Validation of a commercial glanders ELISA as an alternative to the CFT in international trade of equidae

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Glanders – Why we need other tests ?

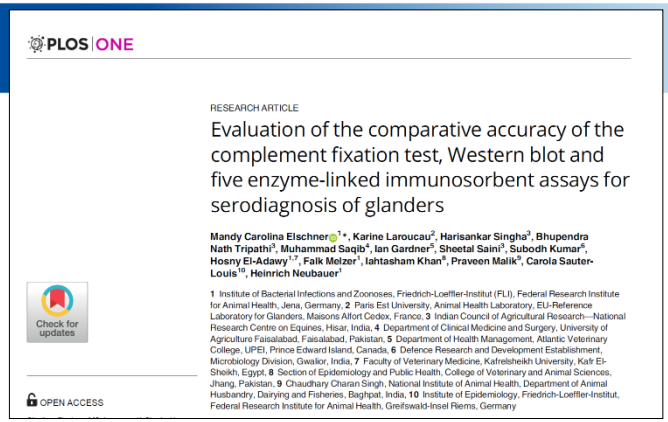
- **CFT – still prescribed test for trade**

Poor specificity 94-96 % → problem of false positives in trade investigations
→ trade restrictions with considerable financial consequences

difficult to standardize → no international standard serum
→ CFT antigens with influences DSe and DSp
→ warm or cold incubation influences DSe and DSp

- **CFT – needs second confirmation**

→ OIE demands confirmatory tests having same- or higher sensitivity and specificity
→ so far in Germany only immunoblot available



PLOS ONE

RESEARCH ARTICLE

Evaluation of the comparative accuracy of the complement fixation test, Western blot and five enzyme-linked immunosorbent assays for serodiagnosis of glanders

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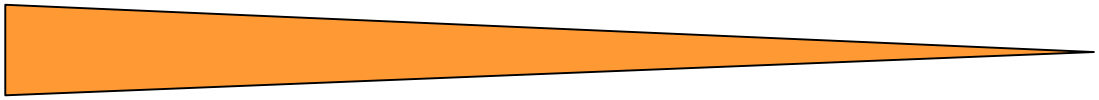
Test	Developed by	Antigen used
Complement fixation test	OIE-Manual	Malleus-CFT antigen; Ccpro GmbH, prepared from B.mallei strain Ivan-NCTC 10230
Western Blot	NRL, Germany	LPS-containing antigen consisting of 3 different B. mallei strains (Bogor, Mukteswar, Bahrain1)
Indirect ELISA	EU_RL, IDvet, France	semi-purified fraction prepared from B. mallei strain ATCC 23344
Indirect ELISA	ICAR-NRCE, India	recombinant protein TssA of Type 6 secretory system of B. mallei
Indirect ELISA	ICAR-NRCE, India	recombinant protein TssB of Type 6 secretory system of B. mallei
Indirect ELISA	ICAR-NRCE, India	recombinant protein HCP1 of Type 6 secretory system of B. mallei
Indirect ELISA	ICAR-NRCE, India	recombinant protein BimA of B.mallei

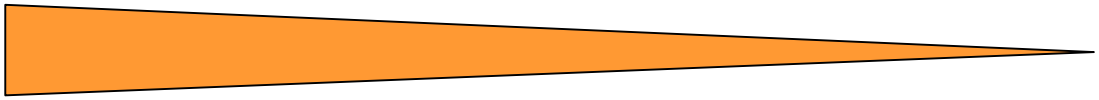
Funding: International Horse Sports Confederation and World Organization of Animal Health (Tender Ref.: AD/SR/2015/1885)

Results of the 2018 study

Assay	TssB	HCP1	IdVet	WB	TssA	BimA	CFT
N	2,959	2,959	2,959	2,959	2,959	2,959	2,959
FP	0	13	14	18	30	76	108
TN	2,959	2,946	2,945	2,941	2,929	2,883	2,851
DSp %	100.00	99.56	99.53	99.39	98.99	97.43	96.35



Assay	CFT	WB	HCP1	IdVet	BimA	TssA	TssB
FN	5	8	12	19	37	43	43
TP	249	246	242	235	217	211	211
DSe %	98.03	96.85	95.28	92.52	85.43	83.07	83.07



CFT was confirmed as test of choice, without alternative

Significance (P values) of differences in DSe

	CFT	WB	IDvet	HCP1	BimA	TssA	TssB	
CFT		0,5488	0,0043	0,1185	< 0,0001	< 0,0001	< 0,0001	CFT
WB			0,0192	0,424	< 0,0001	< 0,0001	< 0,0001	WB
IDVet				0,1671	0,0058	0,0005	0,0005	IDvet
HCP1					< 0,0001	< 0,0001	< 0,0001	HCP1
BimA						0,4404	0,4292	BimA
TssA							0,8551	TssA
TssB								TssB

IDvet, BimA, TssA, TssB are significantly less sensitive than CFT

Differences in DSe between WB and CFT are not significant

WB was proofed as confirmatory test

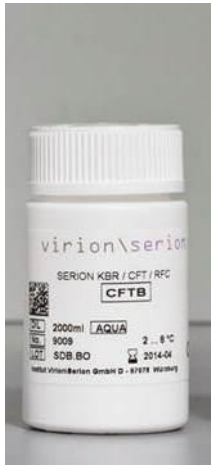
New study 2020-2021

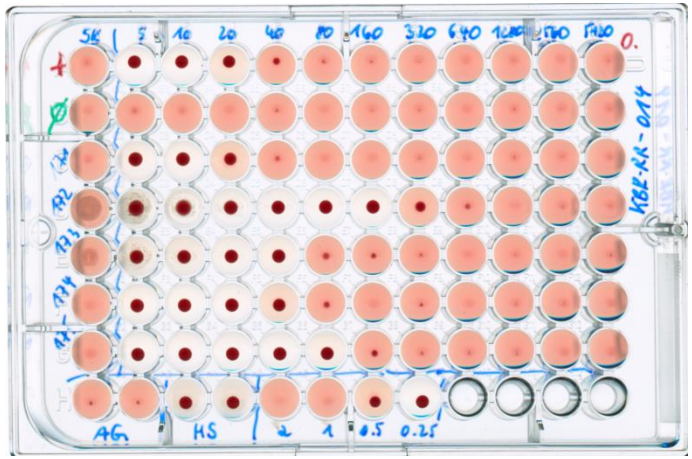
Test	Developer/ protocol used	Antigen used
ID Screen Glanders Double Antigen Multispecies ELISA (GLANDA ELISA)	IDvet, Grabels, France	Recombinant T6SS protein of *B. mallei*; double antigen approach
Western blot (WB)	FLI, Germany [16]	Crude preparations of LPS antigen of 3 different *B. mallei* strains Bogor, Mukteswar, and Bahrain1
Complement fixation test (CFT)	OIE-Manual [6]	Malleus-CFT antigen; Ccpro GmbH (Oberdorla, Germany) prepared from *B. mallei* strain Ivan-NCTC 10230

CFT Antigen: Ccpro-GmbH

incubation 18h, 4°C
5CH_{50}, 2% RBC
duration 20h
needs experience

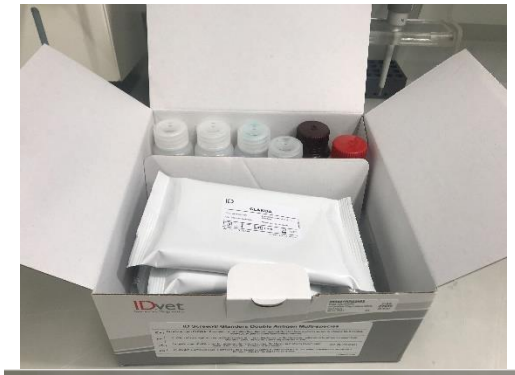
CFT reagents Virion/ Serion Institute





cut-off ≥ 1:5 (25% inhibition of hemolysis)

ID Screen Glanders Double Antigen Multispecies ELISA (GLANDA ELISA)



- duration 2h
- no special experience neccessary

Steps:

- 90µl dilution buffer + 10µl serum, incubation 45 min

→ antibodies bind to rec. antigen on plate

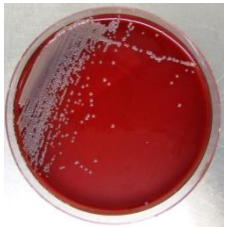
- 3x washing steps
- 100µl rec. *B.mallei* protein HRP-conjugate

→ binds to free Fab of the bound serum anti-*B.mallei* Ab

→ form a antigen-antibody-conjugate-HRP-complex

- incubation 30 min
- 3x washing steps
- 100µl substrate, incubation 15 min
- Stop solution, OD 450nm
- cut-off SP% < 70 negative; > 70 positive

Western Blot

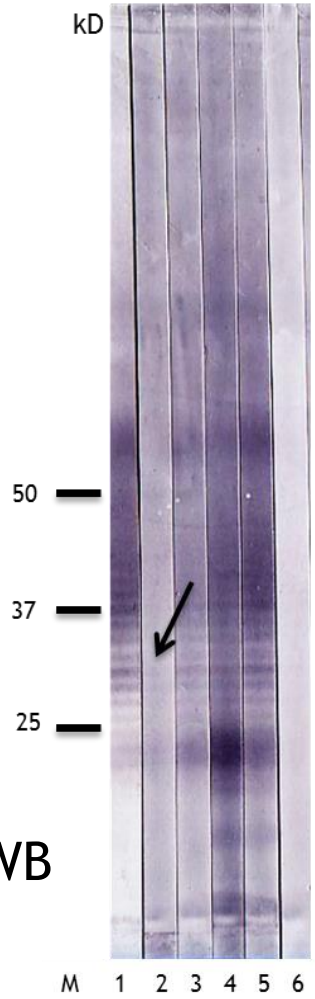


purified LPS-antigen
3 different strains

lyophilized

used for WB

Examples for WB results using horse serum samples:



- in-house method
- highly labourious
- duration IB 6h
- needs experience

M: protein standard
1: positive control
2: suspicious result
3: weak positive serum
4,5: strong positive results
6: negative control

Sample 2 shows not a typical LPS pattern, only slight “shadows” in the area where the LPS ladder would be anticipated (arrow) is seen.

„suspicious“ (rated as positive)

Sera from glanders negative animals (n=400)

… were collected in Germany, which is officially free from glanders.

All samples were collected non-randomly during routine testing for trade or movement.

Sera from glanders positive animals (n=370)

Pakistan, India, Germany, collected 2006-2020

n=117 from clinically positive animals
"clinically positive" on the basis of signs consistent with glanders, and the fact, that they were detected during a cultural confirmed glanders outbreak including close contact to infected animals

n=253 positive by *B. mallei* isolation or molecular detection of *B. mallei* by real-time PCR (n=253)

Species	Horse	Mule	Donkey
Number	338	25	7

Diagnostic specificity (DSp)

DSp testing 400 true-negative samples				
	FP	TN	DSp%	CI 95%
GLANDA-ELISA	1	399	99.8	98.6 - 100.0
WB	3	397	99.2	97.8 - 99.8
CFT	12	388	97.0	94.8 - 98.4

Diagnostic sensitivity (DSe)

DSe testing 370 true-positive samples				
	FN	TP	DSe%	CI 95%
GLANDA-ELISA	7	363	98.1	96.1-99.2
WB	10	360	97.3	95.1-98.7
CFT	13	357	96.5	94.1-98.1

Significance (P values) of differences in DSe and DSp

	Test	CFT	WB
DSe	GLANDA-ELISA	0.210	0.453
	CFT		0.629
DSp	GLANDA-ELISA	0.003	0.625
	CFT		0.035

All test candidates are significantly more specific than CFT

All test show comparable sensitivity

Likelihood ratios (LR) for CFT, WB, GLANDA-ELISA

LR independent of prevalence!

LR+ quantitative indication of the strength of a positive result
highest LR+ best test for ruling in a disease

how much more likely a positive test result occurs in infected than in healthy animals

Assay	GLANDA-ELISA	CI 95%	WB	CI 95%	CFT	CI 95%
LR+	392.4	55.4-2779.3	129.7	42.0-400.6	32.2	18.4-56.2
LR-	0.02	0.01-0.04	0.03	0.01-0.05	0.04	0.02-0.06

LR- quantitative indication of the strength of a negative result
lowest LR- best test for ruling out a disease
means: no false negatives, but detecting all true negatives

how much more likely a negative test result occurs in healthy than in infected animals

What do we need ? Prevention of importation of FN !! We have to rule out the disease.

Summary

CFT is still the prescribed method for trade purposes to certify individual animal free from glanders.

Study data confirmed that the GLANDA-ELISA can identify infected animals with high confidence and demonstrates the freedom from glanders in animals for movement.

The reason for the very good test properties with regard to sensitivity and specificity might be the new double antigen approach of the GLANDA-ELISA, which is hitherto unique to glanders ELISAs.

In particular, the rapid and simple testing protocol qualify the GLANDA-ELISA as a reliable method even for handling large number of samples in standard diagnostic laboratories.

First data indicate that GLANDA-ELISA does not detect immunized animals and also detects animals, infected with *B. pseudomallei*

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