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The current challenges of dourine: difficulties in differentiating *Trypanosoma equiperdum* within the subgenus *Trypanozoon*

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Summary

During its 20th annual meeting in Paris in May 1999, the OIE (World organisation for animal health) Ad Hoc Group on Non-Tsetse Transmitted Animal Trypanosomoses expressed the following concerns about dourine:

– the discrepancies in some of the results of the complement fixation test (CFT), which is the only international diagnostic test officially recognised by the International Organisation for the Transportation of Equidae

– the persistence of suspected cases of dourine in some Asian, European and African countries

– the impossibility of differentiating *Trypanosoma equiperdum* from *Trypanosoma evansi* and of isolating new strains of *T. equiperdum* from clinical cases that have appeared in various parts of the world since 1982.

In the light of these concerns, it was decided, in agreement with the Directorate of the Federal Veterinary Services of Russia in Moscow, to perform comparative trials on the value of CFT/dourine at the OIE Reference Laboratory for dourine in Moscow (The All-Russian Research Institute of Experimental Veterinary Medicine) using reagents (antigens and sera) from seven countries with extensive experience in the field of dourine diagnosis, namely, South Africa, France, Italy, Germany, Russia, the United States of America and the People's Republic of China. It is thanks to the successful co-operation of these countries that the trials were made possible. Results showed an overall concordance and were submitted for consideration to the OIE Biological Standards Commission, the commission which is in charge of the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. These trials serve as a starting point for further study, particularly in the following areas:

– the isolation of new strains of *T. equiperdum* from clinical dourine cases

– the identification of specific markers for *T. equiperdum* which would make it possible to differentiate it from among the other species within the subgenus *Trypanozoon*

– the experimental infection of horses with newly isolated *T. equiperdum* strains to compare their pathogenicity with those currently used in national diagnostic laboratories and with that of *T. evansi*

– phylogenetic studies

– the proposal and validation of new, internationally recognised diagnostic test(s) for dourine.

Keywords
Diagnosis – Differentiation – Dourine – Research – Surra – Trypanosoma equiperdum – Trypanosoma evansi.

Introduction

Among the Non-Tsetse Transmitted Animal Trypanosomoses (NTTAT), dourine is included in List B of the OIE (World organisation for animal health) notifiable diseases lists (42). Due to the strict enforcement of control measures, dourine declined quickly in most parts of the world at the beginning of the 20th Century (16), particularly from the 1950s onwards. However, the disease still exists in some countries (e.g. Botswana, Lesotho, Namibia, Russia, South Africa) and sometimes, the complement fixation test (CFT), the officially prescribed diagnostic test for the international trade in equines, produces false positive results (45).

The problem of false positive results had already been examined by the OIE Ad Hoc Group on NTTAT (Ad Hoc Group) on the occasion of its first international symposium in 1992 (55). However, the need for further study was demonstrated in 1995 when careful investigation proved that a suspected case of the disease in Mexico was not in fact dourine (38, 39). At their annual meeting in 1998, the members of the Ad Hoc Group (40) were particularly concerned about the difficulty of isolating new strains of *Trypanosoma equiperdum* (which had not been achieved since 1982) and differentiating *T. equiperdum* from *Trypanosoma evansi.* Their recommendations were as follows (40):

– to establish both a repertoire of existing *T. equiperdum* strains and an antibody repertoire of dourine positive horses

– to isolate strains of *T. equiperdum* from recent infections

– to look for a facility where research on horses could be carried out

– to evaluate existing diagnostic tests for trypanosome infections of both horses with clinical dourine and healthy horses.

'Once sufficient isolates from clinical dourine horses are available a comparison should be made between *T. equiperdum* and *T. evansi* strains at the molecular level.'

This recommendation was taken up and developed at the 1999 annual meeting of the Ad Hoc Group (41) and, because of the world animal health situation in 1999 (43), again in 2000 (44). In particular, it was decided to undertake a comprehensive study of *T. equiperdum* within the subgenus *Trypanozoon* (12), with the aim of developing improved means for diagnosing and controlling dourine. Before that, it was decided that a study of CF testing in various countries should be performed.

Comparing the complement fixation tests used in seven countries to diagnose dourine

Despite the usefulness and the universal acceptance of the CFT for diagnosing dourine, some discrepancies have been recorded. For example, a progressive fading of CFT titre was observed in the chronic stages of the disease (17) and, more importantly, variability of results (positivity or negativity) was noticed in some horses undergoing repeated testing (22). Similarly, there were false positive reactions in horses exported from Mexico in 1995 (38, 39) and reversible reactions in racehorses imported into France in 1998 (41). These variable results ultimately led to the refusal of the authorities to allow these racehorses to be imported, despite subsequent negative CFT results. These cases illustrate the major difficulties in diagnosing *T. equiperdum* infections, namely, positive serological test results even in the absence of clinical symptoms and detectable parasites. As a first step in clarifying the problem with the way in which the CFT is currently performed, the Director of the OIE Reference Laboratory for dourine in Moscow (the All-Russian Research Institute of Experimental Veterinary Medicine [VIEV]), in agreement with the main Directorate of the Veterinary Services of Russia, submitted a proposal to the Ad Hoc Group which was subsequently accepted. The proposal was to conduct comparative trials of the antigens and other reagents in use in seven national dourine diagnosis laboratories, namely, the National Veterinary Parasitological Institute in the People's Republic of China; the Agence française de sécurité sanitaire des aliments in France; the Federal Institute for Consumer Health-Protection and Veterinary Medicine in Germany; the National Veterinary Services Laboratories in the United States of America; the NPO 'Biocenter' in Russia; the Istituto Zooprofilattico dell' Abruzzo e del Molise in Italy; and the Onderstepoort Veterinary Institute in South Africa.

All the received antigens were in lyophilised form, excluding the antigen from France. The results of this comparative trial are set out in Table I.

Table I
The results of comparative tests on the complement fixation tests used in seven countries to diagnose dourine

Origin of the serum	Antigen of *Trypanosoma equiperdum*						
	People's Republic of China Titre 1:16	United States of America Titre 1:64	Russia Titre 1:50	France Titre 1:20	Germany Titre 1:32	Italy Titre 1:40	South Africa Titre 1:125
United States of America: positive							
Titre 1:20	1:10 2+	1:20 2+	1:20 3+	X	1:20 2+	1:10 3+	1:10 2+
Titre 1:20-1:40	1:40 3+	1:80 2+	1:80 3+	1:20 2+	1:80 2+	1:80 2+	1:40 2+
High titre	1:320 3+	1:160 2+	1:320 2+	1:80 2+	1:160 2+	1:160 2+	1:160 2+
Russia: positive							
Titre 1:10	1:10 2+	1:10 2+	1:10 2+	X	1:10 2+	1:10 2+	1:10 2+
Titre 1:40	1:40 2+	1:40 2+	1:80 2+	1:20 2+	1:40 2+	1:40 2+	1:40 2+
France: positive							
Titre 1:10	1:40 2+	1:20 2+	1:40 2+	1:5 3+	1:20 2+	1:40 2+	1:20 3+
Germany: positive							
Titre 1:80	1:40 2+	1:80 2+	1:80 2+	1:10 2+	1:40 3+	1:40 2+	1:40 2+
Titre 1:320	1:80 3+	1:160 3+	1:160 3+	1:20 3+	1:160 2+	1:80 3+	1:80 2+
Italy: positive							
Titre 1:96	1:40 3+	1:80 2+	1:160 3+	1:5 3+	1:40 2+	1:20 3+	1:20 2+
South Africa: positive							
Titre 1:32 4+	1:40 2+	1:80 3+	1:80 3+	1:20 2+	1:40 2+	1:40 2+	1:80 2+
USA: negative	negative	negative	negative	negative	negative	negative	negative
Germany: negative	negative	negative	negative	negative	negative	negative	negative
Russia: negative	negative	negative	negative	negative	negative	negative	negative
Italy: negative	negative	negative	negative	negative	negative	negative	negative
South Africa: negative	negative	negative	negative	negative	negative	negative	negative

X: does not work
Source: V.T. Zablotsky & Ch. Georgiu, The All-Russian Research Institute of Experimental Veterinary Medicine, Laboratory of Protozoology, 109472, Moscow, Kuzminki, Viev

Comments on these trials

None of the national laboratories which participated in the trials passed comment on their results, but the OIE Reference Laboratory in Moscow reported that: 'As it is visible from Table I, the comparative trials of the diagnostic value of the above mentioned antigens, excluding antigen sent from France, did not show any one antigen to be better than another.

The low sensitivity of the antigen from France was probably as a result of it being sent in dissolved form and being kept in warm conditions for too long.

Overall, the results demonstrated the reliability of the CFT for the diagnosis of dourine in eradication campaigns.

This comparative trial, the result of successful international co-operation, highlighted the difficulties of transporting parasite antigens around the world. The conditions in which they are kept, during transit and at custom storage premises, are particularly problematic. It would be useful to bring this to the attention of the International Air Transport Association to avoid costly redispatching.

Other diagnostic methods

There are many examples of the usefulness of the CFT; it has been used very effectively in numerous national dourine eradication programmes throughout the world, e.g. Canada (16), Belgium (12), Poland (17), Yugoslavia (22), Ethiopia (14), France (50), India (21), Italy (10), Morocco (19), Romania (11), Russia (7, 28, 62), Spain (36), South Africa (4, 66), Tunisia (3) and Turkey (2).

However, the CFT is not species specific, but only specific for the genus *Trypanosoma* (46). The diagnostic significance of this test is therefore doubtful in countries where both *T. equiperdum* and *T. evansi* infection occur in equines. This group reaction of CFT with an antigen of *T. equiperdum* has also been used to diagnose human sleeping sickness (47) due to *T. gambiense* and for the diagnosis of surra in camels due to *T. evansi* (20).

The alternative serological test methods for dourine, as stated in the 4th Edition of the OIE *Manual of standards for diagnostic tests and vaccines* (45), are the indirect fluorescent antibody test and the enzyme-linked immunosorbent assay (ELISA). The

disadvantages of the CFT are that it requires careful continuous titration of numerous labile agents and that it does not function with sera having anticomplementary activity. According to J.B. Katz *et al.*, 'The test interpretation is often subjective, test sensitivity is relatively low compared with more modern assay methods, and the sensitivity of the CFT declines as the serologic responses of exposed animals shift from initial IgM-based reactions to those of other immunoglobulin classes and subclasses' (27).

Several ELISA techniques have been published (6, 53, 65, 67) and/or tested (1, 23) and there are also several other alternative serological tests that are used, such as the agar gel immunodiffusion test (23), the arrayed immunodiffusion method (23) and the competitive immunoassay (cELISA). The cELISA method has several advantages over the CFT: it can be performed in less time than the corresponding CF procedure, it is reproducible, results are objectively measured and calculated and the method is amenable to automation (26).

Recent developments in nucleic acid diagnostic techniques (e.g. polymerase chain reaction [PCR], restriction fragment length polymorphism, random amplification of polymorphic DNA, etc.) have made it possible to reliably identify trypanosomes from among the subgenus (*Trypanozoon*) (34, 70).

Parasitological diagnosis

Direct parasitological demonstration of *T. equiperdum* is unusual as it was always very difficult to isolate the organism directly from either the blood or pathological secretions (œdemas, plaques) of infected horses, even at the earliest stages of the infection (49, 51). However, several authors succeeded in isolating strains either directly from the blood (5, 29, 51) of equines thought to be infected (24, 32, 37) or from laboratory animals injected intraperitoneally [rabbits (47), rats (31) and mice (33)] or subcutaneously [dogs (32)]. The ease with which a strain was established in rats and the absence of localisation in the genitalia of rabbits were considered evidence of *T. evansi* (30).

As far as isolation from blood is concerned the parasites are usually found, after cautious pipetting and careful examination of relevant blood smears (5), in the plasma column just above the buffy-coat layer.

The susceptibility of dogs to *T. equiperdum* is generally high (37, 49, 51) and this means that strains can be sent from remote countries after the animals have been experimentally infected. However, some breeds, like the pariah dogs in India, are less susceptible or sometimes even completely resistant.

Before World War I, there was no record of *T. equiperdum* having been isolated in Russia. However, it was thought that rabbits might be useful in differentiating between *T. evansi* and *T. equiperdum* because the lack of localisation in the genitalia of this animal species clearly shows horse trypanosome to be *T. evansi*. After World War II, strains of *T. equiperdum* were successfully isolated by the intratesticular injection of rabbits with blood or material from infected horses.

The dourine status of other countries and the eradication methods applied

As previously mentioned, there are still some countries reporting dourine, or suspected cases of dourine, to the OIE (41, 43). However, dourine is written about in international publications from Asia to Africa (5, 28) and it is a regular topic of discussion at international meetings (35, 58, 59, 60). In Europe and in the Asian part of Russia, dourine is strictly controlled by measures put in place after CF testing. These measures can include the segregation and quarantine of reactors, treatment with high doses of trypanocides followed by close surveillance for several months, or the slaughtering of the reactors. No recent data are available for the Central Asian republics (28), except for Kyrgyzstan, which reported, at the 69th OIE General Session in May 2001, that there had been 94 cases of dourine in 2000; all the infected animals were slaughtered.

In tropical Africa, dourine is only regularly reported in the southern part of the continent: in Botswana, Lesotho, Namibia and South Africa (43). In the Sudan, the disease was reported in 1961 in the Nyala area, Southern Darfour, in a donkey mare (58). No further observations have been recorded since then because nobody has been looking for it (35), but it may still exist. For a number of years dourine has been suspected in Eritrea and is studied in Ethiopia in the provinces of Shoa and Arsi (1, 14), but it has never been possible to isolate a strain of *T. equiperdum* from clinically affected horses and donkeys after the diagnosis has been established by an ELISA method or PCR. There are 2.75 million horses, 5.02 million donkeys and 0.65 million mules in Ethiopia (18); because of the rugged mountainous terrain of this country these animals are still the main method of transporting both people and agricultural products. Donkeys, which only show discrete signs of the disease when infected, require special consideration as these animals appear to be reservoirs and carriers of the infection. The same is true of donkeys in other African countries and this merits further study.

In Namibia, the disease occurs throughout the country in horses, mules and donkeys (60). The CFT is very reliable and an essential tool in identifying positive animals. Sera from donkeys tend to cause anticomplementary reactions.

Serological surveys, the results of which can be found in Table II, were conducted for six consecutive years. On many farms where animals tested positive for dourine using the CFT the disease was eradicated by slaughtering or castrating the positive reactors. Dourine can be eradicated quite easily and re-introduction can be effectively prevented (coital transmission) provided farms are properly fenced off and the necessary precautions are taken.

Clinically, the disease is absent in wild equidae (zebras), but this needs further investigation as these animals are often exported to other countries. Namibia is collecting and storing frozen zebra sera with a view to screening for dourine and other equidae diseases once a representative sample size has been collected.

It is important to note that castrating adult stallions does not always change the copulatory ability of such animals and it should be performed with caution when attempting an eradication programme. To prevent the introduction of dourine, serum samples should be taken following a period of isolation (quarantine) to ensure that the animals are not in the incubation period.

Beside the traditional method of eradication, various drugs have been used in an attempt to find a treatment for dourine, e.g. neoarsphenamine (44, 11), suramin (68) and quinapyramine (61). Moreover, in the laboratory, *T. equiperdum* has proved sensitive to the trypanocidal drugs diminazene, melarsomine, and isometamidium (8, 25, 56, 69). However, only neoarsphenamine and suramin have been used in large dourine eradication programmes (2, 19, 28, 50, 62, 63, 64). It is recommended that neoarsphenamine be administered twice in high doses of 40 g to 50 g per adult horse. The decline of the CFT titre should then be monitored until it reaches zero (3). This treatment should be repeated once a year. In donkeys, neoarsphenamine was found to be too toxic (15). For horses, even if high doses are debilitating, such doses are needed to prevent the appearance of *T. equiperdum* chemo-resistant carriers.

Differentiation between *Trypanosoma equiperdum* and *Trypanosoma evansi*

Ever since *T. equiperdum* was first discovered (32, 51), the uniqueness of this 'new' trypanosome has been called into question. Clinically, the diseases caused by *T. evansi* (surra in horses) and *T. equiperdum* (dourine) are quite different. The former is acute and can rapidly lead to death but is relatively easy to cure, or at least improve temporarily with trypanocides, and the latter is a chronic disease that causes paralysis and slow death, and is always difficult to cure with trypanocides. Moreover, clinical symptoms of localisation to genitalia are almost always pathognomonic of dourine. Another way of differentiating between the two diseases is by injecting suspected infected horse blood into laboratory animals; the animals will develop surra within four to five days, whereas dourine requires a considerably longer incubation period (48). However, serologically (with or without mild clinical symptoms), differentiation between the two infections is not possible. Recent data on the phylogenetics of causal trypanosomes show that the parasites are closely related (9, 54) and a comparison (9) demonstrated that there is only a tiny difference between these two species after the hybridisation of their kinetoplastic DNA, i.e. there are only minicircles in *T. evansi* but both minicircles and maxicircles in *T. equiperdum*. This comparison can only be done by examining akinetoplastic strains, which frequently occur with *T. evansi* in South America, without them, such a comparison would be impossible. So far, serological procedures have failed to separate the two species. Molecular and immunological research studies are currently in development (13, 34, 57) and could give rise to promising findings in the near future.

Conclusion

Dourine is often described as a slowly disappearing disease of horses and other equines, which is true for most developed

Table II
The number of equines in Namibia that tested positive for dourine using the complement fixation test (1986-1991)

	1986		1987		1988		1989		1990		1991		Total		
	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Rate
Horses	1,987	93	207	10	667	22	775	28	665	38	357	23	3,758	214	4.7
Donkeys	115	0	0	0	8	3	38	1	63	0	11	0	235	4	1.7
Mules	6	0	0	0	3	0	25	0	11	0	19	2	64	2	3.1
Total	2,108	93	207	10	678	25	838	29	739	38	387	25	4,057	220	5.4
Percentage positive		4.4%		4.8%		3.7%		3.5%		5.1%		6.5%		5.4%	

countries, with a few exceptions. For other countries, mainly in Africa, it is difficult to identify the causal agent and, given the indispensable role played by equines in these countries, the disease deserves more attention. In line with the results of the international comparative trials recently carried out at the OIE Reference Laboratory for dourine in Moscow, racehorses should be systematically checked using the CFT when they are transported from country to country.

The findings of these encouraging comparative trials have highlighted the need for the following:

– the standardisation of the CFT

– a careful study of other reliable and easy to perform alternative diagnostic methods, with a view to possible validation

– the isolation of new strains of *T. equiperdum* from clinical cases, for comparison with existing stocks from specialised laboratories

– a meeting of international dourine specialists, either in an OIE Reference Laboratory for dourine or in another specialised laboratory, to examine all the current problems of dourine, particularly the problem of differentiating between *T. equiperdum* and *T. evansi*.

■

Les problèmes actuels liés à la dourine : difficultés de différenciation de *Trypanosoma equiperdum* au sein du sous-genre *Trypanozoon*

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Résumé

Rassemblés à Paris, en mai 1999, pour leur 20e réunion annuelle, les membres du Groupe *ad hoc* de l'OIE (Organisation mondiale de la santé animale) sur les trypanosomoses animales non transmises par les glossines avaient exprimé un certain nombre d'inquiétudes au sujet de la dourine, à savoir :

– l'existence de différences dans les résultats obtenus par réaction de fixation du complément, alors que ce test est la seule épreuve diagnostique internationale reconnue officiellement par l'Organisation internationale pour le transport des équidés ;

– la persistance de cas suspectés de dourine dans certains pays d'Asie, d'Europe et d'Afrique ;

– l'impossibilité de différencier *Trypanosoma equiperdum* de *Trypanosoma evansi* et d'isoler les nouvelles souches de *T. equiperdum* apparues dans plusieurs parties du globe depuis 1982.

À la lumière de ces préoccupations, la décision avait été prise, conjointement avec la direction des Services vétérinaires fédéraux de Russie (Moscou), d'effectuer des essais comparatifs sur la valeur de l'épreuve de la fixation du complément dans le cas de la dourine. Le Laboratoire de référence de l'OIE pour la dourine (Institut pan-russe de recherche en médecine vétérinaire expérimentale de Moscou) chargé de ces essais devait employer les réactifs (antigènes et sérums) des sept pays possédant une solide expérience dans le diagnostic de la dourine, à savoir l'Afrique du Sud, la France, l'Italie, l'Allemagne, la Russie, les États-Unis d'Amérique et la République populaire de Chine. La réalisation de ces essais marque l'aboutissement d'une coopération fructueuse entre ces différents pays. On a observé une concordance générale des résultats,

qui ont ensuite été envoyés pour examen à la Commission des normes biologiques de l'OIE chargée de la rédaction du *Manuel des tests de diagnostic et des vaccins pour les animaux terrestres*. Ces essais serviront de point de départ à d'autres études, notamment dans les domaines suivants :
– l'isolement de nouvelles souches de *T. equiperdum* à partir de cas cliniques de dourine ;
– la recherche de marqueurs spécifiques de *T. equiperdum* qui permettront de différencier cette espèce des autres espèces appartenant au sous-genre *Trypanozoon* ;
– l'inoculation expérimentale de chevaux avec des souches de *T. equiperdum* récemment isolées en vue de comparer leur pathogénicité à celle de *T. evansi* ;
– les études phylogénétiques ;
– la proposition et la validation de nouvelles épreuves diagnostiques reconnues internationalement pour la dourine.

Mots-clés
Diagnostic – Différenciation – Dourine – Recherche – Surra.

Problemática actual de la durina: la dificultad de distinguir entre *Trypanosoma equiperdum* y otras especies del subgénero *Trypanozoon*

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Resumen
En su vigésima reunión anual, celebrada en mayo de 1999 en París, el Grupo *ad hoc* de la OIE (Organización Mundial de Sanidad Animal) sobre las tripanosomosis animales no transmitidas por glosinas expresó los siguientes motivos de inquietud con respecto a la durina:
– las discordancias existentes en los resultados obtenidos con la técnica de fijación del complemento, que es la única prueba internacional de diagnóstico oficialmente reconocida por la Organización Internacional para el Transporte de Équidos;
– la persistencia de presuntos casos de durina en algunos países asiáticos, europeos y africanos;
– la imposibilidad de distinguir entre *Trypanosoma equiperdum* y *Trypanosoma evansi* y de aislar nuevas cepas de *T. equiperdum* que vienen apareciendo en diversas partes del mundo desde 1982.
A la luz de esa preocupante situación, se decidió, de común acuerdo con la Dirección de los Servicios Veterinarios Federales de Rusia (Moscú), que el Laboratorio de Referencia de la OIE para la durina (el Centro Panruso de Investigación en Medicina Veterinaria Experimental, con sede en Moscú) efectuara ensayos comparativos sobre el rendimiento de la prueba de fijación del complemento aplicada a la durina, utilizando reactivos (antígenos y sueros) procedentes de varios países con dilatada experiencia en el diagnóstico de esa enfermedad, a saber: Sudáfrica, Francia, Italia, Alemania, Rusia, los Estados Unidos de América y la República Popular de China. La realización de esos ensayos comparativos fue posible gracias a la fructífera colaboración que se

estableció con todos esos países. Los resultados obtenidos, que muestran un elevado nivel de concordancia general, fueron transmitidos para su estudio a la Comisión de Normas Biológicas de la OIE, que es el órgano encargado de elaborar el *Manual de pruebas de diagnóstico y vacunas para los animales terrestres*. Esos ensayos constituyeron el punto de partida de ulteriores estudios, centrados especialmente en los siguientes temas:
– aislamiento de nuevas cepas de *T. equiperdum* a partir de casos sintomáticos de durina;
– búsqueda de marcadores específicos de *T. equiperdum* que sirvan para distinguir entre esta y otras especies del subgénero *Trypanozoon*;
– infección experimental de caballos con las cepas de *T. equiperdum* recién aisladas para comparar su patogenicidad con la de *T. evansi*;
– estudios filogenéticos;
– propuesta y validación de una (o varias) nueva(s) prueba(s) internacionalmente reconocida(s) para el diagnóstico de la durina.

Palabras clave
Diagnóstico – Diferenciación – Durina – Investigación – Surra.

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