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Serodiagnosis of glanders by dot-ELISA using various antigens

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Glanders is a highly contagious, acute or chronic, usually fatal disease of solipeds (Acha and Szyfres 2003) caused by the gram-negative bacillus *Burkholderia mallei* (Garrity *et al*. 2005). The disease typically has a progressive course and poses a significant human health risk as it is an important occupational disease. The clinical and bacteriological diagnosis of glanders is difficult in a large population of equines, especially in the early stages, or if the disease is latent. There is no proper treatment or vaccine available for glanders (Wagg *et al*. 2006) therefore, its diagnosis, prevention and control are important.

Considering the glanders as a reemerging disease in India (Anonymus 2008), it is important to have a quick and effective reliable diagnostic tool. Complement fixation test is considered as confirmatory test, but many times it failed to detect confirmed cases (Zhang and Lu 1983), particularly for testing sera of mules, hinnies and asses owing to the high degree of anticomplement activity (Verma *et al*. 1990). Therefore, dot-ELISA with various antigens, viz. CFT antigen, mallein PPD and recombinant antigen was standardized for the diagnosis of glanders.

Serum samples (248) belonging to glanders affected and apparently normal equines were used for the present study. These samples were from Punjab, New Delhi, Maharashtra, Karnataka, Andhra Pradesh, Uttar Pradesh, Himachal Pradesh, Haryana and Uttarakhand.

The complement fixation test (CFT) antigen (*Pseudomonas mallei* pro RVK, Bioveta, a.s. Ivanovice na Hane, Czech Republic), Mallein purified protein derivative (PPD) (Indian Veterinary Research Institute, Izatnagar and recombinant antigen (Defence Research Development Establishment, Gwalior, Madhya Pradesh) were used in the present study. Protein content of the antigens was determined as per Lowry *et al*. (1951).

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Dot enzyme-linked immunosorbent assay was standardized with the above antigens for the diagnosis of glanders as per Verma *et al*. (1990) with modifications (John 2008). The optimum concentration of each antigen to be used in the test proper was determined by checker-board titration using known glanders positive and negative horse sera. The optimally diluted antigen was dotted on to the centre of the separate nitro-cellulose membrane bounded on the dipsticks and were dried. Unbound nitro cellulose membrane sites were blocked by albumin bovine serum (3%) -PBS (pH 7.2). After washing with PBST, these strips were immersed in optimally diluted serum samples and then in the optimally diluted conjugate (HRP conjugated rabbit anti-horse IgG). After incubation at 37°C for 30 min these strips were again washed with PBST and were dipped in substrate solution (DAB) and the reactions were stopped using distilled water. Evaluation of diagnostic test was done as per Thrusfield (2005) by preparing 2×2 contingency table (Table 1).

Out of 248 sera, 171 were from horses, 75 from mules and only 2 from donkeys and 88.7% sera were from animals of age 3 years and above (adult) and 11.3% were from animals of age below 3 years (young). Considering a CFT titre of 16 and above as positive and a titre of 8 as suspected, the sera used in the test were classified as positive (11), suspected

Table 1. Classification of dot -ELISA results for the detection of glanders from field equine serum samples

Test (dot-ELISA)		Disease status		Total
		Diseased	Non-diseased	
CFT antigen	Positive	11	6	17
	Negative	0	231	231
Total		11	237	248
Mallein PPD	Positive	10	6	16
	Negative	1	231	232
Total		11	237	248
Recombinant antigen	Positive	11	6	17
	Negative	0	231	231
Total		11	237	248

(45) and negative (192). The glanders positive samples were from Maharashtra (4), Uttarakhand (3), Uttar Pradesh (2) and Punjab (2). All the 11 positive samples were from adult equines. The glanders suspected samples exhibited varying CFT titres ranging from 4 to 32 along with anti-complementary reaction and the negative samples have shown either no titre or a CFT titre less than 4.

In the present study, the optimum dilution of CFT antigen and mallein PPD was 1: 100 and for the recombinant antigen 1: 50, which were used in the test proper for coating the nitrocellulose membrane. The total protein content used was 131.52, 139.98 and 138.72 ng for CFT antigen, mallein PPD and recombinant antigen respectively. The optimum dilution of conjugate was 1: 7000 by checker-board titration. During optimization of antigen and conjugate, it was observed that brown-coloured dot on the nitrocellulose membrane at the antigen coated site was clearly visible up to 1: 200 dilution of known glanders positive serum sample and therefore a dilution of 1: 200 is used for sera for further dot-ELISA test-proper.

Dot-ELISA with CFT antigen and recombinant antigen was positive in all 11 glanders positive serum samples (8 horses and 3 mules), whereas dot-ELISA with mallein PPD did not show positive reaction only in one sample of horse. This particular serum sample was from adult horse of Maharashtra in which IHA with mallein PPD was also negative. This might be due to absence of agglutinating and ELISA antibodies against mallein PPD since it was negative on repeated testing also.

Dot-ELISA with CFT antigen, mallein PPD and recombinant antigen detected 6 serum samples as positive out of 45 suspected field sera of mules. These were of mule's sera which were collected within 1 week of mallein testing leading to transient seroconversion as reported by Hagebock *et al*. (1993). All the 192 negative sera samples (163 horses, 27 mules and 2 donkeys) were also negative by dot-ELISA with CFT antigen, mallein PPD and recombinant antigen.

The analysis of diagnostic test characteristics of dot-ELISA with various antigens for the diagnosis of glanders was evaluated. The sensitivity of dot-ELISA with CFT and recombinant antigens was 100% and with mallein PPD 90.91%, whereas specificity of dot-ELISA remained 97.47% with all the 3 antigens (Table 2). The degree of sensitivity of dot-ELISA was similar to that reported by Verma and Misra (1989) and Verma *et al*. (1990).

Table 2. Diagnostic test characteristics of dot-ELISA with various antigens

Test characteristics (dot-ELISA)	CFT antigen	Mallein PPD	Recombinant antigen
Sensitivity (%)	100	90.91	100
Specificity (%)	97.47	97.47	97.47
Positive predictive value (%)	64.71	62.5	64.71
Negative predictive value (%)	100	99.57	100
True prevalence (%)	4.44	4.44	4.44
Apparent prevalence (%)	6.86	6.45	6.86
Accuracy of prediction (%)	97.58	97.18	97.58
Error of prediction (%)	2.4	2.8	2.4

The present study on serodiagnosis of glanders using various antigens revealed that, the dot-ELISA with CFT or recombinant antigen can be used for field diagnosis of glanders. However, the CFT antigen for the diagnosis of glanders was not readily available in India and was procured from Czech Republic during the present study. The recombinant antigen can be prepared using cloned gene in *E. coli* without involving the risk of handling the zoonotic microbe (*B. mallei*), hence this may be the preferential antigen for dot-ELISA. Moreover dot-ELISA was found to be a simple, rapid, sensitive, economical field test which detects minute levels of antibodies earlier than CFT and IHA (Verma and Misra 1989, Verma *et al.* 1990) as it is a primary binding assay (Nichols and Nakamura 1986).

SUMMARY

Glanders is notifiable in India. The standardization of dot-ELISA with complement fixation test antigen, mallein purified protein derivative and recombinant antigen revealed 131.52 ng, 139.98 ng and 138.72 ng protein as optimum for coating the nitrocellulose membrane with regards to each antigen respectively. A dilution of 1: 200 was optimum for known glanders positive and negative serum samples during optimization of antigens and the conjugate (1: 7000). The analysis of the diagnostic test characteristics of dot- ELISA in reference to conventional complement fixation test, revealed 100% sensitivity with CFT antigen and recombinant antigen and 90.91% sensitivity with mallein PPD and 97.47% specificity with all the 3 antigens used.

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