Antigenic Differentiation of Equine Herpes Virus-1 (EHV-1) Isolates of Indian Origin using Monoclonal Antibodies

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A panel of seven monoclonal antibodies (Mabs) (5 non-neutralizing and 2 neutralizing) raised against 140 kDa polypeptide of EHV-1 strain (Hisar-90-7) were used for antigenic characterization of five Indian isolates and compared with reference strain (592) of EHV-1. Jind-96 and Hisar-90-7 were antigenically indistinguishable from reference strain in indirect enzyme-linked immunosorbent assay (ELISA) using 5 non-neutralizing Mabs. The antigenic differences were observed in three EHV-1 isolates i.e. Tohana-96-2, Delhi-98 and Raj-98 as they failed to react with 5, 4, and 1 of five non-neutralizing Mabs, respectively. While all the six virus isolates were neutralized with Mab 9C4 in virus neutralization test (VNT), two (Tohana-96-2 and Raj-98) of the 6 viral isolates were not neutralized with one Mab, IH6. However, EHV-1 isolates could not be differentiated on western blot analysis as all the Mabs reacted with a 140 kDa protein in these isolates. The findings of the ELISA and VNT using 7 Mabs indicate that initial isolates (Hisar-90-7 and Jind-96) are antigenically closer to reference strain (592) and there is emergence of antigenically different isolates (Tohana-96-2, Delhi-98 and Raj-98) subsequently. The study also establishes that more than one antigenically different strains of EHV-1 are circulating in equines of northern India.

Keywords: equine herpes virus-1, monoclonal antibodies, antigenic differentiation, ELISA, virus neutralization, western blotting

Introduction

Since the first report of equine herpes virus -1 (EHV-1) associated abortion in mares in 1965 (Sharma et al, 1965), the infection with this virus has been well established among horses in India. The virus has been isolated and characterized from the tissue of aborted foetus (Jain et al, 1976; Singh et al, 1991), still birth and foal mortality (Jain & Ram, 1980), neurological disorders (Shankar & Yadav, 1986) and respiratory syndrome (Tewari & Prasad, 1983). During 1989, the National Research Centre on Equines (NRCE), Hisar, reported involvement of EHV-1 in an abortion storm among mares at an army stud in northern India. In this outbreak, among 360 pregnant mares, 54 abortions (15%) occurred (Uppal et al, 1991). In a national assessment of EHV-1 infection among equidae in India, the overall seropositivity to EHV-1 was 13.5% (349/2573) (Singh et al, 1998).

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The antigenic and genetic differences among two previous subtypes of equine herpes virus viz. EHV-1, subtype 1 and EHV-1 subtype -2 led to their classification as separate types i.e. EHV type 1 and EHV type 4 (Thompson et al, 1978; Allen et al, 1983; Sabine et al, 1981; Studdert et al, 1981). Although all isolates of EHV type 1 (EHV-1), are antigenically closely related, minor variations in the neutralizing activity for different isolates with polyclonal sera revealed the existence of antigenic heterogeneity of EHV-1 circulating in the field (Thompson et al, 1978). Conclusive evidence for the existence of antigenic diversity within EHV-1 was established by use of the panel of monoclonal antibodies(Mabs) (Yeargan et al, 1985; Shimizu et al, 1989). The antigenic variations within Indian EHV-1 isolates have not been studied due to non-availability of Mabs. The information about antigenic differences among EHV-1 isolates has direct application for studying the pathogenesis and development of effective vaccine against EHV-1. The present study was aimed to detect antigenic differences among EHV-1 virus isolates of Indian origin using Mabs developed at NRCE.
Materials and Methods

Cells
Equine embryonic dermis (EED) cell line obtained from National Centre for Cell Science, Pune was used for virus cultivation, titration and neutralization experiments. The cell line was cultivated as per standard technique.

EHV-1 Isolates
Five virus isolates were recovered from samples of aborted mares and foal mortality collected from different outbreaks, places and point of times. In addition, a reference strain of EHV-1 (592) was also included in the study (Table 1). All virus isolates were propagated from initial passage level frozen aliquots (−70°C) samples by inoculating EED at a 0.001 multiplicity of infection (m.o.i.) and infected cells were maintained in minimum essential medium supplemented with 2% foetal calf serum and 5% CO₂ incubator till 80% infected cells showed cytopathic effect (CPE). The virus was purified by the discontinuous sucrose gradient method (Sinclair et al., 1989).

Mabs
Mabs used for antigenic differentiation of EHV-1 isolates in the present study were developed against abortigenic EHV-1 strain (Hisar-90-7) (Singh et al., 2001). Properly cloned EHV-1 antibody secretory hybridoma against Hisar-90-7 was inoculated intraperitoneally in BALB/c for development of ascites fluid. This fluid was purified by ammonium sulphate and used as Mabs (Sinclair et al., 1989). Seven Mabs (1H6, 9C4, 6A1, 6A2, 6B1, 6D1 and 9D4), all directed against 140 kDa polypeptide, were used.

ELISA
The indirect ELISA (Allen et al., 1988) was performed using diluted stock of immunoglobulins purified from ascitic fluid (1:500) with purified EHV-1 antigen. Briefly, 96 well flat bottom microassay plate (Falcon, Bectson Dickinson Labware, Oxnard, CA) was coated with approximately 1 µg/well of purified intact virus overnight at 4°C in coating buffer (0.05 M NaHCO₃/Na₂CO₃, pH 9.6). After washing 3 times with phosphate buffer saline (pH 7.2) containing 0.05% Tween 20 (PBS-T), the test Mabs (1:500) were added to the wells and incubated for 90 min. EHV-1 antigen coated wells in which purified ascites of non-secretary clone (6B6) was added, served as negative control. This was followed by successive addition of an optimum dilution of rabbit antimouse horse radish peroxidase (HRPO) IgG-conjugate diluted 1:1200 in 0.5 mg/ml bovine serum albumin. After washing 5 times, 0-phenylenediamine substrate (OPD) was added. All assays were carried out in triplicate wells and mean OD₄₉₂nm value of two separate experiments were recorded. Mabs were considered to be positive when the mean of the positive/negative ratio (P/N) i.e. OD₄₉₂nm for test Mab divided by OD₄₉₂nm negative control was ≥ 2.0.

Micro Virus Neutralization Test (VNT)
In EED cells, VNT was done to detect virus neutralization ability of Mabs by constant virus-variable serum method (Singh et al., 1995). Each test was done in the presence of 100 TCID₅₀ (50% tissue culture infective dose) of virus isolate in 25 µl quantity in quadruplicate wells containing doubling dilution of Mab and in the presence of 16 units of guinea-pig complement (GPC'). The mixture was then incubated at 37°C for 2 hrs. EED cells (1.7×10⁵ cells/ml) were added in minimum essential medium supplemented with 15% FCS. VNT was also performed without GPC'. Each assay consisted of controls for cells, Mabs, anti-EHV-1 positive rabbit and negative control sera. Simultaneously, a virus titration was also performed during each test to ensure a challenge dose of 10¹⁰⁻¹³ TCID₅₀/well. Anti-EHV-1 polyclonal antiserum raised in rabbit was also tested similarly in VNT against all the viral isolates. The plates were then transferred to a 5% CO₂ incubator and incubated at 37°C for 5 days. Finally, neutralizing antibody titre of Mabs was expressed as reciprocal of the dilution of Mab that caused 50% reduction in CPE (Reed & Muench, 1938).

Western Blotting
The proteins of the purified EHV-1 isolates were resolved by SDS-PAGE (Crabb & Studdert, 1990) and were electrophoretically transferred from gel to a 0.45-μm pore size PVD membrane (Millipore) (Towbin et al., 1979) using semi-dry electrophoresis apparatus. Following transfer, the membrane was blocked in 5% bovine serum albumin (BSA), washed 5 times in PBST and incubated with Mabs at optimum dilution at 37°C for 90 min. After washing the membrane, it was incubated with anti-mouse peroxidase-conjugated rabbit immunoglobulin for 90 min at
After washing 3 times, the membrane was developed with freshly prepared diaminobenzidine (DAB). Washing in distilled water stopped the reaction and air-drying preserved the membrane.

**Results**

All the 7 Mabs were directed against a 140 kDa polypeptide of Hisar-90-7 isolate of EHV-1. All the Mabs reacted in indirect ELISA with the homologous viral strain, however, only 2 Mabs (1H6 & 9C4) were able to neutralize the Hisar-90-7 strain in the presence of GPC (Table 1). When different EHV-1 isolates were tested with two neutralizing Mabs in VNT, Mab 9C4 was able to neutralize all the isolates tested giving a neutralizing titre ranging between 8 and 64 (Table 1). However, Mab 1H6 failed to neutralize Tohana-96-2 and Raj-98 isolates indicating that these two isolates lack the neutralizing epitope for Mab 1H6.

A polyclonal anti-EHV-1 rabbit serum could neutralize all the six isolates both in presence or absence of GPC. None of the Mabs neutralized any isolate in absence of GPC. The purified protein of all the six EHV-1 isolates reacted in western blots analysis with all the 7 Mabs. No differences could be detected by western blot analysis as all Mabs reacted equally with 140 kDa protein of different isolates (Fig. 1).

The Hisar-90-7, Jind-96 and reference strain 592 were antigentically indistinguishable in indirect ELISA as all the 7 Mabs reacted with them giving P/N ratio ≥ 2 (Table 1). Delhi-98 could be differentiated from other EHV-1 isolates as it failed to react with 4 of 7 Mabs (6A1, 6A2, 6B1 and 9D4) while Raj-98 did not react with 2 Mabs (1H6 and 9D4) in indirect ELISA (Table 2). Interestingly, Tohana-96-2 isolate could not be identified by any of the Mabs in indirect ELISA, although it was neutralized by one of the neutralizing Mabs in VNT (Table 1 & 2). The re-

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### Table 1 — Complement-dependent neutralization of different EHV-1 isolates using Mabs

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Virus isolates (designation)</th>
<th>Place of origin</th>
<th>Year of isolation</th>
<th>Clinical manifestations</th>
<th>Reciprocal of log_{10} neutralizing titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1H6(IgG2a)</td>
</tr>
<tr>
<td>1</td>
<td>Hisar-90-7</td>
<td>Hisar</td>
<td>1990</td>
<td>Abortion</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>Tohana-96-2</td>
<td>Tohana</td>
<td>1996</td>
<td>Abortion</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Raj-98</td>
<td>Rajasthan</td>
<td>1998</td>
<td>Abortion</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Delhi-98</td>
<td>Delhi</td>
<td>1998</td>
<td>Abortion</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>Jind-96</td>
<td>Jind</td>
<td>1996</td>
<td>Foal mortality</td>
<td>32</td>
</tr>
<tr>
<td>6</td>
<td>592</td>
<td>UK</td>
<td>—</td>
<td>Foal mortality</td>
<td>32</td>
</tr>
</tbody>
</table>

— = negative results; 'Other Mabs did not neutralize any of EHV-1 isolate either in presence or absence of GPC'.

### Table 2 — Antigenic differentiation of 6 EHV-1 isolates using Mabs by indirect ELISA

<table>
<thead>
<tr>
<th>Mabs</th>
<th>ELISA positive/negative ratio with different EHV-1 isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hisar-90-7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1H6*</td>
<td>5.92</td>
<td>0.12*</td>
<td>1.46*</td>
<td>5.52</td>
<td>2.00</td>
<td>2.53</td>
<td></td>
</tr>
<tr>
<td>9C4*</td>
<td>5.85</td>
<td>0.75*</td>
<td>2.95</td>
<td>2.55</td>
<td>2.13</td>
<td>3.91</td>
<td></td>
</tr>
<tr>
<td>6A1*</td>
<td>7.42</td>
<td>0.96*</td>
<td>2.09</td>
<td>0.23*</td>
<td>2.84</td>
<td>4.64</td>
<td></td>
</tr>
<tr>
<td>6A2*</td>
<td>6.55</td>
<td>1.52*</td>
<td>2.38</td>
<td>0.46*</td>
<td>2.75</td>
<td>6.11</td>
<td></td>
</tr>
<tr>
<td>6B1*</td>
<td>3.79</td>
<td>0.01*</td>
<td>2.36</td>
<td>0.69*</td>
<td>2.00</td>
<td>2.63</td>
<td></td>
</tr>
<tr>
<td>6D1*</td>
<td>9.97</td>
<td>0.71*</td>
<td>12.63</td>
<td>4.15</td>
<td>2.60</td>
<td>3.83</td>
<td></td>
</tr>
<tr>
<td>9D4*</td>
<td>4.79</td>
<td>0.12*</td>
<td>1.64*</td>
<td>0.641*</td>
<td>2.81</td>
<td>3.94</td>
<td></td>
</tr>
</tbody>
</table>

* = Neutralizing Mabs; * = negative results; figures in bold letters indicate negative results in VNT also.

The result was considered positive if the mean of the OD_{492 nm} value of the test sample/negative control OD_{492 nm} was ≥ 2.
Discussion

In the present study, all the Mabs reacted with a single band of 140 kDa EHV-1 polypeptide of different isolates in Western blotting. A target antigen of 140 kDa polypeptide under non-reducing condition also reacted with various Mabs of different properties (Allen et al., 1991). Further, it is quite possible that several epitopes may be present in any large protein molecule and Mabs in this study are directed against different epitopes on the same 140 kDa protein.

The virus neutralizing activity of 2 Mabs (1H6 & 9C4) was not observed in absence of guinea-pig complement (GPC'). GPC'-dependent and GPC'-independent virus neutralization has been previously documented with EHV-1 Mabs (Sinclair et al., 1989; Shimizu et al., 1989). Since some of the Mabs directed against the 140 kDa protein are neutralizing, it is likely that these Mabs are directed against the surface glycoprotein (envelope protein) of EHV-1. However, nature of the specific protein against which these Mabs are directed, is being confirmed.

Mab-based ELISA is considered sensitive diagnostic tool for antigenic differentiation of herpes viruses (Yeargan et al., 1985; Balchandran et al., 1982; Peterson et al., 1983). However, Tohana-96-2 isolate was not detected by any of the Mabs in ELISA. Mab (9C4) reacted in VNT but not in ELISA with the purified antigens of Tohana-96-2. It is not unusual to observe variability in the reaction of the same Mab in different test systems. Such variations in the reaction of Mab with different isolates in ELISA and VNT have also been documented against bovine herpes virus-1 (Collins et al., 1984). Mabs giving positive immunofluorescence staining and VNT against EHV-1 gave positive results by FAT against EHV-4 but gave negative VNT (Sinclair et al., 1989). In this study, non-reactivity of Tohana-96-2 isolate in ELISA and VNT with 1H6 suggests its antigenic differences from other EHV-1 isolates. Similarly, Raj-98 isolate did not react with 1H6 neutralizing Mab both in ELISA and VNT indicating that Raj-98 is also antigenically different from other isolates.

Since quantitative serological differences by polyclonal antibodies is difficult to detect and because of the close serological relationship which exists among EHV's, the panel of Mabs to EHV-1 reported here has potential as diagnostic tool to facilitate differentiation among Indian EHV-1 strains. Mabs raised against EHV-1 in Europe and USA showed antigenic differentiation among EHV-1 strains prevalent in these countries (Yeargan et al., 1985; Allen & Bryans, 1986; Edington et al., 1987).

It may be assumed as discussed above that some Indian strains of EHV-1 used in this study also differ at more than one antigenic sites. This study suggests that initial strains (Hisar-90-7 & Jind-96) of EHV-1 are antigenically closer to reference strain (592) while there is emergence of antigenic heterogeneity amongst subsequent EHV-1 strains from northern India (Tohana-96-2, Delhi-98 & Raj-98). It also indicates the prevalence of antigenic heterogeneity among Indian strains.

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