Development of a Reverse Line Blot for simultaneous detection of Tick-Borne Pathogens in equines

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INTRODUCTION

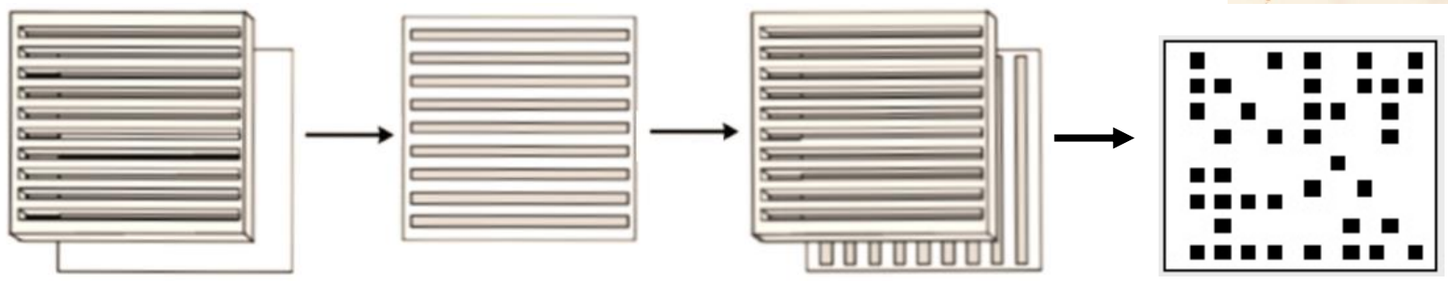
Babesia caballi, *Theileria equi*, *Anaplasma phagocytophilum* and *Rickettsia* spp. are the main etiologic agents of Tick Borne Diseases (TBDs) in equines. Due to the nonspecific symptoms of TBDs, several molecular assays, each specific for a different Tick Borne Pathogen (TBP), are required for the diagnosis. This work aimed at developing a single Reverse Line Blot (RLB) for simultaneous detection and identification of equine TBPs.

MATERIALS AND METHODS

PCRs with biotinylated primers on DNA from equine blood and ticks.

A new optimized multiplex PCR for *Anaplasma* spp. and *Rickettsia* spp. DNA amplification developed.

PCR products were diluted 1:4 in 2XSSPE / 0.1% SDS **(a)**, to a final volume of 150 µl, heated for 10 min at 99°C **(b)**, and immediately cooled on ice **(c)**. Denatured PCR samples were placed in the slots **(d)**. Excess of samples were aspirated **(e)**. Hyperfilm preparation **(f)** and development in the darkroom was carried out **(g)**.

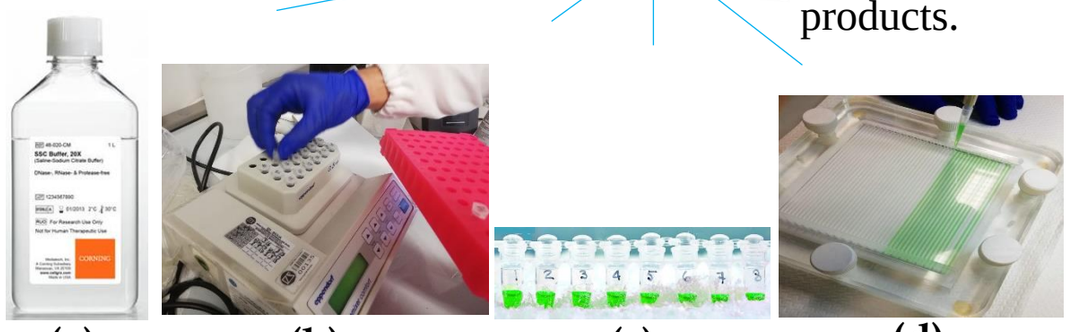


Probe immobilization in a nitrocellulose membrane by a miniblotter.

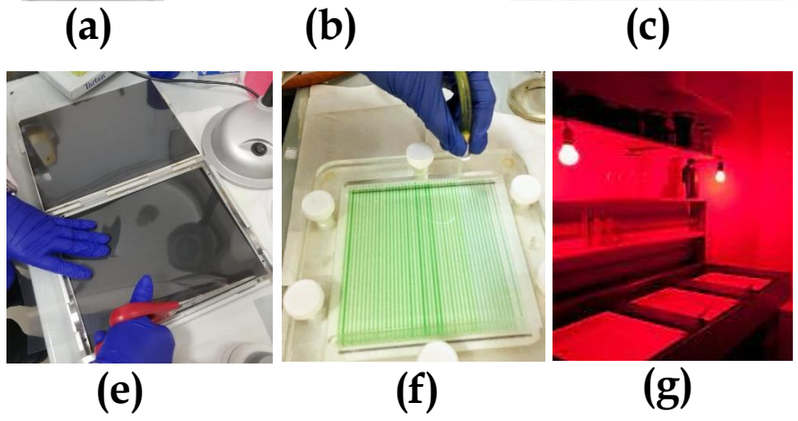
Probes are bound covalently to the membrane

90° membrane rotation and application of biotinylated PCR products.

Hyperfilm exposure and development.

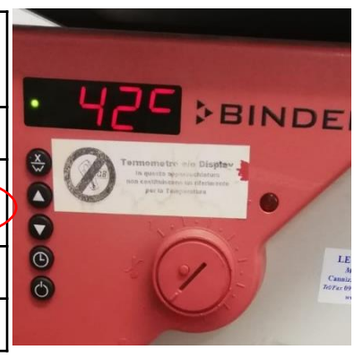


(a) (b) (c) (d)



(e) (f) (g)

INCUBATION TIMES
30 minutes
60 minutes
90 minutes
180 minutes



- Sensitivity was determined by 10-fold serial dilutions of positive samples.
- Specificity was tested with samples positive for other related pathogens.
- Simultaneous detection of DNA from different pathogens was verified with different ratios in a single reaction mixture, loaded into a single channel.

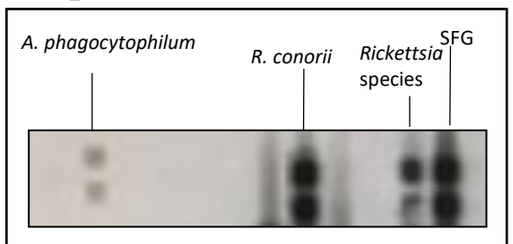
RESULTS AND CONCLUSIONS

The new RLB has been optimized with 15 different probes at two optimal concentrations:

PROBES	SEQUENCE	CONCENTRATION (pmol/150 µl)
T.equi	GTTTCGATTATTCGTTTCCCGG	1200;2400
T.equi-like	GGGGCATGTTTTCATGACTCGA	1200;2400
B.caballi	GTTGCGTTGTTCTTGCTTTTTGCTT	1200;2400
B.caballi-like	CGGGTTATTGACTTCGCTTTTTCTT	1200;2400
*Anaplasma*_HGE	GCTATAAAGAATAGTTAGTGG	1200;2400
Ehrlichia deer	GAATAATCTCTGTAGCTTGCT	1200;2400
R.svolaca	GTAGCCCCTGCCACGATA	400;1200
R.Conorii	GTTATATACTGTAGCCCTG	400;1200
R.aesch	ATATTATACTGTATGTAGCCCC	400;1200
R.helvetica	CATGGCTTGATCCACGGTA	400;1200
Eq.pirol_Catch all	TAATGGTTAATAGGARCRGTTG	1200; 2400
*A.phago*1	TTGCTATAAAGAATAATTAGTGG	1200;2400
*A.phago*2	TTGCTATRAAGAATARTTAGTGG	1200;2400
Rickettsia species	TAGCTCGATTGRTTTACTTTG	400;1200
Spotted Fever Group *Rickettsia* (SFG)	ACTCACAARGTTATCAGGT	400;1200

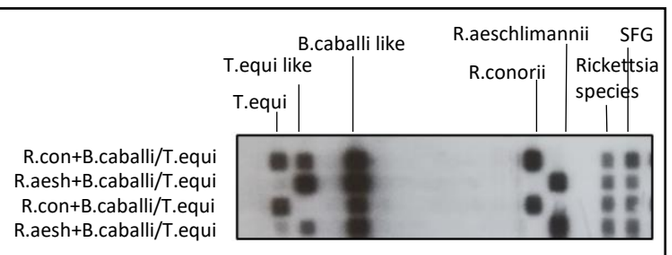
RLB sensitivity →

Sensitivity assays produced a positive signal up to 1:1000 dilution for *Babesia*, *Theileria* and *Rickettsia* probes, and up to 1:10 for *Anaplasma* probes for both the probe concentrations.



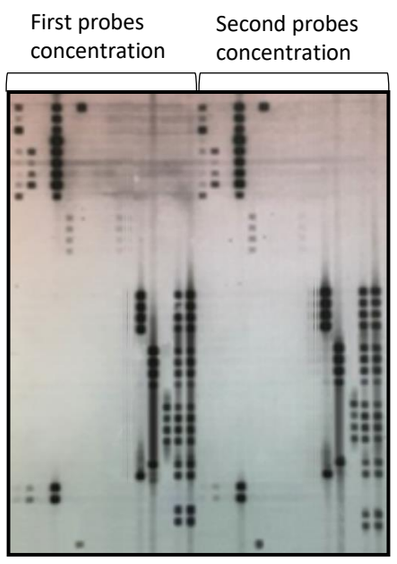
The new multiplex PCR for *Anaplasma* and *Rickettsia* species made the test faster.

Amplicon combination in a single tube obtained by the different PCRs allowed the detection of different TBPs in a unique channel.



25% of both samples positive and 50% 2XSSPE/0.1%SDS

	1200 pmol/150µl									400 pmol/150µl					
	T.equi	*T.equi like*	*B. caballi*	*B.caballi like*	*HGE*	*Catch all*	*Ehrlichia*	*A.phago1*	*A.phago2*	*R.slovaca*	*R.conorii*	*R.aesch*	*R.helv*	*Generic*	*SFG*
T.equi/B.caballi	•			•		•									
T.equi/B.caballi	•			•											
T.equi/B.caballi	•			•											
T.equi/B.caballi				•											
T.equi/B.caballi	•	•		•											
T.equi/B.caballi 1*	•	•		•											
T.equi/B.caballi	•	•		•											
T.equi/B.caballi 1:10	•	•		•											
T.equi/B.caballi 1:100	•	•		•											
T.equi/B.caballi 1:1000	•			•											
T.equi/B.caballi 1:10000															
A.phagocytophilum					•			•							
A.phagocytophilum					•			•							
A.phagocytophilum 1*					•			•							
A.phagocytophilum 1:10					•			•							
A.phagocytophilum 1:100															
A.phagocytophilum 1:1000															
A.phagocytophilum 1:10000															
R.conorii 1*											•			•	•
R.conorii 1:10											•			•	•
R.conorii 1:100											•			•	•
R.conorii 1:1000											•			•	•
R.conorii 1:10000															
R.aesclimannii 1*												•		•	•
R.aesclimannii 1:10												•		•	•
R.aesclimannii 1:100												•		•	•
R.aesclimannii 1:1000												•		•	•
R.aesclimannii 1:10000															
R. helvetica 1*													•	•	•
R. helvetica 1:10													•	•	•
R. helvetica 1:100													•	•	•
R. helvetica 1:1000													•	•	•
R. helvetica 1:10000															
R. aeschlimannii												•		•	•
R.conorii											•			•	•
T.equi/B.caballi	•	•		•											
T.equi/B.caballi	•	•		•											
R.monacensis														•	•
R.felis														•	•
A.marginale															
T.annulata						•									



RLB Specificity

The probes used reacted only in correspondence of the DNA complementary to them demonstrating the specificity of the test.

*Pathogen DNA (10-30 ng)

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