation

- OIE prescribes denaturation with methyl mercuric hydroxide, but highly toxic and no more commercially available

- RNA sample + 10% dimethyl sulfoxyde (DMSO)
- heat at 95°C for 3 min in thermocycler
- place on ice

duration: appr. 15 min

Belgian protocol: overview



1. RNA extraction

2. denaturation

3. reverse transcription (RT)

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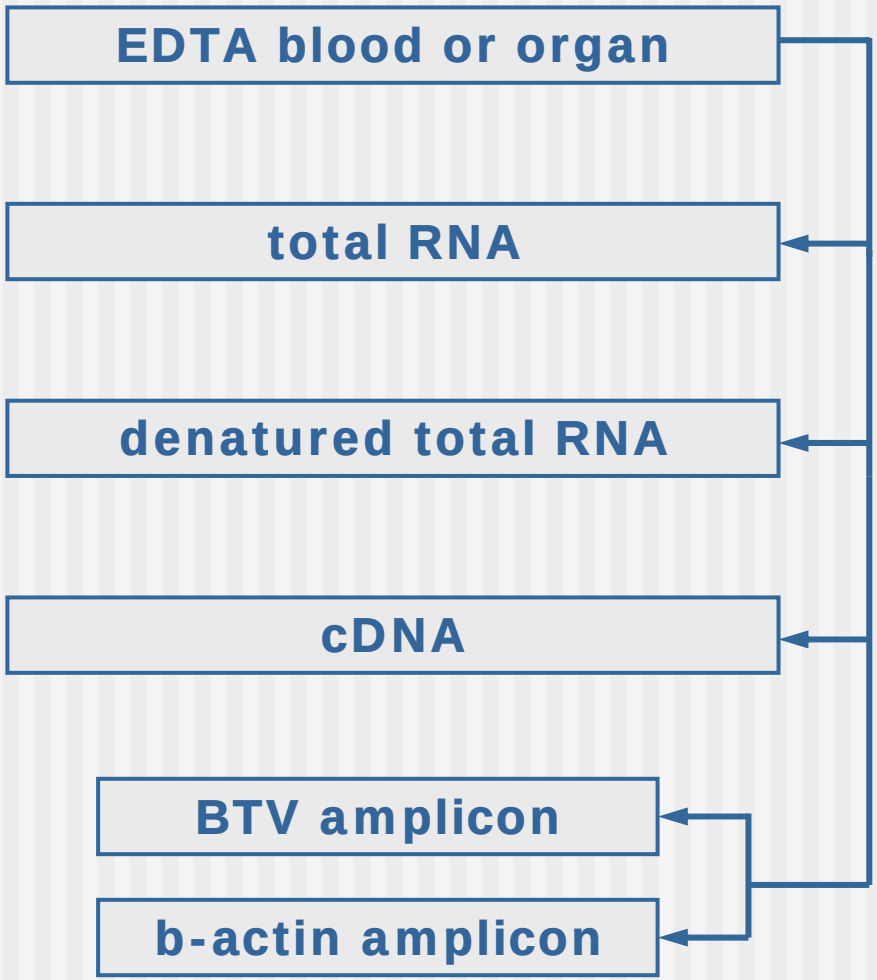
Toussaint et al. 2006

Belgian protocol: reverse transcription

- TaqMan Reverse Transcription Reagents (Applied Biosystems)
- random hexamers
- program thermocycler:
  - 10 min at 25°C
  - 30 min at 48°C
  - 5 min at 95°C
- controls
  - negative control: water sample
  - positive BTV control: synthetic RNA

duration: appr. 1 hour

Belgian protocol: overview



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Toussaint et al. 2006

Belgian protocol: real time PCR

- BTV, segment 5 (NS1)
  - TaqMan technology
  - amplicon of 75 bases
  - recognizes all 24 BTV serotypes
- b-actin mRNA
  - TaqMan technology
  - amplicon of 131 bases
  - internal control
- control
  - negative PCR control: water sample

Belgian protocol: real time PCR

- BTV primers contain degenerate bases (wide recognition)
- BTV TaqMan probe with 6 locked nucleic acid residues (increases annealing temperature)
- b-actin forward primer designed to hybridize at junction between exon 4 & 5

Exon 4 *Exon 5*

GCACC CAGCA CAATGAAGAT CAAG|ATCATC GCGCCCCCTG AGCGCAAGTA CTCCGTGTGG

ACTB_F_1005-1029

Belgian protocol: real time PCR

primer/probe	sequence (5'-3')
BTV forward	GGCAAC**Y**ACCAAACATGGA
BTV reverse	AAAGT**Y**CTCGTGGCATT**W**GC
BTV FAM/TAMRA	CYCCA**C**TG**A**TRTT**G**TA**T**T**T**T**C**TCAA
ACTB forward	CAGCACAATGAAGATCAAGATCATC
ACTB reverse	CGGACTCATCGTACTCCTGCTT
ACTB FAM/TAMRA	TCGCTGTCCACCTTCCAGCAGATGT

locked nucleic acid residues

Y = C/T ; W = A/T

Belgian protocol: real time PCR

- ABI 7900HT standard mode
  - TaqMan Universal PCR Mastermix
  - MicroAmp™ Optical 96-well Reaction Plate
  - program:
    - stage 1: 10 min at 95°C
    - stage 2: 15 sec at 95°C
      1 min at 60°C
      ] 45 repeats

duration: appr. 1 hour 45 min

Belgian protocol: real time PCR

- ABI 7900HT fast mode
  - TaqMan Fast Universal PCR Mastermix
  - MicroAmp™ Fast Optical 96-well Reaction Plate
  - slightly modified mixes
  - program:
    - stage 1: 20 sec at 95°C
    - stage 2: 1 sec at 95°C
      20 sec at 60°C
      ] 45 repeats

duration: appr. 45 min

Belgian protocol: multiplex qPCR

- what?
  - simultaneous amplification of multiple targets in one reaction
- why?
  - increases capacity
  - reduces costs
  - saves time
- multiplex versus singleplex
  - analysis of BTV+ samples in single- and multiplex

Belgian protocol: multiplex qPCR

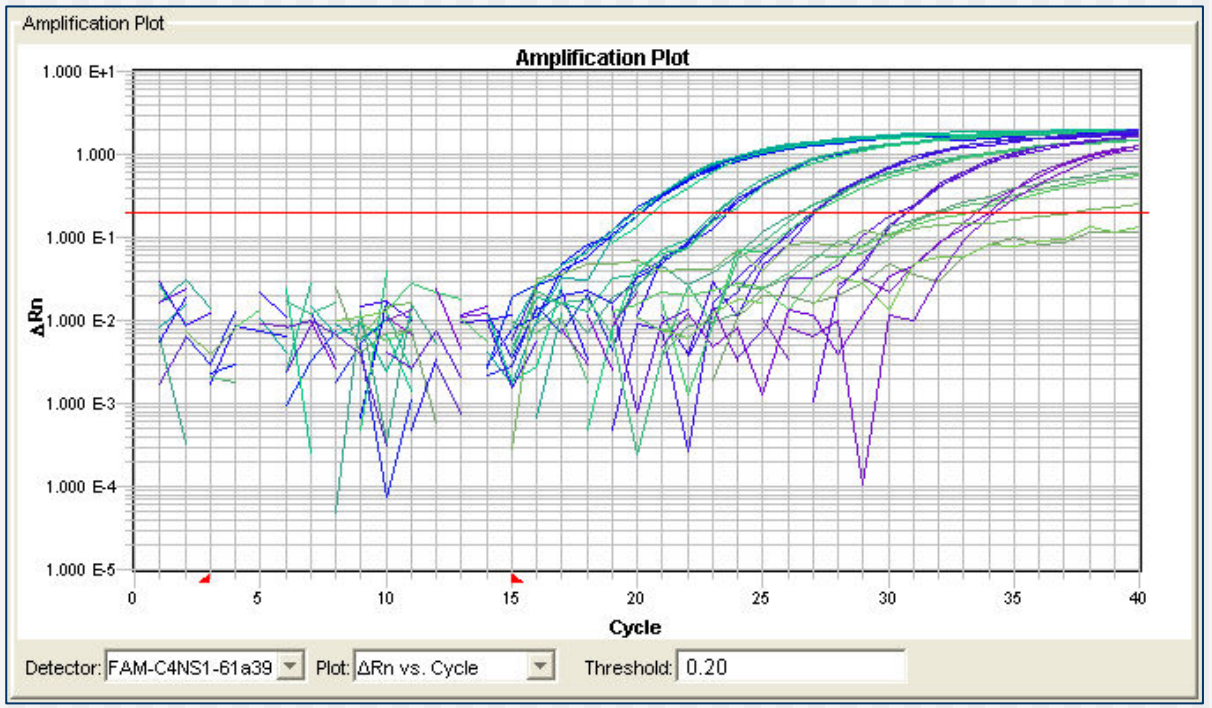
comparison of single- and multiplex by analysis of 70 BTV+ field samples

Ct class	N° samples	mean Ct single	mean Ct multi	△ Ct	stdev △ Ct
20-25	2	24.8	25.7	0.84	0.91
25-30	21	27.7	27.6	-0.15	0.34
30-35	30	32.2	32.2	0.05	0.91
35-40	17	36.1	39.2	3.07	2.85

samples with Ct>35 can be missed by multiplex qPCR!

Belgian protocol: multiplex qPCR

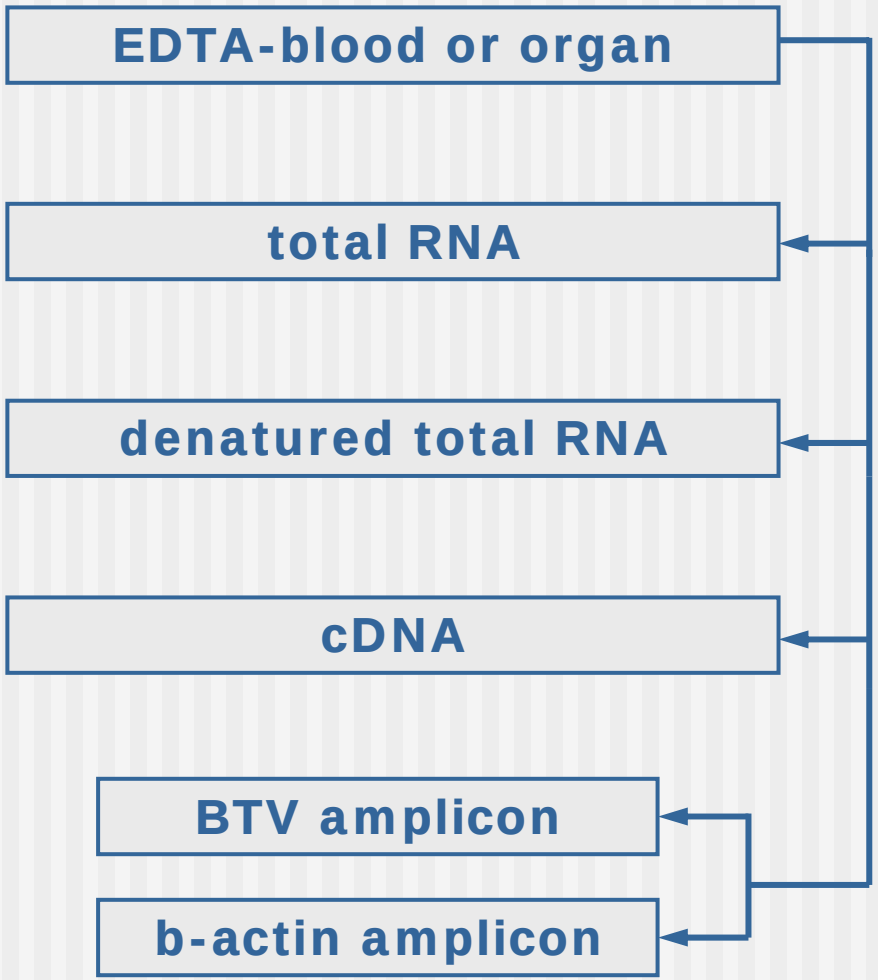
- analysis 10-fold serial dilutions (10^7-10^0 RNA copies/µl)
- singleplex
  multiplex



multiplex less sensitive at higher dilutions

singleplex is only option for reliable BTV diagnostic

Belgian protocol: controls



1. RNA extraction
   + *neg extraction control*
2. denaturation
3. reverse transcription (RT)
   + *neg RT control*
   + *pos BTV control (C1)*
4. real time PCR (qPCR)
   + *neg PCR control*

Toussaint et al. 2006

Belgian protocol: synthetic RNA control

- synthetic RNA for BTV segment 5:
  - TA-cloning in expression vector
  - plasmid purification
  - linearization
  - *in vitro* transcription
  - digestion with RNAse-free DNAse
  - quantification by spectrophotometry
  - diluted and strictly stored at -80°C

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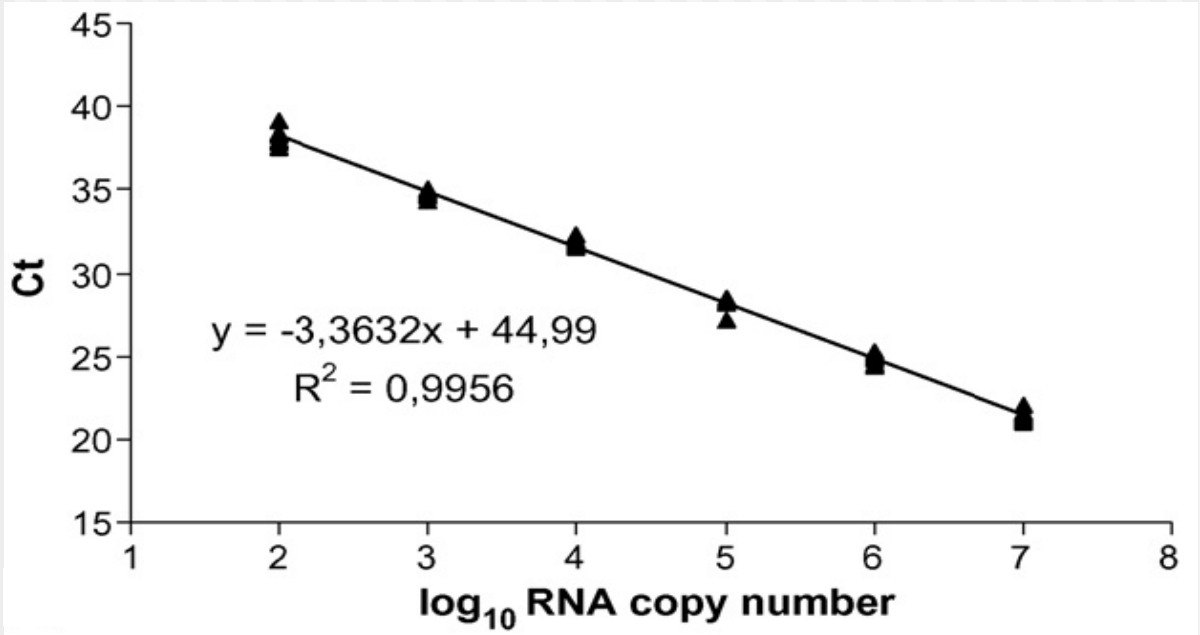
Performance characteristics

- analytical sensitivity
  - 6x 10-fold dilution series of synthetic RNA
  - 2x 10-fold dilution series of virus stock
  - ⇨ detection limit: 100 RNA copies/0.01 ECE_{50}
- analytical specificity
  - 35 BTV strains from 24 serotypes: all pos
  - blood samples (goat, sheep and cattle) from BTV free areas: all neg
  - genetically related viruses EHDV and AHS: all neg
  - ⇨ cut-off for diagnostics: 40 Ct
  - ⇨ cut-off for quantification: 38 Ct

Performance characteristics

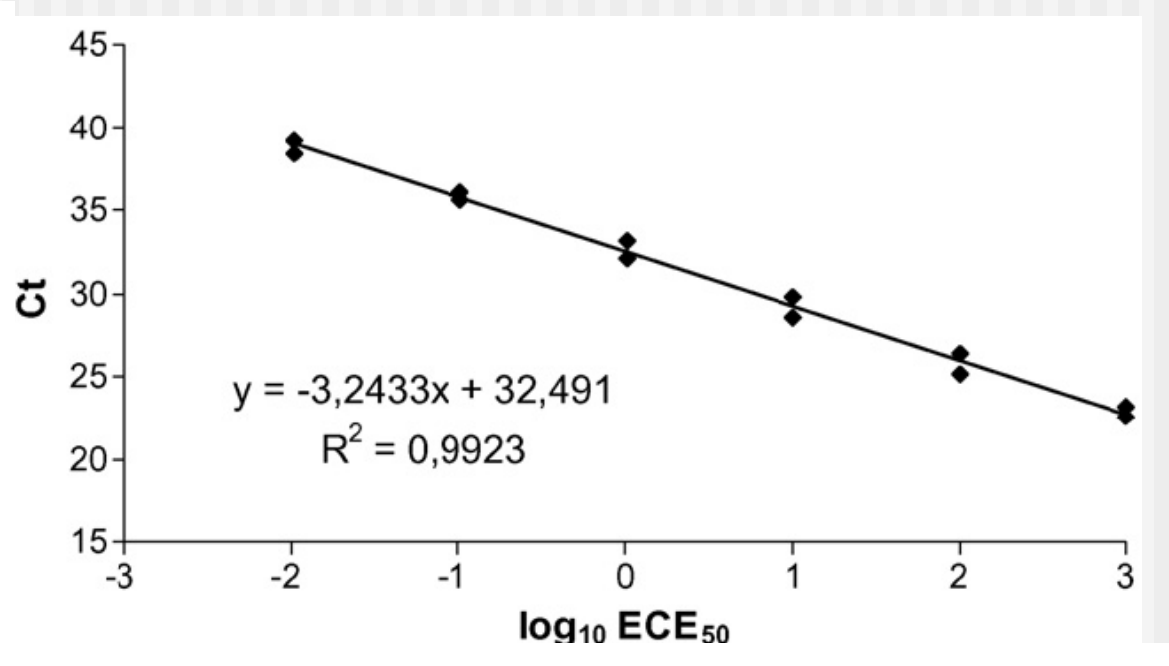
6x 10-fold dilution series of synthetic RNA



- linear distribution
- amplification efficiency = 98%
- highly reproducible (6 repeats on 6 different days)

Performance characteristics

2x 10-fold dilution series of virus stock



- linear distribution
- amplification efficiency = 103%
- highly reproducible (2 repeats on 2 different days)

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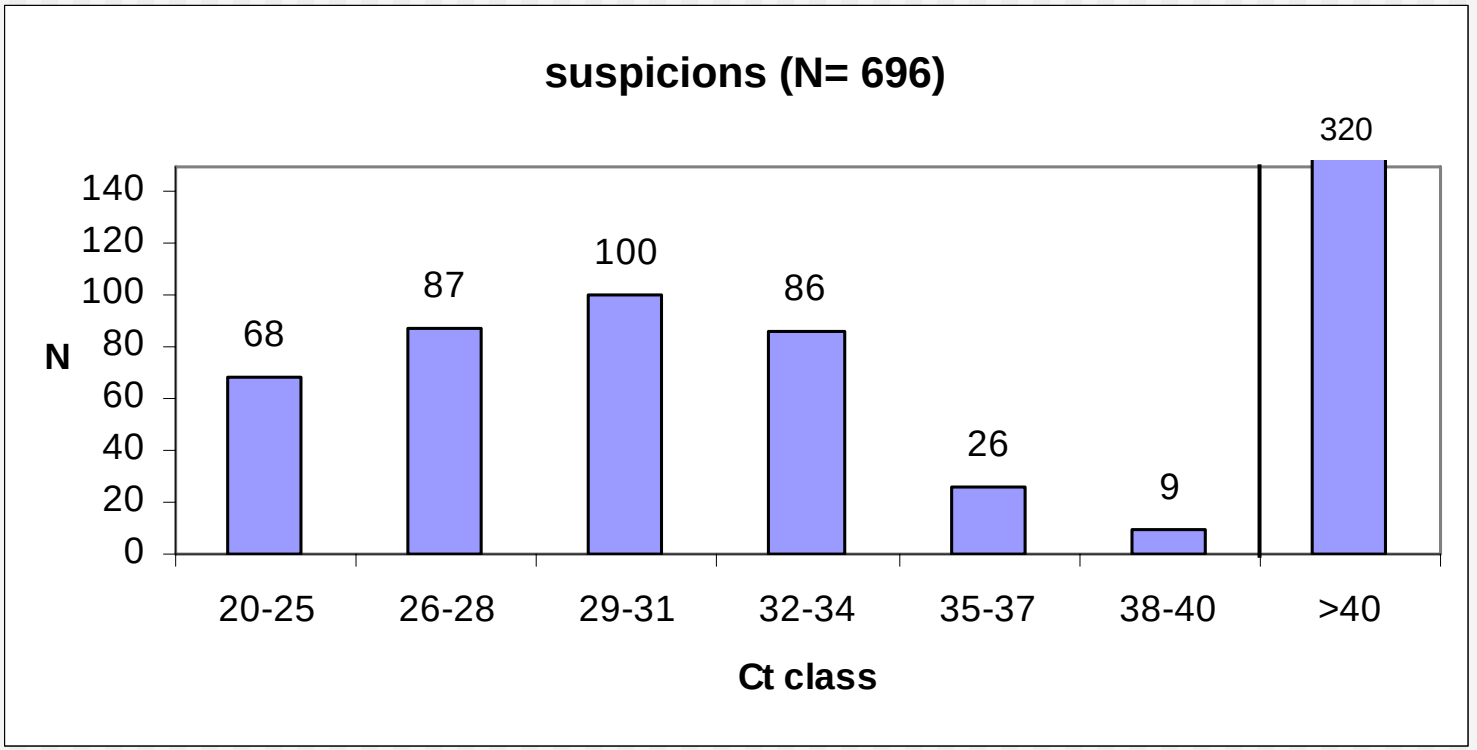
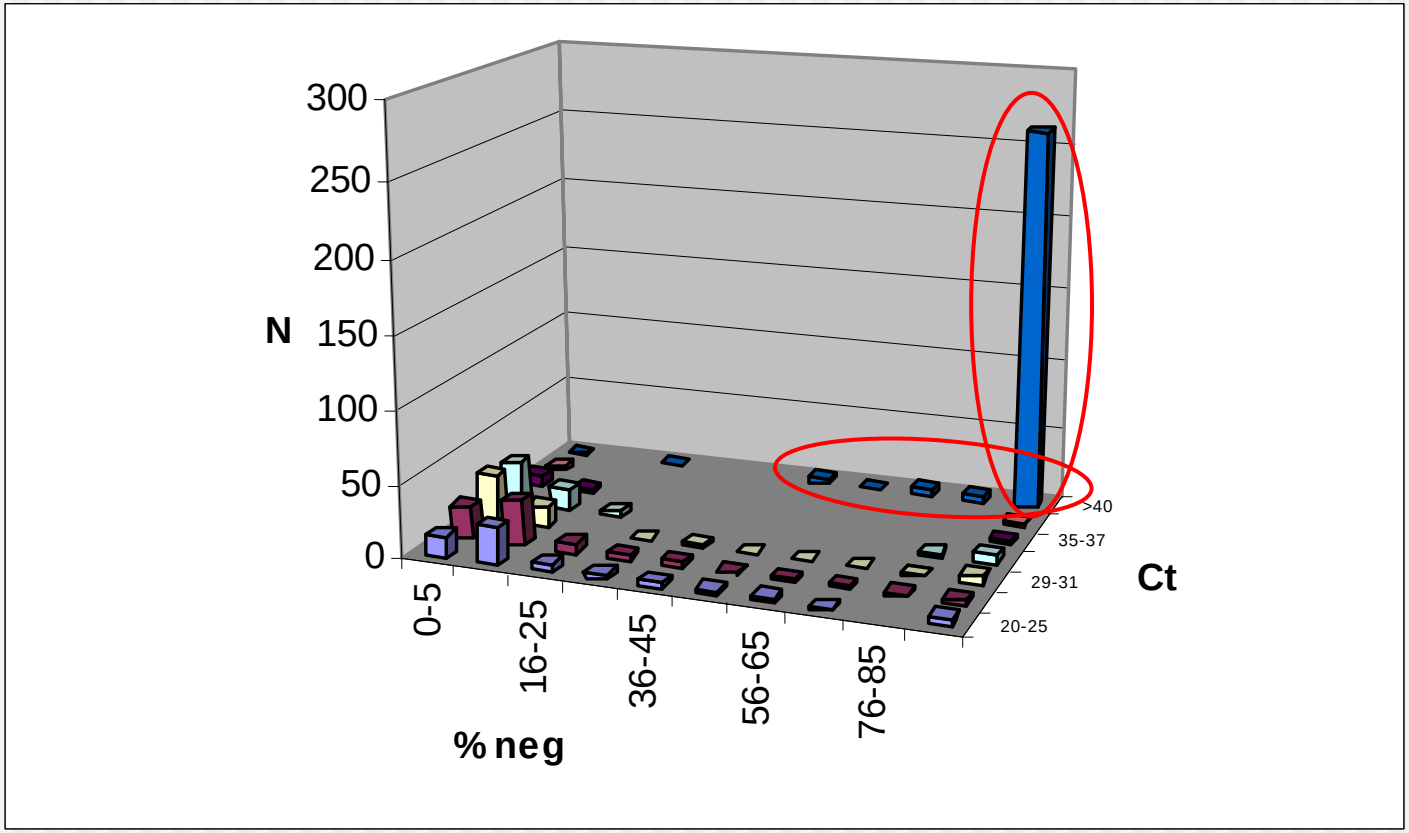
coffee break

Results BT crisis: blood samples



Results BT crisis: blood samples



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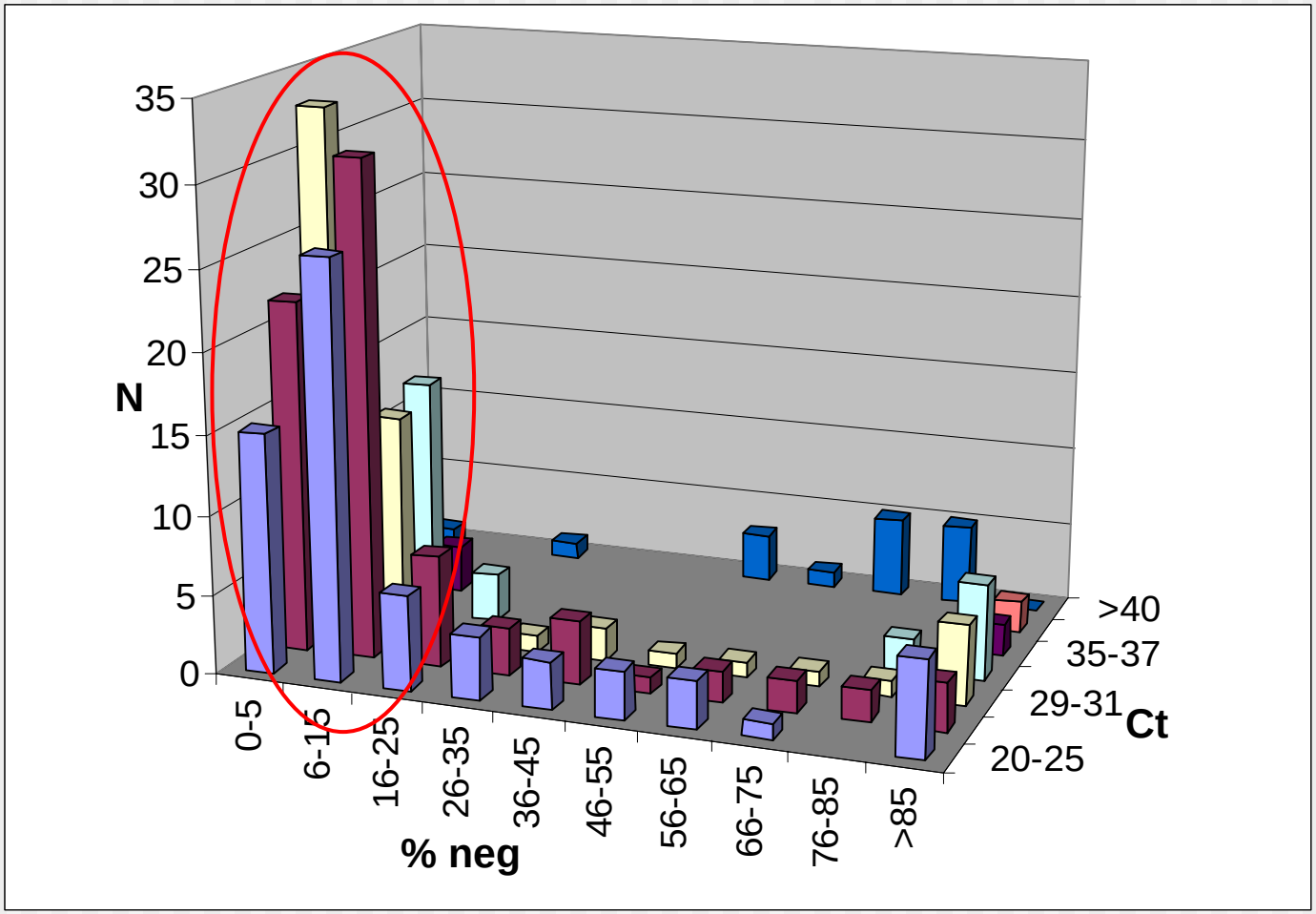
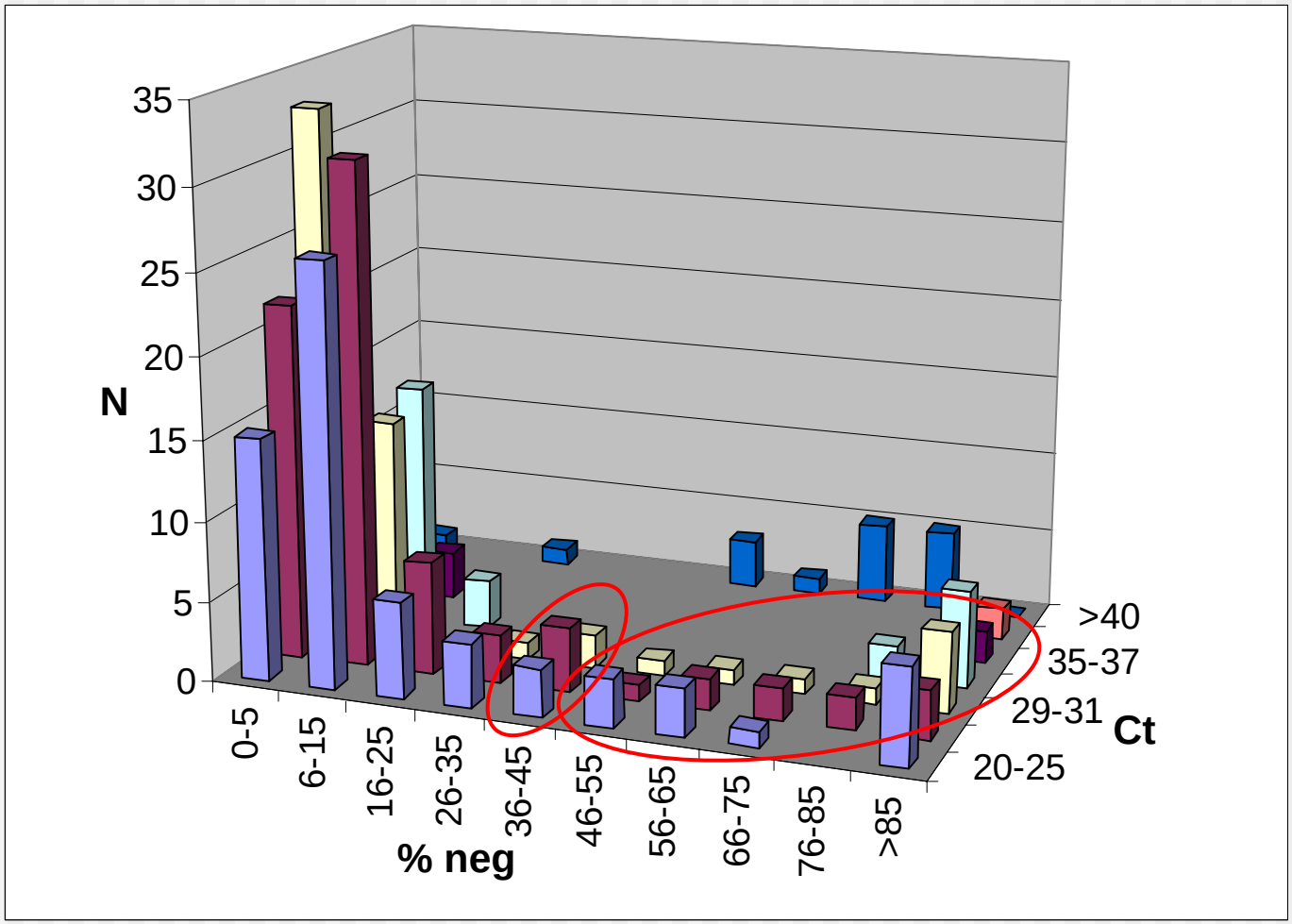
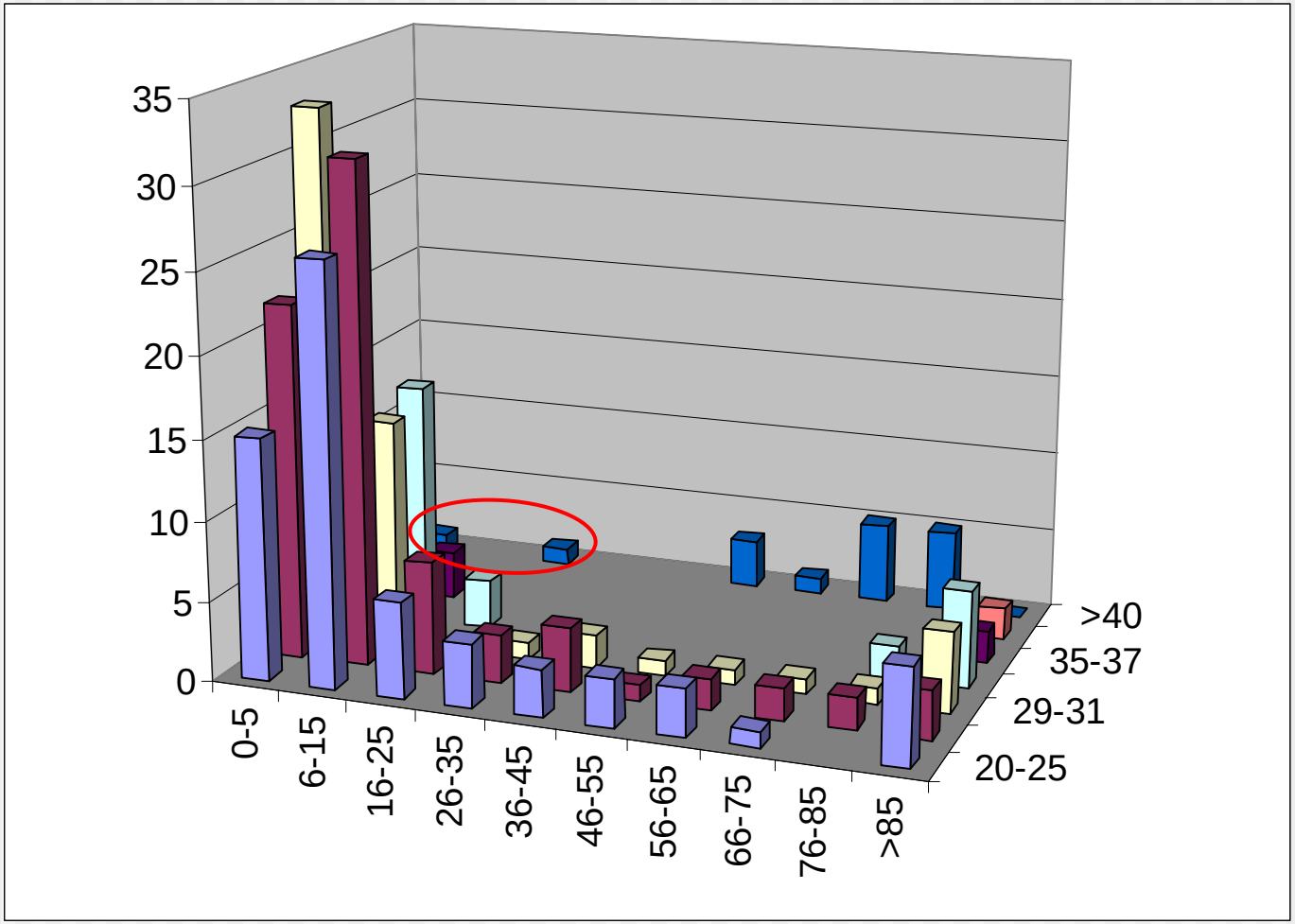


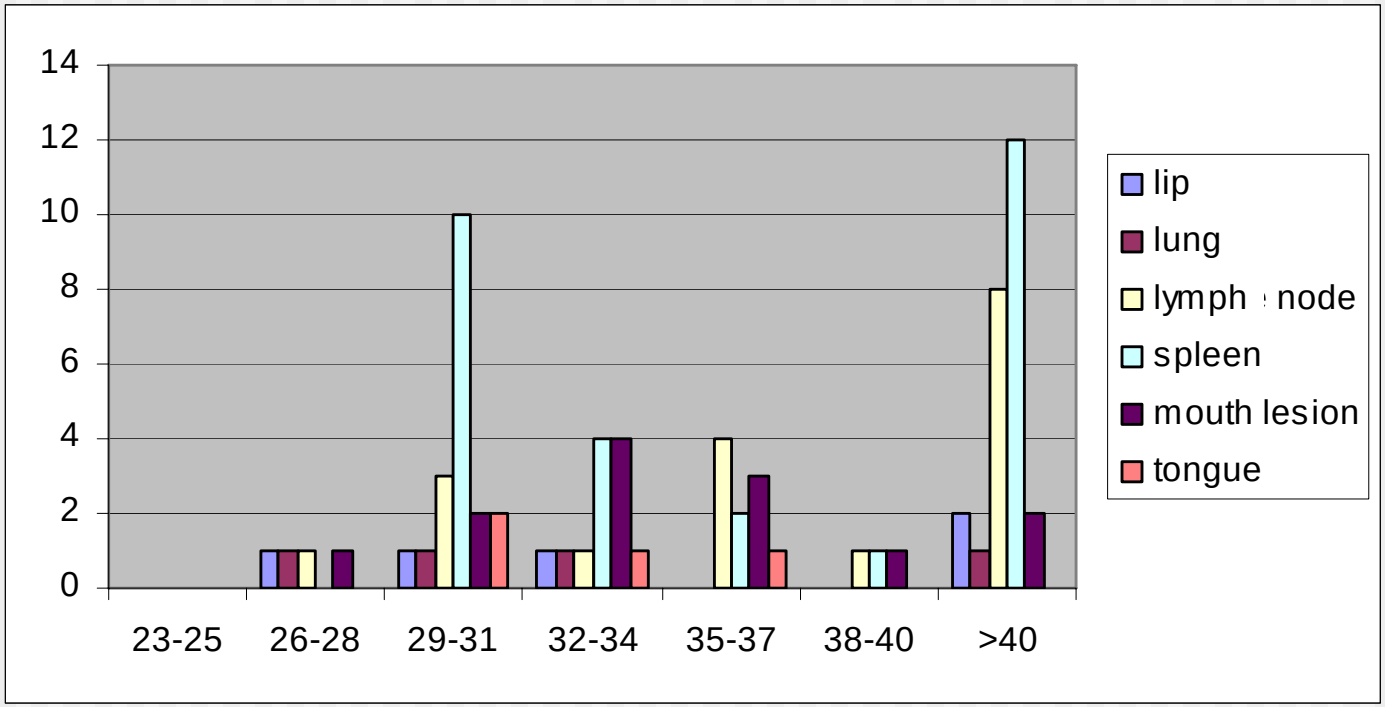
Results BT crisis: blood samples



Results BT crisis: blood samples



Results BT crisis: organs



Results BT crisis: pooled blood samples

- what?
  - blood samples pooled for animal certification (max 10/pool)
- why?
  - increases capacity
  - reduces cost
  - saves time

Results BT crisis: pooled blood samples

Comparison individual BT+ samples (Ct pure) with the same sample diluted in 9 BT- blood samples (Ct 1/10)

N° samples	Ct Class	mean Ct pure	mean Ct 1/10	△Ct	stdev △Ct
2	20<Ct<25	22,8	26,2	3,4	0,42
32	25<Ct<30	27,5	31,8	4,2	0,97
29	30<Ct<35	32,2	35,8	3,6	0,95
26	35<Ct<40	37,9	43,3	5,5	2,19
89	**total**			**4,4**	**1,60**

samples from class 35<Ct<40 were retested in pools of 5

mean △Ct = 3.7

stdev △Ct = 2.14

Thank you

Next ...

practical course
on 06-09-2007
at VAR