

# Caratterizzazione molecolare di stipiti di *Theileria equi* e *Babesia caballi* circolanti nel centro Italia (LT 11/14 RC)

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# PIROPLASMOSI EQUINA

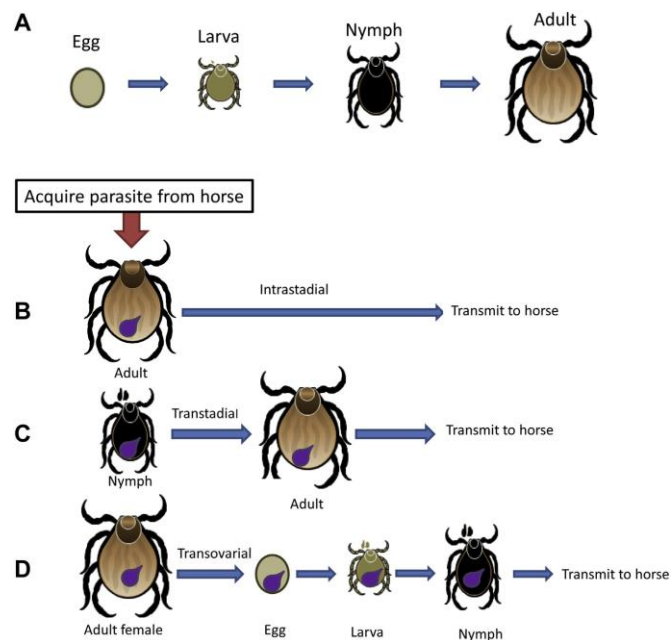
- *Babesia caballi* and *Theileria equi* (ex *B.equi*)
- Presente sulla lista dell'OIE
- Equidae



# PIROPLASMOSI EQUINA

- Trasmessa da zecche:

*Dermacentor, Rhipicephalus e Hyalomma*

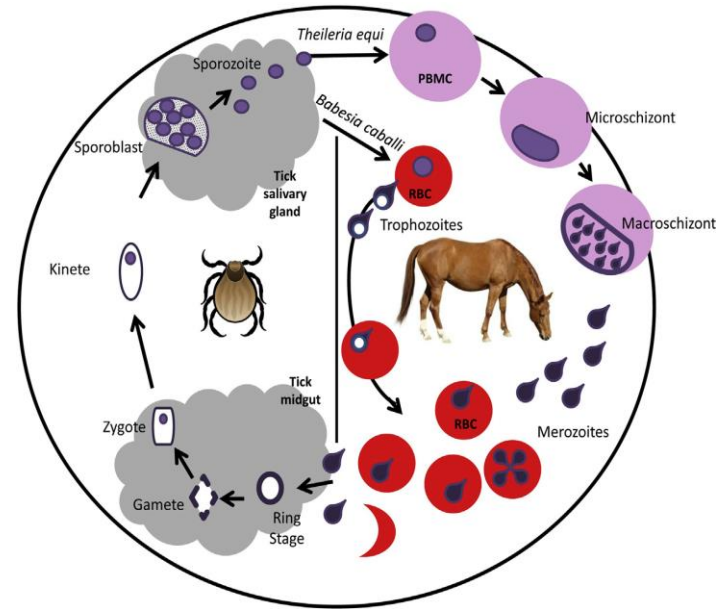
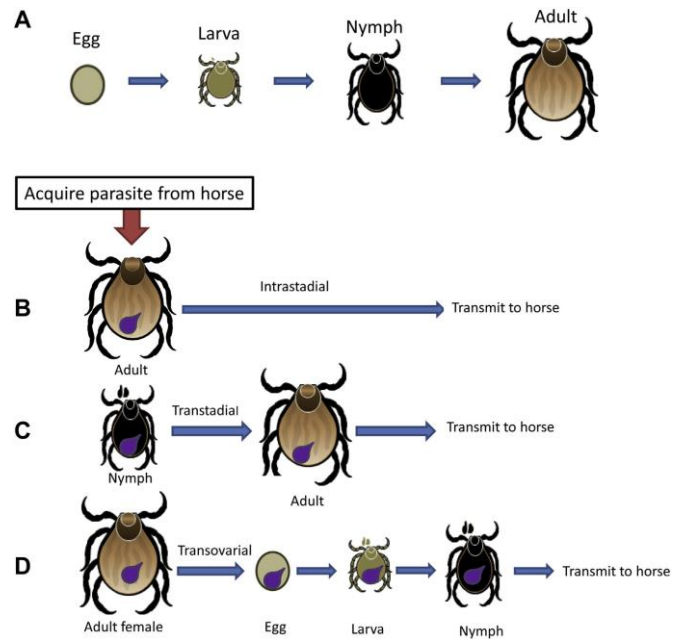




# PIROPLASMOSI EQUINA

- Trasmessa da zecche:

*Dermacentor, Rhipicephalus e Hyalomma*



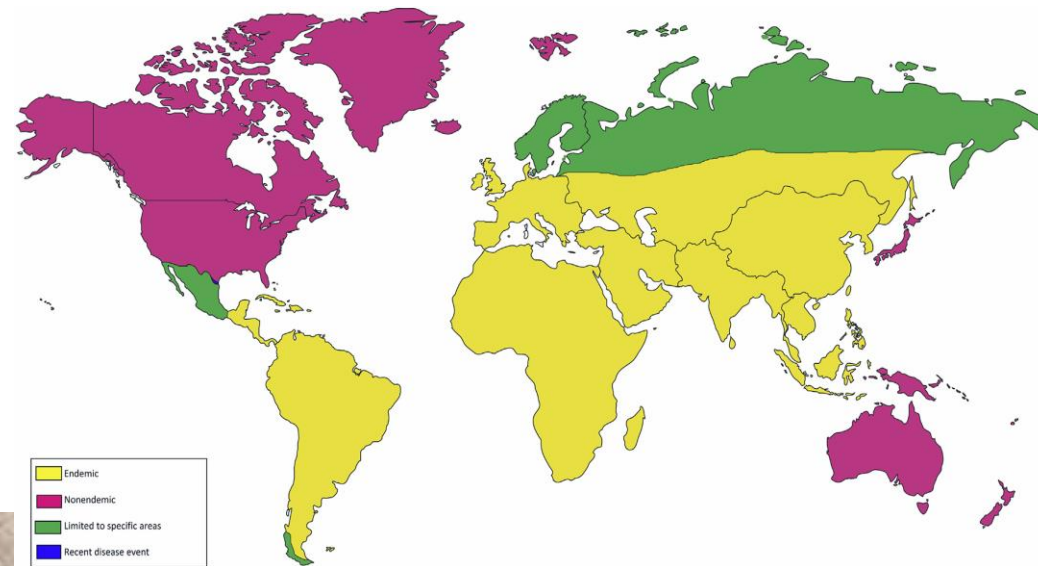
# PIROPLASMOSI EQUINA

- Iperacuta, acuta, subacuta o cronica
- Febbre, anemia e ittero. Segni aspecifici, calo rendimento



# PIROPLASMOSI EQUINA

- Distribuzione mondiale
- Importanti perdite economiche

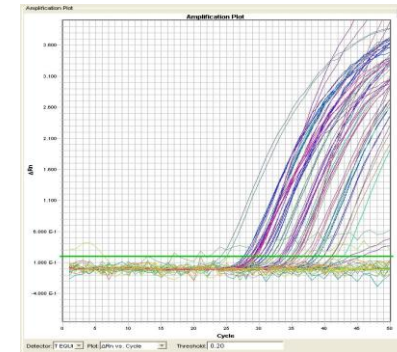
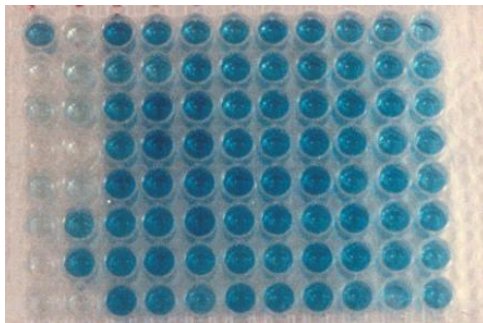




# STUDIO EPIDEMIOLOGICO PRECEDENTE (LT 16/11 RC)

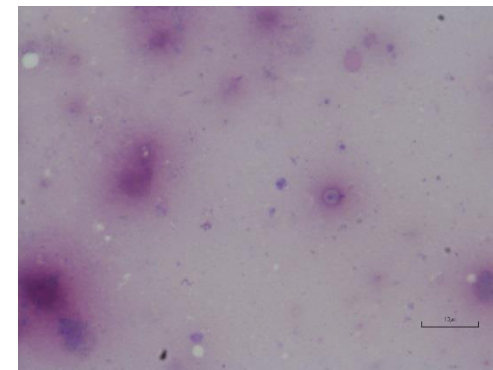
- Stima delle prevalenze delle infezioni da *Babesia caballi*, *Theileria equi* ed *Anaplasma phagocytophilum*
- Analisi e messa a punto di metodi diagnostici quantitativi

## EVIDENZIATE DELLE DISCORDANZE TRA GLI ESITI DEI TEST SIEROLOGICI E BIOMOLECOLARI



## OBIETTIVI (LT 11/14 RC)

- Determinare la presenza e caratterizzazione dei diversi stipiti circolanti.
- Distribuzione nel territorio.
- Evidenziare il grado di patogenicità.
- Relazione tra varianti e difficoltà diagnostica.
- Investigare le relazioni tassonomiche.





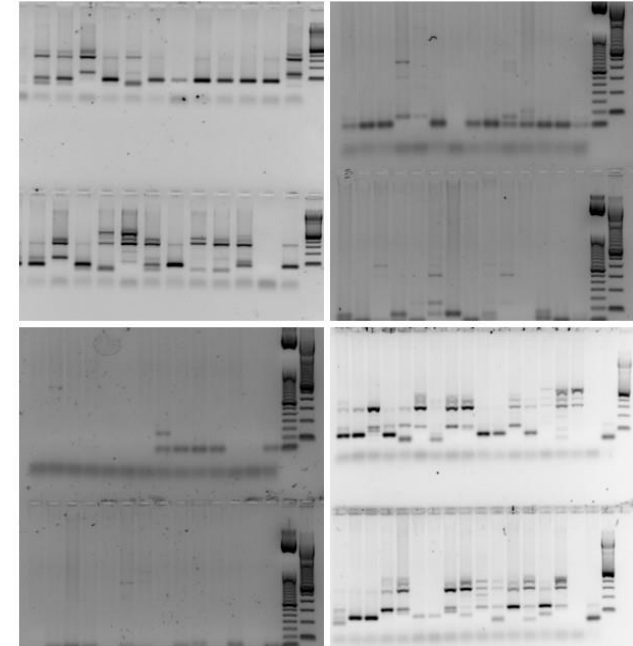
# TIPIZZAZIONE DEGLI STIPITI

Target ideale:

- Regione conservata con aree variabili

Permette:

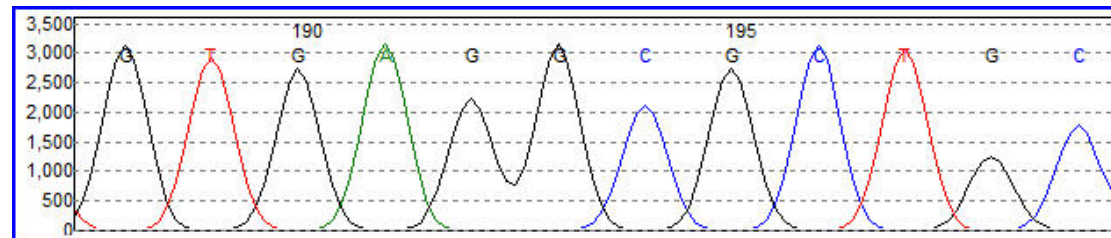
- Allineamenti inequivoci
- Discriminazione filogenetica



# TIPIZZAZIONE DEGLI STIPITI

Target più usati

- ★ • 18S rRNA - V4 >> sequenziamento per determinare specie diverse
- Gene antigene del merozoita (EMA-1) di *T. equi*
- Gene della proteina associate al rhoptry 1 (RAP-1) di *B.caballi*



## MATERIALI E METODI

- 100 campioni da equidi sintomatici e asintomatici  
Real Time Ct<24. Serologia discordante (ELISA)
- 26 campioni di DNA da zecche
- PCR (V4,EMA)
- Sequenziamenti e analisi  
Omologia di sequenza effettuata con BLAST  
Sequenze assemblate e allineate (Geneious)





# MATERIALI E METODI

- 100 SEQUENZE

- Cavalli sintomatici: 35
- Cavalli asintomatici: 42
- Asini sintomatici: 2
- Asini asintomatici: 21



\*ELISA discordante

(*Babesia* 6/9 e *Theileria* 32/92)

\*Real Time 18S (*Babesia* e *Theileria*)  
Ct<24

- V4: Ipervariabile 18 S (diverse specie)
- EMA (*Theileria*)
- Sequenziamento
- Analisi sequenze



# MATERIALI E METODI

## ZECCHIE – Monti Aurunci

- *Dermacentor marginatus*

V4 ibridazione del genoma della zecca

15/26 Positive a *T. equi* (PCR EMA)

Nessuna a *B. caballi* (PCR RAP)

- Sequenziamento
- Analisi sequenze

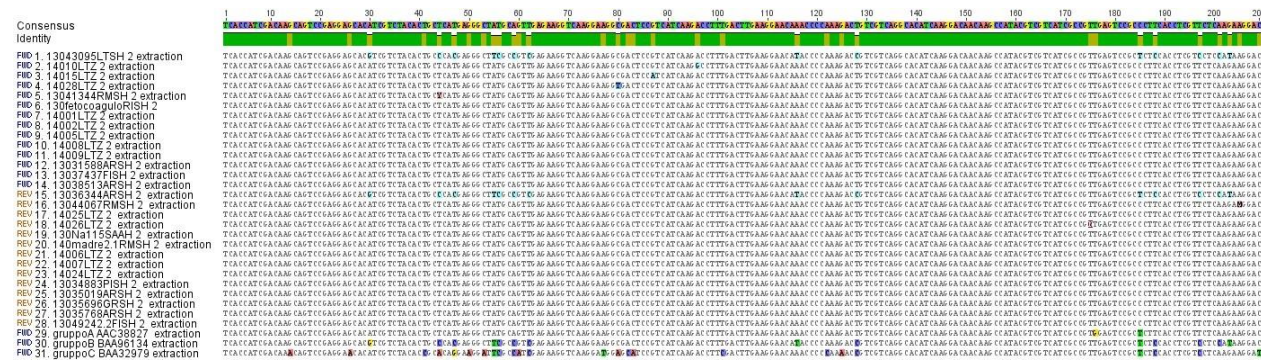




# MATERIALI E METODI

- Sequenziamenti e analisi

Omologia di sequenza effettuata con BLAST  
Sequenze assemblate e allineate con Geneious





# MATERIALI E METODI

- Relazioni statistiche tra gruppi V4 e:  
coinfezione  
patogenicità  
discordanze sierologiche  
risultato debole o negativo a PCR EMA



# RISULTATI

- In V4: 94 *Theileria*, 9 *Babesia*
- 15/26 zecche positive a EMA-PCR
- 49/91 equidi infettati con *Theileria* positivi a EMA-PCR (42 discordanti)
- Sequenze ottenute omologia e query coverage >98%



# RISULTATI

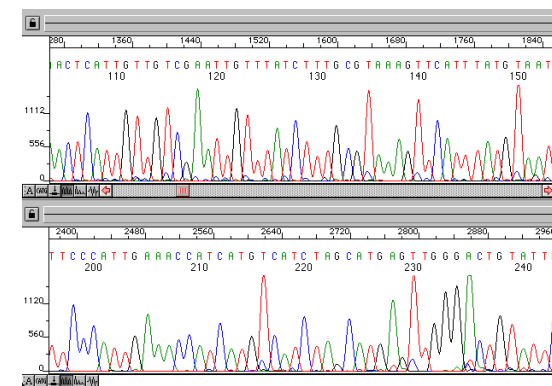
- Insieme alle sequenze dei gruppi ipotizzati da Bhoora *et al.* (2010)

*B.caballi*  
V4 { Z15104  
AY534883  
EU642514

*T.equi*  
V4 { Z15105  
AY534882  
AB515315

*T.equi*  
EMA { AAC38827  
BAA96134  
BAA32979

- 3 gruppi/3 per ogni parassita con V4
- 2 gruppi/3 *T. equi* EMA PCR (zecche e cavalli)
- No relazione netta tra i gruppi V4 e EMA





# Sequenze registrate su GenBank per *B.caballi* V4 (6/9)

- **Gruppo 1: Identità media 100%**  
KU923669, KU923668, KU923667
- **Gruppo 2: Identità media 99,7%**  
KU923664
- **Gruppo 3: Identità media 99,8%**  
KU923665, KU923666

Consensus	1	10	20	30	40	50	60
	GATGGCGACT	TAAACCCCTCG	CCAGAGTAAC	AATTGGAGGG	CAAGTCTGGT	GCCAGCAGCC	
1. Group 3 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
2. Group 2 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
3. Group 1 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
Consensus	70	80	90	100	110	120	
	GCGGTAATTG	CAGCTCCAAT	AGCGTATATT	AACTTGTTG	CAGTTAAAAA	GCTCGTAGTT	
1. Group 3 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
2. Group 2 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
3. Group 1 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
Consensus	130	140	150	160	170	180	
	GAATTTCTGC	GTTGCGTTT	TCTTGCTTT	TGCTTGATT	TCGCTTCGCT	TTTBTTTT	
1. Group 3 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
2. Group 2 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
3. Group 1 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
Consensus	190	200	210	220	230	240	
	ACTTTGAGAA	AATTAGAGTG	TTTATCGCAG	ACTTTTGCT	TGAATACTTC	AGCATGGAAT	
1. Group 3 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
2. Group 2 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
3. Group 1 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
Consensus	250	260	270	280	290	300	
	AATAGAGTAG	GACCTTGGTT	CTATTTTGTT	GGTTTGGAN	CCTTGGTAAT	GGTTAATAG	
1. Group 3 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
2. Group 2 alignment consensus sequence	.....	.....	.....	.....	.....	.....	
3. Group 1 alignment consensus sequence	.....	.....	.....	.....	.....	.....	



# Sequenze registrate su GenBank per *B.caballi* V4 (6/9)

299 bp

Consensus

1. Group 3 alignment consensus sequence
2. Group 2 alignment consensus sequence
3. Group 1 alignment consensus sequence

Consensus

1. Group 3 alignment consensus sequence
2. Group 2 alignment consensus sequence
3. Group 1 alignment consensus sequence

Consensus

1. Group 3 alignment consensus sequence
2. Group 2 alignment consensus sequence
3. Group 1 alignment consensus sequence

Consensus

1. Group 3 alignment consensus sequence
2. Group 2 alignment consensus sequence
3. Group 1 alignment consensus sequence

Consensus

1. Group 3 alignment consensus sequence
2. Group 2 alignment consensus sequence
3. Group 1 alignment consensus sequence

1 GATGGCGACT	10 TAAACCCCTCG	20 CCAGAGTAAC	30 AATTGGAGGG	40 CAAGTCTGGT	50 GCCAGCAGCC
60 GCGGTAATTC	70 CAGCTCCAAT	80 AGCGTATATT	90 AAACTTGTTG	100 CAGTTAAAAA	110 GCTCGTAGTT
120 GAATTTCTGC	130 GTTGCGTTTT	140 TCTTGCTTTT	150 TGCTTGATTT	160 TCGCTTCGCT	170 TTTTBTTTTT
180 ACTTTGAGAA	190 AATTAGAGTG	200 TTTATCGCAG	210 ACTTTTGTCT	220 TGAATACTTC	230 AGCATGGAAT
240 AATAGAGTAG	250 GACCTTGGTT	260 CTATTTTGTT	270 GGTTTTGGAN	280 CCTTGGTAAT	290 GGTTAATAG
300 ..C.T..	310 ..T..	320 ..	330 ..T..G	340 ..A	350 ..
360 ..	370 ..	380 ..	390 ..G..-	400 ..	410 ..



# Sequenze registrate su GenBank per *T. equi* V4 (49/94)

- **Gruppo 1: Identità media 99,8%**

KU923625, KU923627, KU923638, KU923639, KU923640, KU923641, KU923642, KU923643, KU923644, KU923645, KU923646, KU923647, KU923648, KU923649, KU923650, KU923651, KU923652, KU923653, KU923654, KU923655.

- **Gruppo 2: Identità media 99,5%**

KU923615, KU923616, KU923617, KU923618, KU923619, KU923620, KU923621, KU923622, KU923623, KU923624, KU923626, KU923628, KU923656, KU923657, KU923658, KU923659, KU923660, KU923661, KU923662, KU923663.

- **Gruppo 3: Identità media 99,1%**

KU923629, KU923630, KU923631, KU923632, KU923633, KU923634, KU923635, KU923636, KU923637.

```

Consensus  AAGAATTTC CCTCTGACA TCAATACGA ATGCCCCCA
1. consensus "group 2"
2. consensus "group 2"
3. consensus "group 1"

Consensus  CTGTTCCCTA TAACCATTAC TCGGGCTCCT AAAACCAACA
1. consensus "group 2"
2. consensus "group 2"
3. consensus "group 1"

Consensus  AAATAGAACC AAAGTCCTAC TCCATTATTC CATGCTAAAG
1. consensus "group 2"
2. consensus "group 2"
3. consensus "group 1"

Consensus  TATTC AAGG AAAAGCCTGC TTGAAGCAC CTAATTTTC
1. consensus "group 2"
2. consensus "group 2"
3. consensus "group 1"

Consensus  CAAAGTAAAC GTGCGTMAV GAAAHWTGN AHCCCAACTA
1. consensus "group 2"
2. consensus "group 2"
3. consensus "group 1"

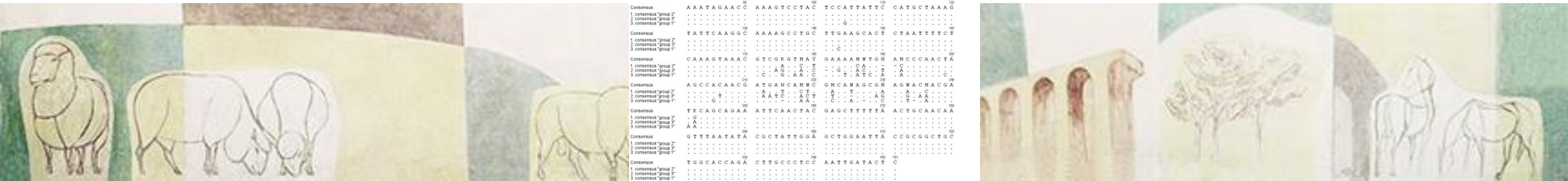
Consensus  AGCCACAACG ATGANCAMWC GRCAWAGCGR AGWACHACGA
1. consensus "group 2"
2. consensus "group 2"
3. consensus "group 1"

Consensus  TFCAGCAGAA ATTCAACTAC GAGCTTTTTT ACTGCAACAA
1. consensus "group 2"
2. consensus "group 2"
3. consensus "group 1"

Consensus  GTTAAATATA CGCTATTGGA GCTGGAATTA CCGGGGCTGC
1. consensus "group 2"
2. consensus "group 2"
3. consensus "group 1"

Consensus  TGGCACCAGA CTGGCCCTCC AATTGATACT C
1. consensus "group 2"
2. consensus "group 2"
3. consensus "group 1"

```





# Sequenze registrate su GenBank per *T. equi* V4 (49/94)

351 bp

Consensus	A A G A A T T T C A	C C T C T G A C A G	T C A A A T A C G A	A T G C C C C C A A
1. consensus "group 2"	.	.	.	.
2. consensus "group 3"	.	.	.	.
3. consensus "group 1"	.	.	.	.
Consensus	C T G T T C C T A T	T A A C C A T T A C	T C Y G G C T C C T	A A A A C C A A C A
1. consensus "group 2"	.	.	.	.
2. consensus "group 3"	.	.	.	.
3. consensus "group 1"	.	.	.	.
Consensus	A A A T A G A A C C	A A A G T C C T A C	T C C A T T A T T C	C A T G C T A A A G
1. consensus "group 2"	.	.	.	.
2. consensus "group 3"	.	.	.	.
3. consensus "group 1"	.	.	.	.
Consensus	T A T T C A A G G C	A A A A G C C T G C	T T G A A G C A C T	C T A A T T T T C T
1. consensus "group 2"	.	.	.	.
2. consensus "group 3"	.	.	.	.
3. consensus "group 1"	.	.	.	.
Consensus	C A A A G T A A A C	G T C G R G T M A Y	G A A A A M W T G N	A M C C C A A C T A
1. consensus "group 2"	.	.	.	.
2. consensus "group 3"	.	.	.	.
3. consensus "group 1"	.	.	.	.
Consensus	A G C C A C A A C G	A T G A N C A M W C	G M C A W A G C G M	A G W A C M A C G A
1. consensus "group 2"	.	.	.	.
2. consensus "group 3"	.	.	.	.
3. consensus "group 1"	.	.	.	.
Consensus	T R C A G C A G A A	A T T C A A C T A C	G A G C T T T T T A	A C T G C A A C A A
1. consensus "group 2"	.	.	.	.
2. consensus "group 3"	.	.	.	.
3. consensus "group 1"	.	.	.	.
Consensus	G T T T A A T A T A	C G C T A T T G G A	G C T G G A A T T A	C C G C G G C T G C
1. consensus "group 2"	.	.	.	.
2. consensus "group 3"	.	.	.	.
3. consensus "group 1"	.	.	.	.
Consensus	T G G C A C C A G A	C T T G C C C T C C	A A T T G A T A C T	C
1. consensus "group 2"	.	.	.	.
2. consensus "group 3"	.	.	.	.
3. consensus "group 1"	.	.	.	.



1 10 20 30 40 50 60 69  
TIDKQSEEHIVYTAHEGFAVEKVKEGDSVIKTFDLKEQTPKTVVRHIKDNKPYVVI AVE\$ALHLVLKKD  
.....Y.....  
.....V.....H.....H.....  
.....Q.....I.....D.A.....



69 aa

	1	10	20	30	40	50	60	69
Consensus	TIDKQSEEHIVYTAHEGFAVEKVKEGDSVIKTFDLKEQTPKTVVRHIKDÑKPYYVVI AVEŠALHLVLKKD							
1. Group 1	.	.	.	.	.	.	.	.
2. Group 2	.	V.	.	.	.	H.	.	H.
3.BAA32979	.	.	Q.	I.	D.A.	.	.	.



## ANALISI STATISTICA V4 ( $p < 0,05$ )

<b>V4 <i>Theileria equi</i></b>					
Variabili	Categorie	Gruppo 1	Gruppo 2	Gruppo 3	p
Sintomi N=94	Asintomatici	11	36	13	<u>&lt;0,0001</u>
	Sintomatici	30	2	2	
ELISA N=92	Negativo	24	5	3	<u>&lt;0,0001</u>
	Positivo	16	33	11	
PCR EMA N=91	Debole/negativo	10	23	9	<u>&lt;0,05</u>
	Positivo	28	15	6	
Coinfezione N=94	No	33	26	13	0,2710
	Si	8	12	2	





## ANALISI STATISTICA V4 ( $p < 0,05$ )

<b>V4 <i>Babesia caballi</i></b>					
Variabili	Categorie	Gruppo 1	Gruppo 2	Gruppo 3	p
Sintomi N=9	Asintomatici	3	2	1	1,000
	Sintomatici	2	0	1	
ELISA N=9	Negativo	4	0	2	0,1667
	Positivo	1	2	0	
Coinfezione N=9	No	1	1	1	1,000
	Si	4	1	1	



## V4 *Theileria equi* e *Babesia caballi*

G1

41 campioni

40 cavalli

1 asino

Discordanti ELISA

Animali sintomatici

G2

38 campioni

26 cavalli

12 asini

Discordanti EMA PCR

Animali asintomatici

G3

15 campioni

5 cavalli

10 asini

Discordanti EMA PCR

Animali asintomatici

G1

5 sequenze

cavalli

G2

2 sequenze

cavalli

G3

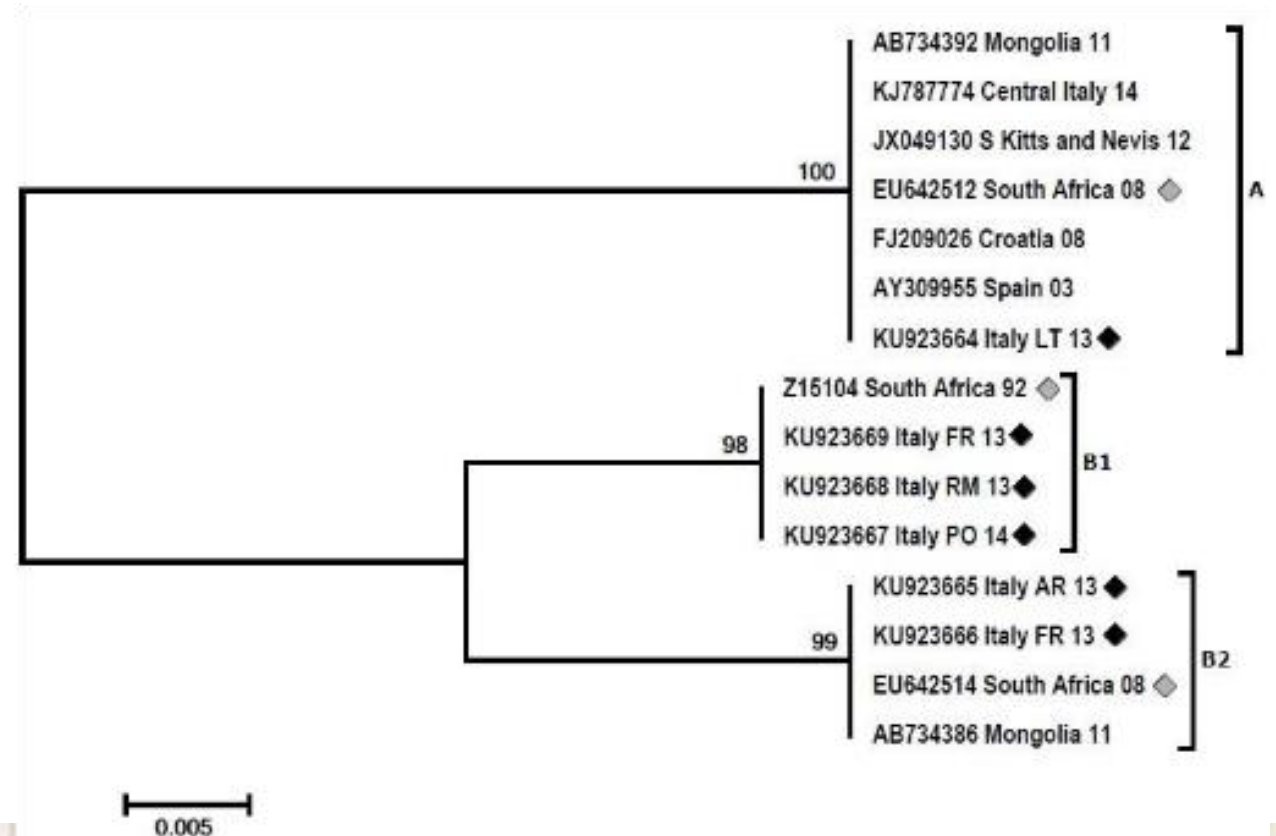
2 sequenze

cavalli



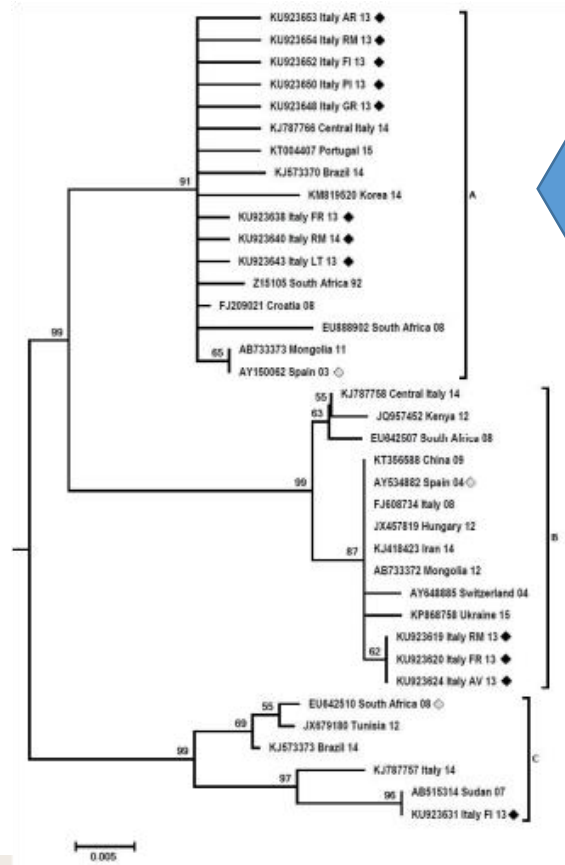
# Albero filogenetico della regione ipervariabile RNA 18S di *B.caballi*

- 15 sequenze
  - 3 da Bhoora *et al.*, (2010)
  - 6 da GenBank
  - 6 ottenute dallo studio
- 3 GRUPPI (A, B1, B2)





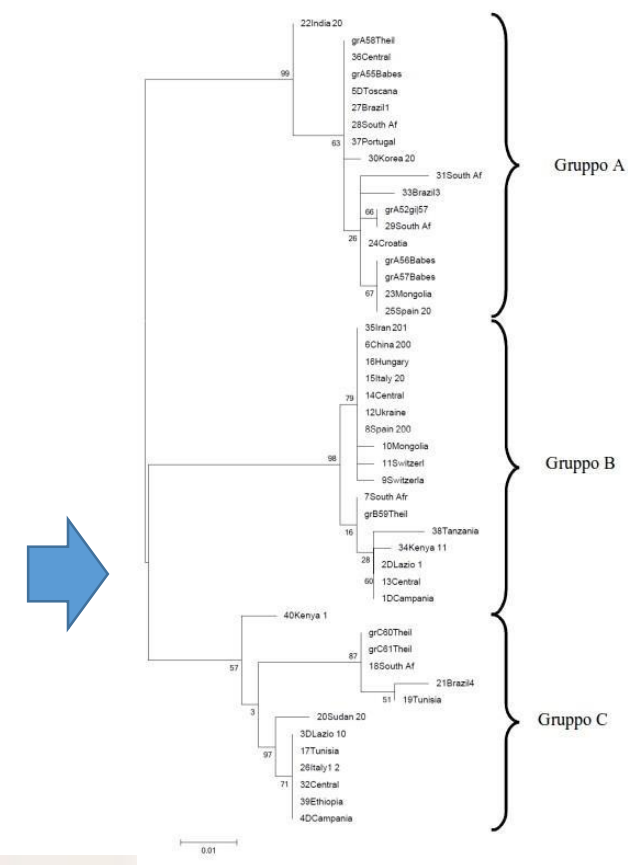
# Albero filogenetico della regione ipervariabile RNA 18S di *T. equi*



- Cavalli (37 sequenze):  
3 da Bhoora *et al.*, (2010)  
22 da GenBank  
12 ottenute dallo studio

- Asini (49 sequenze):  
44 sequenze da GenBank  
5 dal presente studio

3 GRUPPI (A, B, C)



- 22 sequenze:
  - 3 da Bhoora *et al.*, (2010)
  - 11 da GenBank
  - 8 ottenute dallo studio.

Phylogenetic tree of H5N1 virus sequences. The tree shows relationships between various sequences, with bootstrap values at nodes. Sequences are grouped into clusters A, B, C, and D. Cluster A includes sequences from USA, Brazil, Italy, India, South Africa, Japan, and Morocco. Cluster B includes sequences from Brazil, USA, South Africa, and Italy. Cluster C includes sequences from Japan and South Africa. Cluster D includes sequences from Mongolia. A scale bar of 0.02 is shown at the bottom.

Sequences and their locations:

- L13784 USA 93
- U97167 Brazil 97
- KU923612 Italy GR 13
- KU923610 Italy PI 13
- KU923605 Italy SA 13
- KU923600 Italy AR 13
- KU923599 Italy FI 13
- KU923591 Italy RM 13
- KC347576 India 12
- JQ782603 South Africa 10
- AB015211 Japan 98
- U97168 Morocco 97
- AB731193 Mongolia 11
- AF255730 Brazil 00
- AB015235 USA 98
- JQ782604 South Africa 10
- KU923587 Italy LT 13
- KU923601 Italy AR 13
- AB015208 Japan 98
- JQ782607 South Africa 10
- AB713965 Mongolia 11
- AB731194 Mongolia 12

Scale bar: 0.02



# DISCUSSIONE E CONCLUSIONI

- Tre genotipi per ogni parassita (V4)
  - = Bhoora *et al.*, 2009-2010 in Sudafrica
  - 4 gruppi: Salim *et al.*, 2010 in Sudan; Veronesi *et al.*, 2014 in Italia
  - 5 gruppi: Qablan *et al.*, 2013 in Giordania
- Differenze nella patogenicità (V4 *Theileria* Gruppo 1 vs 2 e 3)
- Indipendenti dall'ospite (zecca, asino, cavallo)
- Indipendenti dall'area geografica
  - \*movimentazioni animali
  - \*divergenza genetica parallela
  - \*diversi stadi di infezione





## DISCUSSIONE E CONCLUSIONI

- Relazione tra patogenicità e discordanze in ELISA e PCR EMA (=Hall *et al.*, 2013)
  - ELISA: \*periodo finestra
    - \*sensibilità (test su 1 epitope -> antigene merozoita variabile)
  - EMA: \*sensibilità
    - \*cambiamenti nell'espressione del gene
- Ulteriori indagini sulla biologia molecolare dei parassiti e metodi diagnostici



# RACCOMANDAZIONI

- Considerare la piroplasmosi anche in caso di sintomatologia lieve.
- Abbinare test sierologici e molecolari.
- Adeguare i test diagnostici alla situazione (asintomatico, esportazione).
- Introdurre i test sierologici e biomolecolari per piroplasmosi tra le analisi.
- Obbligo di effettuare analisi per piroplasmosi in caso di movimentazione internazionale.



# GRAZIE

