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

WAVLD 17th International Symposium Saskatoon, Canada 2015 June 15-18

CENTRO REFERENZA NAZIONALE MALATTIE DEGLI EQUINI

Addition of PCR to the Conventional Serological Routine Diagnosis of Equine Piroplasmosis

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Equine piroplasmosis (EP)

Present in tropical and subtropical parts with prevalence related to the distribution of its tick vectors, **ixodid spp.**

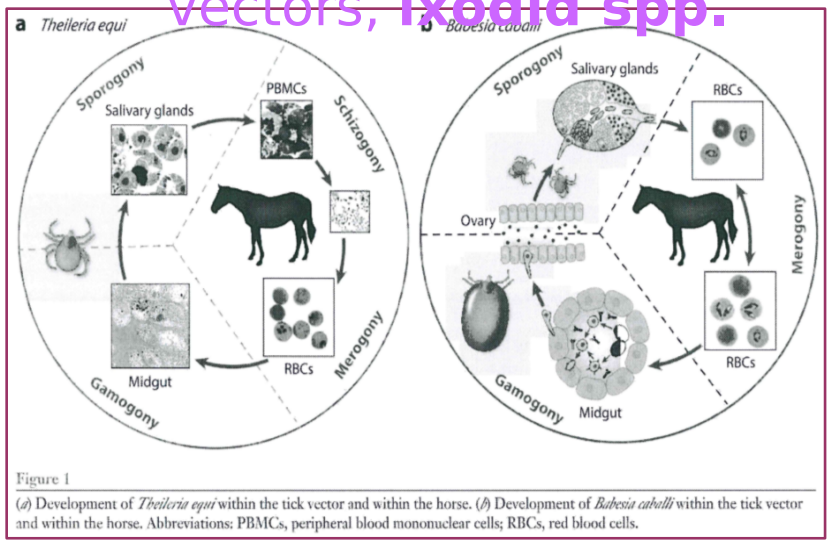

Figure 1

(*a*) Development of *Theileria equi* within the tick vector and within the horse. (*b*) Development of *Babesia caballi* within the tick vector and within the horse. Abbreviations: PBMCs, peripheral blood mononuclear cells; RBCs, red blood cells.

Scoles GA, Ueti MW. Vector ecology of equine piroplasmosis. Annu Rev Entomol. 2015 Jan 7;60:561-80

Affects ***Equus* spp.**

Acute, subacute or chronic disease **characterised by erythrolysis**

Occasionally fatal, in particular in acute *T.equi* infections.

Signs are fever, **progressive anaemia**, **icterus**, **haemoglobinuria (in advanced stages)** in which case prognosis is poor.

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- **Clinical diagnosis uncertain–** non-specific clinical signs?

- Equivalence of **serological and parassitological status?**

Constraints of EP diagnosis

- **Reliable definition of sanitary status** for therapeutic and regulatory requirements?

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For **international movement**, OIE prescribes the **sole use** of the **serological tests**

Reliability of serological assays is restricted by **antibody detection limits** and **cross-reactivity** (Bruning et al., 1997)

Table 1. *Test methods available for the diagnosis of equine piroplasmosis and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
Microscopic examination	–	+	–	++	+	n/a
PCR	+++	+++	+++	+++	+++	n/a
Detection of immune response[2]						
IFAT	++	++	++	+++	++	n/a
C-ELISA	+++	+++	+++	+++	+++	n/a
CFT	+	+	+	+	+	n/a

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable.
Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
PCR = polymerase chain reaction; IFAT = indirect fluorescent antibody test;
C-ELISA = competitive enzyme-linked immunosorbent assay; CFT = complement fixation test.

OIE Terrestrial Manual 2014 Chapter 2.5.8. Equine Piroplasmosis

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Is serological diagnosis for EP sufficient to assess the sanitary status of an equid ?

Aim of the present study

Combination of molecular and serological methods to evaluate the efficacy of the diagnosis of EP

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PCR protocols considered

Choice based on highest **sensitivity** (LOD & n° of positves) & **specificty** (verified by sequencing)

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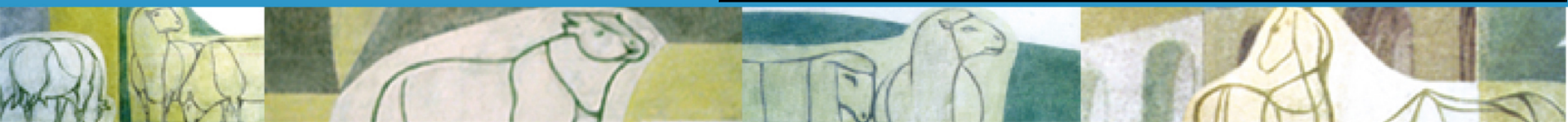

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Study set-up

Study population: 274 (whole and EDTA) blood samples from horses of riding stables from Central Italy (endemic area) and classified as:

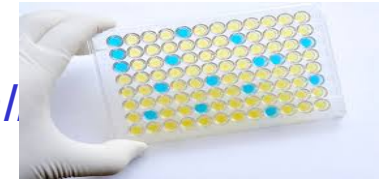
suspect cases (n=79)

non-suspects (n=195)

Definition of suspect case: t >38°C (100.4°F) a
at least one of the following signs: **jaundice**, **ana**
or **petecchial haemorrages**

Serological methods employed

C-ELISA: *B. caballi/B. equi* Antibody Test Kit C-ELISA VMRD®, USA. Recombinant antigens employed derived from:
EMA 1 gene for *T. equi*
RAP 1 gene for *B. caball*
(data kindly provided by VMRD).



IFAT: slides spotted with *B. caballi* or **T. equi** infected erythrocytes & IgG Control Set Fuller Laboratories®, USA. Presence of IgG in sample tested at 1/80 dil. detected by FITC-labeled ar
molecule)

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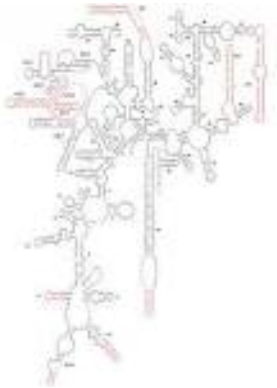
qRT-PCRs based on **Small subunit (18S) ribosomal rRNA genes**



For ***B. Caballi*** Bhoora et al, 2010
95 bp DNA fragment in the **V4 hypervariable region of the 18S rRNA** gene – **only forward primer** and probe are **B. caballi specific**:

Forward: 5' -GTA ATT GGA ATG ATG GCG ACT TAA-3';
Reverse: 5' -CGC TAT TGG AGC TGG AAT TAC C-3 ';
Probe: 5'-6-**FAM**-CCT CGC CAG AGT AA-**MGB**™ 3'

Robust and quantitative assay, **a TaqMan MGB™ probe** used in a **"conserved target sequence"** of the V4 hypervariable region of the B. caballi 18S rRNA gene.

For ***H. Equi*** Nunn et al, 2008

81bp DNA fragment of the 18S rRNA gene outside the V4 hypervariable region:

Forward: 5'-GCG GTG TTT CGG TGA TTC ATA-*3' ;*
Reverse: 5'-TGA TAG GTC AGA AAC TTG AAT GAT ACA TC-*3';*
Probe: 5'-**VIC**-AAA TTA GCG AAT CGC ATG GCT T-3'

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qRT-PCR protocol

DNA extraction **– *from 200µl EDTA blood*** employing QIAamp cador Pathogen Mini kit using automated robotic workstation QIAcube HT (Qiagen, GmbH, Hilden, Germany) and DNA was eluted in 150 µof buffer and stored at –20°C.

PCR mixture composition **– *total vol of 25µl*** for both Real Time PCRs, TaqMan® Universal PCR Master Mix kit (Applied Biosystems, USA) was used with the following -:

12.5µl of TaqMan® 2X Universal PCR Master Mix (A. Biosystems),
900 nM of each primer
250nM of each probe
5µl of DNA template (100ng)

Thermal profiles using ABIPRISM 7900 HT Sequence Detection System - Applied Biosystems
50°C for 2 min,
95°C for 10 min
50 cycles of 95°C for 20 sec
60°C for 1 min (*B. caballi*)/55°C for 1 min (*T. equi*).

Pos ctrls obtained from plasmid vectors pCRII®-TOPO TA Cloning® Invitrogen, Carlsbad, CA, USA) in which *T. equi* and *B. caballi* PCR targets were cloned.

Threshold: 40 Ct

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Outcome of serological tests

	B. CABALLI (274)	
	PCR + (14)	**PCR- (260)**
ELISA+	0	0
ELISA -	14	260
IFAT+	7	32
IFAT-	7	228

	T. EQUI (274)	
	PCR + (137)	**PCR- (137)**
ELISA+	92	23
ELISA -	45	114
IFAT+	118	26
IFAT-	19	111

Sensitivity (Se) and **specifity** (Sp) of serological assays for *B. caballi* and *T. equi* relative to PCR

		B. CABALLI	***T. EQUI***
ELISA	Se (%)	0	67.2
	Sp (%)	100	83.2
IFAT	Se (%)	50	86.1
	Sp (%)	87.7	81

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Agreement between methods

	B.caballi		
Agreement	PCR	ELISA	IFAT
PCR		0,95	0,86
ELISA	0,95		0,86
IFAT	0,86	0,86	

	T. equi		
Agreement	PCR	ELISA	IFAT
PCR		0,75	0,84
ELISA	0,75		0,86
IFAT	0,84	0,86	

	ELISA +	**IFAT +**	**PCR +**	**+ to at least 1 assay**
Proportion of + for B. caballi (%)	0	14,2	5,1	**16,8**
Proportion of + for T.equi %	42	52,6	50	**59,5**

Proportion of test positive samples detected using a **single or combined diagnostic system**.

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	B. CABALLI PCR + (14)	T. EQUI PCR + (137)
SUSPECT	4	27
NON-SUSPECT	10	110
Proportion of suspects (%)	**28.6**	**19.7**

	B. CABALLI IFAT + (39)	T. EQUI IFAT + (144)
SUSPECT	6	28
NON-SUSPECT	33	116
Proportion of suspects (%)	**15.4**	**19.4**

	B. CABALLI ELISA + (0)	T. EQUI ELISA + (115)
SUSPECT	/	14
NON-SUSPECT	/	101
Proportion of suspects (%)	**/**	**12.2**

Agreement of + results of 3 tests with proportion of suspects

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What about the discordant results?

B. caballi infections occur at very low parasitaemias rarely exceeding 1% (Hanafusa et al., 1998)

T. equi infections are not self-limiting and once infected, horses remain life-long carriers of the parasite (Bhoora et al., 2010)

Sero pos/qPCR neg

• antibody titres remain at detectable levels after the parasite has been cleared spontaneusly or by treatment for *B. caballi* and/or below the detection limit of the assay

• possible presence of sequence variation, in the region of the 18S rRNA gene where primers and probes were designed (Bhoora et al., 2009).

Sero pos/qPCR pos

• earliest stages of infections prior to antibody production (Bhoora et al., 2010).

Use the best tools in the worst situations!!!!!!!

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What considerations?

Incorporation of **sensitive/specific molecular methods** increase **diagnostic capibilities for EP** especially in case symptoms are non-specific/absent

Accurate definition of EP **carrier status** is essential for **effective control measures**, including animal movements.

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Acknowledgements