



**Veterinary
Laboratories
Agency®**

Development of an automated, real-time multiplex PCR for simultaneous detection of Classical and African swine fever viruses

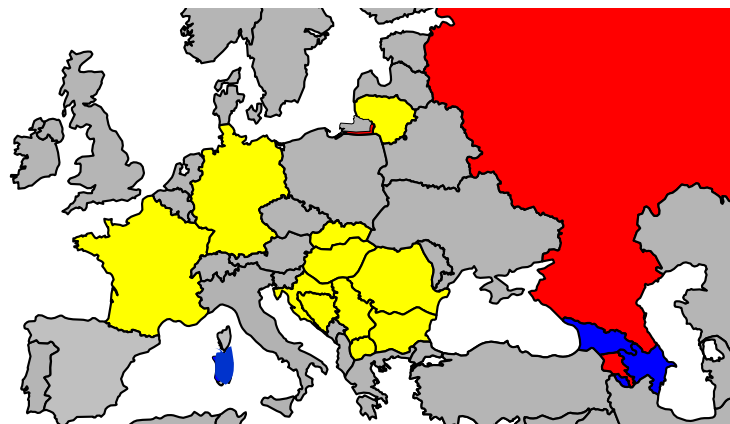
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Our viruses are getting closer:

Countries with swine fever incidents 2004-2010



- CSF
- ASF
- Both CSF/ASF

VLA -CSF

IAH -ASF

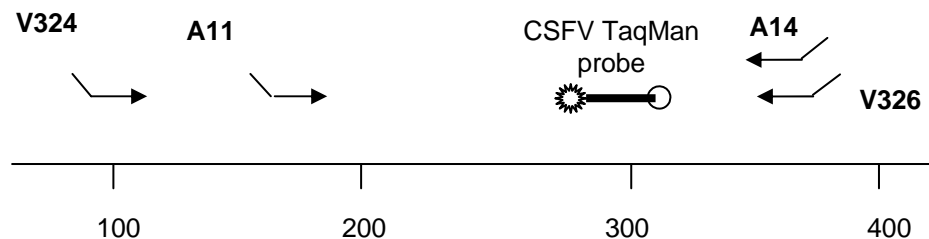


Aim

- To develop a multiplex, real-time PCR to detect both ASFV and CSFV in one assay
- To increase throughput capacity by automating nucleic acid extraction
- To include an internal control to monitor for false negatives
- Validate assay in comparison to existing PCRs as gold standards

Established tests

- VLA: CSFV – manual RNA extraction, one-tube, nested, real-time RT-PCR (Mc Goldrick et al 1998/9)
 - EDTA blood pooled up to 1 in 10



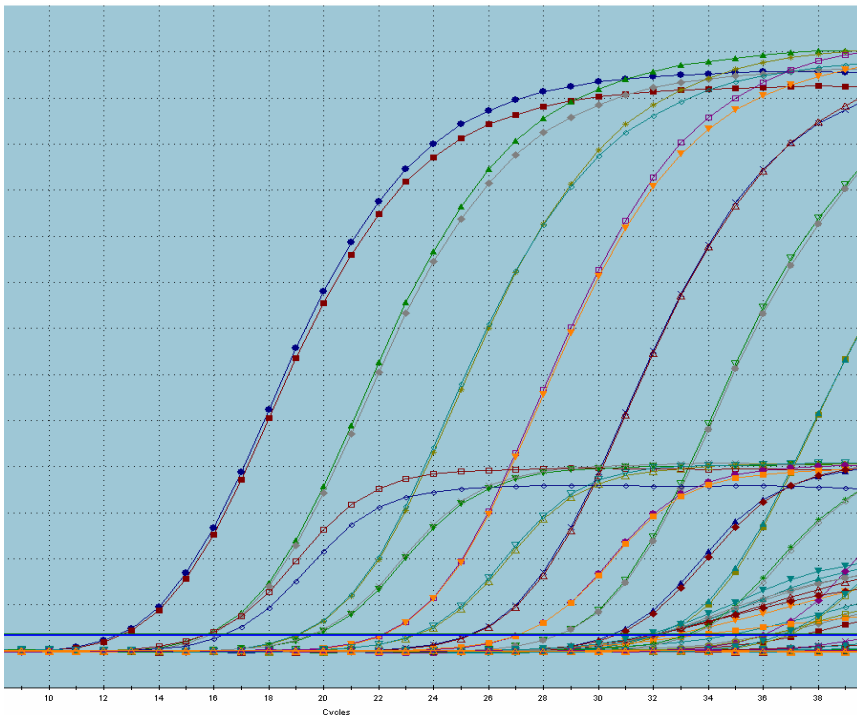
- IAH: ASFV – manual DNA extraction, (King et al 2003)
 - ASF sense and antisense primers, 250bp target VP72
- Same extraction method (Viral RNA kit QIAgen), but different PCR parameters

Development of a multiplex ASFV/CSFV/IC PCR assay

- Tried combining primers and probes from the established King and McGoldrick assays
 - Too many primers, long amplicons, probe “not optimum”, no internal control
- Examined primers and probes from other real-time CSFV and ASFV PCR assays:
 - ASFV (Martin Hofmann *et al*)
 - Primers: ASFVp72IVI_L, ASFVp72IVI_R Probe: King et al (Cy5)
 - CSFV (Bernd Hoffmann *et al*)
 - Primers: CSF100-F, CSF192-R
 - CSF-Probe 1 (FAM)
 - Internal control
 - EGFP: INTYPE IC RNA, primers and probe (Hex) (B.Hoffmann *et al*)

Multiplex PCR Optimisation

qRT-PCR kit	CSF sensitivity	ASF sensitivity
QuantiTect Multiplex RT-PCR NR (Qiagen)	+	+
QuantiTect Probe RT-PCR (Qiagen)	++	+
QuantiTect Virus (Qiagen)	++	++
SuperScript Platinum III One-Step (Invitrogen)	+++	+++



- Optimised [primer, probe, Mg²⁺]
- The multiplex “triplex” assay has similar efficacy and analytical sensitivity to duplex and single assays
- High ASFV/CSFV template affects detection of IC RNA
- Detects
 - 5x10² copies CSFV RNA
 - 1 copy ASFV DNA

Specificity

- CSFV

Isolate	Genotype	Final Call
Alfort 187	1.1	CSFV positive
Brescia	1.2	CSFV positive
Guatemala HC/#4409	1.3	CSFV positive
UK2000	2.1	CSFV positive
SP399-96	2.2	CSFV positive
Rostock	2.3	CSFV positive
Congenital Tremor	3.1	CSFV positive
CBR93	3.3	CSFV positive
Kanagawa	3.4	CSFV positive
Isolate	Virus	Final Call
C24V	BVDVI	CSFV/ASFV Negative
502643	BVDVII	CSFV/ASFV Negative
S137/1	BDV	CSFV/ASFV Negative

- ASFV
- Tested DNA/tissues from 46 different ASFV provided by IAH and CISA-INIA
- Included representatives of genotypes I, II, VII, IX, X
- Detected all 46 as ASFV positive

Automated nucleic acid extraction

- Qiagen BioRobot Universal System
- 96 well format, 2-3 hours for one plate, vacuum based
- Qiagen One for all UNIV programme and All nucleic acid MDx kit
- Problems initially with clogging of wells with porcine EDTA blood
- Assessed different lysis buffers (Roche Magna Pure, Qiagen AL, AVL), varying EDTA blood volumes, inclusion of an on deck proteinase K treatment.
- 50ul blood volume, Qiagen AVL lysis buffer, with a proteinase K treatment most successful for porcine EDTA blood samples
- Used extensively for characterisation of CSFV experimental infections
 - But reduced sensitivity compared to manual extraction



Dilution CSFV Positive blood	Triplex PCR Universal extraction	Triplex PCR Manual extraction
10^{-1}	+	+
10^{-2}	+	+
10^{-3}	+	+
10^{-4}	-	+
10^{-5}	-	+
10^{-6}	-	-
10^{-7}	-	-

Nucleic acid extraction with QIAcube

- Same chemistry as manual QIAmp Viral RNA mini kit
- 12 samples in 30-60 min, 140µl EDTA blood
- No cross contamination
- Equivalent sensitivity to manual extraction
- If pool samples 120 samples in 1 h verse 96 in 3h in universal

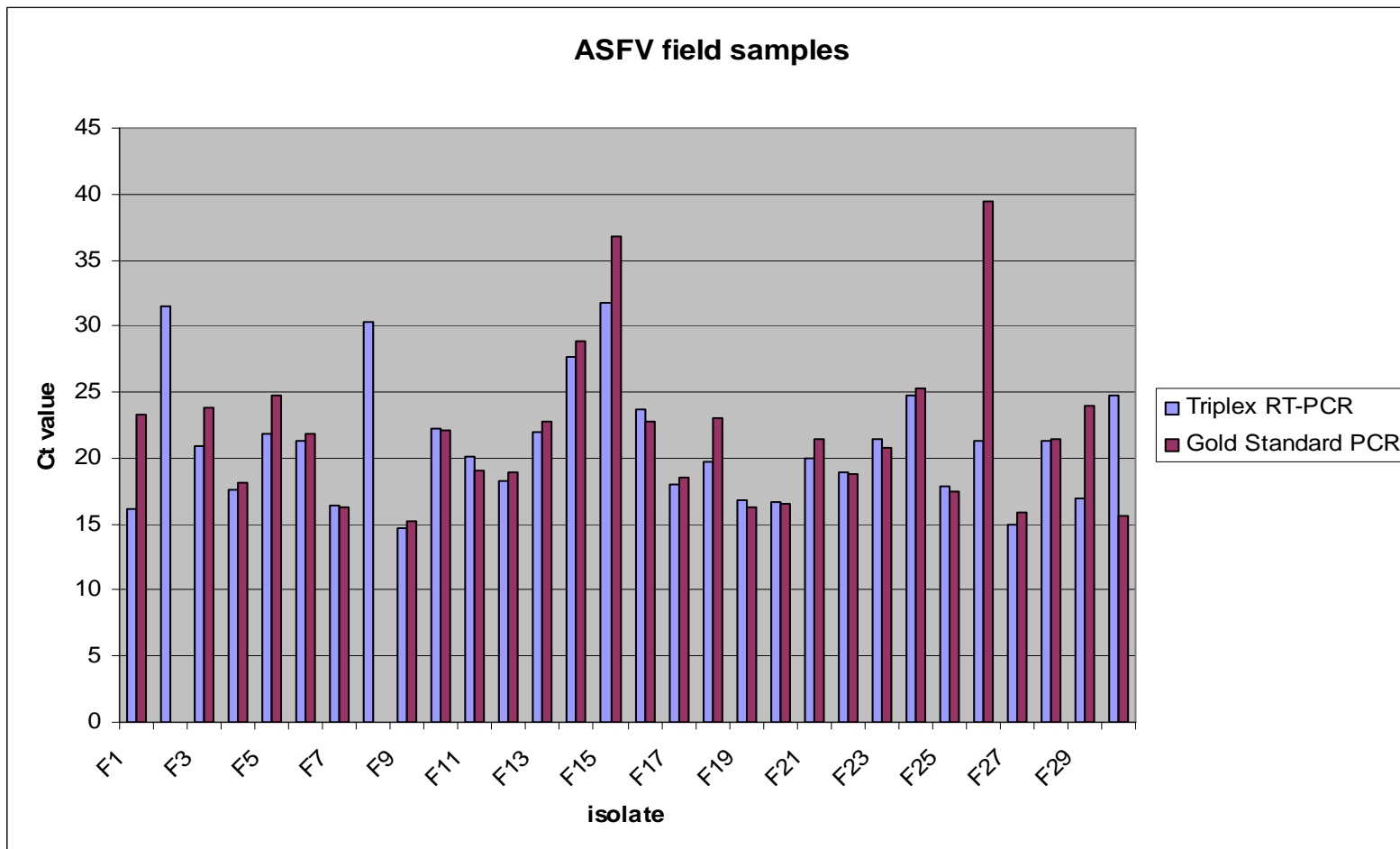


Dilution CSFV Positive blood	QIAcube extraction	Manual extraction
10⁻¹	+	+
10⁻²	+	+
10⁻³	+	+
10⁻⁴	+	+
10⁻⁵	+	+
10⁻⁶	+	+
10⁻⁷	+	+

Validation of triplex assay with Qiacube

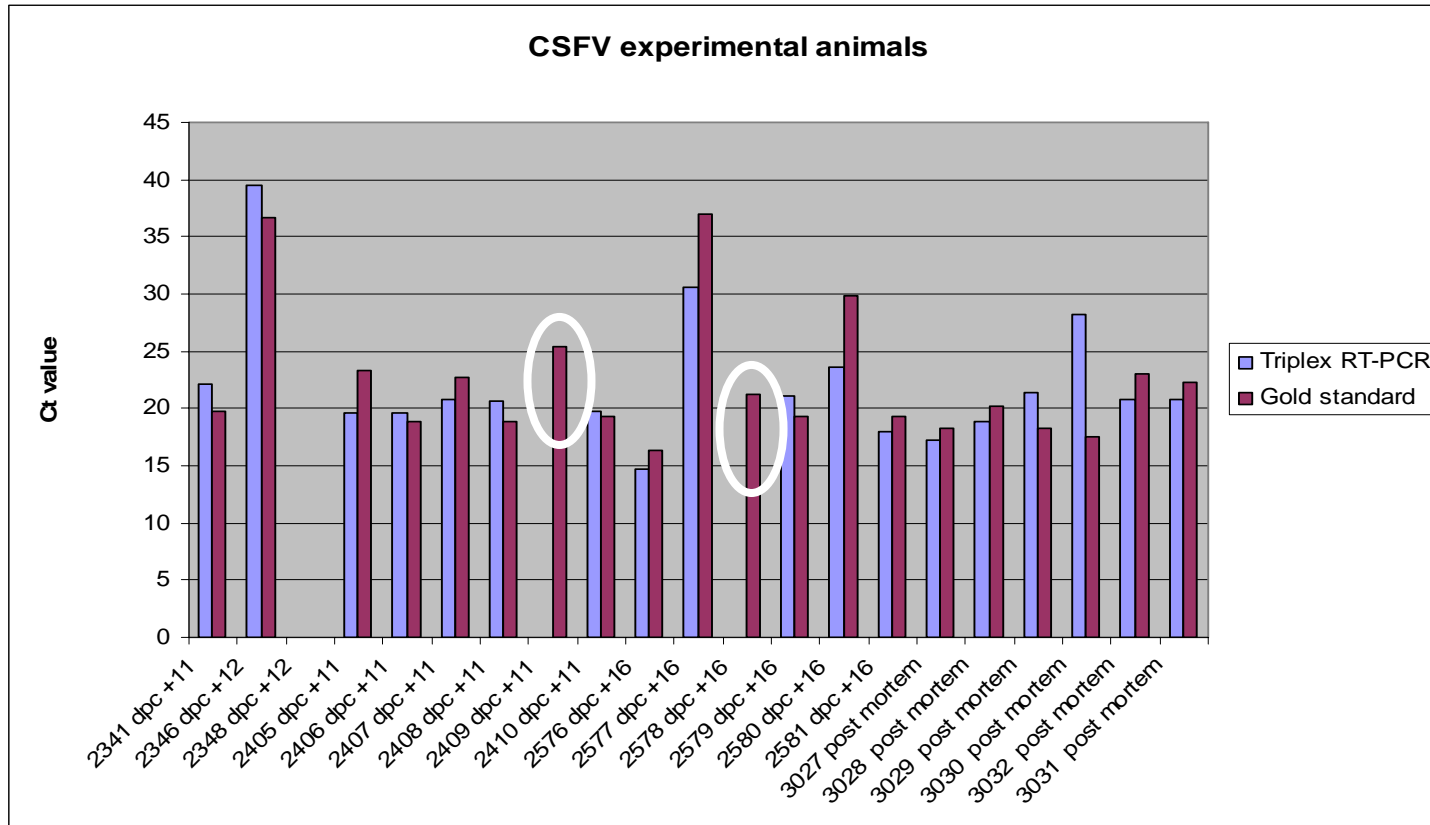
- Compared the new triplex/Qiacube assay to both singleplex/manual extraction assays as gold standards
 - CSFV EDTA blood and tissue samples from experimental infections with UK2000 and CBR/93
 - Archived field samples from UK2000 outbreak
 - ASFV EDTA blood and tissue samples from experimental infection
 - ASFV field samples
 - Samples from recent CSFV and ASFV ring trials, and Epizone ASFV ring trial
 - Currently only tested limited number of negative samples

Detection of ASFV in field samples



- ASFV triplex assay slightly more sensitive than gold standard

Detection of CSFV in samples from experimental animals



- Some CSFV positive samples not detected by triplex but were identified as inconclusive/false negative with the Internal control

Conclusions

- Multiplex assay detects both CSFV and ASFV nucleic acid in same reaction
- A few samples inconclusive with triplex assay but detected by gold standard methods
- Internal control detects false negatives
 - These inconclusive samples would be retested
 - If assume inconclusive result positive, diagnostic sensitivity for CSFV and ASFV = 100%
- Qiacube: equivalent to manual methods – easily adapt existing assays. Provides an economical automated solution with medium throughput
- Method for extraction of porcine EDTA blood on Universal BioRobot developed: minimal clogging but reduced sensitivity compared to manual extraction

Acknowledgements

- Martin Hofmann for providing ASFV primer sequences
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- 1 year position available

