THE RELEVANCE OF VIRAL CULTURE BASED METHODS FOR THE DETECTION OF RAT CYTOMEGALOVIRUS (RCMV ALL-03) IN THE MODERN ERA OF TECHNOLOGY

Siti-Nazrina Camalxaman,[1] Nazariah Allaudin Zeenathul,[1*]. Yi-Wan Quah,[1] Hwei-San Loh,[2] Hassan Zuridah,[3] Abdul Rahman Sheikh-Omar[1], Mohd Lila Mohd-Azmi[1]

[1]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM), 43400 UPM, Serdang, Selangor, Malaysia.
[2]School of Biosciences, The University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor, Malaysia.
[3]Department of Medical Laboratory Technology, Faculty of Health Sciences, Universiti Teknologi MARA (UiTM), Kampus Puncak Alam, 42300 Bandar Puncak Alam, Selangor, Malaysia.

SUMMARY

In spite of the unequivocal advantages of traditional virological methods, its utilization seems to have taken a back seat coincident with the concurrent advances in science and technology. This study examined the impact of such techniques in the groundwork identification of rat cytomegalovirus ALL-03 strain. Virus was validated in terms of its intrauterine novelty, cytopathogenicity and was identified based on morphologic criteria classical of herpesvirus. Our findings demonstrate the suitability of primary brain endothelial cells for the propagation of the virus resulting in concomitant production of cytopathic effects and formation of plaques comparable to the ones produced in established secondary cell lines. In the absence of any known sequences for molecular based detection method, such findings provided critical clues crucial for the preliminary identification of the virus. This paper concludes that conventional methods such as tissue culture and electron microscopy are still in the forefront in the field of virology and remains relevant, but should ideally be used in conjunction with more advanced methodologies for a more comprehensive evaluation of novel viruses.

Keywords: Cytopathogenicity, host specificity, rat cytomegalovirus.

INTRODUCTION

Cytomegalovirus (CMV) is a complex member of the *Herpesviridae* family. The virus is infamous and its infection legendary. Individuals with immature or impaired immune systems are burdened by its notoriety, often linked to a wide array of congenital and opportunistic infections (Varani and Landini, 2011). Due to its worldwide prevalence, CMV exerts an interest in global research and consequently accurate identification of the viral agent is of utmost importance. Choosing the optimal method for virus identification and its subsequent implementation, whether it is by use of modern technological driven strategies or by conventional means is largely empirical, often determined in terms of practicality, reliability, reproducibility and cost-effectiveness of the method.

In many cases, it may be intricate to revive and recover a newly isolated virus without losing its potency and adaptability with its host cell. This obscurity is particularly distressing if the virus is of great importance, as in the case of rat CMV (RCMV) ALL-03 strain. Following its isolation from the uterus and placenta of *Rattus rattus diardii* (Loh *et al*., 2003), the virus remains undisclosed in terms of its evolution and molecular background. Its genome is currently completely uncharacterized and no sequence data is available with the minor exception of two transcripts of major immediate early genes (Loh *et al*., 2007). Available primers for the RCMV Maastricht (RCMV-M), RCMV English (RCMV-E) and the other RCMV isolate from Australia (Sandford *et al*., 1993, Beisser *et al*., 1998, Smith *et al*., 2004) could not amplify the genes of RCMV ALL-03, making the detection solely on molecular analysis ineffective. Furthermore, most RCMV studies have been carried out using well established Maastricht and English strains rather than RCMV ALL-03 and in general make use of continuous cell lines. Here, we describe the functional merits of non-molecular approaches in identifying RCMV ALL-03 by recovering the virus from its natural host and propagating it in various cell lines.

*Corresponding author: Assof. Prof. Dr. N.A. Zeenathul
E-mail: zeenathul@putra.upm.edu.my

MATERIALS AND METHODS

Virus Recovery from Infected Tissues

Two-month old female Sprague Dawley rats were subjected to cyclophosphamide (Sigma Chemicals Co., Missouri, USA) injection at a dosage of 40 mg/rat subcutaneously 48 h prior to virus inoculation. Following this, rats were injected with RCMV ALL-03 (10^4 $TCID_{50}$), mated and sacrificed following four weeks of challenge.

Virus Isolation

The salivary gland, lung, uterus, brain, placenta and fetal tissues were collected in serum free Dulbecco's Minimum Essential Medium (DMEM,

Gibco, USA) containing 8% (v/v) antibiotic-antimycotic solution and 5% (v/v) anti-pleuro pneumonia-like organisms (PPLO) agent. Tissues were homogenized and virus particles sonicated before being stored at - 70°C.

Mycoplasma Screening

Tissue homogenates were cultured onto Difco™ PPLO Agar with Difco™ Mycoplasma supplements (Becton Dickinson, USA) and incubated in 5% CO_2 at 37°C for up to 21 days in aerobic and anaerobic conditions before they were rendered negative for mycoplasmas.

Host Range Specificity Studies

Rat embryonic fibroblast (REF), Crandall-Reese feline kidney (CRFK), Felis catus whole-fetus 4 (Fcwf-4), Madin-Darby canine kidney (MDCK), Madin-Darby bovine kidney (MDBK), Vero (African green monkey kidney), HeLa (human cervical cancer), MCF7 (human breast cancer) and primary rat brain endothelial cells (RBEC) were tested against the tissue homogenates. Cells were observed daily for the presence of CPE and scored based on the degree of cellular destruction using standard procedures as previously described (Pindak and Schmidt, 1969). In addition, inoculation via the allantoic cavity and the chorioallantoic membrane (CAM) route in 10 days embryonated eggs was performed, followed by standard haemaglutinin assays (HA).

Virus purification

Infected REF cells exhibiting advanced cytopathic effects (CPE) were harvested, clarified and concentrated. Pellets were re-suspended in a small volume of phosphate buffered saline (PBS, 7.4), layered on top of a 20-60 % (w/v) sucrose gradient and ultracentrifuged at 40,000 rpm for 16 h at 4°C in a Sorvall® WX Ultra-100 centrifuge (Thermo Electron Corp.,USA). Visible bands containing purified virus were collected and pelleted at 40,000 rpm for 3 h at 4°C before being re-suspended in a small volume of PBS.

Negative contrast electron microscopy (NCEM)

Purified viral suspension was dropped onto the coated face of carbon-coated formvar grid for 5 mins. The grid was withdrawn, blotted and placed on a fresh drop of 1 % (w/v) uranyl acetate for 5 mins. The stained sample was air dried and examined in a Hitachi H710 electron microscope.

RESULTS

Virus Recovery

Cell disintegration was characteristically slow but progressed to involve the entire monolayer (Figure 1). CPE initially began as foci noticeable within 4-6 days post infection (p.i). At 12-14 days p.i, cytopathological changes were severe, indicated by the expansion of plaques, emergence of cytoplasmic extension and subsequent detachment from the substrate. Mock infected cells remained intact and revealed no signs of cell damages. *Mycoplasma screening*

No mycoplasma colonies were detected on mycoplasma agar plates following three weeks of culture, rendering the rat host and tissue samples free from mycoplasma contamination.

Host Range

The results of infecting cell cultures of human, feline, canine, bovine, monkey and rodent origin are shown in Table 1. Only rat associated cells were susceptible, revealing cytopathological features following infection with the viral agent.. The extent of CPE formation is summarized in Table 2. In contrast, other cell lines were non permissive with no irregular abnormalities or cellular alterations observed. Similarly, virus was undetected in the allantoic fluid by HA test and no lesion were apparent following inoculation in embryonated eggs.

Cell type	CPE	Note
HeLa	Negative	No cytopathological changes
MCF-7	Negative	No cytopathological changes
CRFK	Negative	No cytopathological changes
Fcwf-4	Negative	No cytopathological changes
MDCK	Negative	No cytopathological changes
MDBK	Negative	No cytopathological changes
Vero	Negative	No cytopathological changes
REF	Positive	Degenerative changes noted
RBEC	Positive	Degenerative changes noted
CAM route	N/A	No lesions detected
Allantoic fluid	N/A	No haemaglutination observed

Table 1: Receptiveness and susceptibility of different cell types to the viral agent.
Note: Conclusions were drawn following four passages.

NCEM

Several naked virus consisting only of hexagonal-like ring structures in void of enveloped membranes were observed, while others displayed the characteristic "fried egg" appearance associated with herpesvirus. Due to its pleiomorphic nature, the size of the viruses varied, but in general displayed an average measurement of 120 - 150 nm, with the capsid sizes in the range of 95-98nm (Figure 2).

.

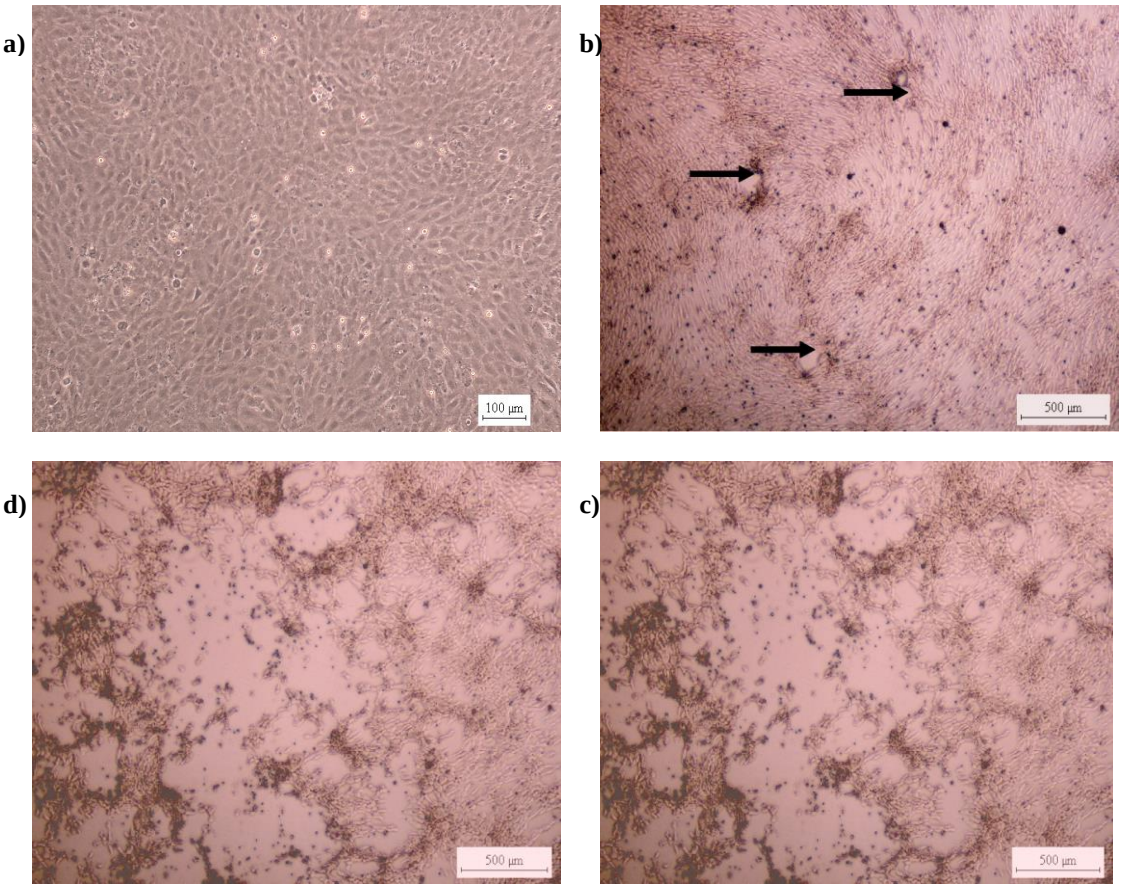

Figure 1: CPE development of the virus retrieved from infected tissues.
(a) Mock infected REF cells showing intact monolayer at day 10,
(b) Infected REF cells showing plaque formation (arrows) at day 5 p.i.,
(c) Progression of CPE as part of cellular degeneration at day 9 p.i. and;
(d) Extensive cellular deterioration throughout the monolayer by day 14 p.i.

	CPE on days upon inoculation											
Inoculum dilution	Primary RBEC						Secondary REF					
Undiluted	4	6	8	10	12	14	4	6	8	10	12	14
10^{-1}	+	+	2+	3+	3+	4+	2+	2+	3+	3+	4+	4+
10^{-2}	+	+	+	2+	3+	3+	+	+	+	2+	3+	3+
10^{-3}	+	+	+	1+	2+	2+	+	+	+	2+	3+	3+
10^{-4}	+	+	+	1+	2+	2+	+	+	+	2+	3+	3+
10^{-5}	0	0	0	0	2+	2+	0	0	+	2+	2+	3+
10^{-6} *	0	0	0	0	2+	+	0	0	0	0	2+	2+

Table 2: CPE caused in primary RBEC and secondary REF cell lines upon infection with RCMV ALL-03.

Grading of the CPE: 0, no CPE; +, small focal area of plaque/ cellular necrosis.
2+, larger areas of cellular necrosis/ spread of plaque formation. 3+, necrosis in approximately 50% of the monolayer. 4+, necrosis in approximately 80% of the monolayer.
* At 10-7 dilution, no CPE was observed in both monolayers.

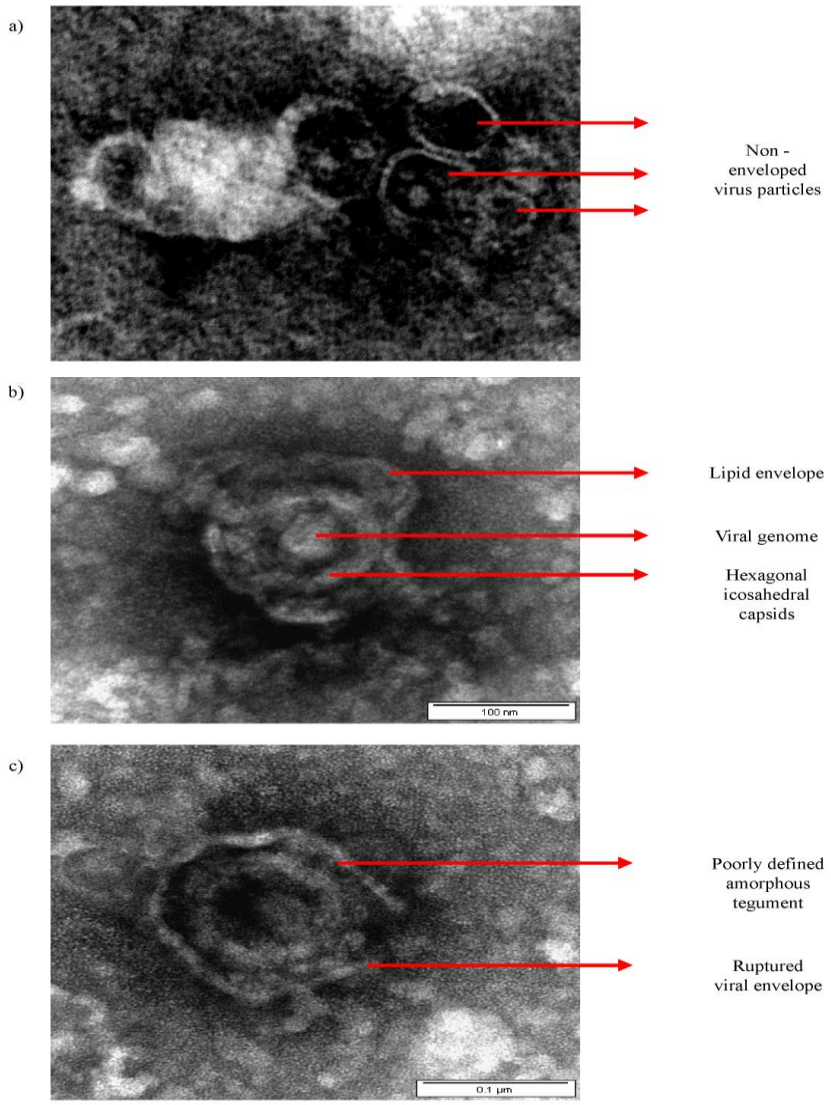

Figure 2: Virus structure revealed by negative staining of virions purified from REF cell line.
(a) Cluster of virions exhibiting capsids in void of outer viral envelope.
(b) Virion displaying intact viral envelope with nucleocapsid core.
(c) Fully mature herpesvirus-like virion showing ruptured viral envelope.

DICUSSION

Morphological identification methods have been customarily used for uncovering unknown viruses. Nevertheless, the demand of such methods has declined steadily over the several years concurrent with the development, application and expediency of modern techniques (Kellam, 1998). The exponential increase in studies utilizing molecular techniques and the equally rapid expansion of knowledge in this field are undoubtedly fascinating. Nevertheless, the single approach of using such techniques alone may be insufficient to meet the needs of all situations in viral diagnosis. The methods employed may have fundamental issues which limit its implementation and may demand technological understanding of the sample to be tested that could impede the progression of newly discovered viruses. For CMV, genomic content may vary according to species and thus, sequence similarity of RCMV with that of other CMVs may be limited. For example, when comparing the homology of Mmus CMV-2 using DPOL and gB sequences (GenBank: GUO17485) with murine CMV (GenBank: NC_004065), RCMV-M (GenBank: AF232689), RCMV-E (GenBank: GU018179) and HCMV (GenBank: AY446894), the pairwise identity percentages were 57.6 %, 56.2 %, 65.9 % and 46.9 %, respectively (Teterina *et al.*, 2009). Alternatively, genome based research strategies although plausible (Cunningham *et al.*, 2010), come with additional costs complexities which could restrict its use. For these reasons, pathogens are still been discovered using non-molecular approaches (Ginocchio, 2007).

The retrieved ALL-03 virus retained the timing and cultural characteristics typical of RCMV. Virus was recovered from all the tissues including the

placenta and uterus using the concept of Koch's postulate, validating its intrauterine novelty as previously claimed. Furthermore, screening of mycoplasma in our study was negative, exemplifying the legitimacy and authenticity of the CPE features as a direct consequence of viral infection and not due to other morphological, biochemical and immunological changes (Drexler and Uphoff, 2002; Chiew *et al*., 2008).

The nature of CPE and the time required for it to manifest are often indicative of the virus group and thus serves as a preliminary clue in the classification of isolates to at least be assigned to a family. Strains of pseudorabies virus for example, have been shown to demonstrate the first signs of CPE within 18-36 hrs p.i (Zeenathul, 1999) characteristic of alphaherpesvirus. In marked contrast, the growth of betaherpesviruses such as RCMV, are disreputably slower with initial signs of morphologic changes apparent at 3-6 days p.i (Bruggeman *et al*., 1982). Despite the timing differences, both PRV and RCMV exhibits comparable morphologic changes typical of herpesvirus which includes the rounding of cells, formation of syncytia and the development of plaques. This highlights the value of CPE for providing preliminary clues and for this reason; it remains the preferential gold standard for virus detection in many circumstances.

Our results connote the narrow host range nature archetypal of beta-herpes virus (Sinzger and Jahn, 1996) showing adaptability only to rat associated strains (REF and RBEC), while HeLa, MCF-7, CRFK, Fcwf-4, MDCK and MDBK cell lines remained un-receptive. Previous studies on host susceptibility of RCMV were restricted to other strains and did not employ RCMV ALL-03. In addition, available literature on RCMV ALL-03 focused solely on REF secondary cells, but did not take in account the adaptability of other rat-associated cells to this particular strain. For this reason, there was a critical need to determine what other cells, other than REF could be used as alternatives for viral propagation. Our findings demonstrated that primary RBEC was found to be equally compatible for the isolation and propagation of RCMV ALL-03 from infected tissues, which in turn could be beneficial for future exploration. Comparable plaques were obtained in both the primary and secondary cell lines when inoculated with the tissue homogenate, with minimal discrepancies as evidenced by slightly slower development of CPE in RBEC. Such findings is of significance, as endothelial cells have been cited as critical sites for CMV persistence and latency (Jarvis and Nelson, 2007) using other models.

Ultrastructural features of the retrieved RCMV ALL-03 virus were morphologically consistent with previous findings, and our sizes were comparable to the published sizes of different strains (Bruggeman *et al*., 1982; Priscott and Tyrrell, 1982; Smith *et al*., 2004). Historically, electron microscopy (EM) has made a major contribution to virology and continues to do so (Singh *et al*., 2006; Roingeard, 2008; Goldsmith and Miller, 2009). The simplicity of the test, its rapid preparation and instant evaluation makes it a practical diagnostic tool. For this reason, in the field of veterinary medicine, EM remains a mainstay for identifying previously un-described or re-emerging agents and viral outbreaks (Coyne *et al*., 2006; Maeda *et al*., 2007; Smith *et al*., 2008; Woolcocka and Shivaprasad, 2008). Likewise our results demonstrate that EM remains and continues to be useful for supporting initial viral diagnosis. For a more inclusive identification, further analytical tests will focus on tests relevant to RCMV, saving valuable time, money and diagnostic efforts.

CONCLUSIONS

While it is conceivable that modern analytical techniques will eventually replace traditional ones, virus isolation in permissive cells and its examination *in vitro* continues to yield desirable outcomes. In contrast to more contemporary assays which may necessitate specific viral probes, direct visualization and morphological scrutiny can provide fundamental clues for viral existence and proliferation without a preconceived notion of the aetiological agent. The slowly progressing CPE, the narrow host range exhibited and distinct herpesvirus morphological characteristics provided virological evidence that are still valid, relevant and should not be dismissed. Our findings prove that despite improvements in technology, viral culture based methods remain to be invaluable tools for viral diagnosis, particularly in the cases where there are no probes available. Such techniques are neither substitutes nor alternatives to modern test systems, but should ideally be used in combination with non-culture methods to provide optimal diagnostics.

ACKNOWLEDGEMENTS

The authors would like to thank and acknowledge the Ministry of Higher Education (MOHE), Malaysia for providing financial support under the Fundamental Research Grant Scheme (03-10-07-337FR). In addition, we thank Universiti Putra Malaysia (UPM) for providing the research environment and necessary facilities.

REFERENCES

Beisser, P.S., Vink, C., Van Dam, J.G., Grauls, G., Vanherle, S.J.V. and Bruggeman, C.A. (1998). The R33 G protein-coupled receptor gene of rat cytomegalovirus plays an essential role in the pathogenesis of viral infection. *J. Virol.* **72(3)**: 2352-2363.

Bruggeman, C.A., Meijer, H., Dormans, P.H., Debie, W.M., Grauls, G.E. and Van Boven, C.P.A. (1982). Isolation of a cytomegalovirus-like agent from wild rats. *Arch. Virol.* **73**: 231-241.

Chiew, L. S., Zeenathul, N. A., Aini, I., Tan, C. S., Nazrina, C., Noraini, A.S., Chan, P. S., Tan, S. S., Mohd Azmi, M. L. (2008). Cytopathic features in rat tissue-inoculated cell culture. Proceedings of the 20th Veterinary Association Malaysia Congress.

Coyne, K.P., Jones, B.R., Kipar, A., Chantrey, J., Porter, C.J., Barber, P.J., Dawson, S., Gaskell, R.M. and Radford, A.D. (2006). Lethal outbreak of disease associated with feline calicivirus infection in cats. *Vet. Rec.* **158**: 544–550.

Cunningham, C., Gatherer, D., Hilfrich, B., Baluchova, K., Dargan, D.J., Thomson, M., Griffiths, P.D., Wilkinson, G.W.G., Schulz, T.F. and Davison, A.J. (2010). Sequences of complete human cytomegalovirus genomes from infected cell cultures and clinical specimens. *J. Gen. Virol.* **91**: 605–615.

Drexler, H.G. and Uphoff, C.C. (2002). Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. *Cytotechnology*. **39(2)**: 75-90.

Ginocchio, C.C. (2007). Detection of respiratory viruses using non-molecular based methods. *J. Clin. Virol.* **40(1)**: S11-S14.

Goldsmith, C.S. and Miller, S.E. (2009). Modern uses of electron microscopy for detection of viruses. *Clin. Microbiol. Rev.* **22(4)**: 552-563.

Jarvis M.A. and Nelson J.A. (2007). Human cytomegalovirus tropism for endothelial cells: not all endothelial cells are created equal. *J. Virol*. **81**:2095-2101.

Kellam, P. (1998). Molecular identification of novel viruses. *Trends Microbiol*. **6(4)**: 160-165.

Loh, H.S., Mohd-Azmi, M.L., Lai, K.Y., Sheikh-Omar, A.R. and Zamri-Saad, M. (2003). Characterization of a novel rat cytomegalovirus (RCMV) infecting placenta-uterus o*f rattus rattus diardii*. *Arch. Virol.* **148(12)**: 2353-2367.

Loh, H.S., Mohd-Azmi, M.L., Sheikh-Omar, A.R., Zamri-Saad, M., Zeenathul, N.A. (2007). Molecular characterization and identification of the major immediate-early (MIE) genes of rat cytomegalovirus. *J. Vet. Malaysia*. **19(1)**: 21-32.

Maeda, Y., Shibahara, T., Wada, Y., Kadota, K., Kanno, T., Uchida, I. and Hatama, S. (2007). An outbreak of teat papillomatosis in cattle caused by bovine papilloma virus (BPV) type 6 and unclassified BPVs. *Vet. Microbiol*. **121**: 242–248.

Pindak, F.F and Schmidt, J.P. (1969). Propagation of MM virus in continuous cell lines. *Appl. Microbiol.* **17(6):** 815-818.

Priscott, P.K. and Tyrrell, D.A.J. (1982). The isolation and partial characterisation of a cytomegalovirus from the brown rat, *rattus norvegicus*. *Arch. Virol*. **73(2)**: 145-160.

Roingeard, P. (2008). Viral detection by electron microscopy: past, present and future. *Biol. Cell.* **100**: 491–501.

Sandford, G. R., Ho, K. and Burns, W. H. (1993). Characterization of the major locus of immediate-early genes of rat cytomegalovirus. *J. Virol*. **67(7)**: 4093-4103.

Singh, H. K., Madden, V., Shen, Y.J., Thompson, B.D. and Nickeleit, V. (2006). Negative-staining electron microscopy of the urine for the detection of polyomavirus infections. *Ultrastruc. Pathol.* **30**: 329–338.

Sinzger, C. and Jahn, G. (1996). Human cytomegalovirus cell tropism and pathogenesis. *Intervirology*. **39**: 302-319.

Smith, L.M., Tonkin, J.N., Lawson, M.A. and Shellam, G.R. (2004). Isolates of cytomegalovirus (CMV) from the black rat *rattus rattus* form a distinct group of rat CMV. *J. Gen. Virol.* **85(5)**: 1313-1317.

Smith, J. A., Wellehan, J.F Jr., Pogranichniy, R.M., Childress, A.L., Landolfi, J.A. and Terio, K.A. (2008). Identification and isolation of a novel herpesvirus in a captive mob of eastern grey kangaroos (Macropus giganteus). *Vet. Microbiol.* **129**: 236–245.

Teterina, A., Richter,D., Matuschka, F.R., Ehlers, B. and Voigt, S. Identification of a
novel betaherpesvirus in *Mus musculus. Virol. J*. **6:** 225.

Varani, S. and Landini, M.P. (2011). Cytomegalovirus-induced immunopathology and its clinical consequences. *Herpesviridae.* **2**: 6.

Woolcocka, P.R. and Shivaprasad, H.L. (2008). Electron microscopic identification of viruses associated with poult enteritis in turkeys grown in California 1993–2003. *Avian Dis.* **52**: 209–213.

Zeenathul, N.A. (1999). Characterisation of pseudorabies virus and its mutants. Master of Science thesis, Universiti Putra Malaysia.