In-vitro production of *Trypanosoma equiperdum* antigen for CFT – time consuming, expensive, but possible

Our experiences

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https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.06.03_DOURINE.pdf

CHAPTER 3.6.3.

DOURINE IN HORSES (*TRYPANOSOMA EQUIPERDUM* INFECTION)

(version adopted in May 2021)

2.1. Complement fixation test

Standard or microplate techniques may be used. Guinea-pig serum (available commercially) is used as a source of complement. Other reagents are sheep red blood cells (RBCs) washed in veronal buffer, and rabbit haemolytic serum (i.e. rabbit anti-sheep RBC) (commercial) as well as known negative and positive control sera.

2.1.1. Antigen production

Because of lack of solid serological or molecular markers to differentiate *T. equiperdum* from the other *Trypanozoon* taxa (Büscher *et al.*, 2019; Cuypers *et al.*, 2017), it is important to indicate which *T. equiperdum* strain is used for any antigen preparation. Strains that easily grow in rodents are *T. equiperdum* OVI, BoTat, Dodola and TeAp-N/D1. Strains that are adapted to *in-vitro* culture are *T. equiperdum* OVI and IVM-t1. It should be kept in mind that crude antigen preparations such as described below, are not dourine-specific and will cross-react with sera from horses infected with *T. brucei* and *T. evansi*.

2.1.1.1 Antigen preparation from *in-vitro* propagated parasites

The procedure described below is based on Bassarak *et al.* (2016) with some modifications. *Trypanosoma equiperdum* OVI (ITMAS 241199C, purchased from Institute of Tropical Medicine, Antwerp, in 2008) was adapted to *in-vitro* culture conditions. Culture-adapted trypanosome stocks in liquid nitrogen are available on request[1].

i) Reagents and solutions to prepare medium

Substance	Identification System	Number
MEM powder for 1 litre with Earle's salts & L-glutamine, without $NaHCO_3$ (Sigma-Aldrich M0268)		
2-Mercapto-ethanol	CAS	60-24-2
Adenosine	CAS	58-61-7
Antibiotic-antimycotic solution (100×)		
Bathocuproine disulfonate	CAS	52698-84-7
Cysteine	CAS	52-90-4
D(+)-Glucose × 1 H_2O	CAS	50-99-7
Glycerol	CAS	51-81-5
HEPES	CAS	7365-45-9
Isopropanol	CAS	67-63-0
Hypoxanthine	CAS	68-94-0
New-born calf serum, heat-inactivated (NCS)		
Potassium chloride	CAS	7447-40-7
Magnesium sulfate × 7 H_2O	CAS	10034-99-8
MEM non-essential (100×)		
Sodium pyruvate	CAS	113-24-6
Sodium chloride	CAS	7647-14-5
Sodium hydrogene carbonate	CAS	144-55-8
Na_2HPO_3 × 12 H_2O	CAS	10039-32-4
NaH_2PO_3 × 2 H_2O	CAS	10049-21-5
Ornithine/HCl	CAS	3184-13-2
Thymidine	CAS	50-89-5
Hypoxanthine 100× stock solution		225 ml H_2O, 340 mg hypoxanthine, 25 ml 1 M NaOH. Stir in water bath for 20 min at 55°C. Filter through 0.22 µm filter; store at 4°C.
Cysteine/bathocuproine-disulfonate 100× stock solution		225 ml H_2O, 705 mg bathocuproine disulfonate, 4550 mg cysteine, 25 ml 2 M HCl. Stir for 20 min at 55°C. Filter through 0.22 µm filter. Store at 4°C.

ii) Preparation of the culture medium with 15% NCS (example for 3 litre)

Using a fume hood, add 47 µl 2-mercapto-ethanol to 10 ml H_2O. In a 5 litre beaker with 2430 ml H_2O, add: 3 MEM powder packs, 6.6 g $NaHCO_3$, 17.85 g HEPES, 3 g glucose, 0.66 g sodium pyruvate, 0.15 g ornithine, 0.012 g thymidine, 0.039 g adenosine, 30 ml MEM non-ess 100× stock solution, 15 ml antibiotic-antimycotic 100× stock solution, and 10 ml of the 2-mercapto-ethanol dilution. Adjust to pH 7.4 with NaOH and HCl and stir for 10 minutes. Add 30 ml hypoxanthine 100× stock solution and 30 ml cysteine/bathocuproine-disulfonate 100× stock solution. Adjust to pH 7.4 with NaOH and HCl and add H_2O up to 2550 ml.

In three 1 litre flasks, dispense 150 ml NCS. Fill the flasks with 850 ml culture medium filtered over a 0.22 µm filter. Mix gently and store at 4°C. The culture medium is stable for at least 8 weeks.

iii) Preparation of the trypanosome dilution buffer (TDB), pH 7.7

Dissolve 3.23 g Na_2HPO_4 × 12 H_2O, 0.14 g NaH_2PO_4 × H_2O, 0.19 g KCl, 2.34 g NaCl, 0.13 g $MgSO_4$ × 7 H_2O, 1.80 g D(+)-Glucose × H_2O in 450 ml H_2O. Adjust to pH 7.7 with NaOH & HCl. Adjust to 500 ml with H_2O. Filter through 0.22 µm filter. Store at 4°C (stable for at least 8 weeks).

iv) Preparation of a 5% PVP (polyvinylpyrrolidone), 0.01% merthiolate-NaCl solution

Prepare a 1% merthiolate-NaCl solution by dissolving 4.25 mg NaCl and 5 mg sodium ethylmercurithiosalicylate in 0.5 ml H_2O. In a 50 ml beaker, dissolve 425 mg NaCl and 2.5 g PVP 25 in 40 ml H_2O. Add the 0.5 ml 1% merthiolate-NaCl solution and adjust to 50 ml with H_2O. Filter through 0.22 µm filter. Store at 4°C.

v) Prepare a trypanosome culture with 1×10^5 trypanosomes/ml respecting a surface-volume ratio of 3.25 cm^2 per ml, e.g. in three-level T-500 culture flasks filled with 154 ml culture medium, and incubate at 37°C in a CO_2 incubator.

vi) Harvest the trypanosomes at concentrations of $1.5–2 \times 10^6$/ml once or twice a week in batches of 400 ml cell culture medium. Keep trypanosomes on ice during the whole process. Trypanosome containing medium is filled in a set of 50 ml tubes and centrifuged (10 minutes, 4°C, 1300 ***g***). Pellets of 8 tubes are resuspended carefully with a small volume of ice-cold TDB and transferred to one new, sterile 50 ml tube. The trypanosomes are washed twice with TDB (10 minutes, 4°C, 1300 ***g***) and the supernatant is removed completely. Pellets are stored at –20°C. It is advisable to confirm sterility of preparations using blood agar plates.

vii) The total number of cells of all pellets is determined. Prepare a fresh PVP-merthiolate solution (1 ml per 1×10^9 trypanosomes). Thaw frozen pellets on ice, resuspend the pellets with 50% of the calculated volume of ice-cold 5% PVP in 0.01% merthiolate-NaCl solution and pool them in a new sterile 50 ml tube. Fill ice-cold 5% PVP in 0.01% merthiolate-NaCl solution to 100% of the calculated volume. Fill 200 µl antigen solution each in sterile beaded rim bottles (mix thoroughly several times during process), and place them in the biosafety transport box on ice for transport to the –80°C freezer. The lyophilisation apparatus is started and after 90 minutes the frozen antigen containing bottles are placed into the lyophilisation apparatus. Lyophilisation is performed overnight. The next day, lyophilisation is completed and immediately the cap is closed tightly and the antigen stored at –20°C. Alternatively, the antigen solution can be stored in small volumes at –80°C. The working dilution of antigen is standardised by titration against a 1/5 dilution of a standard low-titre antiserum.

1 From the OIE Reference Laboratory for dourine: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

A new paragraph on *in-vitro* antigen preparation

Veterinary Parasitology 223 (2016) 133–140

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Research paper

In vitro production of *Trypanosoma equiperdum* antigen and its evaluation for use in serodiagnosis of dourine

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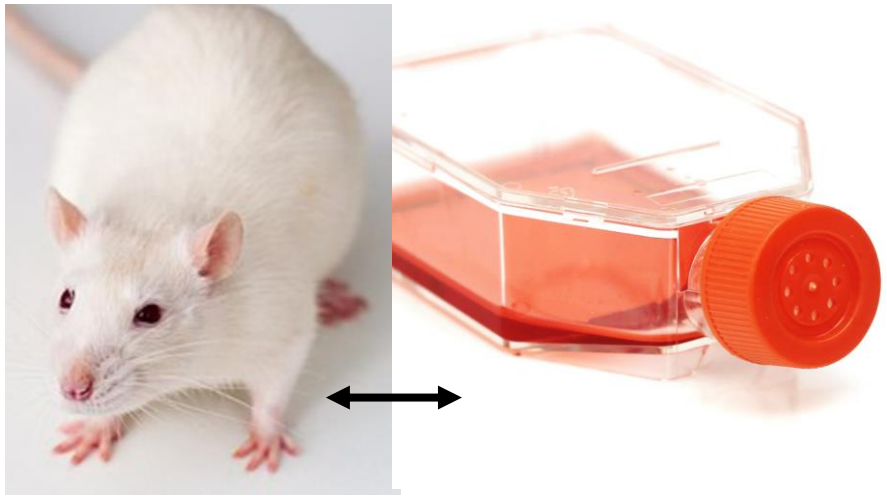

Replacement,
Reduction and
Refinement

→ FLI has to supply regional laboratories with CFT antigen
→ We should try to use in-vitro production….

German NRL Dourine moved to Island Riems in 2016 when Dr. I. Moser retired

MEM - medium based on Baltz et al., 1985 // Trypanosome *T. equiperdum* strain OVI (ITMAS 241199C)

Pre-paring medium

Substance	Identification System	Number
Medium		
MEM powder packs for 1 litre with Earle's salts & L-glutamine, without NaHCO3 (Sigma-Aldrich M0268)		
2-Mercapto-ethanol	CAS	60-24-2
Adenosine	CAS	58-61-7
Antibiotic-antimycotic solution (100×)		
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Cysteine	CAS	52-90-4
D(+)-Glucose × 1 H2O	CAS	50-99-7
HEPES	CAS	7365-45-9
Hypoxanthine	CAS	68-94-0
New-born calf serum, heat-inactivated (NCS)		
Sodium pyruvate	CAS	113-24-6
Sodium hydrogene carbonate	CAS	144-55-8
Ornithine/HCl	CAS	3184-13-2
Thymidine	CAS	50-89-5
Hypoxanthine 100× stock solution	225 ml H2O, 340 mg hypoxanthine, 25 ml 1 M NaOH. Stir in water bath for 20 min at 55°C. Filter through 0.22 µm filter; Store at 4°C.	
Cysteine/bathocuproine-disulfonate 100× stock solution	225 ml H2O, 705 mg bathocuproine disulfonate, 4550 mg cysteine, 25 ml 2 M HCl. Stir for 20 min at 55°C. Filter through 0.22 µm filter. Store at 4°C.	

Pre-paring antigen

TDB		
Potassium chloride	CAS	7447-40-7
Magnesium sulfate × 7 H2O	CAS	10034-99-8
Sodium chloride	CAS	7647-14-5
Na_2HPO_3 × 12 H2O	CAS	10039-32-4
NaH_2PO_3 × 2 H2O	CAS	10049-21-5
D(+)-Glukose x1 H2O		

Stabilate preparation		
Glycerol for freezing medium	CAS	51-81-5
Isopropanol for freezing device	CAS	67-63-0

Hypoxanthin Stock 100x	
Hypoxanthin	40.8 mg
Aquabidest.	27 ml
NaOH, 1M	3 ml
stirr 20 min at 55°C (water bath)	
sterile filtration	
store at 4°C	

Cystein/Bathocuproine disulfonate Stock 100x	
Bathocuproine Disulfonate (BCS)	90 mg
Cystein	546 mg
Aqua bidest.	27 ml
HCl, 2 M	3 ml
stirr 20 min at 55°C (water bath)	
sterile filtration	
store at 4°C	

Medium to prepare stabilates	
TDB	30.0 ml
Glycerin	7.5 ml
vortex	
sterile filtration	
store at 4°C (up to 8 weeks)	

Nalgene CryoBox

OIE proposes / Bassarak et al. 2016 used:
Three-level T-500 culture flasks filled with 154 ml culture medium, and incubate at 37°C in a CO2 incubator

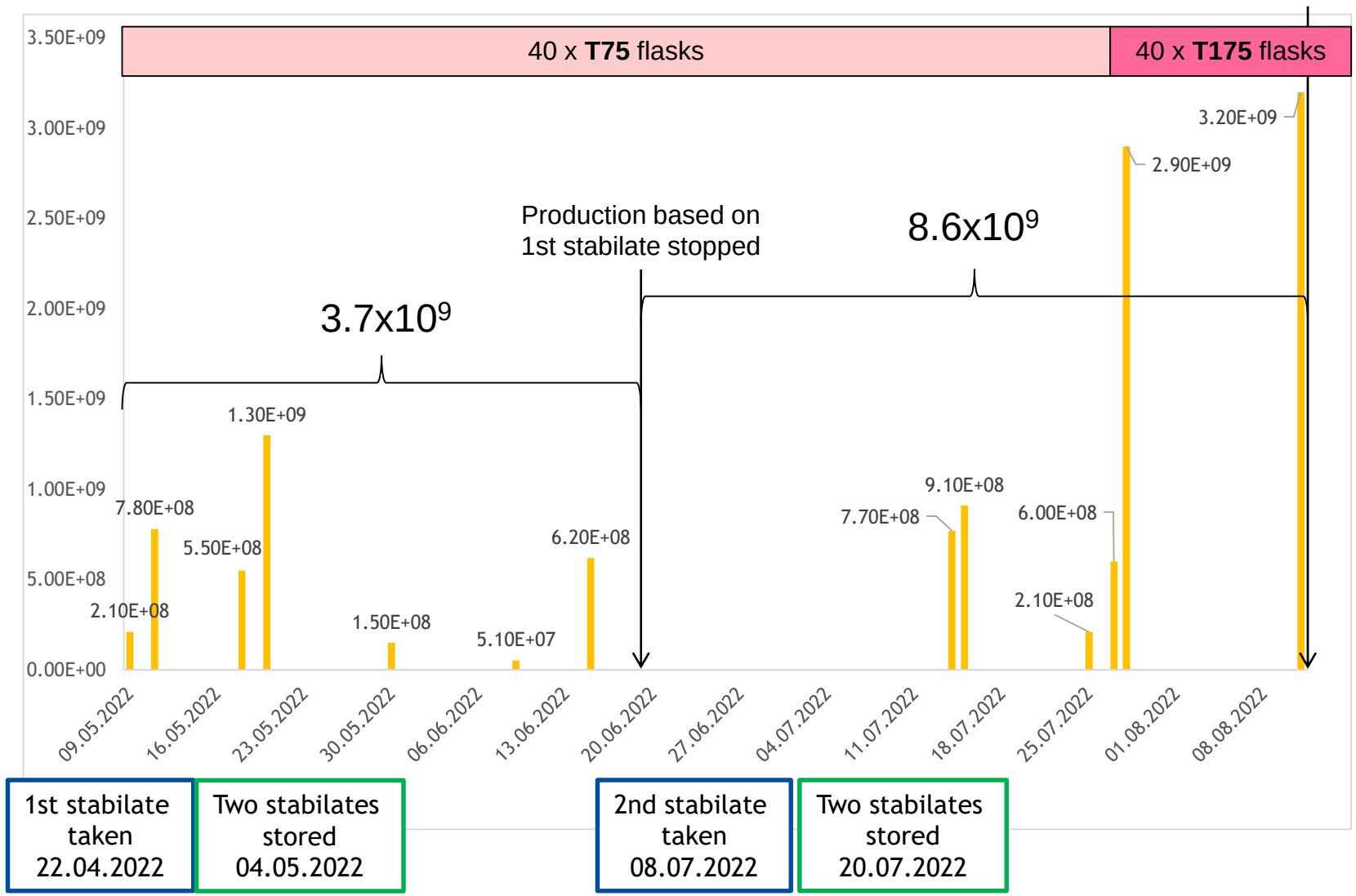
We :
T-75/one level (~25 ml medium) or
T-175/one level (~45 ml medium)

Trypanosoma equiperdum OVI (ITMAS 241199C, purchased from Institute of Tropical Medicine, Antwerp, in 2008)

Total production, 3 months
1.23×10^{10}

N=53 doses of lyophilized antigen á 200 µl

We never reached densties of
$> 2 \times 10^{6}$
(as reported ty Bassarak et al. 2016)



Cultivation needs time

Often trypanosome culture got too concentrated…

Wednesday

fresh medium/split

Friday

fresh medium/split

Optimal day for harvesting

Weekend

Monday

fresh medium/split

Proviciency testing - Antigen produced 2020 is working well

ILPT REPORT # Anses_LSAn_22_08_EURL_Dourine_Surra_ V01

Annex 2. Dourine CFT and CATT/T.evansi qualitative results reported by the ILPT participants.

Dourine CFT		Samples									
		1	2	3	4	5	6	7	8	9	10
		Negative serum			Teva Pure		Teva 1/4		Rotat 1.2 1/2	Trypeq Pure	
Laboratory code	Expected result	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos
	1	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	2	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	3	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	5	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	6	NEG	NEG	NEG	POS	POS	POS	POS	POS	SUS	POS
	7	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	8	NEG	NEG	NEG	POS	POS	NEG	NEG	SUS	POS	POS
	9	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	10	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	11	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	12	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	13	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	14	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	15	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	16	POS	NEG	POS	POS	POS	NEG	NEG	POS	SUS	SUS
	17	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	18	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	19	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
	20	SUS	SUS	SUS	POS	POS	POS	POS	POS	POS	POS

	1
Date of sample analysis	29/06/2022
Results date	05/07/2022
Method	CFTB (OIE)
HT control: batch / supplier	02/95/ FLI PKS
LT control: batch / supplier	
NEG control: batch / supplier	06/13/ FLI NKS
Antigens: Batch / suplier / strain	02/20/ FLI Ag
Antigens dilution :	1:10
Complement batch/supplier	IDvet/H48
Complement: dilution	1:50
Haemolytic serum: Batch:supplier	IDvet/HS-007
Haemolytic serum: dilution	1:1000
RBC final %	1%
RBC supplier	Boehringer Ingelheim/11855
Buffer type	
Buffer supplier	H72
Buffer pH	7,2
Reading after	centrifugation
Difference with OIE	

Laboratory 1							
Sample	#	1/5	1/10	1/20	1/40	1/80	Qualitative
1	106	trace	trace	trace	trace	trace	Negative
2	10	trace	trace	trace	trace	trace	Negative
3	379	trace	trace	trace	trace	trace	Negative
4	255	4+	4+	4+	3+	trace	Positive
5	74	4+	4+	4+	3+	trace	Positive
6	118	4+	3+	trace	trace	trace	Positive
7	324	4+	3+	trace	trace	trace	Positive
8	29	4+	4+	4+	3+	trace	Positive
9	399	4+	4+	4+	4+	3+	Positive
10	122	4+	4+	4+	4+	2+	Positive

Our antigen used together with reagents from ID.vet (Complement, Haemolytic serum) and Boehringer Ingelheim (RBC)

Conclusion

- In-vitro production of *T. equiperdum* antigen for CFT is time consuming, expensive, but possible.
- FLI-CFT based on in-vitro generated antigen passed proviciency testing offered by ANSES in 2022

Thank you for your attention.