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CENTRO DI REFERENZA NAZIONALE CESME

Development of new tests to increase the performance of dourine's diagnosis

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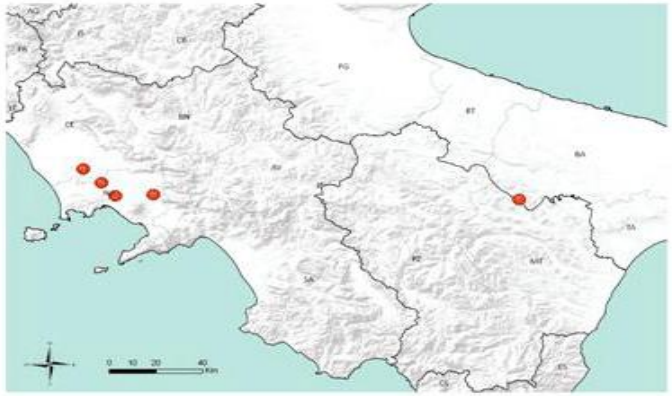
Italian epidemiological situation

- IZS Teramo is the National Reference Laboratory for Exotic Diseases
- The disease was first eradicated in Italy in the 1940s, but a serious epidemic reoccurred between the 1970s and 1980s. After sporadic reports at the end of the 1990s , there was a new outbreak in May 2011
- Before the 2011 outbreak, in Italy there was a surveillance plan to test equine for reproduction and about 3600 samples/year were tested by CFT for dourine. Using this test some doubt cases were routinely found



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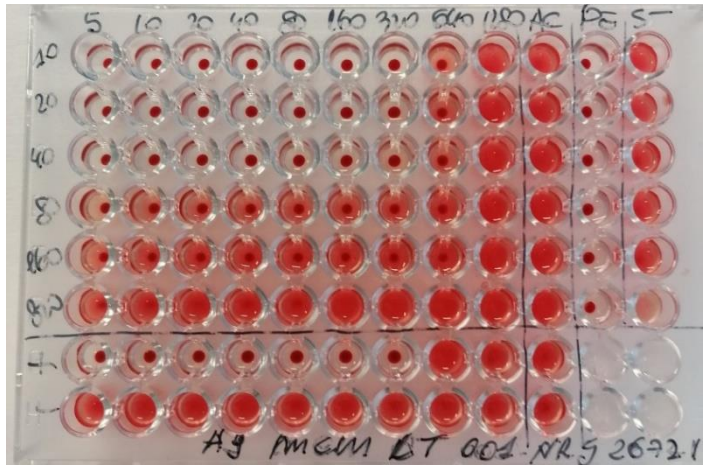
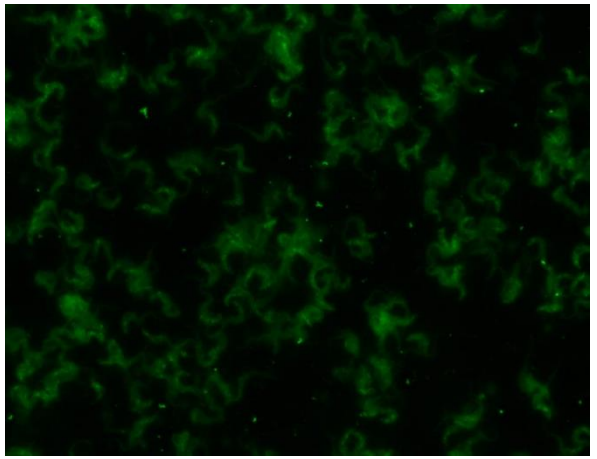
Serological tests

- Complement fixation test (CFT) is the official test for the diagnosis of Dourine and for import/export of animals. It is used to detect latent infections, also donkeys and mules, sometimes it gives inconsistent or nonspecific reactions because of the anti complementary effects of sera
- Indirect fluorescent antibody (IFAT) can be used in the case of anti complementary sera
- Cross-reactions are possible due to the presence in some countries of other trypanosomes
- Methodologies are described in the WOAH Terrestrial Manual





Disavantages of serological tests

- The major drawbacks of CFT are the need for careful continuous titration of several labile reagents and the anti-complement effect of sera frequently observed in uninfected animals, particularly donkeys and mules, but also reported in horses, which results in inconsistent or nonspecific reactions
- IFAT is frequently used as a confirmatory test for CFT results, but its interpretation is both labour intensive and operator-dependent, therefore it is preferable to use it to test a small number of sera
- In these conditions confirming positive serological cases or clarifying inconclusive or discrepant cases can be challenging

Improving studies

- Development of immunoblotting to increase the performances of serological test for dourine **(Objective 1)**
- Development of an animal model to study the pathogenesis of infection using the rabbit instead of horse and use of infected animals to evaluate humoral immune response and delayed –type hypersensitivity using an experimental "**tripanosomina**" **(Objective 2)**

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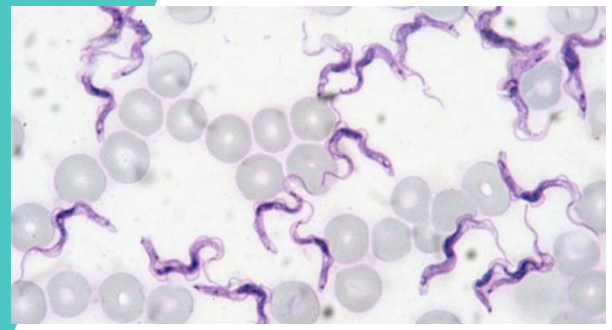
Objective 1

Trypanosoma antigen purification

T. equiperdum strain, provided by Onderstepoort Veterinary Institute, Pretoria, South Africa (OVI *T. equiperdum*) was used as antigen

A two-step protocol was optimised according to obtain a purified OVI T.e. antigen.

- Blood from rats infected intra peritoneally was collected in sodium citrate tubes
- The parasite concentration in whole blood was estimated 1.8×10^8 trypanosomes/ml
- Blood was mixed with an equal volume of Percoll density gradient solution containing sucrose and glucose at pH 7.4
- The suspension was separated in three different phases by centrifuging and Trypanosomes were recovered from the upper and intermediate phases
- The parasite suspension was purified using DEAE-cellulose chromatographic column
- The DEAE column retains rat blood leukocytes, while the motility of the trypanosomes facilitates quick elution of the parasite



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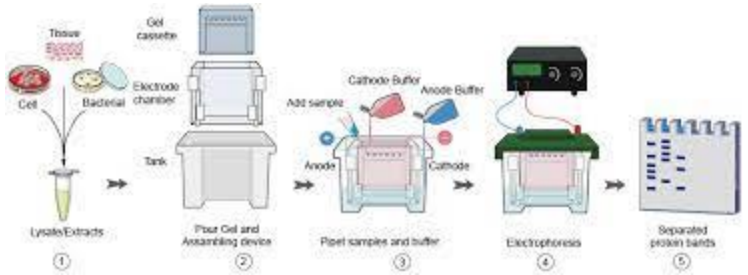
Objective 1
Immunoblotting

- Parasite concentration was calculated and adjusted to 1 × 10^8 Tryp/ml
- The purified OVI T.e. antigen was separated by SDS PAGE (12% Bis-Tris pre-cast gel) and gels were then transferred to a nitrocellulose membranes
- The membrane strips were saturated with PBS containing 0.05% Tween 20 (PBST) and 5% skimmed milk and incubated for 2 hours
- The test sera were diluted 1:10 in PBST containing 2.5% skimmed milk, added to the membrane and incubated over night
- The strips were incubated with a monoclonal antibody anti-horse IgG-HRP-conjugate home made for 1 our and they were then analysed by chemiluminescence



Objective 1

Sera used

- Positive sera: 8 naturally infected horses from Italian outbreaks (Istituto Zooprofilattico Sperimentale Abruzzo & Molise, 2012), 2 experimentally infected mares (Animal experimentation was carried out in compliance with Italian national law implementing Directive 86/609/EEC of the Council of the European Communities on the protection of animals used for experimental and other scientific purposes).
- Negative sera: 10 healthy animals from the field in disease free regions

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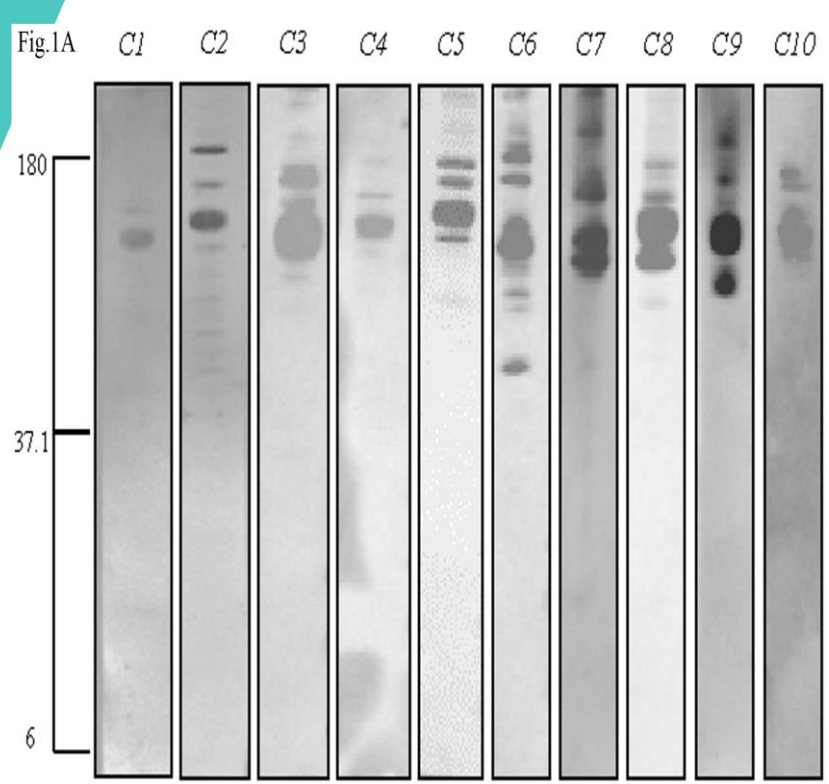
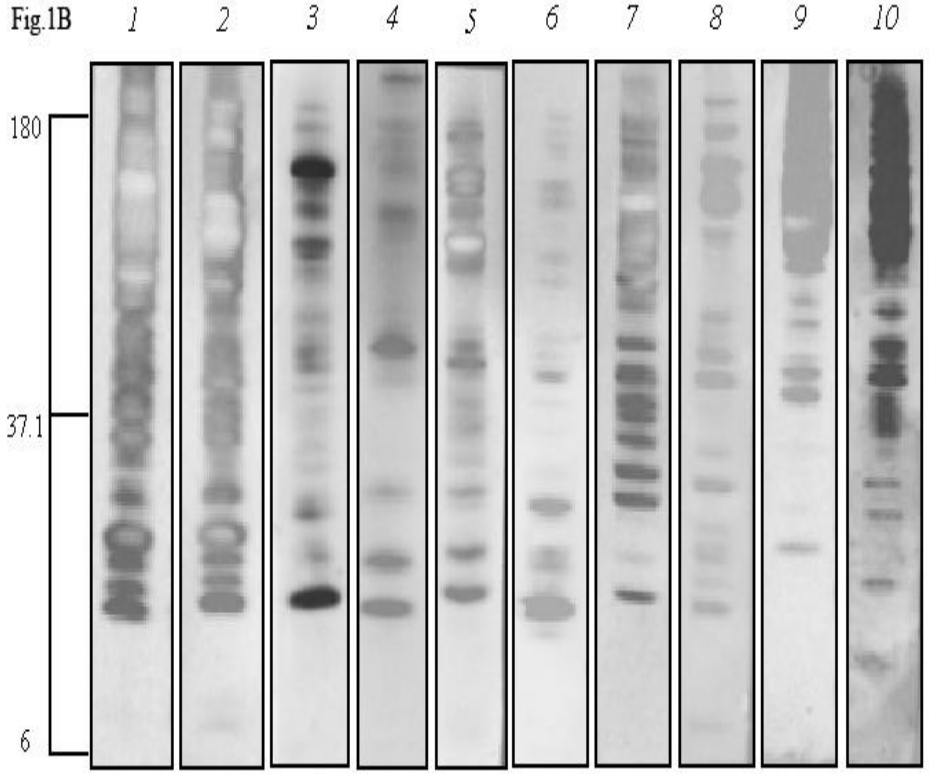
Objective 1 Results





- IgGs from confirmed cases, specifically recognise a antigenic profile with low molecular weight bands ranging between 10 and 37 kDa

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Research paper

IgG antibodies from dourine infected horses identify a distinctive *Trypanosoma equiperdum* antigenic pattern of low molecular weight molecules

M. Luciani [a], C. Di Pancrazio [a], T. Di Febo [a], M. Tittarelli [a], M. Podaliri Vulpiani [a], M.O. Puglielli [a], J. Naessens [b], F. Sacchini [a,*]

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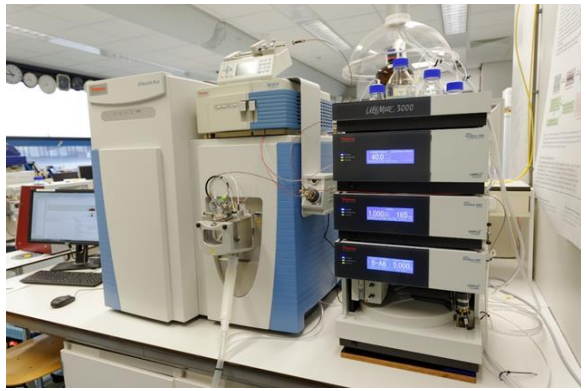
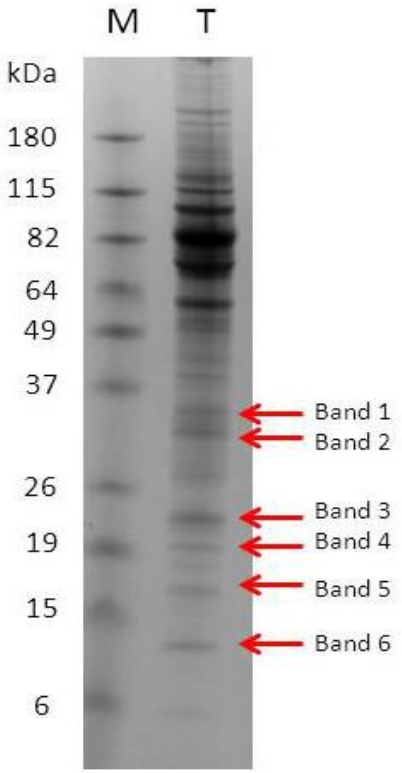
Objective 1

Identification of *T.equiperdum* potential biomarkers

- Six SDS-PAGE bands, recognised by antibodies from infected horses with molecular weight ranging between 37 and 10 kDa, were selected for analysis
- Analysis was performed using an UPLC-Easy n-LC 1000 system coupled to a Q Exactive HF mass spectrometer
- Identified proteins were annotated in terms of topological and immunological features using bioinformatic softwares





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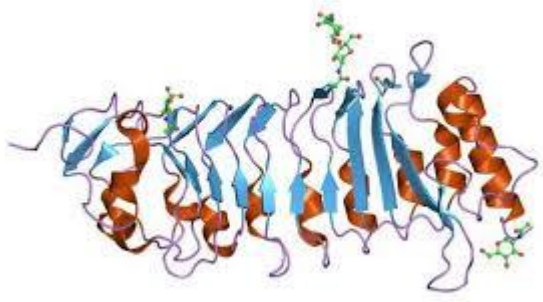
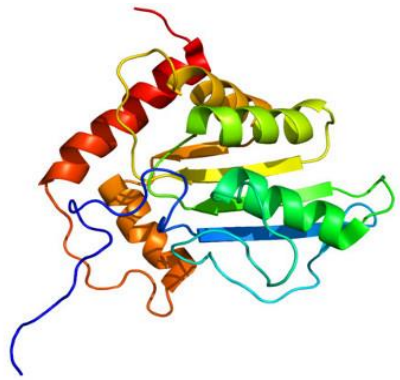
Objective 1

Bioinformatic analysis

- A total of 167 proteins were identified
- Subcellular localization was predicted by using different softwares
- Non-cytoplasmic proteins with B-cell solvent exposed epitopes were analized by BLASTp (Aminoacid sequences of identified proteins were compared to all sequenced proteins of Trypanosoma available *T. evansi, T. brucei brucei, T. b. gambiense, T. b. rhodesiense, T. congolense, T. cruzi, T. rangeli, and T. vivax*)
- Among them, 37 were found unique for *T. equiperdum*. 24 of them could represent possible candidate diagnostic antigens for the development of serological tests more specific for *T. equiperdum*





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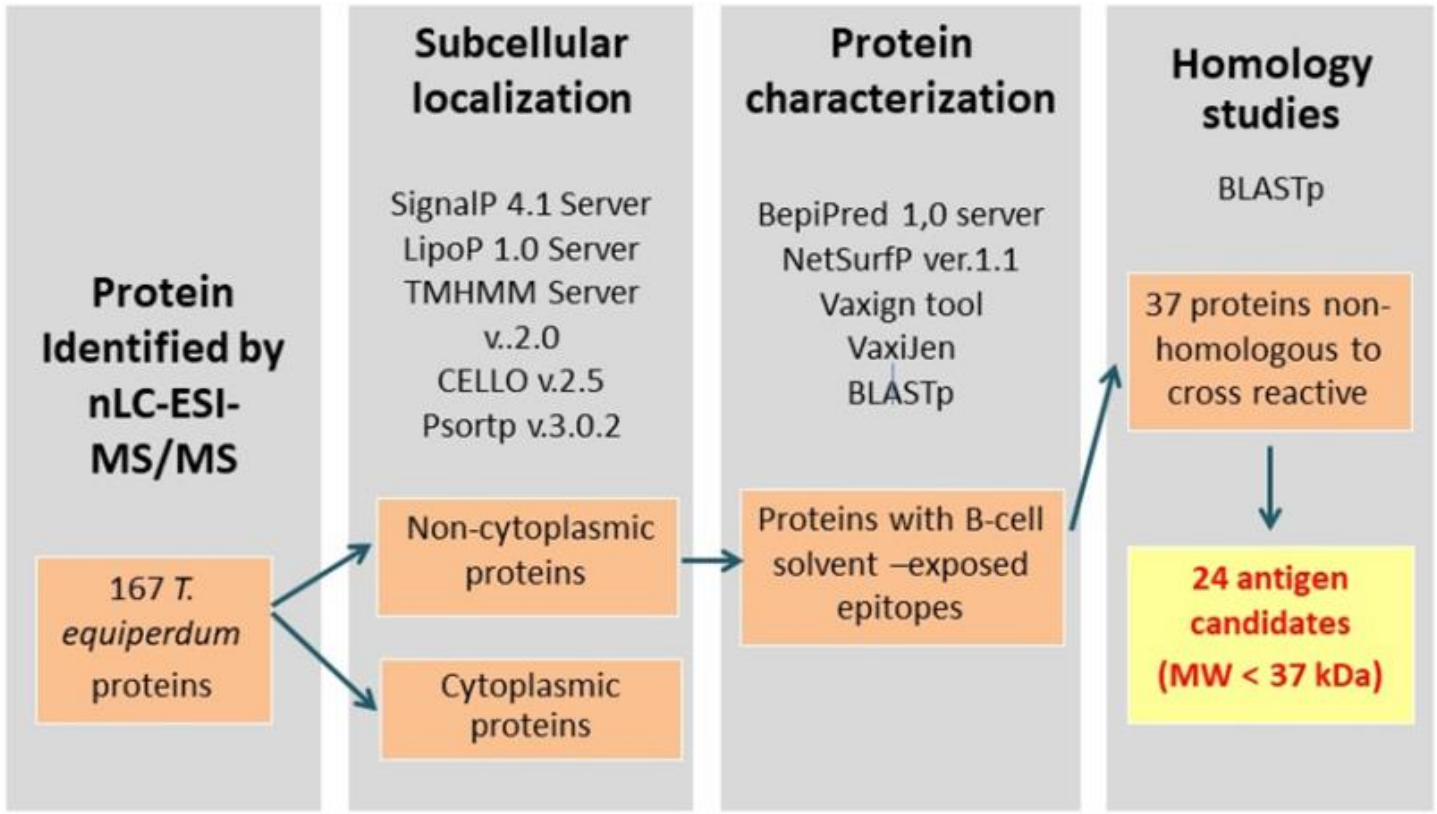
Objective 1

Bioinformatic analysis Workflow



frontiers in Veterinary Science

ORIGINAL RESEARCH
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Trypanosoma equiperdum Low Molecular Weight Proteins As Candidates for Specific Serological Diagnosis of Dourine

Mirella Luciani[1†], Tiziana Di Febo[1†], Massimiliano Orsini[1], Ivanka Krasteva[1], Angela Cattaneo[2], Michele Podaliri Vulpiani[1], Chiara Di Pancrazio[1], Angela Bachi[2] and Manuela Tittarelli[1]*

[1] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale, Teramo, Italy, [2] Istituto FIRC di Oncologia Molecolare (IFOM), Milan, Italy

Objective 1

Selected proteins

3 proteins were selected for the development of immunoblotting:

- Peptidyl-prolyl cis-trans isomerase (UniProt accession number: A0A1G4I8N3)
- GrpE protein homolog (UniProt accession number: A0A1G4I464)
- Transport protein particle (TRAPP) component (UniProt accession number: A0A1G4I740)

These proteins were selected on the base of bioinformatics analysis because:

- no cross reactive with *T.evansi*
- have the high percentage of B-cell epitopes

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Objective 1

Sera used

Sera	Origine	Number	Title CFT(MCM)
Reference positive sera	Italian	1	1:320
Reference negative sera	Italian	2	Negative
Positive sera	Italian and African (Namibia)	15	1:10 – 1:640
Negative sera for MCM (Italian farms)	Italian	80	Negative
Positive sera for Theileria equi	Italian	3	Negative
Positive sera for Babesia caballi	Italian	3	Negative

- Using these sera to evaluate diagnostic sensitivity, specificity and accuracy by means of double entry tables (WOAH Manual)

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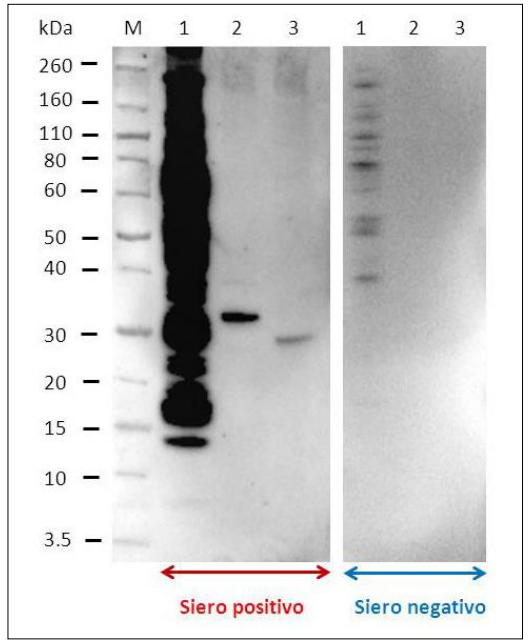
Objective 1

Results of immunoblotting



Peptidyl-prolyl cis-trans isomerase (A0A1G4I8N3) gave the best sensitivity and specificity in immunoblotting.

	%	U.C.L	S.C.L.
Diagnostic sensitivity	86.7	61.7	96.0
Diagnostic specificity	92.5	84.6	96.5
Diagnostic accuracy	91.6	84.2	95.6
Upper Confidence Limit (probability of 95%)			
Lower confidence limit (probability of 95%)			

M Novex Sharp Prestained Protein Standard
Lane 1 Purified OVI T.e
Lane 2 Rec protein A0A1G4I8N3
Lane 3 Rec protein A0A1G4I464

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Objective 1

Results of immunoblotting

- **GrpE protein homolog** (A0A1G4I464) gave the minor sensitivity and specificity in immunoblotting

	%	L.C.I. (95%)*	L.C.S. (95%)§
Diagnostic sensitivity	46.7	24.7	70.1
Diagnostic specificity	81.3	71.3	88.3
Diagnostic accuracy	75.8	66.3	83.3
Upper Confidence Limit (probability of 95%) **Lower confidence limit (probability of 95%)**			

- **Transport protein particle** (TRAPP) component (A0A1G4I740) gave low protein expression

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Objective 1

Conclusion

- Need to identify other unique *T. equiperdum* proteins as antigen in serological tests to improve the diagnosis of dourine
- In parallel, in vitro experimental production of proteins of *T. equiperdum* should be useful in order to reduce the use of experimental animals in the production of antigen for CFT
- Very few data are present in the literature relating to the production and diagnostic use of recombinant proteins of *T. equiperdum* (genome sequencing published in 2017)

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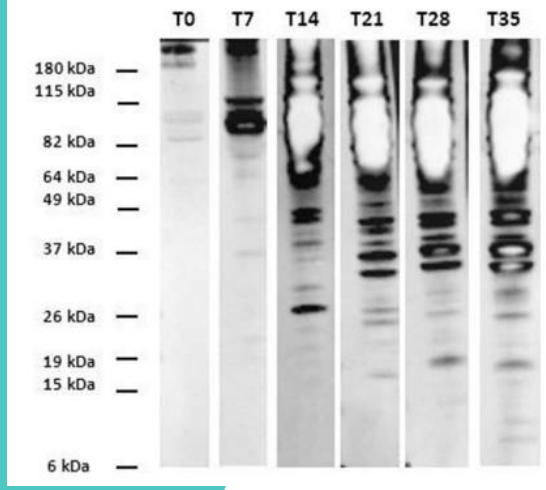
Objective 2

Development of the rabbit model to know humoral immune response and delayed –type hypersensitivity

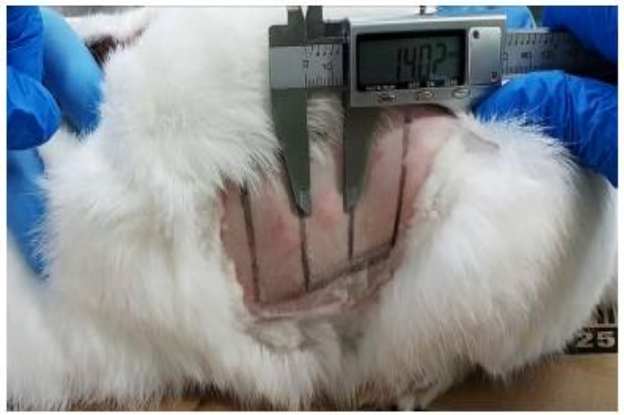
- A better comprehension of the host immune response following infection could allow to identify targets that could improve the diagnosis of the disease
- Rabbits were experimentally infected with *T. equiperdum* strain OVI and their humoral response was studied in vivo using serological tests (complement fixation test and immunoblotting) currently used for the diagnosis of dourine in horses
- A protein extract of *T. equiperdum* strain OVI (the procedure used was similar to tuberculin one – PPD antigen) was produced and used as skin test antigen on rabbits in order to evaluate its performances and its safety. The skin test antigen could be used in the field diagnosis of dourine in horses, in particular in endemic areas

Objective 2

Results



- All the rabbits infected with OVI *T. equiperdum* were positive by CFT after 7 days from inoculation (T7) and maintained high antibody titres for the entire duration of the experiment. The control animals remained negative
- Immunoblotting results showed that rabbit serum antibodies from the control group at times from T0 to T35 identified bands with MW ranging from 40 to 180 kDa. On the contrary, starting from time T7, antibodies in the sera of infected rabbits showed an increasing reaction with *Trypanosoma* proteins (purified OVI T.e) , and in particular with low MW proteins (6–37 kDa)
- Skin reactions to *T. equiperdum* skin test antigen were observed in 8 out of 9 infected rabbits. In particular, a positive reaction (diameters of erythema of 8–25 mm) was observed at 24 hours. Negative reaction was observed in the skin negative control areas



Objective 2
Conclusions

- The rabbit model can be used as horse replacement because it gave similar results in both CFT and in immunoblotting
- The “tripanosomina” could help to develop more effective diagnostic tools
- Further studies should be done to evaluate diagnostic performances of skin test antigens on *T. equiperdum* infected horses in areas where dourine is endemic and to compare the cell-mediated response induced by *T. equiperdum* in both rabbits and horses
- OVI *T. equiperdum* skin test antigen should also be tested on *T. evansi* infected horses or other animals like camel to evaluate possible cross-reactions

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Objective 2

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Humoral immune response and delayed-type hypersensitivity in rabbits infected with *Trypanosoma equiperdum*

Tiziana Di Febo✉, Ivanka Krasteva, Barbara Bonfini, Manuela Tittarelli, Osvaldo Matteucci, Gianluca Orsini, Emanuela Rossi, Michele Podaliri Vulpiani, Diamante Rodomonti, Luigi Iannetti & Mirella Luciani

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Thanks for the attention

- Serology and immunology department
- Production of viral vaccines and diagnostic tools department
- Production of bacterial vaccines department