Summary: The authors describe a rapid and simple dot immunobinding assay (DIA) for detection and identification of equine herpesvirus-1 antigen in field samples from cases of abortion, stillbirth, perinatal foal mortality and paralysis. The assay employs a nitrocellulose membrane to which antigen is adsorbed as a dot. Antigen is identified as a coloured dot using a procedure based on the principle of enzyme-linked immunosorbent assay (ELISA). In all, 61 samples were tested by DIA and the test was compared with conventional agar gel immunodiffusion (AGID). With DIA, 44 (72%) samples gave positive results, while only 22 of 61 (36%) samples tested positive by AGID. DIA was observed to be rapid, more sensitive and more specific than AGID, in addition to the obvious advantage of being reagent-conservative, inexpensive and simple to perform.


INTRODUCTION

Equine herpesvirus-1 (EHV-1) is an alphaherpesvirus and belongs to the family Herpesviridae. It is the most important cause of abortion in equines and may also cause paralysis, stillbirth, perinatal foal mortality and respiratory disease in young foals (5, 7, 12, 13, 18, 25). The epidemiology of the virus is complicated by the establishment of latency in the host; the site of latency of the virus is still unknown (15). One of the most serious aspects of EHV-1 infection is that natural immunity is incomplete or short-lived, and re-infection with the same or related strains has been documented (9). Although vaccines are available, these do not provide an adequate spectrum or duration of protection and require repeated administration (10, 11, 14) which renders disease control programmes much less effective. In order to formulate an effective control strategy against EHV-1 infection, the development of a definitive diagnostic test is of paramount importance. Diagnosis may be performed by histopathological examination of infected tissues; the demonstration of Cowdry type A inclusion bodies, the isolation of virus in cell culture and the detection of viral antigen by direct immunofluorescence.
have all been found useful to a certain extent (27). Serological tests including agar gel immunodiffusion (AGID), serum neutralisation, complement fixation (CF) and indirect haemagglutination (IHA) have been employed by various workers to demonstrate antibodies against EHV-1 (16, 26, 30, 31).

However, the demonstration of antibodies alone has limited significance with respect to diagnosis of EHV-1, since the virus is highly endemic and establishes latent infection without showing any clinical signs and symptoms. The development of a simple, rapid test where virus and/or viral antigen could be demonstrated in field samples would be very useful. The present communication describes a simple, rapid and sensitive dot immunobinding assay (DIA) which uses “dipsticks” as the reagent carrier. The performance of the DIA has been evaluated by comparing the test with agar gel immunodiffusion, which is conventionally used to identify EHV-1 antigen in infected tissue materials in many laboratories.

MATERIALS AND METHODS

Field samples

Tissue samples from 61 cases of abortion and stillbirth were supplied in 50% phosphate glycerol buffer for the diagnosis of EHV-1. Samples included pieces of lung, liver, spleen, kidney and heart from aborted foetuses and dead foals. Of 61 samples tested, 85% were from cases of abortion and 15% from stillborn foals or perinatal deaths.

Antigen preparation

To prepare the antigen, pieces of tissue were triturated as a 10% (w/v) suspension in phosphate-buffered saline (PBS), precipitated by PEG 6000 and eluted in tris buffer (pH 7.6) (30).

Hyperimmune serum

EHV-1 hyperimmune antiserum was prepared in guinea-pigs by giving three intramuscular injections (0.5 ml/inoculation) of the standard “Pneumabort-K” vaccine at intervals of two weeks. Serum obtained from a pre-immunised guinea-pig served as the negative control.

Dot immunobinding assay procedure

The protocol for DIA described by Hawkes (19) was followed, with some modifications. Antigen (1 µl) was adsorbed on the nitrocellulose membrane of the dipsticks and air-dried. The antigen was dotted three to four times at the same spot. The dipsticks were wetted in PBS (pH 7.2) containing 0.05% Tween 20 (PBST) and the remaining non-specific adsorptive sites were saturated by keeping the dipsticks in a solution of 5% spray-dried skimmed milk powder in PBST for two hours at 37°C. This was followed by three washings in PBST (5 min each). The dipsticks were then immersed in hyperimmune anti-EHV-1 guinea-pig serum (1:10 in PBST) for two hours at 37°C, followed by three washings in PBST (5 min each). The dipsticks were incubated with peroxidase-conjugated anti-guinea-pig immunoglobulin (Ig)G (1:1,000 in PBST) for 30 min at 37°C. After a final washing in PBST, the dipsticks were placed in the substrate solution (5 mg diaminobenzidine tetrahydrochloride [DAB] dissolved in 10 ml PBS [pH 7.2] with 10 µl 30% H₂O₂). Colour development was allowed to occur for 15-20 min.
at room temperature and the enzyme reaction was stopped by washing the dipsticks in distilled water. The dipsticks were air-dried and the colour development was recorded.

**Agar gel immunodiffusion**

AGID was performed following the technique described by Tewari and Prasad (30). Briefly, the gel medium consisted of 1% agarose (w/v) in a gel buffer (3.0 g PEG 6000, 37 mg ethylenediamine tetra-acetic acid, 3.5 ml 4 M NaCl, 1 ml 1 M tris HCl [pH 8.3] and 20 mg sodium azide per 100 ml of distilled water). Hyperimmune anti-EHV-1 guinea-pig serum (30 μl) was placed in the central well and positive antigens in the two opposite peripheral wells. The remainder of the peripheral wells were filled with test antigens (70 μl each). The slide was incubated for 24-48 h at 25°C. Test antigens showing a precipitin line identical to that of the positive antigen were considered positive. The bands were stained with 0.1% Coomassie brilliant blue (28).

**RESULTS AND DISCUSSION**

In order to determine the optimal dilution of anti EHV-1 hyperimmune serum and enzyme antibody conjugate, various dilutions were employed. Optimal results with respect to intensity of dot colour were obtained using serum at a dilution of 1:10 with antibody conjugate at 1:1,000 dilution.

Correlation and comparison between the results of DIA and AGID for the detection of EHV-1 antigen is shown in Figure 1. Of 22 samples positive by AGID, 20 samples also tested positive by DIA. A total of 44 samples tested positive by DIA, which means that 24 samples which tested negative by AGID were positive by DIA. However, two samples (3.2%) which tested positive by AGID gave negative results by the DIA (Table I). This difference cannot be explained without recourse to extensive testing by other procedures, such as virus isolation or Western blotting. As with all methods, variation occurs and in this case it may be due to the fact that in the AGID test, antigens react by diffusion through the gel medium, while in DIA the solid nitrocellulose membrane immobilises all the antigens. It is also possible that different types of antigen may react in each test. The results show a 57.4% correlation in DIA and AGID specificity. In addition, DIA is 39.4% more sensitive than AGID under identical conditions (Fig. 1). Furthermore, the results of AGID could not be always obtained after incubation for 24 h, while DIA results were obtained within four to five hours.

**Table I**

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIA</td>
<td>44 (72%)</td>
<td>17</td>
<td>61</td>
</tr>
<tr>
<td>AGID</td>
<td>22 (36%)</td>
<td>39</td>
<td>61</td>
</tr>
</tbody>
</table>

Although other methods exist which are also specific and sensitive for EHV-1 diagnosis, DIA is favourable in terms of time, labour, specificity, sensitivity and economy. In earlier studies, Tewari and Prasad (30) reported AGID to be more rapid
a) correlation = 57.4% (20 + 15/61)
b) sensitivity = 39.4% (24/61)
c) false positive and/or cross-reaction = 3.2% (2/61)

**FIG. 1**

**Correlation and comparison between a dot immunobinding assay (DIA) and the agar gel immunodiffusion (AGID) test for the detection of equine herpesvirus-1 antigen**

A total of 61 samples were tested by both tests and the figure indicates the number of samples in each category

and simple than viral isolation in cell culture and identification by virus neutralisation. However, the present results show that DIA is even more rapid and less complex than AGID. In addition, the amount of reagents required for DIA is quite small compared to AGID. Since DIA uses 1:10 diluted serum to give a clearly visible dot, correspondingly smaller amounts of the antigen can be detected (because AGID uses the same serum undiluted). The dipsticks can be stored for a long time without any loss of the brown colour dot, while AGID slides cannot be stored without staining.
In a similar comparison between AGID and DIA for the detection of bluetongue virus (BTV) antibodies, DIA has been found to be superior (3, 17). Heberling and Kalter (20) reported a good correlation between the immunofluorescence technique and DIA in determining the antibody status of vaccinated humans and dogs in relation to rabies virus. Sensitivity of DIA was comparable to immunofluorescence, but DIA was more rapid. Several workers have compared DIA with the microplate ELISA for a number of different antigens including pseudorabies virus, rinderpest virus, BTV, lymphatic filariasis, retrovirus and Entamoeba histolytica (1, 2, 4, 6, 21, 22, 23, 29). The advantages of DIA over microplate ELISA include the fact that the nitrocellulose membrane is able to bind antigen more rapidly and efficiently than polystyrene plates (1, 24); DIA also gives fewer false-positive results and requires shorter incubation periods (4). DIA has been found to be a more promising test for the diagnosis of human immunodeficiency virus (HIV) antigen than the reverse transcriptase assay (8). Recently, Verma and colleagues (32) reported that even whole blood can be used for DIA instead of serum to diagnose glanders in equines. They also found that DIA showed positive titres in most of the animals earlier than CF and IHA. This implies that DIA may be a better test for detecting glanders at an early stage.

ACKNOWLEDGEMENTS

This work forms a part of MVSc thesis for Dr Richa, and is also supported by grants from the University Grants Commission in New Delhi (India) to Dr S. Charan, under the Research Scientist Scheme. Assistance rendered by Mr Rajpat and K. Prasad is gratefully acknowledged. The authors also wish to thank the Head of the Department for providing the necessary facilities for this study.

* *


Résumé : Les auteurs décrivent une méthode d'immuno-adsorption sur filtre (dot immunobinding assay : DIA), rapide et simple, appliquée à la détection et à l'identification de l'antigène herpès-virus-1 équin dans des prélèvements de terrain à partir de cas d'avortement, de mortalité prénatale du poulain et de paralysie. La méthode utilise une membrane de nitrocellulose où l'antigène adsorbé sert de marqueur. Le marqueur de couleur permet de reconnaître l'antigène à l'aide de la technique enzyme-linked immunosorbent assay (ELISA). En tout, 61 prélèvements ont été soumis à l'épreuve DIA et les résultats comparés à ceux de la technique classique de l'immunodiffusion en gélose (IDG). Avec l'épreuve DIA, 44 (72 %) des prélèvements ont donné des résultats positifs contre 22 (36 %) avec la technique IDG. La méthode DIA s'est avérée rapide, plus sensible et plus spécifique que l'IDG ; elle offre en outre l'avantage incontestable d'utiliser peu de réactifs biologiques, d'être peu chère et simple à réaliser.

Resumen: Los autores describen una prueba de inmunoadsorción sobre filtro (dot immunobinding assay: DIA), rápida y sencilla, que se aplica a la detección e identificación del antígeno herpesvirus-1 equino en muestras obtenidas en el terreno a partir de casos de aborto, de mortalidad perinatal, de mortalidad perinatal del potrillo y de parálisis. Esta técnica se vale de una membrana de nitrocelulosa en que el antígeno adsorbido sirve de marcador; el marcador de color permite reconocer el antígeno según la técnica ELISA (enzyme-linked immunosorbent assay). Sesenta y una muestras en total se sometieron a esta prueba DIA, y sus resultados se compararon con los obtenidos a partir de la técnica clásica de inmunodifusión en gelosa (IDG). Cuarenta y cuatro (72%) de las muestras sometidas a la prueba DIA arrojaron resultados positivos, contra 22 (36%) con la IDG. La prueba DIA se mostró rápida, más sensible y más específica que la IDG, a lo que debe agregarse la ventaja evidente de necesitar pocos reactivos biológicos y ser barata y de realización sencilla.


REFERENCES


