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COMPARISON OF CLINICAL SIGNS AND MORTALITY RATE IN MURINE MODELS INFECTED WITH HERPES VIRUS ISOLATES FROM CAPTIVE WILDLIFE

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SUMMARY

Clinical signs, cellular infectivity and mortality rate of two closely related alpha-herpesvirus from a gaur (UPMV5/05) and a bear (UPMV19/05) were studied in mice experimentally infected via the nasal route. The grade of viral infection was divided into low ($10^3$and 10^4 pfu/ml), medium ($10^5$and 10^6 pfu/ml) and high (10^7 pfu/ml). Clinically, all animals with the exception of controls, showed respiratory and neurological signs starting from 36 hpi. Although there were a number of differences in the onset time of clinical signs, these were not significant and the types of clinical signs shown were common. Nevertheless, there was a significant difference in the mortality rate, in which isolate UPMV19/05 from a bear was more pathogenic (83%) than the gaur isolate, UPMV5/05 (16.7%). These results strongly suggest that the consequences of host adaptation of herpesviruses may have been derived from interspecies transmission and underscore the need for extreme caution when managing wild or captive animals in close proximity to bovines.

Keywords: Captive wildlife, bear, gaur, clinical signs, alpha-herpesvirus, pathogenicity, mortality rate

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INTRODUCTION

The family herpesviridae consists of viruses that have been isolated from a wide range of animal species, human beings, catfish, and invertebrates such as oysters (Ardans, 1992), and can be divided into three major subfamilies: alpha-, beta-, and gamma-herpesvirinae. Within this group of viruses, there is wide variation in biological properties including cytopathology, pathogenicity, a propensity to form latent infections, and oncogenic potential.

In most natural hosts, animal herpesvirus produces a highly fatal infection that can affect all types of animals either pets, livestock or wildlife. Some important diseases caused by this virus include Infectious Bovine Rhinotracheitis (IBR) and Malignant Catarrhal Fever (MCF) in bovine, Equine Laryngotracheitis, Equine Abortion and Equine Coital Exanthema (ECE) in equine, Canine Herpesvirus in canines and Feline Rhinotracheitis in felines. However, despite the infectivity of this virus in various species, its infection in wildlife has rarely been detected and reported.

This study was initiated to investigate two different hespesvirus isolates (UPMV5/05 and UPMV19/05) from two different captive wildlife species (gaur and bear respectively) from two different areas in Malaysia. Preliminary genome investigation using Bam*HI* digested restriction enzyme (RE) pattern revealed close similarity within their genomes. In addition, the RE pattern of both isolates also resembled that of a bovine herpesvirus (BHV) giving an indication of the same origin. To gather further information on their pathogenicity and clinical manifestation, the same experimental model, mice, was chosen for comparison.

MATERIALS AND METHODS

Viruses

Two purified cell culture herpesvirus isolates were obtained from the Virology Laboratory of Faculty of Veterinary Medicine, Universiti Putra Malaysia. They were isolated from cases of captive wildlife animals from different areas in Malaysia. Isolate UPMV5/05 was obtained from a blood sample of a gaur whilst isolate UPMV19/05 was obtained from an oral swab of a growth on a mandibular region of a bear.

Virus stock preparation

Each viral isolate was propagated in Vero cell line and prepared in stock. The stock titre was determined by plaque forming assay (pfu). Different concentrations of titre were produced for each herpesvirus isolate: 10^7, 10^6, 10^5, 10^4 and 10^3 pfu/ml.

Negative Contrast Electron Microscopy (NCEM)

The purified virus was examined under an electron microscope. One drop of the purified virus was placed

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on wax paper, and carbon-formvar coated copper grid was layered with the carbon side down on the drop for five minutes. The grid was picked up with forceps and excess fluid was removed by filter paper. This step was followed by placing the grid with its carbon side down on a drop of 2% (w/v) methylamine tungstate for another five minutes. The excess fluid was removed as described above and the grid was dried overnight before being examined under a Hitachi 7100 Transmission Electron Microscope (TEM).

Intranasal inoculation of virus into mice

Balb/c inbred mice (5-8 weeks old) were obtained from the Institute for Medical Research, Kuala Lumpur, Malaysia. Each isolate was intranasally inoculated into 5 groups of mice at 5 different concentrations, of 10^3, 10^4, 10^5, 10^6 and 10^7 pfu/ml. Six uninfected mice served as controls.

Monitoring of clinical signs

In order to ensure proper clinical signs monitoring throughout the experiment, several methods were employed. These included visualisation with naked eyes, video recording and photography. All finding were recorded and tabulated. Monitoring commenced after the animals were infected. Protocols in management of the mice were in accordance to the standard laboratory animal husbandry which conforms to standard ethical practices. In this study, the onset of clinical signs was very subtle because of individual variation in immune status. It was therefore more convenient to observe the signs in groups of low (10^3/10^4pfu/ml), medium (10^5/10^6 pfu/ml) and high (10^7 pfu/ml) viral doses.

Mortality rate

The mortality rate, also known as fatality rate, is summed as the ratio of the total number of deaths to the total number of the population. Mortality rate in this trial is expressed in percentage as shown below:

$$\text{Mortality rate} = \frac{\text{No. of dead mice (each dose)}}{\text{No. of mice inoculated (each dose)}} \times 100\%$$

Statistical analysis

All data were analysed using SPSS version 16.0. Student *t*-test was used and the significant level was set at $p < 0.05$.

RESULTS

Viral propagation in cell culture

The cytopathic effect (CPE) was first observed at 24 hours post-infection (hpi) and continued progressively towards completion at 72 hpi (Figure 1A). CPE types of different characteristics were produced by each isolate. Vero cells infected with UPMV19/05 showed a large syncytium (Figure 1B) whilst infection with UPMV5/05 produced plaque containing vacuoles, rounded polykaryocytes and multiple peripheral cytoplasmic extensions (Figure 1C).

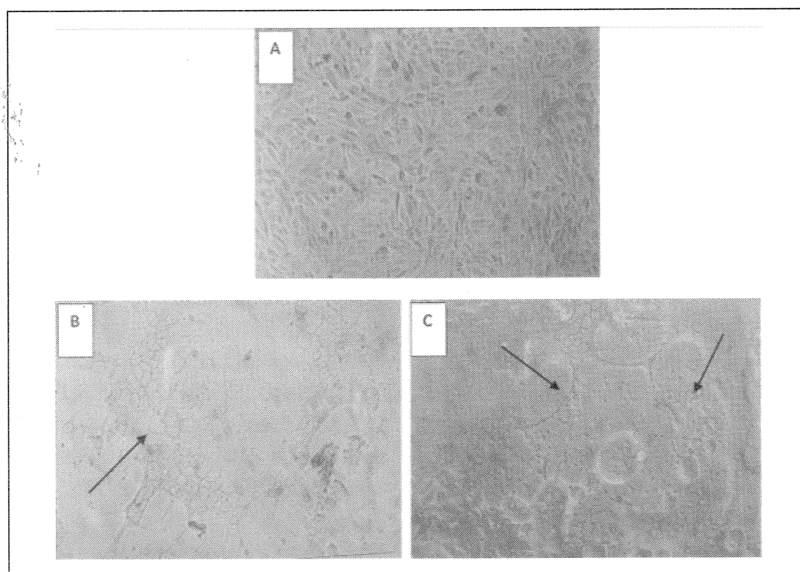

Figure 1: Normal uninfected and infected Vero cells at 72 hpi. (A) The spindle shape of the normal uinfected Vero cells; (B) UPMV19/05 infected Vero cells showed extensive fusion of cells and syncytium formation; (C) Isolate V5/05/UPM on Vero cells at 72 hpi with arrows showing CPE characterised by a large plaque containing vacuoles and rounded polykaryocytes with multiple peripheral cytoplasmic extensions (magnification 20x).

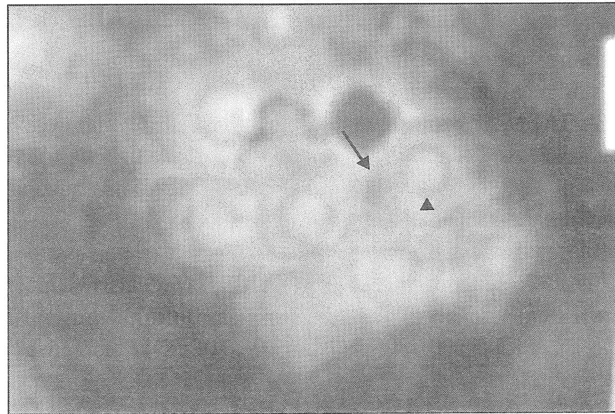

Figure 2: Electron micrograph of isolate UPMV5/05. The envelope (arrow) of the herpesvirus was intact and covering the capsid (arrowhead) (magnification 10000x).

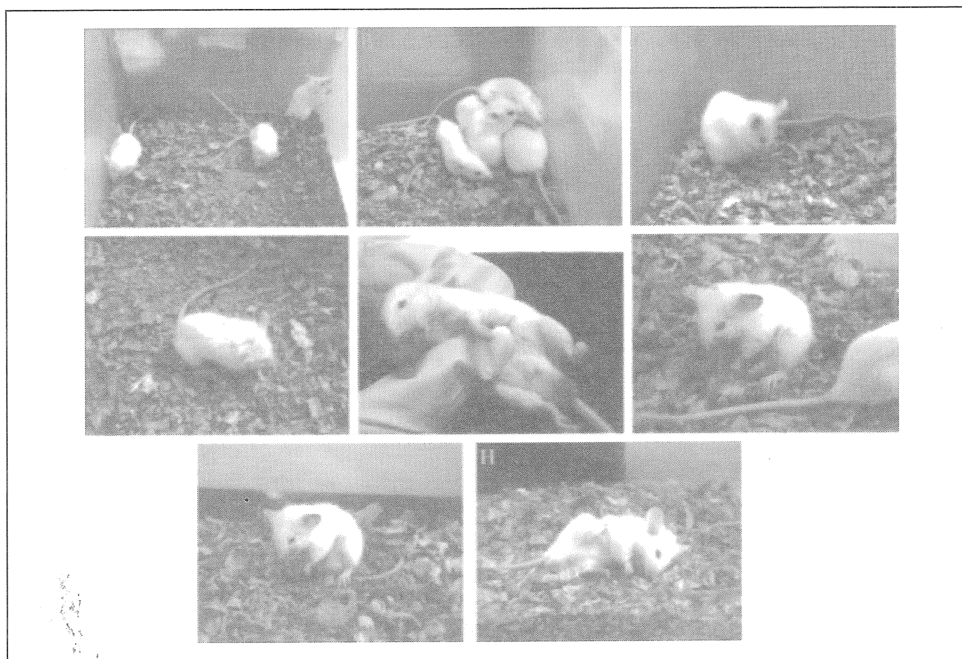

Figure 3: General observation of clinical signs after herpesvirus infection in mice at different time frames. Mice became depressed (A) and huddling together (B) after 5 hpi. At 10 hpi, mice started to rub their mouth and nose intensively (C). On day 2 (36 hpi), mice showed signs of ruffled hair (D). Bleeding nose (E) and ears (F) were observed at 60 hpi (day 3). Mice showed hunched back position (G) at 84 hpi and lastly they showed tilted head (H) prior to death.

Negative Contrast Electron Microscopy (NCEM)

Each concentrated viral stock was prepared for NCEM. Figure 2, based on UPMV5/05, represents the characteristic icosahedral feature of herpesvirus. Under NCEM, the herpesvirus was easily identified and confirmed by the presence of the capsid of the virion which appeared as a hexagonal ring, and enveloped by an electron-translucent shell ranging from 100-102 nm in diameter.

General observation of clinical signs

The first clinical signs after infection with herpesvirus were observed at 5 hpi (Day 1) when mice became depressed (Figure 3A) and huddled together (Figure 3B) at one corner. Depression was presented with decreasing functional activity, decreased interest in surroundings or decreased response to external stimuli while huddling means crowding closely. At the subsequent 5 hpi, the animals were observed to rub their mouth and nose (Figure

3C) intensively due to itchiness. At 36 hpi (day 2), they showed signs of ruffled hair (Figure 3D), trembling and shivering. Ruffled hair is denoted as disturbed smoothness or tranquillity of hair. Trembling refers to visible muscle tremor and shivering as involuntary shaking of the body.

Heavy dyspnoea with tachypnoea was observed at 60 hpi (Day 3). Dyspnoea is denoted as laboured or difficult breathing whereas tachypnoea is very rapid respiration, the rate being fast and the depth shallow. Frequent attacks were characterised by extensive scratching of the face, nose, maxillary region (Figure 3E) and ears (Figure 3F). These were observed to cause severe hemorrhagic dermal erosions and ulcerations at 60 hpi (Day 3).

At 72 hpi (Day 3), signs of uncoordinated gait and circling movement were observed. Uncoordination is denoted as a lack of normal adjustment of muscular action whereas circling is persistent walking in circles. Lastly, at 84 hpi (Day 4), the animals showed a hunched position (Figure 3G) as indicated by their abdomen being drawn up with head down with their four feet underneath and prior to death, they showed a tilted head (Figure 3H) which includes rotation and deviation.

Onset of clinical signs

Table 1 summarises the onset of clinical signs for isolates UPMV5/05 and UPMV19/05. Briefly, the medium and high viral doses triggered earlier clinical signs compared to lower grade infection; however, no significant difference was observed in infections between the two isolates.

Mortality rate induced by isolate UPMV5/05 and UPMV19/05 in murine models

Table 2 presents the mortality rate caused by isolates UPMV5/05 and UPMV19/05 during the first week of post-viral inoculation. The mortality rate was 100% from a concentration of 10^5 pfu/ml up to10^7 pfu/ml for both isolates. A significant difference was observed when inoculated with a lower viral concentration (10^3 pfu/ml) which produced 16.7% and 83.3% mortality rates, respectively at Day 7 post infection (pi) for UPMV5/05 and UPMV19/05. At medium and high dose infection (10^5 pfu/ml to 10^7 pfu/ml), death of mice started from Day 2 pi with all mice being dead by Day 5 pi. For the lower concentration (10^4 pfu/ml and 10^3 pfu/ml), murine mortality was from Day 4 onwards till Day 7 pi. All control mice were alive throughout the entire experimentation period.

DISCUSSION

Herpesviridae sub-families can be distinguished by cytopathology and also host range, duration of replicative cycle, and latent infection characteristics (Ardans, 1990). The Vero cell line supported the replication of both UPMV5/05 and UPMV19/05 isolates and subsequently showed first signs of cytopathic effect (CPE) at 24 hpi which were completed by 72 hpi. The rapid CPE formation strongly suggests alpha-herpesvirus, which has a relatively short replication cycle (<24 hpi). Although, the UPMV5/05 isolate from the gaur was suspected initially of malignant cattarhal disease (gamma-herpesvirus), the extensive spread of CPE within a short period of time contradicted the slow progressive cytopathic pattern of either beta or gamma-herpesvirus (Browning and Studdert, 1988). While alpha-herpesviruses are notably cytopathic, most lymphotropic herpesviruses (gamma-herpesvirus) do not cause significant degree of cellular injury. The Vero cell line allows higher replication titre of the herpesvirus, specifically alpha-herpesvirus, in comparison to other used cell lines at a similar passage (Warda, 2007). Both herpesvirus isolates have the tendency to induce fusion

Table 1: Onset of clinical signs in mice inoculated with different doses of herpesvirus isolates UPMV5/05 and UPMV19/05

Clinical Signs (h.p.i.)	V5 (pfu/ml)			V19 (pfu/ml)		
	$10^3/10^{4(low)}$	$10^5/10^{6(medium)}$	$10^{7(high)}$	$10^3/10^{4(low)}$	$10^5/10^{6(medium)}$	$10^{7(high)}$
Depression	10	5	5	12	5	5
Huddling	10	5	5	12	5	5
Rubbing mouth/ nose	5	5	5	10	5	5
Ruffled hair	41	36	36	41	36	36
Trembling/ shivering	41	36	36	41	36	36
Dyspnea/ tachypnea	65	60	60	65	60	60
Bleeding nose/ ears	65	60	60	65	60	60
Uncoordinated gait	77	72	72	77	72	72
Circling movement	77	72	72	77	72	72
Hunched position	89	84	84	89	84	84
Tilted head	89	84	84	89	84	84

Table 2: Mortality rate of Balb/c mice infected with different doses of herpesvirus isolates UPMV5/05 and UPMV19/05

Days post inoculation	V5 (pfu/ml)						V19 (pfu/ml)					
	C	10^3	10^4	10^5	10^6	10^7	C	10^3	10^4	10^5	10^6	10^7
Day 1	-	-	-	-	-	-	-	-	-	-	-	-
Day 2	-	-	-	1	-	2	-	-	-	1	-	4
Day 3	-	-	-	-	4	3	-	-	1	7	5	2
Day 4	-	-	2	5	3	3	-	1	1	-	3	2
Day 5	-	-	3	2	1	-	-	2	1	-	-	-
Day 6	-	-	-	-	-	-	-	1	1	-	-	-
Day 7	-	1	1	-	-	-	-	1	1	-	-	-
Total Dead	0	1	6	8	8	8	0	5	5	8	8	8
Total Alive	3	5	0	0	0	0	3	1	1	0	0	0
Total Animals Used	3	6	6	8	8	8	3	6	6	8	8	8
Mortality Rate (%)	0.0	16.7[a]	100.0	100.0	100.0	100.0	0.0	83.3[b]	83.3	100.0	100.0	100.0

Different alphabets in the same row indicate significant difference ($p<0.05$).
C = Control
– = None

of membranes of adjacent cells, merging to a mass of cytoplasm containing more than one nucleus, known as a syncytium. However, fused cells are short lived and subsequently lyse; apart from direct effects of the virus, they cannot tolerate more than one non-synchronised nucleus per cell.

As both virus isolates showed similar replication time frames in the same cell line, the possibility that both viruses are closely related is high. Further characterisation was performed to investigate the clinical signs in the infected murine models. The observed clinical symptoms included non-specific and specific signs. Non-specific signs were signs associated with the animal undergoing the disease process such as depression, huddling, ruffled hair, closed eyelids, loss of appetite (anoretic) and inactive (apathetic) (Takafumi, 1996), whereas the specific signs were herpesvirus related clinical signs after introduction to the body system. Two main body systems involved in herpesvirus pathogenicity are the respiratory system and nervous system (Ritchey *et al.*, 2005). Signs of the respiratory system of the disease process are rubbing mouth and nose intensively and also respiratory distress (dyspnoea and tachypnoea) whilst neurological signs include trembling and shivering, uncoordinated gait, circling movement, muscle tremor, tilted head, seizure and paralysis (Rizvi *et al.*, 1997; Ardans *et al.*, 1992). Both UPMV5/05 and UPMV19/05 presented similar clinical manifestation with some slight differences in the time of onset which were not significant.

Viral pathogenesis is a complex, variable, and relatively rare state; however, like the course of a virus infection, pathogenesis is determined by a balance of host and virus factors. All mice infected with UPMV5/05 and UPMV19/05 died when inoculated with viral doses of 10^5 pfu/ml to 10^7 pfu/ml. The first observation of death began on Day 2 pi and all mice died by Day 5 pi. However, death started a day later in low grade infection (10^3 pfu/ml - 10^4 pfu/ml) and resulted in a mortality rate of 16.7% and 83.3% at Day 7 pi for UPMV5/05 and UPMV19/05 respectively. There was a significant difference ($p<0.05$) in the mortality rate of these two viral isolates at lower grade infection. The variation in pathogenicity implies the consequence of an adaptation to a new host, although both isolates appeared similar genetically and in the manner of triggering clinical symptoms.

The ability of BHV to infect different animal species may have important implications for control or eradication efforts. Incidence of IBR (an alpha-herpesvirus) in buffalo in Malaysia is an example of a virus infecting related species in close contact (Ibrahim *et al.*, 1983). Other alpha-herpesvirus, such as equine herpesvirus 9 (EHV9) have managed to cross species and infect a polar bear and the case resembled a fatal herpesvirus encephalitis (Schrenzel *et al.* 2008; Donovan *et al.*, 2009). Likewise, the Bam *HI* DNA restriction pattern of the bear isolate was almost similar to the gaur isolate, UPM V5/05 (unpublished data). Both resembled a BHV origin. A characteristic herpesvirus, exclusive to the bear, has yet to be identified. It may have not been caused by a completely new or unknown virus but by viruses that were well known in the geographical areas. The variation in the pathogenicity of the seemingly similar isolates of UPM V5/05 and UPMV19/05, apparently from known BHV origin might have crossed the species barrier and eventually appear to have changed their behaviour. In other words, host adaptation may have triggered genetic

changes which altered their behaviour with time, with significant effects on their pathogenesis.

CONCLUSION

In conclusion, isolates UPMV5/05 and UPMV19/05 showed similar clinical presentation and minimal differences in terms of onset of clinical signs and significant variation in mortality at lower grade infection in the murine model. Although, the initial thought was that both isolates were the same virus strain due to the close similarity in genetic fingerprinting to BHV, this study found otherwise. Thus, it is reasonable to expect that animals raised in close contact with cattle in areas where BHV is endemic are vulnerable to interspecies viral transmission and therefore should be considered potential reservoirs of the virus. Due to these concerns, a geographically targeted survey of potentially infected animals for BHV is highly recommended especially in zoo, zoological gardens and animal sanctuaries.

REFERENCES

Ardans, A. (1992). A Herpesviridae. In: Review of Veterinary Microbiology. Biberstein, E.L.(Ed.). Blackwell Scientific Publications. pp. 198-210.

Browning, G. F. and Studdert, M. J. (1988). Equine herpesvirus 2 (equine cytomegalovirus). *Vet. Bull.* **58**: 775-790.

Donovan, T.A., Schrenzel, M.D., Tucker, T., Pessier, A.P., Bicknese, B., Busch, M.D.M., Wise, A.G., Maes, R., Kiupel, M., McKnight, C. and Nordhausen, R.W. (2009). Meningoencephalitis in a polar bear caused by equine herpesvirus 9 (EHV-9). *Vet. Pathol.* **46**: 1138–1143.

Ibrahim A, Saw SP, Fatimah I and Saharee AA. (1983) Isolation of infectious bovine rhinotracheitis virus from buffalo in Malaysia. *Vet. Record.* **112(13)**: 303-4.

Ritchey, J.W., Payton, M.E. and Eberle, R. (2005). Clinicopathological characterisation of monkey B virus (*Cercopithecine herpesvirus* 1) infection in mice. *J. Comparative Pathol.* **132**: 202-217.

Rizvi, S.M., Slater, J.M., Slade, A.J. and Field, H.J. (1997). Transmission of equine herpesvirus 2 to the mouse: characterization of a new laboratory infection model. *J. General Viro*l. **78**: 1119-1124.

Schrenzel, M.D., Tucker, T.A., Donovan, T.A., Busch, M.D.M., Wise, A.G., Maes, R.K. and Kiupel, M. (2008). New hosts for equine herpesvirus 9. *Emerging Infect. Dis.* **14(10)**: 1616-1619.

Takafumi Watanabe (1996). Pathogenesis of equine herpesvirus-1 infection in the mouse in reference to analysis of viral distribution. *Japan J. Vet. Res.* **44(1):** 44-44

Warda, S.A. (2007). Adaptation of locally isolatedequine herpesvirus (EHV-1) on different cell lines. BS Veterinary Medical Journal 5th Scientific Conference, pp. 52-56.