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Workshop of the EU-RL for equine diseases for EIA
November 9th, 2022
Anses laboratory for Animal Health in Normandy- PhEED unit

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European Union reference laboratory for equine diseases

MOLECULAR BIOLOGY TEST FOR EIAV DETECTION

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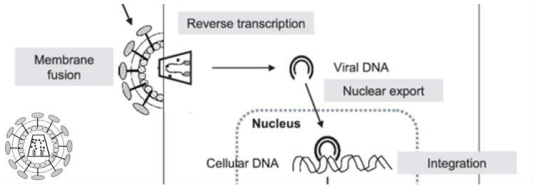
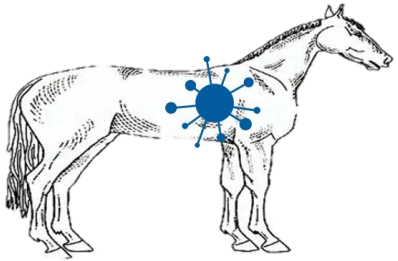
PhEED Unit

1. Introduction

Equine Infectious Anemia

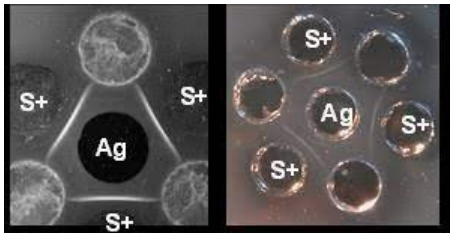
RNAss +
Order: Ortervirales
Familly: Retroviridae
Genus: Lentivirus





Diagnostics Tools

Serological (AGID)



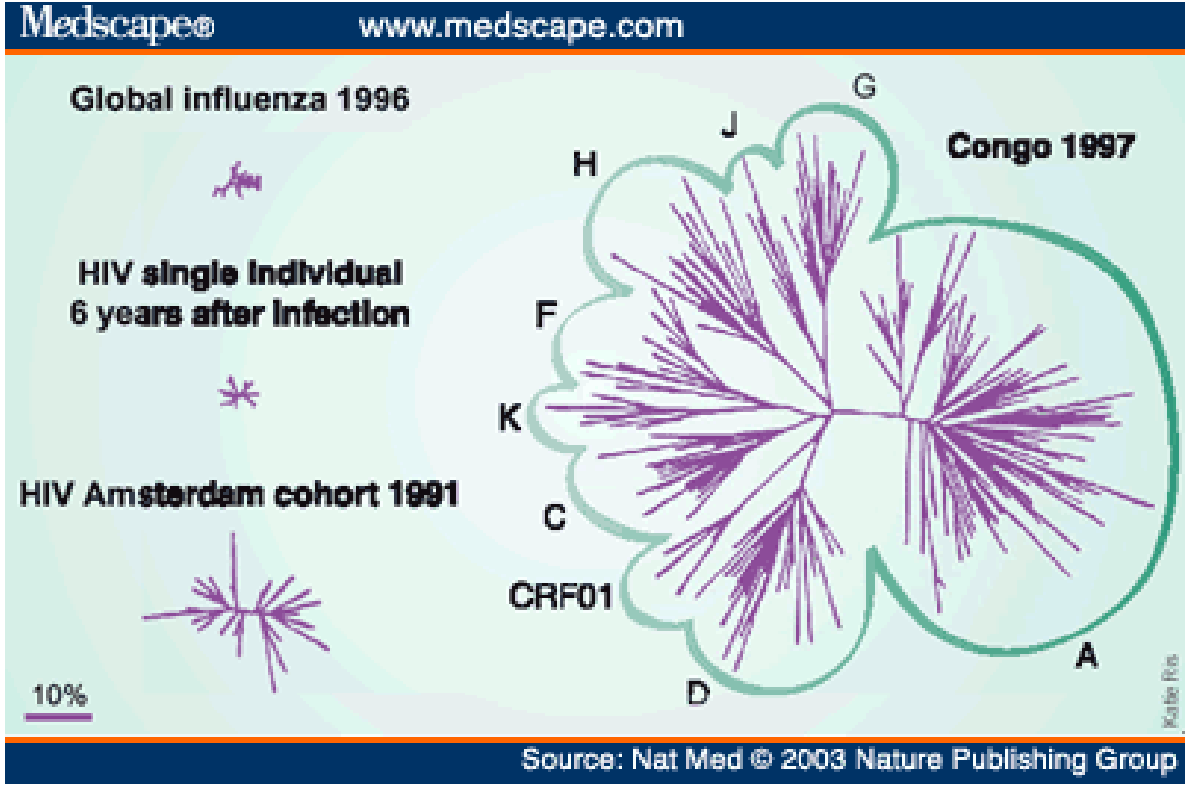
Molecular Biology RT-qPCR / qPCR

Universal molecular biology tools do not exist

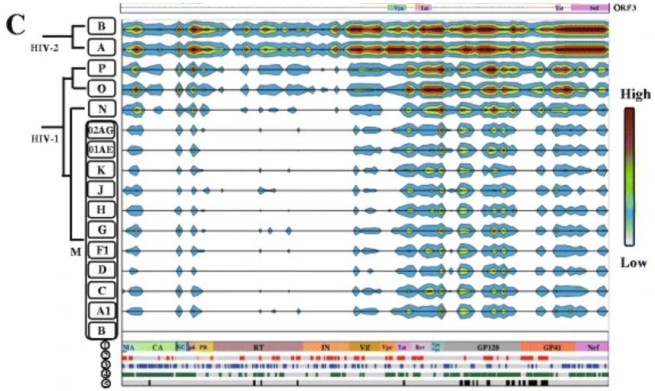
* High mutation rate

* No conserved regions

Lentivirus & High mutation rate



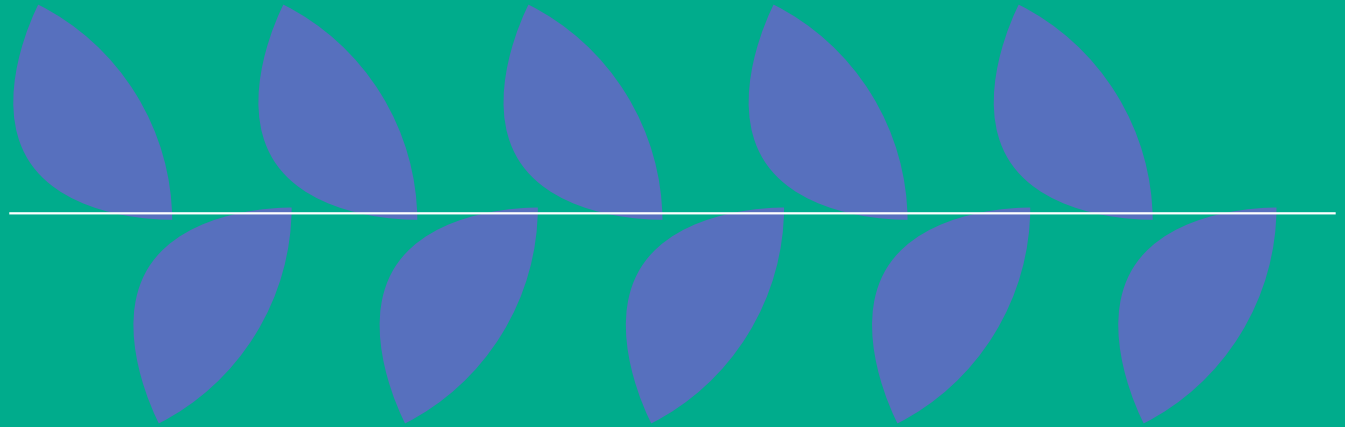
EIAV & Conserved regions



In the full-length genome, we identified conserved regions: Capsid, Nucleocapsid, Protease, RT, Integrase, Vpr and N-terminal domain of GP41. (2015)

29 EIAV Complete sequence genomes

2. Objectives



Objectives

I) Test the commercial kits for EIAV detection by RT-qPCR amplification

II) Identify conserved regions to develop new sets of “universal primers “

2. Commercial kits for molecular biology EIAV analysis

Market study

4 kits

We order :

2 kits

We tested

2 kits

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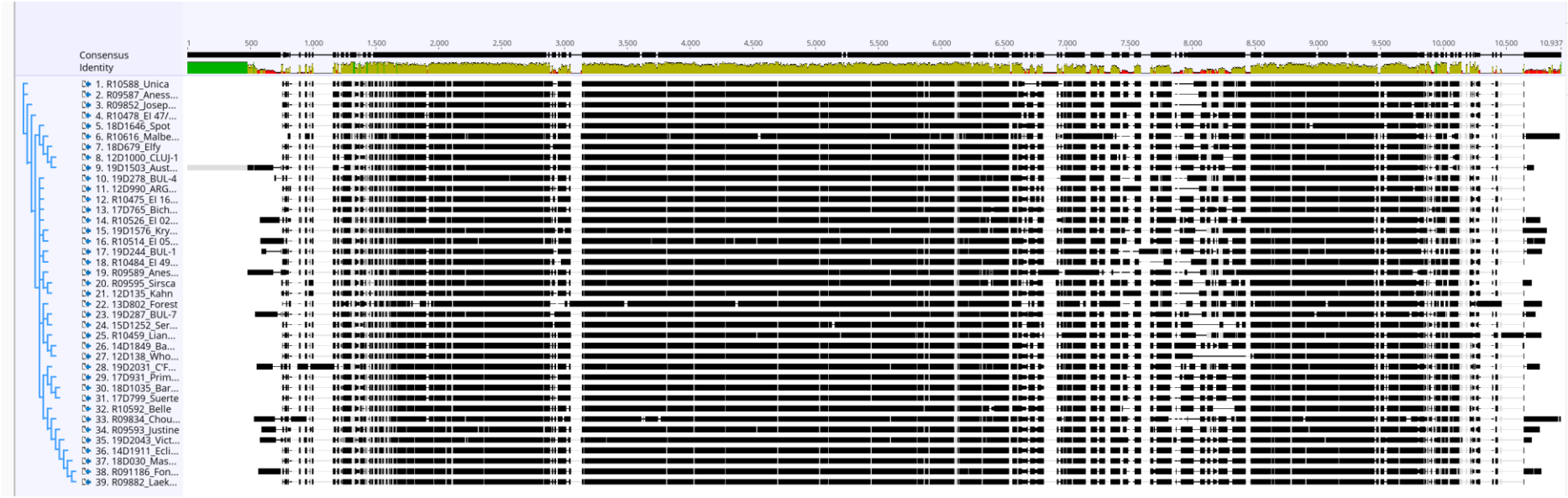
42
EIAV Positive Samples

Study Samples : Panel RT-qPCR



KIT – 1

* We found some problems with the protocols kit that was included in the notice.

- The company changes the protocol to use the kit between the day that we order and the reception of the product. (But not the products inside the kit)

We tested both protocols and none of them works with their and our control samples

KIT – 2

PCRmax Ltd™ qPCR test

Equine infectious anemia virus

Polymerase (pol) gene

150 tests

Kit contents

- **EIAV specific primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **EIAV positive control template (for Standard curve RED)**
- **Internal extraction control primer/probe mix (150 reactions BROWN)**
  VIC labelled as standard
- **Internal extraction control RNA (150 reactions BLUE)**
- **Endogenous control primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **EIAV/Internal extraction control/endogenous control RT primer mix (150 reactions GREEN)**
  Required for two step protocol only
- **RNase/DNase free water (WHITE)**
  for resuspension of primer/probe mixes
- **Template preparation buffer (YELLOW)**
  for resuspension of internal extraction control template, positive control template and standard curve preparation

oasig™ lyophilised OneStep qRT-PCR Mastermix

Instructions for use of Primerdesign oasig lyophilised OneStep Mastermix

Kit Contents

- 3 x Lyophilised Mastermix (50 reactions per glass ampule)
- 1 x Lyophilised ROX (BROWN)
- 4 x Re-suspension buffer (BLUE)

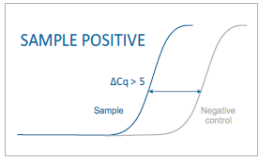
KIT – 2 - Results

Interpretation of Results

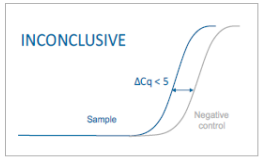
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	+ / -	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	+	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
> 30	-	+	-	**POSITIVE QUALITATIVE RESULT** do not report copy number as this may be due to poor sample extraction
-	+	+	-	**NEGATIVE RESULT**
+ / -	+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+ / -	+	> 35	*
-	-	+	-	**SAMPLE PREPARATION FAILED**
+ / -	+ / -	-	+ / -	**EXPERIMENT FAILED**

Positive control template (RED) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

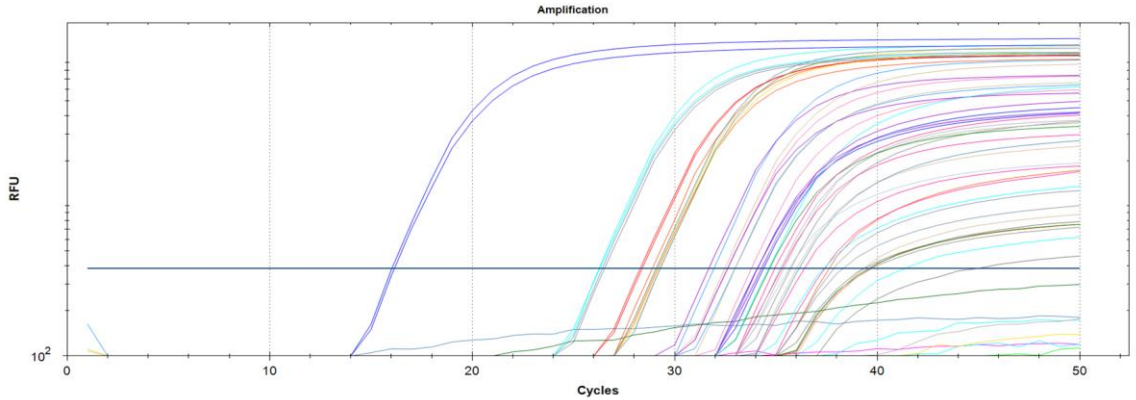
*Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 5 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.



RT-qPCR

Only 22 / 42 samples shown an amplification curve

52%

We would like to confirm the data, but the kit is not available since this summer!!

3. Non-Commercial molecular biology protocols for EIAV detection.

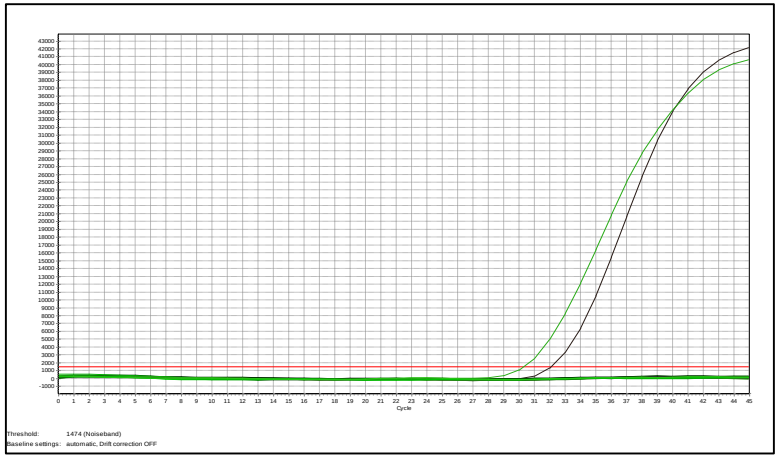
EIAV RT-qPCR related publications

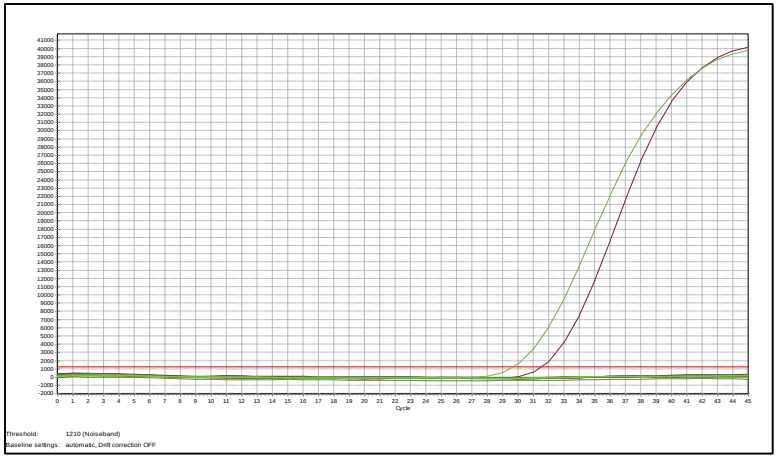
Detection of Equine Infectious Anemia Viral RNA in Plasma Samples from Recently Infected and Long-Term Inapparent Carrier Animals by PCR	J L Langemeier, S J Cook, R F Cook, K E Rushlow, R C Montelaro, and C J Issel	1996	JOURNAL OF CLINICAL MICROBIOLOGY	2-step RT-nested PCR; plasma; gag; early stages of infection
Development of a multiplex real-time reverse transcriptase-polymerase chain reacion for equine infectious anemia virus	R. Frank Cook, S.J. Cook, F. Li, R.C. Montelaro, C.J. Issel	2002	Journal of virological methods	real time RT-PCR; multiplex PCR; gag; plasma
Real-time quantitative RT-PCR and PCR assays for a novel European field isolate of equine infectious anaemia virus based on sequence determination of the gag gene	M. Quinlivan, RF. Cook, A. Cullinane	2007	Veterinary recod	2 step RT-qPCR Taqman; 1-step RT-qPCR Sybr Green; gag; tissues, plasma, serum and nasal, buccal, conjunctival and genital swabs; viral RNA (cDNA synthesis) and DNA
Molecular Detection, Epidemiology, and Genetic Characterization of Novel European Field Isolates of Equine Infectious Anemia Virus	Katia Cappelli,1,† Stefano Capomaccio,1,† Frank R. Cook,2 Michela Felicetti,1 Maria Luisa Marenzoni,1 Giacomo Coppola,1 Andrea Verini-Supplizi,1 Mauro Coletti,1 and Fabrizio Passamonti1,*	2011	J Clin Microbiol	Spleen, liver, bone marrow, and lymph node tissues, blood; RNA (cDNA synthesis) and DNA; nested PCR; (gag and full-lenght gag)
Development of a nested PCR assay to detect equine infectious anemia proviral DNA from peripheral blood of naturally infected horses	JB. Dong, W. Zhu, F.R. Cook, Y. Goto, Y. Horii, T. Haga	2012	Archives of Virology	nested PCR; LTR; tat; peripheral blood cells; DNA
Detection, molecular characterization and phylogenetic analysis of full-length equine infectious anemia (EIAV) gag genes isolated from Shackleford Banks wild horses	S.Capomaccio; Z.A.Willand; S.J.Cook; C.J.Issel; E.M.Santos; J.K.P.Reis; R.F.Cook	2012	Veterinary Microbiology	blood (EDTA); RNA extraction; cDNA synthesis; nested PCR; from exon 1 of Tat to the Pol gene
Is a diagnostic system based exclusively on agar gel immunodiffusion adequate for controlling the spread of equine infectious anaemia?	Maria Teresa Scicluna a,*, Charles J. Issel b, Frank R. Cook b, Giuseppe Manna a, Antonella Cersini a, Francesca Rosone a, Raffaele Frontoso a, Andrea Caprioli a, Valeria Antonetti a, Gian Luca Autorino a	2013	Veterinary Microbiology	exon 1 tat; plasma; qRT-PCR; RNA (cDNA synthesis)
Detection and molecular characterization of equine infectious anemia virus in Mongolian horses	T. Sharav, S. Konnai, N. Ochirkuhuu, E.O. Ts, H. Mekata, Y. Sakoda, T. Umemura, S. Murata, T. Chultemdorj, K. Ohashi	2017	The journal of veterinary medical science	nested PCR; LTR; tat; DNA; EDTA blood samples
Rapid detection of equine infectious anemia virus nucleic acid by insulated isothermal RT-PCR assay to aid diagnosis under field conditions	RF Cook, M Barrandeguy, PYA Lee, CF Tsai, YH Shen, YL Tsai, HFG Chang, HTT Wang, UBR Balasuriya	2018	Equine veterinary journal	reverse transcription-insulated isothermal pcr (RT-iiPCR); tat gene; plasma; spleen; buffy coat; whole blood; RNA and DNA
One-Step RT-qPCR assay for detection and quantification of equine infectious anemia virus in-vitro	B.L. Bueno, F.G. Oliveira, G.K. Lima, A.A. Fonseca Júnior, T.C. Kassar, R.J.F. Câmara, R.C. Leite and J.K.P. Reis	2018	Genetics and Molecular Research 17 (3): gmr18027	EIAV; viral load; RNA; synthesis; gag; real-time OneStep RT-qPCR
Molecular characterization of the major Open Reading Frames (ORFs) and enhancer elements from four geographically distinct north american equine infectious anemia virus (EIAV) isolates	S.J. Cook, G. Li, Y. Zheng, Z.A. Willand, C.J. Issel, R.F. Cook	2020	Journal of equine veterinary science	long range PCR; proviral DNA

EIAV RT-qPCR selected primers sets

Primers and probe(s)						
	Primer name	Direction	Primer sequence (5'-3')	Start	End	Amplicon size (bp)
qPCR_1 (2002)	LateRTproduct_EIAV-F	F	GGAGCCTTGAAAGGAGGGCCACTAAA	1595	1620	226
	LateRTproduct_EIAV-R	R	TTGTTGTGCTGACTCTTCTGTTGTATCGGG	1792	1821	
	LateRT_EIAV-Probe	Probe	ACGGGAAGCAAGGGGCTCAAGGGAGGCC	1749	1776	
qPCR_2 (2002)	EIAV_1572 PV	F	GGAGCCTTAAAAGGAGGGCCACTAAA	1595	1620	226
	LateRTproduct_EIAV-R	R	TTGTTGTGCTGACTCTTCTGTTGTATCGGG	1792	1821	
	LateRT_EIAV-Probe	Probe	ACGGGAAGCAAGGGGCTCAAGGGAGGCC	1749	1776	
qPCR_3 (2007)	1689	F	CAATGCAGAAATGCGCCAAAA			0
	1754	R	GCTGACCCTTCTGCTGTATGG			
	TMPr_1772	Probe	CCTCCCCTGAGCCCC			
qPCR_4 (2013)	MkIII Forward	F	GGCGCCCGAACAGGGACC			0
	MkIII Reverse	R	TGGCCAGGAACACCTCCAGAAGAC			
	MkIII_Probe	Probe	T[+G]AACCT[+G]G[+C]TGATCG[+T]AG[+G]A			
qPCR_5 (2018)	EIAV F352	F	CTGCCTGCTGAACCTGGCT			0
	EIAV R463	R	CTCCCATCTTACCTGTCTTCCTGT			
	EIAV_P	Probe	ACCTCCAGAAGACGTCTG			
qPCR_6 (2018)	EIAVPV1458	F	TTCAGAACGCAAATGAGGAA			0
	EIAVPV1988	R	TGTTACTACCACAAACTGTCCA			
	LateRT_EIAV-Probe	Probe	ACGGGAAGCAAGGGGCTCAAGGGAGGCC	1749	1776	

	Cq qPCR_1		Cq qPCR_2	
Sample	DNA	RNA	DNA	RNA
R0	N/A	N/A	N/A	N/A
R0	N/A	N/A	N/A	N/A
R0	N/A	N/A	N/A	N/A
R1	N/A	N/A	N/A	N/A
R1	N/A	N/A	N/A	N/A
R1	N/A	N/A	N/A	N/A
R1	N/A	N/A	N/A	N/A
12	N/A	N/A	N/A	N/A
12	N/A	N/A	N/A	N/A
17	35,23	32,12	34,25	31,6
18	N/A	N/A	N/A	N/A
19	N/A	N/A	N/A	N/A
19	N/A	N/A	N/A	N/A
CN	N/A	N/A	N/A	N/A
Ce	33,28	30,36	32,81	29,74
H2	N/A	N/A	N/A	N/A





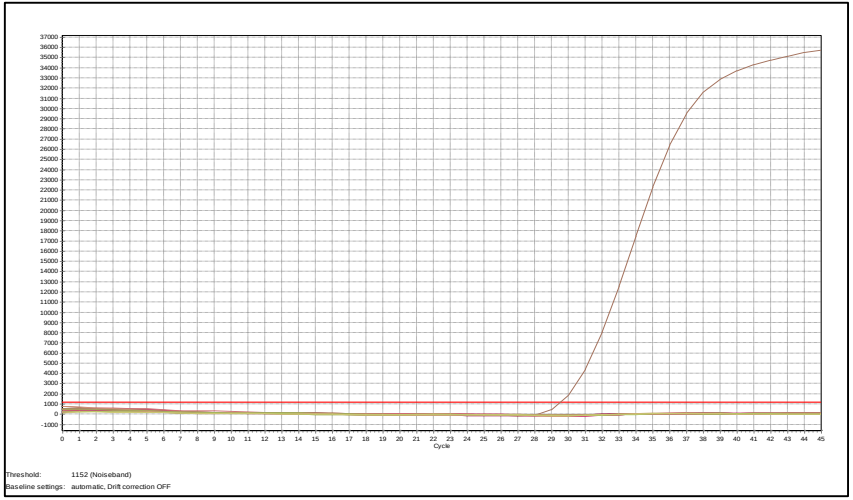
Sample	Cq qPCR_3		Cq qPCR_4	
	DNA	RNA	DNA	RNA
R(	N/A	N/A	N/A	N/A
R(	N/A	N/A	N/A	N/A
R(	N/A	N/A	N/A	41,01
R:	N/A	N/A	32,37	29,67
R:	35,58	36,75	39,29	32,83
R:	N/A	N/A	40,46	40,74
R:	N/A	N/A	33,69	31,97
1:	N/A	N/A	N/A	N/A
1:	43,46 non rep.	N/A	39,36	36,76
1:	N/A	N/A	35,97	32,7
1:	N/A	N/A	N/A	35,36
1:	N/A	N/A	N/A	N/A
1:	N/A	N/A	N/A	N/A
C:	N/A	N/A	N/A	N/A
C(	N/A	N/A	35,03	33,2
H2O	N/A	N/A	N/A	N/A

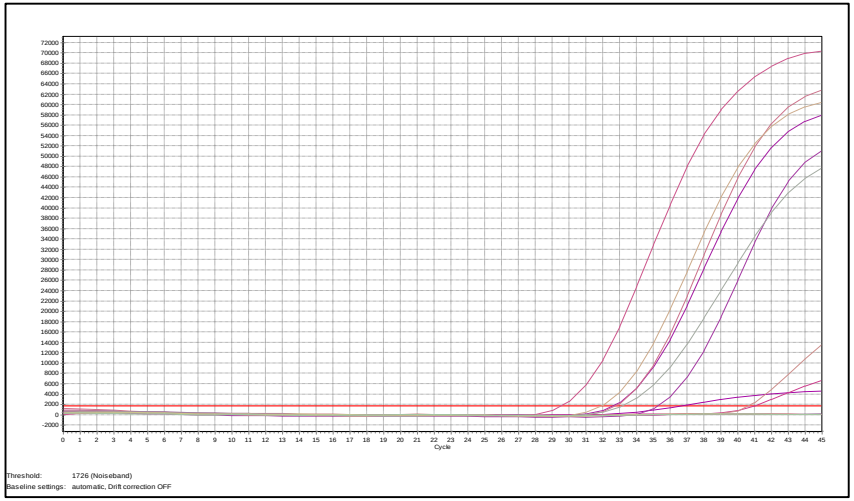
6 / 13 DNA (46 %)

8 / 13 RNA **(61%)**

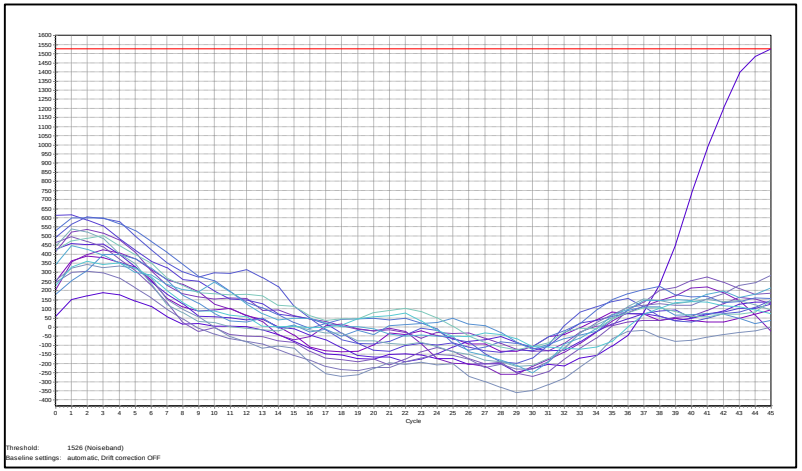
(Scicluna et al. 2013)

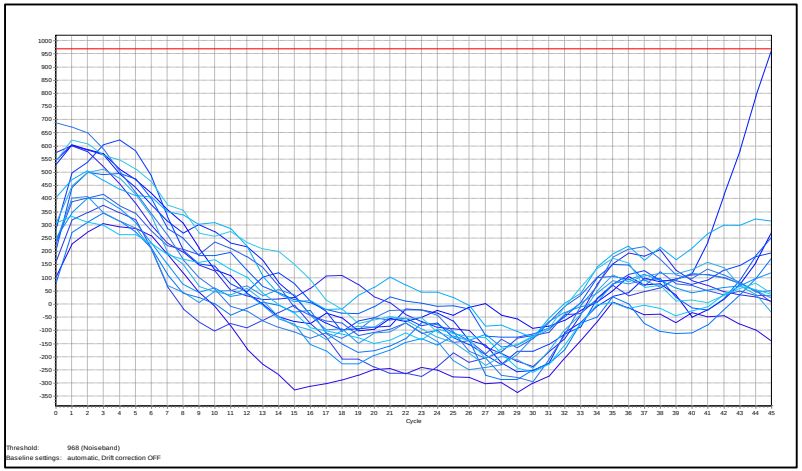
Test in larger panel





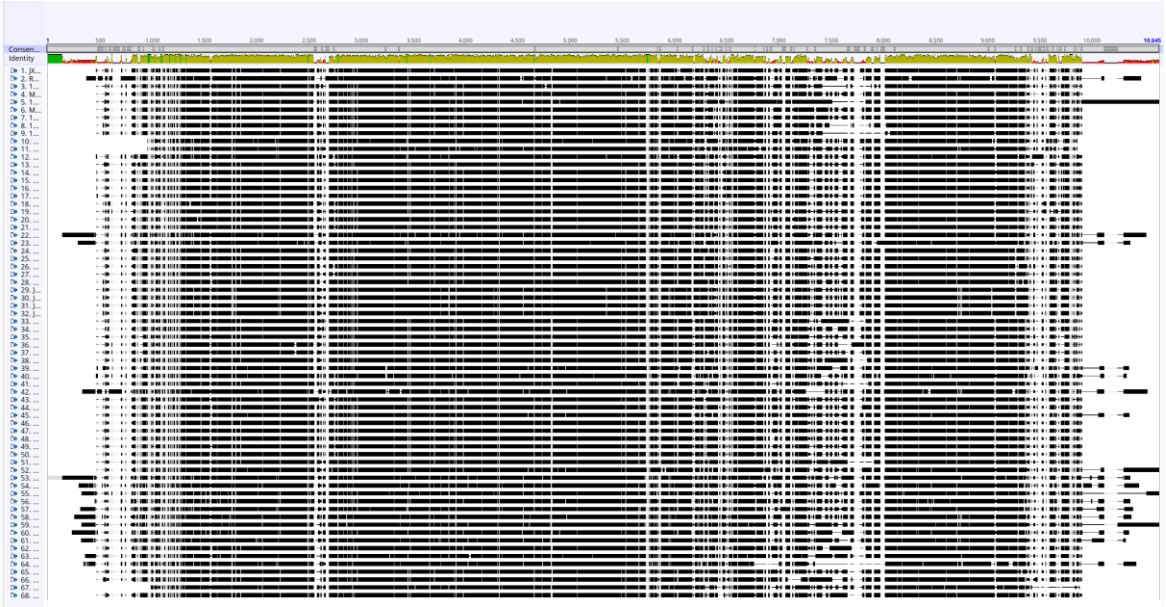
	Cq qPCR_5		Cq qPCR_6	
	DNA	RNA	DNA	RNA
R	N/A	N/A	N/A	N/A
R	N/A	N/A	N/A	N/A
R	39,91 non rep.	N/A	N/A	N/A
R	13,09 non rep.	N/A	N/A	N/A
R	9,88 non rep.	N/A	N/A	N/A
R	38,24 non rep.	N/A	N/A	N/A
R	8,51 non rep.	N/A	N/A	N/A
R	41,81 non rep.	N/A	30,93	N/A
1	41,1 non rep.	N/A	N/A	N/A
1	8,59 non rep.	N/A	40,35 non rep.	N/A
1	41,12 non rep.	N/A	39,97 non rep.	N/A
1	12,14 non rep.	N/A	43,68 non rep.	N/A
1	12,28 non rep.	N/A	44,92 non rep.	N/A
1	44,05 non rep.	N/A	39,58 non rep.	N/A
C	39,78 non rep.	N/A	27,38	N/A
C	N/A	N/A	N/A	N/A
H				





Complete EIAV genomes + EIAV genomes > 85%

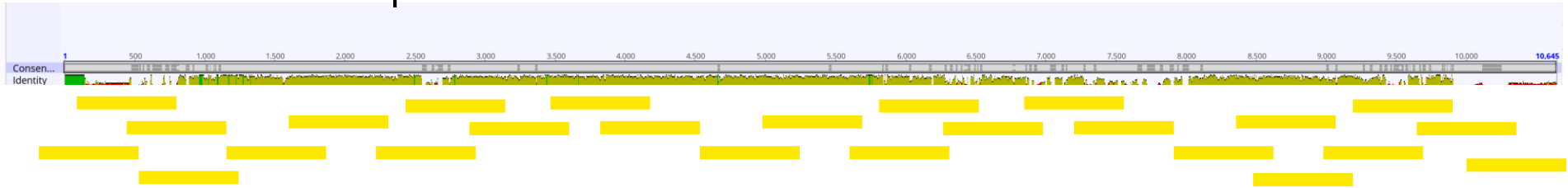
Were aligned and a consensus sequence was obtained



EIAV RT-qPCR assay development

Warning !! Preliminary RESULTS

EIAV consensus sequence

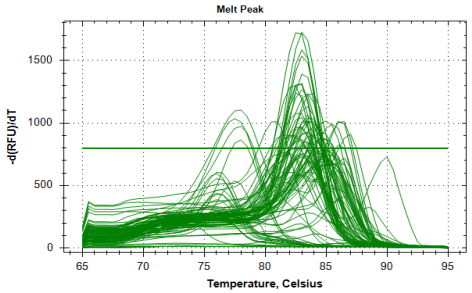


25 primers pairs (including degenerated primers)

30 / 42
71%

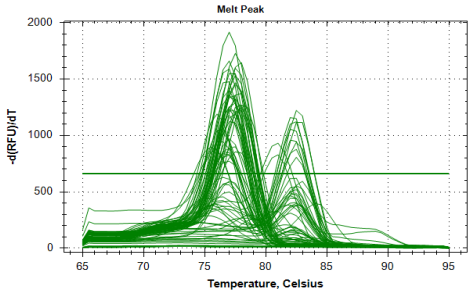
Pair 1

SYBR green test



38 / 42
90,5%

SYBR green test



35 / 42
83%

Pair 2

3. Conclusion

Conclusion & Perspectives

I) A molecular biology kit for EIAV detection was tested in a panel of 42 EIAV positive samples and have an detects 22 / 42 (52%)

II) 6 molecular biology protocols for EIAV detection were tested in a panel of 13 EIAV positive samples and the best of them identified 8/13 (61%)

III) Taking advantage from the new EIAV sequences completed in our lab and those with more than the 85% of the sequence complete a consensus sequence was built, and 25 primer set were designed.

IV) **Warning !!!** Warning !! Preliminary RESULTS

2 set of primers give to us satisfactory results in a panel of 42 EIAV positive samples and detects 30 and 35 / 42 respectively (71% - 83%). Indeed combining both sets we can reach 38 / 42 (90,5 %).

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European Union Reference Laboratory for
EQUINE DISEASES

Anthony

Cécile

Fanny

Gabrielle

José-Carlos

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Thank you for your attention