Effect of incubation temperature on the diagnostic sensitivity of the glanders complement fixation test

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Summary

The complement fixation test (CFT) is the only serological test prescribed by the World Organisation for Animal Health (OIE) for the diagnosis of glanders in international trading of equids. However, false-positive reactions have caused financial losses to the animal owners in the past, and false-negative tests have resulted in the introduction of glanders into

healthy equine populations in previously glanders-free areas. Both warm (incubation at 37°C for 1 h) and cold (overnight incubation at 4°C) procedures are recommended by the OIE for serodiagnosis of glanders. In a comparison of the sensitivity and specificity of the two techniques, using the United States Department of Agriculture antigen, warm CFT was found to be significantly less sensitive (56.8%; $p < 0.0005$) than the cold CFT (83.6%). Cold CFT thus increases the detection rate of glanders but a lower diagnostic specificity has to be accepted. The immunoblot was used as the gold standard.

Keywords

Burkholderia mallei – Complement fixation test – Equids – Glanders – Immunoblot – Incubation temperature – Sensitivity – Serodiagnosis.

Introduction

Glanders is a zoonotic disease of solipeds and is caused by the Gram-negative pathogen *Burkholderia mallei.* The disease is notifiable to the World Organisation for Animal Health (OIE) (1, 2). Glanders manifests as nodules on the skin (farcy) and on the mucous membranes of the upper respiratory tract. It can also cause high mortality (95%) in untreated humans and is still endemic in many countries, including Turkey, Iraq, Iran, India, Pakistan, Mongolia, the People's Republic of China and Brazil. Outbreaks have also been reported in the United Arab Emirates, Lebanon, Bahrain, Kuwait and Afghanistan (3, 4, 5, 6, 7, 8, 9, 10, 11, 12).

Although several conventional serological tests are currently used for diagnosis of glanders, the complement fixation test (CFT), because of its ability to detect chronic carrier animals, is the only serological test recommended by the OIE for use in the international transport of equids (13, 14, 15, 16, 17). However, false-positive CFT results can cause large-scale economic losses to animal owners and undue transboundary restrictions on valuable animals. False-negative or equivocal results, on the other hand, can occur in tests on old, debilitated or pregnant animals

and also in well-kept clinically healthy carriers (1, 16). Improved serodiagnostic tests with higher diagnostic specificity and sensitivity are therefore regularly called for (18). Both warm and cold CFT procedures are described in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Terrestrial Manual*) (18). According to the OIE (2008), the temperature and duration of incubation can interfere with the analytical performance of an assay (19). Nevertheless, as the glanders CFT is a widely used and cheap test, the effect of incubation temperature does not attract sufficient attention. No literature on the effect of temperature on the glanders CFT is available, although there is some old literature on the Wassermann reaction (CFT) for diagnosis of syphilis. In 1909, Satta and Donati were the first to address this subject and to publish their observations (20).

In the present study, the warm and cold CFT procedures were compared and the recently evaluated immunoblot was used as the gold standard (21). The immunoblot is the most specific and sensitive test and can also be used to test sera that show anti-complementary activity in CFT (21, 22).

Materials and methods

Origin of serum samples

The 342 equid sera included in this study were divided into two groups. In group I, 196 sera were randomly collected (Mersenne Twister software, SPSS Inc., Chicago, USA) from among 2,282 horse and donkey sera in Germany. These sera were declared true negative because glanders was eradicated in Germany more than 50 years ago and the sera were immunoblot negative (21, 23).

To compare the sensitivity of warm and cold CFT procedures, a set of 146 true-positive sera were selected (group II). This group included 123 samples from Brazil (Pernambuco) and Pakistan (Punjab) that were collected during glanders outbreaks between 2007 and 2009 from

microbiologically or clinically (mallein test) positive glanderous animals. The Pakistani sera were collected from clinically positive and culture-positive equids during two separate outbreaks of glanders among police horses (Faisalabad Metropolis), polo ponies (Lahore Polo Club, Lahore), animals on private farms (Sargodha district) and draught equines (Faisalabad City). The samples from Brazil were collected from clinically positive glanderous animals in different endemic areas of Pernambuco state (National Animal and Plant Laboratory, Ministry of Agriculture, Livestock and Food Supply, Recife, Brazil). In addition, 25 sera from *B. mallei*-immunised horses were collected (21): of these, 23 sera tested positive in immunoblot and were included in group II; the remaining two sera were immunoblot negative and were excluded from the study (21, 23). The animal experiment was authorised by the government of Thuringia, Germany (registration no. 04-105/07).

Complement fixation test procedures

All serum samples were tested using the CFT method according to the OIE *Terrestrial Manual* (18). To minimise the inherent errors of the CFT, a commercially available CFT antigen (United States Department of Agriculture, Ames, Iowa, USA) (18) and a ready-to-use complement and haemolytic system (Institut Virion/Serion GmbH, Würzburg, Germany) were used (19).

The detailed procedure was as follows. Sera were diluted 1/5 in veronal-buffered saline containing 0.1% gelatin and inactivated in a water bath for 30 min at 58°C for horse sera and 63°C for sera from mules, donkeys and non-equids. The inactivated test sera were mixed well on a shaker before pipetting into 96-well round-bottomed microtitre plates. Two-fold dilutions of the sera were then prepared. First, 25 µl of veronal buffer was added to all rows except the second row, then 25 µl of diluted inactivated test serum was pipetted into the first, second and third rows. Two-fold serial dilutions were made, starting from the third row until the end, and discarding the final 25 µl from the last row. Antigen (25 µl) at a working

dilution was then added to all rows except the first. Lastly, 25 µl of complement, diluted to the number of units required, was added to each well. Negative control wells containing diluent only, complement/diluent, or complement/diluent/antigen were prepared (75 µl in each well). Positive and negative serum control wells were also prepared. Plates were shaken for 60 s on a shaker before incubation. For cold CFT, plates were covered and incubated at 4°C for 16 to 20 h; for warm CFT, plates were incubated at 37°C for 1 h only.

In a second step, the haemolytic system was prepared by careful mixing of amboceptor at the working dilution 1:1 with 1% erythrocyte suspension, followed by incubation for 30 min in a 37°C water bath. The plates from the first day were also pre-warmed for 30 min in a 37°C incubator to ensure that the haemolytic system and plates were at the same temperature. Freshly prepared haemolytic system (50 µl) was added to each well and shaken carefully. Plates were moist-incubated at 37°C for 15 to 30 min.

Incubation was stopped when the complement control wells with 2 and 1 units showed complete haemolysis. Plates were centrifuged for 5 min at 670 *g*. The absence of anti-complementary activity was checked for each serum in the first row. The first row was regarded as a serum control (no antigen). Sera were considered negative when 100% haemolysis occurred at 1:5 dilution, 25% to 75% haemolysis was considered equivocal and absence of haemolysis was considered to indicate a positive serum (18). CFT titres were defined as the step at which the serum dilution showed inhibition of haemolysis.

Statistical analysis

The diagnostic specificity and sensitivity of the warm and cold CFT procedures were calculated using standard formulae (24) and software R. The McNemar test was used for comparison of the two CFT procedures and the Wilcoxon test for comparison of the CFT titres (Predictive

Analyse Software version 17.0 SPSS). Confidence intervals (CI) were calculated.

Results

A total of 342 equid sera were evaluated in warm and cold CFTs (Table I). In group I (true negatives; 196 sera), all sera (100%) were negative in the warm CFT; in the cold CFT 192 sera (97.95%) tested negative and four (2.04%) were positive.

In group II (146 sera), 74 sera (50.68%) tested positive, nine sera (6.16%) were equivocal and 63 (43.15%) were negative in the warm CFT. In the cold CFT, 117 (80.13%) tested positive, five (3.42%) were equivocal and 24 (16.44%) were negative (Table I). In further calculations the equivocal samples were considered positive. Weekly samples collected from an immunised horse up to six weeks after the application of mallein tested negative in the warm CFT but positive in the cold test (Fig. 1). In the group of immunised horse sera, differences in cold CFT titres between 12 and 13 days post-immunisation showed a difference of only one two-fold dilution step. Most group II sera that tested positive in the warm CFT showed significantly higher (one- to two-fold) antibody titres in the cold test ($p < 0.0005$).

Overall, in group I the warm CFT showed 100% specificity (CI 0.98; 1) and the cold CFT, 97.95% (CI 0.95; 0.99) specificity. In group II, the warm CFT showed 56.8% sensitivity (CI 0.48; 0.65) and the cold CFT, 83.6% sensitivity (CI 0.76; 0.89), which was significantly higher ($p < 0.0005$).

Discussion

Both temperature and duration of the primary incubation of the test serum have an important effect on the performance of the CFT. This effect was first demonstrated more than 90 years ago in the Wassermann reaction, a CFT for syphilis diagnosis. In most syphilitic sera, primary incubation for 18 h at 2°C to 10°C resulted in a more efficient and sensitive complement

fixation reaction than water-bath or incubator incubation at 38°C for 1 h (25). It was later experimentally proven that more complement is gradually fixed at 0°C than at 37°C. This finding is based on the assumption that the process depends on the formation of an antigen-antibody-complement complex that creates a triple adsorption compound. The smaller particles in the cold mixture present a larger surface for adsorption and therefore the amount of fixed complement increases (20).

While working on brucellosis, Ris demonstrated in 1974 that diagnostic sensitivity in the tube CFT could be increased by incubation at 4°C for 16 h. In a preliminary study, Ris also found that animals that shed brucellosis organisms in their semen tested negative in the warm CFT but positive in the cold test (26). Burgess and Norris (27), also working on brucellosis, were able to demonstrate that the cold CFT was more sensitive than the warm test. On examining 11,922 sera for brucellosis, they observed that 187 (1.57%) were positive in the cold CFT but only 38 (0.32%) were positive in the warm test.

In the present paper, overnight (16 h to 20 h) incubation at 4°C was used to achieve maximum fixation of complement and the results are in line with other findings indicating that cold CFT is indeed more sensitive (83.6%) than the warm test (56.8%) in the diagnosis of glanders. The differences observed between the two tests in terms of antibody titres of positive sera are also in agreement with the findings of Ris (26) (Fig. 1). Most of the sera that were positive in both CFTs showed one- to two-fold higher antibody titres in the cold test. Moreover, four immunoblot-positive samples collected from a horse after malleinisation tested negative in the warm CFT but positive in the cold test. This observation was also made by Burgess and Norris (27), who found that cold CFT detected brucellosis antibodies in experimentally infected animals up to one week earlier than the warm CFT. Similarly, Ris (26) found that sera with titres between 1:10 and 1:20 in the cold CFT had no measurable titres in the warm test. He concluded that in ovine brucellosis an estimated 1.6% of sera tested false positive with cold CFT.

In the present study, among the 196 true-negative sera in group I, no serum sample tested false positive in the warm CFT; however, in the cold CFT four (2%) sera tested false positive. One possible reason for the slightly increased percentage of false-positive results in the cold CFT might be cross-reactivity with other Gram-negative bacteria, such as *Burkholderia cepacia* and *Pseudomonas* spp. (28, 29, 30).

Conclusion

The CFT has the advantage of being cheap and easy to handle, and this should be borne in mind as current budget cuts in veterinary public health are a concern not only for developing countries. However, further investigation should focus on the production of better quality antigens for use in the test. The cold CFT, with its greater diagnostic sensitivity, is recommended for surveillance and screening programmes; nevertheless, its use depends on the epidemic situation of a country and on the availability of laboratory capacity for identification of the higher number of expected false positives. For import and export testing, the CFT should be supported with use of the highly specific immunoblot in order to avoid inconvenience for private owners and public veterinary health authorities.

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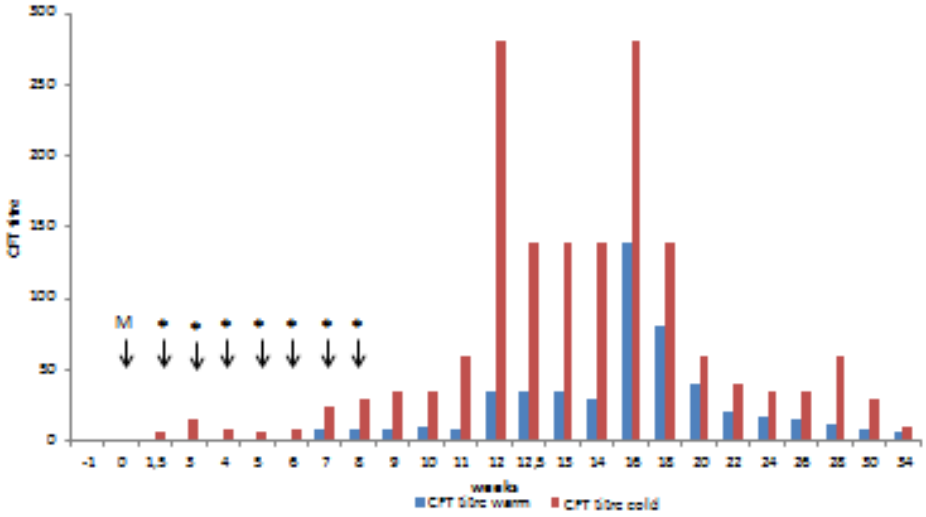
Table I

Warm and cold procedures for complement-fixation testing of sera from glanders-free (Group I) and glanderous and immunised animals (Group II)

Group	No. sera	Nature/origin of sera	Species			Warm CFT			Cold CFT		
			Horse	Donkey	Mule	Positive	Equivocal	Negative	Positive	Equivocal	Negative
I	196	True negative	195	1	0	0	0	196	4[a]	0	192
II	123	Microbiologically and clinically (mallein test) positive glanderous animals from Brazil and Pakistan, immunoblot positive	117	2	4	57	8	58	95	4	24
						53[a]+1[b]+3[c]	8[a]	56[a]+1[b] +1[c]	90[a]+1[b]+4[c]	4[a]	23[a]+1[b]
	23	Immunised horse	23	0	0	17	1	5	22	1	0

a) Horse
b) Donkey
c) Mule



M: application of mallein

*immunisation weekly for 7 weeks

Fig. 1

Comparison of warm and cold complement-fixation titres of serum from a horse immunised against *B. mallei*