Veterinary Parasitology 205 (2014) 70–76

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Inter-laboratory ring trials to evaluate serological methods for dourine diagnosis

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ARTICLE INFO

Article history:
Received 18 April 2014
Received in revised form 13 June 2014
Accepted 19 June 2014

Keywords:
Dourine
Surra
Ring trial
Serology
Trypanosoma equiperdum
Trypanosoma evansi

ABSTRACT

To evaluate the reproducibility of routine serological methods to detect *Trypanosoma equiperdum* antibodies in equine sera, two inter-laboratory ring trials were organized involving 22 European and 4 non-European reference laboratories for dourine. The serological methods were the complement fixation test (CFT; 25 laboratories) and the indirect fluorescent antibody test (IFAT; 4 laboratories). Three of the laboratories applied both these methods. The sample panels were composed of sera that were negative, positive or suspected for dourine. Of the negative sera, one was from a donkey naturally infected with *Trypanosoma evansi*. This study confirmed the reliability of CFT and highlighted its inter-laboratory reproducibility for known *T. equiperdum* positive and negative sera. However the reproducibility was less good for sera positive for *T. evansi* or of unknown status, e.i. nine out of 22 laboratories observed a false-positive result with the *T. evansi*-positive serum, whether by CFT or IFAT. This interesting result suggests that the specificity of dourine serodiagnosis may be improved by standardizing the critical reagents, including antigens and by developing a standard *T. equiperdum* serum which could be used calibrate test systems across multiple laboratories. Trial data confirmed seropositivity in one of the three horses suspected of dourine. It may be beneficial to generalize the use of a suitable low-titer serum control, derived from a standard serum in order to standardize the method's detection limit.

1. Introduction

Dourine is a chronic or acute contagious disease of breeding *Equidae* directly transmitted from animal to animal during mating. It is notifiable to the World Organisation for Animal Health (OIE) (2013). An infected mare may also transmit the disease to its foal in the milk or through udder lesions (Brun et al., 1998). Dourine is widespread throughout Asia (Clausen et al., 2003), Africa (e.g. Ethiopia; Hagos et al., 2010a), the Middle East, South America and Eastern Europe (e.g. Russia) (Zablotskij et al., 2003; WAHID database). The only European country where it has been observed in recent decades is Italy, where the last confirmed outbreaks were reported between the 1970s and 1980s and in 2011. There were also sporadic reports of isolated cases in the late 1990s (Pascucci et al., 2013). For effective disease control, the OIE recommends slaughtering infected animals. The causative agent of dourine is *Trypanosoma equiperdum* (World Organisation for Animal Health (OIE), 2013), a protozoan parasite of the subgenus

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http://dx.doi.org/10.1016/j.vetpar.2014.06.025
0304-4017/

Trypanozoon that also includes *Trypanosoma brucei* and *Trypanosoma evansi*. *T. brucei* is cyclically transmitted by tsetse flies and causes "nagana" in domestic and various wild animals, and "sleeping sickness" in humans. *T. evansi* is mainly spread by biting insects through mechanical transmission, and causes "surra" in a very wide range of domestic and wild animals (Brun et al., 1998).

The diagnosis of dourine is usually based on clinical signs (urticarial plaques, edema, genital depigmentation and cachexia) and/or an epidemiologic link to a confirmed case of dourine, together with serological evidence from complement fixation tests (CFTs) (Pascucci et al., 2013; World Organisation for Animal Health (OIE), 2013). However, diagnosis is difficult to establish in areas where both dourine and surra coexist because (i) the clinical signs of dourine are neither pathognomonic nor constant, and are very similar to those of surra (Pascucci et al., 2013; World Organisation for Animal Health (OIE), 2013), and (ii) there are very significant antigenic and genetic similarities between *T. equiperdum* and *T. evansi* (Brun et al., 1998; Claes et al., 2005; Giardina et al., 2003). A relevant distinction is that *T. equiperdum* contains maxicircle kinetoplast DNA while *T. evansi* does not (Brun et al., 1998). This observation could be used to differentiate the two trypanosomes through PCR, as previously described (Li et al., 2007). However, it is often impossible to confirm the presence of maxicircles in *T. equiperdum* since, unlike *T. evansi*, very few *T. equiperdum* parasites are found in infected tissues and parasitemia is both short-lived and mild (World Organisation for Animal Health (OIE), 2013).

According to literature, the dourine CFT is not specific to *T. equiperdum* antibodies. Moreover, CFT requires the careful continuous titration of numerous labile reagents; the interpretation of results is often subjective and it is sometimes not possible to test anticomplementary sera (Zablotskij et al., 2003). Nevertheless, Zablotskij et al.'s (2003) comparative study on antigens and control sera from seven countries demonstrated the reliability of CFT when diagnosing dourine. In addition to the OIE-prescribed CFT, indirect fluorescent antibody test (IFAT) or enzyme-linked immunosorbent assay (ELISA) can be performed as alternative tests for the diagnosis of dourine within a local setting and can be used in the import/export of animals after bilateral agreement (World Organisation for Animal Health (OIE), 2012). IFAT can also be used as a confirmatory test or to resolve cases with inconclusive CFT results (World Organisation for Animal Health (OIE), 2013). In this context, the aims of the present study were to evaluate test reproducibility when the same serological methods (CFT and IFAT) were performed in different laboratories on the same serum panels, and to assess the specificity and sensitivity of these serological methods.

2. Materials and methods

2.1. Participating laboratories

The European Union Reference Laboratory for dourine (ANSES, Dozulé laboratory for equine diseases, France) organized two successive inter-laboratory ring trials. The first, organized jointly with the Health and Veterinary Laboratories Agency (United Kingdom) in 2009, involved 22 laboratories, and the second, in 2012, 23. In total, 26 reference laboratories for dourine from 26 countries participated in one ring trial, and 19 participated in both. Twenty-two represented countries are European Union Member States, while four are non-European. Each laboratory was identified with a number from 1 to 26.

2.2. Sample panel composition and distribution

All the serum samples selected to perform the ring trials were obtained from horses except serum 09-S3, which was from a donkey (Table 1). Sera were stored at 4 °C (lyophilized sera from the United States Department of Agriculture (USDA)) or −20 °C (sera 09-S2 and 12-S1) or −75 °C (all other sera). In order to evaluate repeatability, some sera were included up to four times in the same sample panel (Table 1). In total, 20 samples for the 2009 ring trial and 10 samples for the 2012 ring trial were randomly coded, and 100 μl of each sample was shipped on dry ice to each participating laboratory. It should be noted that the 2009 sample panel was previously decomplemented by heating at 58 °C for 30 min before shipping.

Dourine-negative sera: sera 09-S1 and 12-S2 were obtained from the USDA (National Veterinary Services Laboratories, USA). Sera 09-S2 and 12.S1 were two aliquots of the same 2009-sampled serum from an uninfected French mare (ANSES, Dozulé laboratory for equine diseases, France). Serum 09-S3 was from a Spanish donkey naturally infected with *T. evansi* (University of Gran Canaria, Spain; Institute of Tropical Medicine, Belgium).

Dourine-positive sera: sera 09-S5 and 12-S3 were USDA standard high-titer and USDA low-titer sera, respectively. Serum 12-S4 was prepared by diluting the USDA standard medium-titer serum twice with the negative serum 09-S2. Sera 09-S4 and 12-S5 were two aliquots of the same 2009-sampled serum from a horse experimentally infected with the *T. equiperdum* Dodola 943 strain (Hagos et al., 2010b) (Institute of Tropical Medicine, Belgium; ANSES, Dozulé laboratory for equine diseases, France). Serum 12-S7 was sampled from a naturally infected stallion during the 2011 Italian dourine outbreak (Istituto Zooprofilatico Sperimentale Teramo, Italy), and serum 12-S6 was an equal v/v mix of sera 09-S4 and 12-S7.

Dourine-suspected sera: sera from three Mongolian horses (designated A–C) imported into Germany in 2006 and suspected of dourine were also added to the 2009 and 2012 sample panels (ANSES, Dozulé laboratory for equine diseases, France). These dourine cases had not been confirmed because the serological results from several different testing laboratories were inconsistent and because the animals showed no clinical manifestation of dourine (unpublished data presented in 2007 during the annual meeting of the OIE ad hoc group on non-tsetse transmitted animal trypanosomes in Paris, France). Sera 09-S6 and 09-S7 were sampled in 2009 from mare A and its foal B, respectively, foal B being born in Germany that year. Sera 09-S8 and 12-S8 were sampled from another mare, designated mare C in 2009 and in 2012. Serum 12-S9 corresponds to serum 12-S8 diluted twice with negative serum 09-S2.