Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri"

Evaluation of PCR methods for the molecular detection of *Babesia caballi* and *Theileria equi* on field samples

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INTRODUCTION

Equine piroplasmosis

-is a tick-borne disease caused by **protozoans *Babesia caballi*** and ***Theileria equi***

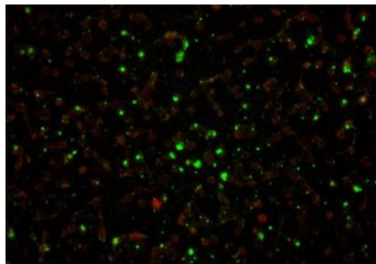
- affects **equids**

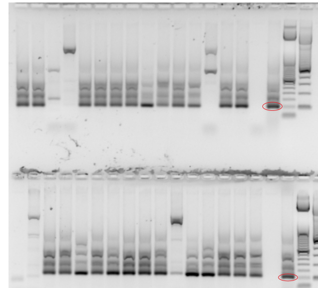
- **endemic** in Europe

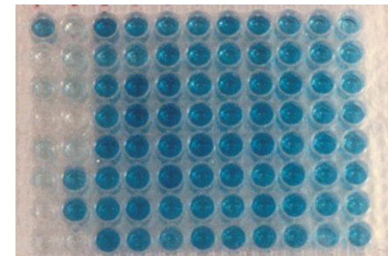
-is subject to international movement **restrictions** (OIE tests are serologically based).

- **long-lasting antibodies** (up to 4 years in *B.caballi* infections and lifelong in *T. equi*).

AIM: Is to define a method able to **differentiate seropositive animals from carriers**







MATERIALS AND METHODS

-**103 whole blood** samples of clinically suspect equids (CERME research programme)

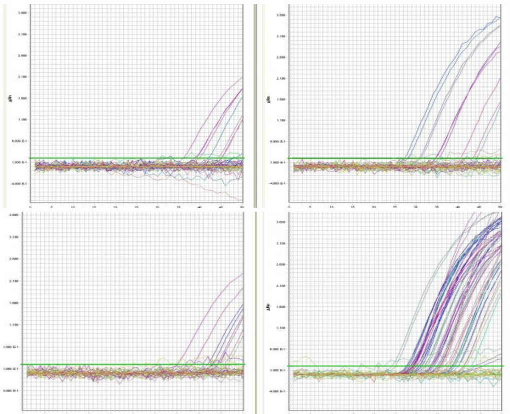
-Genomic **DNA extraction**: Cador Pathogen 96 QIAcube HT Kit (Qiagen®).

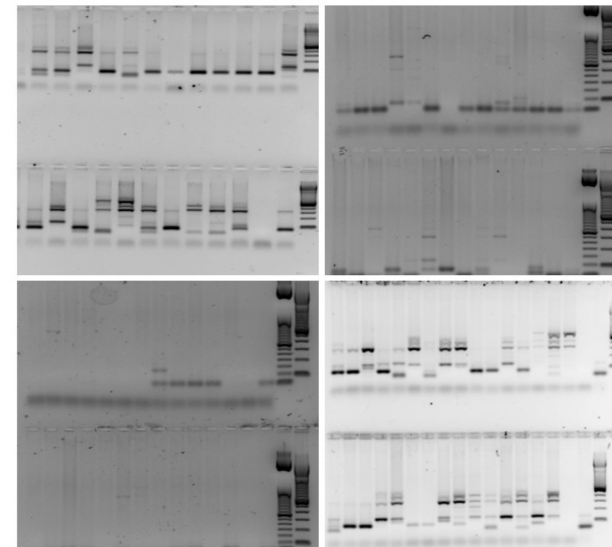
-**4 different PCRs** for each protozoan.

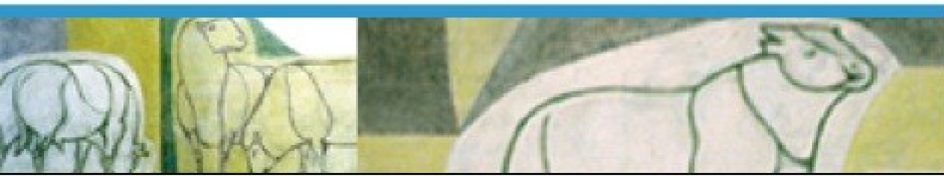
-Discordant results were verified by **sequencing** using different primers: RLB, EMA,18SRNA.

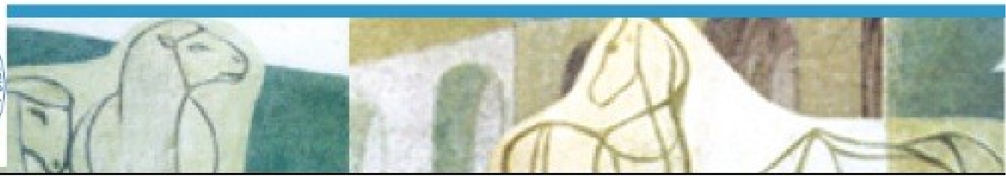
- Assesment of relative sensitivity **(rSe)** and relative specificity **(rSp)** using the PCR detecting the greatest number of positives.

-**Agreement** among the PCRs was estimated.









MATERIALS AND METHODS

T. equi PCR	PCR TECHNIQUE	TARGET	AMPLICONS	PRIMERS
T 1	End point	Equine merozoite antigen gene	268 bp	EMA-5/6 *(Battsetseg B. et al. 2001)*
T 2	Nested	Equine merozoite antigen gene	102 bp	EMAE-F/R EMAI-F/R *(Nicolaiewsky T.B. et al 2001)*
T 3	Real Time	V 4 Hypervariable region 18S RNA gene	81 bp	BE 18S-F/R BE 18S-P *(Kim C. et al. 2008)*
T 4	Real Time (Commercial kit)	Equine merozoite antigen gene	~120 bp	Mix

MATERIALS AND METHODS

B. caballi PCR	PCR	TARGET	AMPLICONS	PRIMERS
B 1	End point	Rhoptry associated protein complex gene	825 bp	BC RAP-F/R *(Battsetseg B. et al. 2001)*
B 2	Nested	Rhoptry associated protein complex gene	430 bp	BC 48-F1/R3 BC 48-F11/R31 *(Bhoora R. et al 2010)*
B 3	Real Time	V 4 Hypervariable region 18S RNA gene	95 bp	BC 18S-F/R BC 18S-P *(Bhoora R. et al 2010)*
B 4	Real Time (Commercial kit)	18S RNA gene	~100 bp	Mix

RESULTS

Number of **positives** for *Theileria equi* per method

SAMPLES	T1 END POINT (EMA5/6)	T2 NESTED (EMAI)	T3 REAL TIME (18S)	T4 COMMERCIAL KIT
103	29	29	35	27

Number of **positives** for *Babesia caballi* per method

SAMPLES	B1 END POINT (RAP)	B2 NESTED (BC48)	B3 REAL TIME (18S)	B4 COMMERCIAL KIT
103	4	8	4	2

RESULTS

<table>
<tr><td></td><td colspan="6">Number of PCRs in agreement</td></tr>
<tr><td></td><td colspan="3">Babesia Caballi</td><td colspan="3">Theileria equi</td></tr>
<tr><td></td><td>4</td><td>3</td><td>2</td><td>4</td><td>3</td><td>2</td></tr>
<tr><td>Positive</td><td>1</td><td>0</td><td rowspan="2">5</td><td>26</td><td>1</td><td rowspan="2">4</td></tr>
<tr><td>Negative</td><td>93</td><td>4</td><td>67</td><td>5</td></tr>
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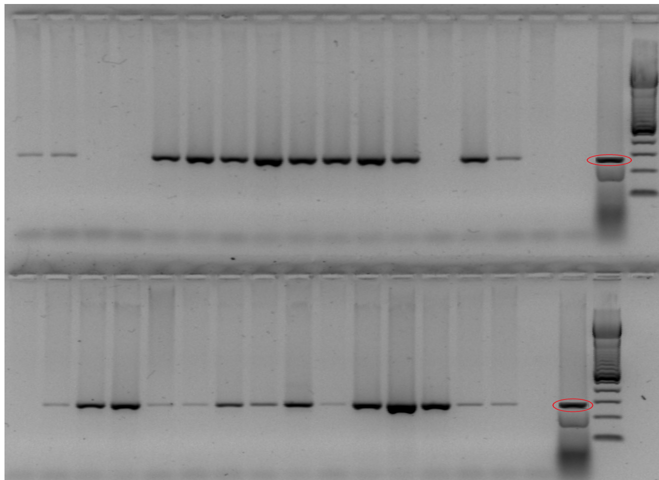
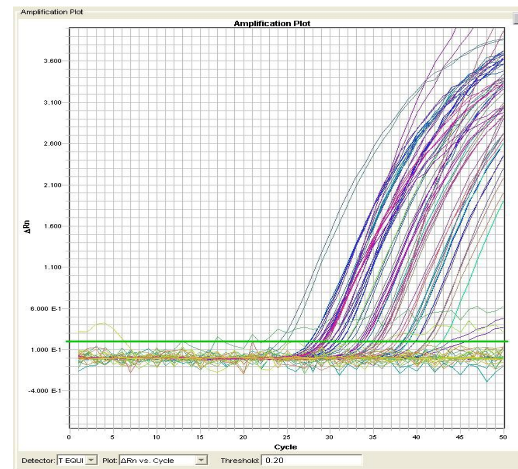
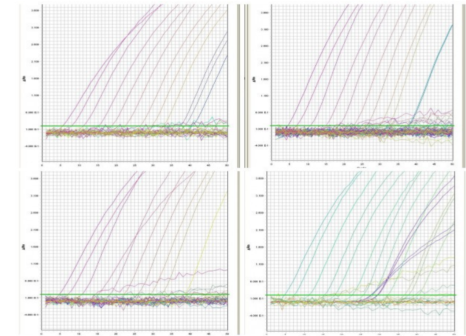
An overall agreement of **91.3**% was observed for ***B. caballi*** and **90.3**% for ***T equi***.





RESULTS



B3 and **T3** detected the highest number of confirmed positive samples and they were used as reference tests to estimate rSe and rSp.

B.caballi data were obtained on a very small number of positives, Recruitment of a major number of positives is necessary to verify the results.

	T3	
	rSe	rSp
T1	80,00	98,53
T2	82,86	100,00
T4	77,14	100,00

	B3	
	rSe	rSp
B1	25,00	96,97
B2	50,00	93,94
B4	50,00	100,00

RESULTS

THEILERIA EQUI **SEQUENCING**

V4 (RLB)

100% AB515314.1 *Theileria* equi
99% AB515315.1 *Theileria* equi
98% KF597074.1 *Theileria* equi
98% EU642509.1 *Theileria* equi
98% JX177672.1 *Theileria* equi
98% AB733373.2 *Theileria* equi
98% EU642508.1 *Theileria* equi

EMA5/6

100% JQ782603.1 *Theileria* equi

Be 18s

100% KJ573374.1 *Theileria* equi
100% KJ549664.1 *Theileria* equi

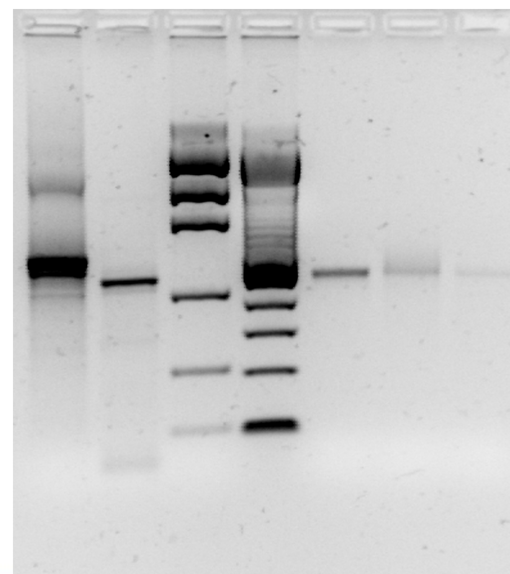
BABESIA CABALLI **SEQUENCING**

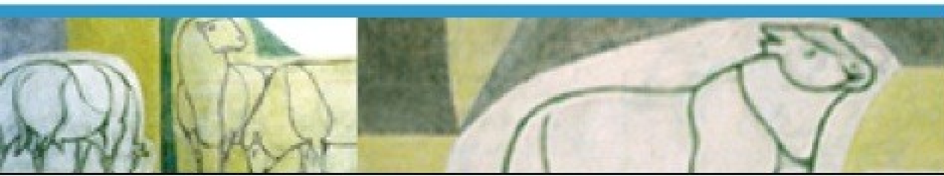
V4 (RLB)

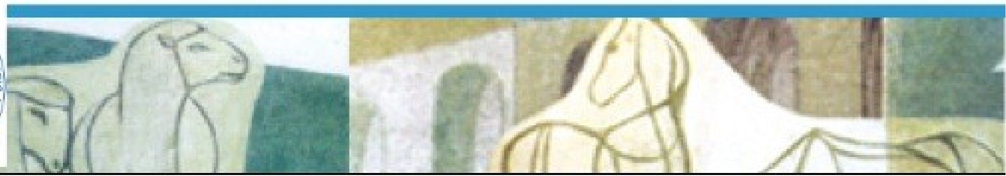
99% EU888904.1 *Babesia caballi*
99% EU642513.1 *Babesia caballi*

Bc 18s

100% KJ787774.1 *Babesia caballi*
99% AB734392.2 *Babesia caballi*
99% JX049130.1 *Babesia caballi*







RESULTS

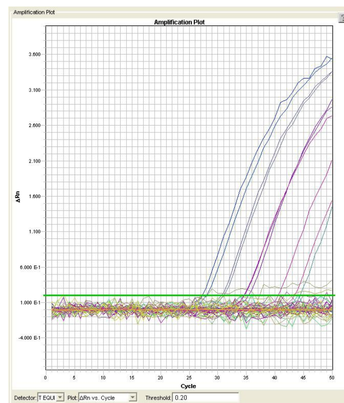
When compared to the serological tests:

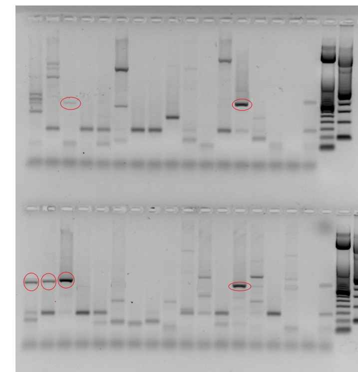
PCRs identified **non-carriers** among the **seropositives**.

PCRs identified **carriers** among the **seronegatives**:

-*T. equi:* 36 PCR positive 17 were seronegative.

-*B. caballi:* all PCR positives were seronegative.





DISCUSSION AND CONCLUSIONS

T.EQUI IN HOUSE PCRs CHARACTERISTICS

	SENSIBILITY	SPECIFICITY	TARGET LENGTH	PRIMER EFFICIENCY	OTHER
T1	Quite high	High	Quite long	High Conserved sequences in the designed primer region	
T2	Quite high	High	Short	High Conserved sequences in the designed primer region	
T3	**Very high**	**Very high**	**Short**	**High**	**Taqman probe designed in a high conserved region**

DISCUSSION AND CONCLUSIONS

B. CABALLI IN HOUSE PCRs CHARACTERISTICS

	SENSIBILITY	SPECIFICITY	TARGET LENGTH	PRIMER EFFICIENCY	OTHER
B1	Low	High	Too long	Low High mutation frequency in 5' RAP gene	
B2	High	Low	Quite long	Low Homology between portions of equine genome and PCR target	
B3	**Very high**	**Very high**	**Short**	**High**	**Taqman MGB make up high mutation frequency, amplicon 81 less problems related to target degradation.**

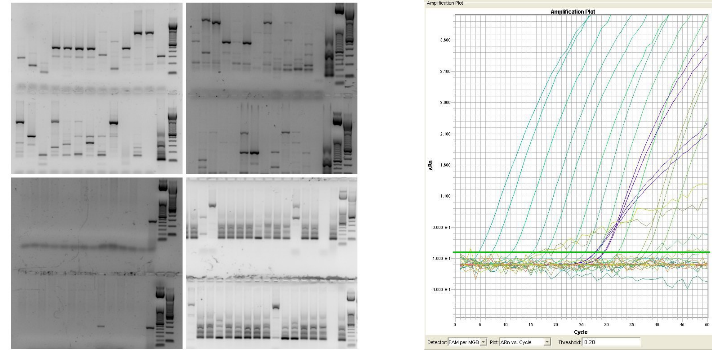
FINAL CONCLUSIONS

-Ideal target characteristcs:

-Short length (Length of target could make up poor extraction efficiency or DNA degradation)

- High preserved regions (18 S)

-Constitutive genes.



-Molecular tests

- Use In routine diagnosis.

- Could be developed as quantitative methods to assess correlation between parasitemia and the clinical phase of infection to aid the clinician, in deciding or verifying treatment.

-Recommendable for international movement control to include PCR, in adjunct to sero-methods in use.

REFERENCES :

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Thank you for your attention!!