PHYLOGENETIC ANALYSIS OF WATERFOWL PARVOVIRUS ISOLATED FROM DUCKS IN MALAYSIA FROM 1995-2014

SYAMSIAH AINI S.*, LEOW B.L., FAIZUL FIKRI M.Y., KHOO C.K., MUHAMMAD REDZWAN S., ONG G.H., BASIRAH M.A. AND SOHAYATI A.R.

Veterinary Research Institute, 59 Jalan Sultan Azlan Shah, 31400 Ipoh, Perak, Malaysia.
* *Corresponding author:* syamsiah@dvs.gov.my

ABSTRACT. Waterfowl parvoviruses are grouped into Muscovy duck parvovirus (MDPV) and goose parvovirus (GPV). Both MDPV and GPV can cause high morbidity and mortality in ducks and geese. In this study of samples received by the Veterinary Research Institute Malaysia in the years 1995 to 2014, the viruses were isolated from the liver and intestine of parvovirus cases and confirmed using polymerase chain reaction (PCR). A total of six parvoviruses (five from Muscovy duck and one from Pekin duck) were isolated by inoculation into 10 to 12-day-old Muscovy duck embryos. Phylogenetic analysis was conducted using a partial region of VP3 gene, amplified and sequenced from each of the isolates. Sequence analysis showed that all five isolates from Muscovy duck shared 99% to 100% sequence similarity with the MDPV isolate from Taiwan (V443/TW05). Contrarily, the isolate from Pekin duck shared 99% sequence similarity with GPV strain of YBLJ and YZYZ20130304 from China. Interestingly, phylogenetic analysis revealed that the isolates from 1995 to 2003 were grouped under MDPV of the Taiwan strain. In contrast, isolates from 2014 was clustered under the Asian strain of GPV. Based on these results, it might indicate that MDPV has not circulated after year 2003. However, more studies should be conducted since the reported cases for this infection are few. Importantly, it serves as a baseline information for waterfowl parvoviruses epidemiology and disease control management in Malaysia.

Keywords: goose parvovirus, Muscovy duck parvovirus, phylogenetic analysis, ducks, Taiwan, China

INTRODUCTION

Waterfowl parvovirus infection causes Derzy's disease with clinical signs which include watery diarrhoea, enteric symptoms, anorexia, prostration and death. Infection with waterfowl parvovirus can bring serious loss in mass waterfowl production (Wozniakowski *et al*., 2009). Surviving young birds and infected older birds show degenerative skeletal muscle myopathy and growth retardation (Deemagarn *et al.,* 2015; Chang *et al.,* 2000). High morbidity and mortality in goslings and ducklings have been reported, with mortality rates between 10% and 80%, respectively and even up to 95% (Chen *et al.,* 2015; Shien *et al*., 2008).

The waterfowl parvovirus species, *Anseriform dependoparvovirus 1*, of the Parvoviridae family, is made up of a linear, single-stranded DNA genome of about 5 kb in length (Chen *et al*., 2015). Its genome

contains two major open reading frames (ORF): the left ORF that encodes for the regulatory (rep) protein, and the right ORF that encodes for three capsid proteins (VP1, VP2, and VP3). Among the capsid proteins, VP3 is the most abundant and can induce neutralising antibodies (Woźniakowski *et al.*, 2009). Based on molecular analysis and virus neutralisation tests, waterfowl parvoviruses can be divided into two groups: the goose parvovirus (GPV) and the Muscovy duck parvovirus (MDPV) (Shen *et al.*, 2015; Shien *et al.,* 2008). The sequence between VP2 and VP3 regions were found to be the most variable in GPV and MDPV genomes (Wozniakowski *et al.*, 2009). As such, many phylogenetic studies of GPV have been conducted based on VP3 gene (Deemagarn *et al.,* 2015).

GPV infection was first described in the mid-1960s in several European and Asian countries. Since then, many countries such as France, Hungary, Poland, Taiwan, Thailand, and China have reported the isolation of this virus (Chen *et al.,* 2015; Deemagarn *et al.*, 2015; Wozniakowski *et al.,* 2009; Palya *et al.*, 2009; Tatar-kis *et al.,* 2004; Tsai *et al.,* 2004; Sirivan *et al.,* 1998). Though various strains have been reported from many countries, they were found to be closely related or have identical antigenicity (Tsai *et al.,* 2004; Kisary, 1974).

MDPV was discovered in France in 1989 where it had caused up to 80% mortality in Muscovy ducks (Le Gall-Reculé and Jestin, 1994). Subsequent outbreaks of MDPV were later reported in Taiwan, USA, China, and Indonesia (Mahardika *et al.,* 2015; Wan *et al.,* 2015; Poonia *et al.*, 2006; Chang *et al.*, 2000). Although belonging to the same genus, GPV and MDPV differ in terms of host ranges, antigenicity and nucleotide sequences. GPV can cause highly contagious and fatal disease in goslings and Muscovy ducklings whereas MDPV only cause disease in Muscovy ducklings (Wan *et al.*, 2015).

In this study of duck parvoviruses (DPV) cases from samples received by the Veterinary Research Institute Malaysia in the years 1995 to 2014, the viruses were isolated in embryonated duck eggs and confirmed using polymerase chain reaction (PCR). The sequence and phylogenetic tree was then analysed by comparing with other published DPV strains from different parts of the world.

MATERIALS

Virus Isolation

Six duck parvovirus (five from Muscovy duck and one from Pekin duck) were isolated from samples submitted to Veterinary Research Institute, Malaysia, between the year 1995 and 2014. They were designated as DPV/Malaysia 1442/1995, DPV/Malaysia/2820/1995, DPV/Malaysia/553/2000, DPV/Malaysia/6619/2000, DPV/Malaysia /10244/2003 and DPV/Malaysia/11936/2014.

The samples received were from livers and intestines. The cases were based on clinical signs such as watery diarrhoea, enteric symptoms, growth retardation and death. The infected farms of these cases were also reported to have more than 50% morbidity and mortality.

The isolates were propagated in the allantoic cavity of 10 to 12-day-old embryonated Muscovy duck eggs. The

allantoic fluid was collected after five days of incubation at 37 °C (Sirivan *et al.,* 1998).

DNA Extraction and Polymerase Chain Reaction

Viral DNA was extracted from the 200 µl of allantoic fluid of each isolates using QIAamp Cador Pathogen Kit (Qiagen, USA) according to the manufacturer's instructions. PCR was carried out using GoTaq® Green Master Mix (Promega, USA). A specific primer pair AL18F2/AL18R2 (Sirivan *et al.,* 1998) was used to amplify 806 bp amplicon that covers a partial region of the VP3 gene. After PCR, the reaction mixture was loaded into 1.5% agarose gel containing SyBr Safe (Invitrogen, USA) for electrophoresis and visualised using a UV transilluminator.

Nucleotide Sequencing

PCR products were excised from agarose gel and purified using QIAQuick Gel Extraction Kit (Qiagen) prior to Sanger sequencing. Sequencing was performed by First Base Laboratories Sdn Bhd, Malaysia. The primers used for sequence analysis were the same as those used for PCR amplification. The raw sequences were manually edited and assembled using Seqman (DNAStar Lasergene, USA). The sequences were compared with sequences accessible in the NCBI GenBank® database (International Nucleotide Sequence Database Collaboration, 2018) using BLAST algorithm (National Center for Biotechnology Information) (Benson D.A. *et al.*, 2017).

Phylogenetic and sequence similarity analysis

Nucleotide sequences were then aligned with Clustal W multiple alignment method (Thomson J.D., Higgins D.G. and Gibson T.J., 1994) and the percentage of similarities of the nucleotide and amino acid sequences were calculated using the BioEdit program version 7.2.5 (Tom Hall/Ibis Therapeutics, USA). The isolates in this study together with another 42 duck parvovirus sequences from the GenBank® were included for phylogenetic analysis. A phylogenetic tree was constructed with MEGA v6.06 using neighbour joining Kimura 2 parameter model with 1,000 bootstrapped replications (Tamura *et al.;* 2013). Phylogenetic analysis of the GPV isolates was generated based on partial VP3 (495bp) from nucleotide 163 to 657.

RESULTS

All waterfowl parvovirus isolates in this study were harvested from the allantoic fluid after inoculation into duck embryonated eggs. Using primer pairs AL18F2 and AL18R2, the partial VP3 gene was successfully amplified at the expected size of 806 bp from the extracted DNA of the isolates.

The percentage of similarities of nucleotide and amino acid sequences based on the partial VP3 gene is tabulated in Table 1. All five Malaysian isolates from Muscovy ducks designated as DPV/Malaysia/1442/1995, DPV/Malaysia/2820/1995, DPV/Malaysia/553/2000, DPV/Malaysia/6619/2000 and DPV/Malaysia/10244/2003 were found to be

Table 1. Similarity analysis of the nucleotide sequences of partial VP3 gene between six Malaysian isolates and other strains from Genbank. Values in the upper right and lower left indicate percent similarities of the nucleotide and deduced amino acid sequences, respectively.

Percent similarity of nucleotide sequences (%)																	
	Seq->	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1	GPV YZYZ20130304(China)		100.0	97.9	97.3	98.7	96.9	99.7	83.0	83.0	82.4	82.4	82.4	82.4	82.4	82.8	82.8	1
2	GPV YBLJ (China)	100.0		97.9	97.3	98.7	96.9	99.7	83.0	83.0	82.4	82.4	82.4	82.4	82.4	82.8	82.8	2
3	GPV T-/2012 (Thailand)	100.0	100.0		98.9	97.1	95.3	97.7	82.6	82.6	82.0	82.0	82.0	82.0	82.0	82.4	82.4	3
4	GPV 98-1218 (Taiwan (P)	100.0	100.0	100.0		96.5	94.7	97.1	82.8	82.8	82.2	82.2	82.2	82.2	82.2	82.6	83.0	4
5	GPV Virulent B(Hungary)	100.0	100.0	100.0	100.0		97.7	98.5	84.0	84.0	83.4	83.4	83.4	83.4	83.4	83.8	83.8	5
6	GPV Hoekstra-France (V)	100.0	100.0	100.0	100.0	100.0		96.7	83.6	83.6	83.0	83.0	83.0	83.0	83.0	83.4	83.4	6
7	DPV/Malaysia /11936/2014	100.0	100.0	100.0	100.0	100.0	100.0		83.2	83.2	82.6	82.6	82.6	82.6	82.6	83.0	82.6	7
8	MDPV-Hungary	93.9	93.9	93.9	93.9	93.9	93.9	93.9		100.0	98.9	98.9	98.9	98.9	98.9	99.3	98.7	8
9	MDPV 89384/France	93.9	93.9	93.9	93.9	93.9	93.9	93.9	100.0		98.9	98.9	98.9	98.9	98.9	99.3	98.7	9
10	MDPV V443/TW05 (Taiwan)	93.9	93.9	93.9	93.9	93.9	93.9	93.9	99.3	99.3		100.0	100.0	100.0	100.0	99.5	98.9	10
11	MDPV 85-103(Taiwan)	93.9	93.9	93.9	93.9	93.9	93.9	93.9	99.3	99.3	100.0		100.0	100.0	100.0	99.5	98.9	11
12	DPV/Malaysia/553/2000	93.9	93.9	93.9	93.9	93.9	93.9	93.9	99.3	99.3	100.0	100.0		100.0	100.0	99.5	98.9	12
13	DPV/Malaysia/1442/1995	93.9	93.9	93.9	93.9	93.9	93.9	93.9	99.3	99.3	100.0	100.0	100.0		100.0	99.5	98.9	13
14	DPV/Malaysia/2820/1995	93.9	93.9	93.9	93.9	93.9	93.9	93.9	99.3	99.3	100.0	100.0	100.0	100.0		99.5	98.9	14
15	DPV/Malaysia/6619/2000	93.9	93.9	93.9	93.9	93.9	93.9	93.9	99.3	99.3	100.0	100.0	100.0	100.0	100.0		99.3	15
16	DPV/Malaysia/10244/2003	93.9	93.9	93.9	93.9	93.9	93.9	93.9	98.7	98.7	99.3	99.3	99.3	99.3	99.3	99.3		16
Percent similarity of the amino acid sequences (%)																		

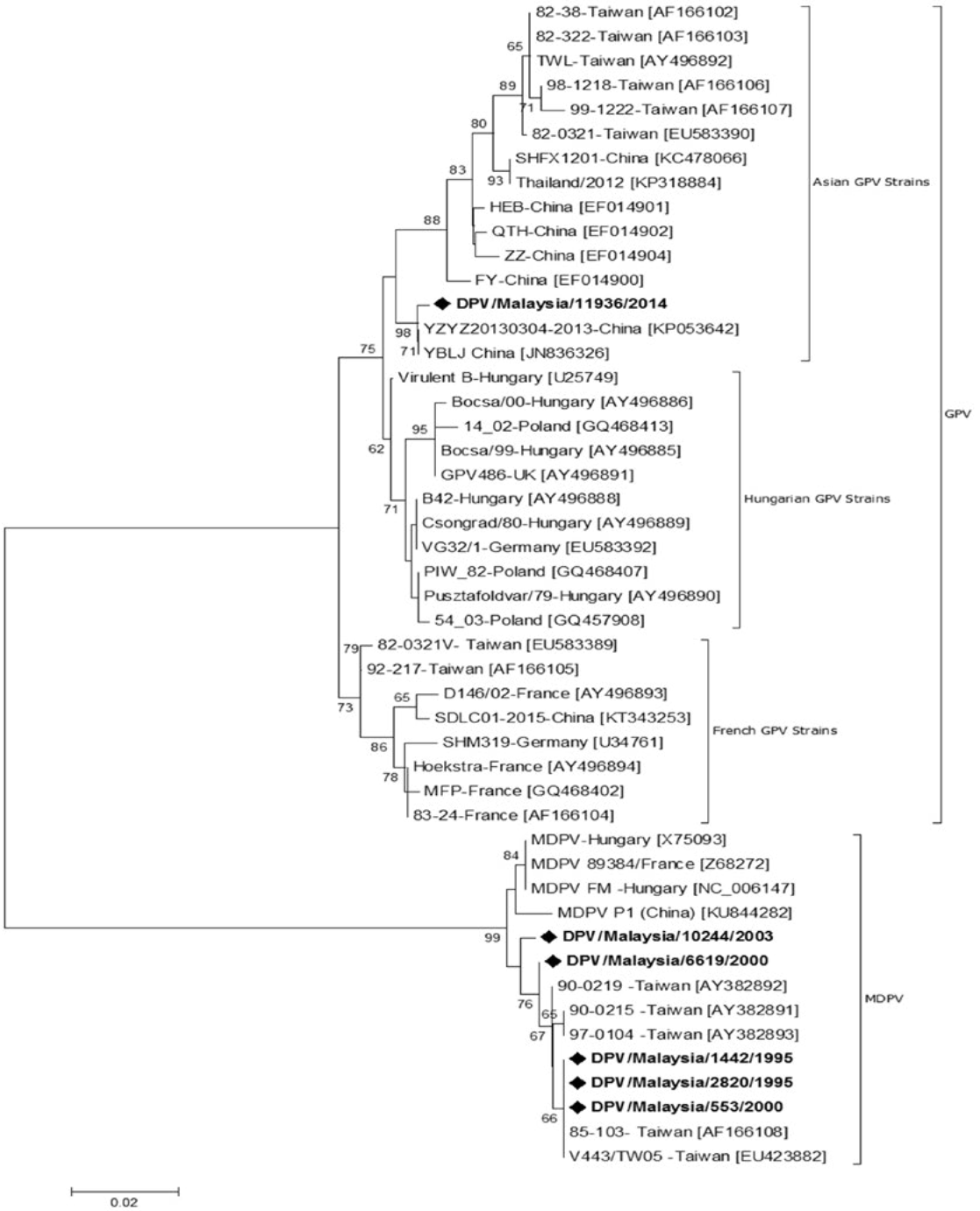

Figure 1. Phylogenetic tree constructed based on 495-bp long nucleotide sequences of VP3 gene of Malaysia parvovirus isolates and other parvovirus isolates from several countries showing their relationship among parvovirus strains. The isolates obtained in the present study are in bold with rotated square. Accession numbers of the sequences from GenBank are shown in parenthesis.

Table 2. Amino acid variation of partial VP3 protein of GPV and MDPV. The consensus sequence is shown in the first row of the table. Amino acid identical to consensus are shown as a dash (-), while (*) indicates isolates in this study.

Isolates	N63	P64	S66	T68	A108	I140	N145	S183	S213	E215
MDPV FM (Hungary)Vacc	-	-	-	-	-	-	-	-	-	-
MDPV P1 (China)	-	-	-	-	-	-	-	-	-	-
MDPV 89384 (France)	-	-	-	-	-	-	-	-	-	-
MDPV 90-0215 (Taiwan)	-	-	T	-	-	-	-	-	-	-
MDPV V443/TW05 (Taiwan)	-	-	T	-	-	-	-	-	-	-
MDPV 85-103 (Taiwan)	-	-	T	-	-	-	-	-	-	-
*DPV/Malaysia/1442/1995	-	-	T	-	-	-	-	-	-	-
*DPV/Malaysia/2820/1995	-	-	T	-	-	-	-	-	-	-
*DPV/Malaysia/553/2000	-	-	T	-	-	-	-	-	-	-
*DPV/Malaysia/6619/2000	-	-	T	-	-	-	-	-	-	-
*DPV/Malaysia/10244/2003	-		T	-	T	-	-	-	-	-
GPV YBLJ (China)	S	Q	A	V	S	V	D	N	T	D
GPV YZYZ20130304 (China)	S	Q	A	V	S	V	D	N	T	D
GPV T-2012 (Thailand)	S	Q	A	V	S	V	D	N	T	D
GPV Virulent B-(Hungary)	S	Q	A	V	S	V	D	N	T	D
GPV SDLC01 (China)	S	Q	A	V	S	V	D	N	T	D
GPV Hoekstra (France)	S	Q	A	V	S	V	D	N	T	D
*DPV/Malaysia/11936/2014	S	Q	A	V	S	V	D	N	T	D

closely related to the Taiwan strain of MDPV V443/TW05 with 99% to 100% nucleotide identity. The isolate from Pekin duck, DPV/Malaysia/11936/2014 was found to have 99% nucleotide identity with the China strains (YBLJ and YZYZ20130304).

Nucleotide and amino acids similarities between the Malaysian isolates were in the range of 82.6% to 83.0% and 93.9% to 99.3%, respectively (Table 1). In comparison, high similarities of both nucleotides and amino acids, between 98.7% to 100%, were observed in the five Malaysian isolates of the MDPV group. Isolate from Pekin duck shared 96.7% to 99.7% similarities with the GPV group. Though there were some differences in nucleotides, similarities of VP3 amino acid sequences showed that all selected GPV strains from GenBank® were 100% identical to the Malaysian isolate.

Amino acid variation of partial VP3 protein of GPV and MDPV strains including all Malaysian isolates are shown in Table 2. There are 10 unique positions present in MDPV and GPV. The substitution positions of GPV to MDPV were at S63N, Q64P, A66S, V68T, S108A, V140I, D145N, N183S, T213S and D215E. Interestingly, instead of serine (S) at

position 66 like in other MDPV reference strains, all the Malaysian isolates of Muscovy duck origin have threonine (T), similar to all Taiwan MDPV strains.

The phylogenetic tree, based on partial VP3 nucleotide sequences, divided the isolates into two main clusters: MDPV and GPV (Figure 1). It indicates that Malaysian isolates were found in both groups of MDPV and GPV. All isolates from Muscovy ducks fell under the MDPV group while one isolate from Pekin duck fell under the GPV group. During 1995 to 2003, only MDPV were isolated in Malaysia while no GPV were isolated before 2014.

Under the MDPV group, there are two sub-clusters: the first subgroup contains European strains while the second group contains Taiwan strains. The five Malaysian isolates from Muscovy duck fell under MDPV Taiwan group. In the GPV group, there are three subgroups: Asian strains, Hungarian strains and French strains. The Malaysian isolate 11936/2014 from Pekin duck was grouped in the Asian strains and placed in a cluster together with China strains (YBLJ and YZYZ20130304). All Malaysian isolates were clustered within pathogenic strains of both MDPV and GPV, differentiated from the subgroup of vaccine strains.

DISCUSSION

Waterfowl parvoviruses from both GPV and MDPV have been isolated in Malaysia from year 1995 and 2014. Five isolates of Muscovy duck origin (isolated in 1995, 2000 and 2003) were MDPV while one isolate from Pekin duck (isolated in 2014) was GPV. The nucleic acid percentage similarities (82.6% to 83.0%) between GPV and MDPV isolates in this study show that they were clustered in different groups. This is in agreement with previous studies conducted in Taiwan and China (Wan *et al.*, 2015; Chang *et al.*, 2000).

Malaysian MDPV isolates have 99% to 100% nucleic acid similarities with the Taiwan isolate V443/TW05. V443/TW05 was isolated in 2005 and was found to be closely related with another Taiwan isolate (85-103) that was identified in year 1985. Even though MDPV was first discovered in France in 1989 (Le Gall-Reculé and Jestin, 1994), Chang *et al.* (2000) reported an MDPV outbreak in 1989/1990 in Taiwan. Interestingly, he had also further identified that the first MDPV isolate in Taiwan was as early as in 1985 (isolate 85-103). Therefore, the finding by Chang *et al.* (2000) implies that MDPV might have been prevalent earlier than first reported in France in 1989. In addition, according to Chen (1990), duck production in Asian developing countries started to grow from the 1980s. As a result, breeding farms in Asian coutries including Malaysia imported half-hatched eggs from Taiwan. One of the imported duck breeds of half-hatched eggs duck was Muscovy duck. Therefore, the high similarity between Malaysian MDPV isolates with the Taiwan isolate V443/TW05 in this study implies that the origin of Malaysian MDPV is from Taiwan. It suggests that the import of half-hatched Muscovy duck eggs from Taiwan had contributed to the introduction of MDPV to Malaysia.

In contrast, Malaysian GPV isolated from Pekin duck in 2014 was believed to be originated from China as the YBLJ (NCBI, 2012) and YZYZ20130304 (NCBI, 2013) strains were isolated from geese in years 2006

and 2013, respectively. Based on previous studies, GPV can cause disease in goslings and Muscovy ducklings as reported in several countries such as Taiwan, Thailand, China, Hungary, France, Germany and Poland (Chang *et al.,* 2000; Deemagarn *et al.,* 2015; Shien *et al.,* 2008; Tatar-kis *et al.,* 2004; Wozniakowski *et al.*, 2012). The Malaysian GPV strain was first reported from Pekin duck while other isolates related to this strain were from goslings except one from Muscovy duckling (Cheng, 2008).

Based on the amino acid sequence of the partial VP3 protein alignment, it was found that MDPV and GPV have unique substitution of residues at certain positions. This is in agreement with previous studies (Poonia *et al.,* 2006; Tsai *et al.,* 2004; Zádori *et al.*, 1994). At position 66, all the Malaysian MDPV isolates from Muscovy duck origin had threonine (T), similar to all Taiwan MDPV strains, instead of serine (S). This finding can be considered as a unique molecular marker for Taiwan MDPV related strains.

In order to control and prevent this viral disease, treatment of young animals with hyperimmune serum or vaccination is recommended (Tatar-kis *et al.,* 2004). Two types of inactivated vaccine were approved in Malaysia from GM and Hoekstra strains of GPV (DVS, 2018). The VP1 polypeptides of GPV and MDPV share 88% amino acid sequence identity (Zádori *et al.,* 1994), allowing cross protection of Muscovy ducks by vaccination with attenuated GPV against MDPV infection (Tatar-kis *et al.,* 2004). Based on the findings of this research, it is recommended to vaccinate not only geese and Muscovy ducks but also Pekin or Cherry Valley ducks as these breeds are also susceptible to the viral infection. Though MDPV has not been isolated or detected since 2003 in Malaysia, precaution should be taken against it spreading from the neighbouring country of Indonesia which reported an outbreak of MDPV for the first time in 2014 (Mahardika *et al.,* 2016),

CONCLUSION

In conclusion, two types of waterfowl parvoviruses are present in Malaysia; MDPV and GPV. This study serves as a baseline information for waterfowl parvoviruses epidemiology and could be useful for disease control management in Malaysia.

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