Acta Scientiae Veterinariae, 2016. 44: 1419.

RESEARCH ARTICLE
Pub. 1419

ISSN 1679-9216

Relationship between Virus Replication and Apoptosis Events in IgM + Cells from Chicken Spleen and Bursa of Fabricius Infected with Malaysia Strain of Very Virulent Infectious Bursal Disease Virus*

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ABSTRACT

***Background*:** Infection of IBDV was reported to be endemic in worldwide including Malaysia and can be spread orally thru polluted fodder and water source, thus causing economic losses especially in commercial poultry industry. The infection resulted in depletion of B lymphocytes and subsequently destruction of the bursa which leaded to immunosuppression of the bird and it was postulated that the depletion of cells in the bursa was due to induction of apoptosis. In the current study, the infection of Malaysia isolated very virulent IBDV UPM0081 on IgM bearing B lymphocytes (IgM+ cells) from chicken spleen and bursa was compared.

***Materials, Methods & Results*:** A total of sixty eggs were obtained and raised until the age of 3 weeks old. The birds were divided into two groups (n = 30), which one of them served as control while IBDV strain UPM0081 was used to infect another group of birds at the concentration of 10^3 ELD50. The birds were observed and sacrificed at day 2, 4 and 5 post infections. Spleen and bursa of Fabricius were harvested and subjected to IgM+ cell enrichment using microbeads. The cell viability of enriched cells was assayed using MTT and cell cycle was analyzed using propidium iodide. Annexin V FITC and acridine orange/propidium iodide double stain assays were used to determine the event of apoptosis in the enriched IgM+ cells. Also, the IBDV viral load was also quantified by using real time PCR to evaluate the relationship between virus replication and apoptosis events in the infected chickens. Current results showed that the apoptotic events were observed to be significantly higher in IgM+ cells isolated from chicken bursa as compared to the cells isolated from spleen. The bursal B lymphocytes cell viability was observed to be decreasing following the infection of very virulent IBDV. The cells were then investigated of their apoptotic rate and data showed that increasing apoptotic cells (early and late apoptosis) were observed in AO/PI double stain as well as increment of SubG0/G1 population in the cell cycle analysis and also increment of Annexin V FITC bound cells in the apoptosis study. As for B lymphocytes from chicken spleen, the magnitude of damage caused by very virulent IBDV was not as severe as what being observed in the chicken bursa, with the cell viability drastically decreased on day 4 following IBDV infection.

***Discussion*:** IBDV caused severe destruction in bursa of Fabricius compared to spleen, in which cell death events in the former was reported to be directly caused by the virus. Apoptotic event in chicken spleen following IBDV infection was observed to be caused by oxidative stress. Thus, viral replication played a role in inducing bursal IgM+ cells death while such phenomenon was not observed in spleen isolated IgM+ cells. In summary, the cell death events of IgM+ cells in chicken spleen and bursa of Fabricius may be accounted by different factors upon infection with Malaysia strain of IBDV UPM0081. It is obvious that IgM+ cells from chicken bursa suffered from apoptotic cell death in an increasing manner considerably with time of infection and RNA load detected in the cells, which supported by previous literature that IBDV induces host cells apoptosis, with both VP2 and VP5 playing a role in binding and apoptosis. Meanwhile, the cell death events of B lymphocytes in chicken spleen was observed to be more relevant to other factors such as the oxidative stress or proinflammatory cytokines that caused by the virus infection rather than the viral RNA load.

Keywords: infectious bursal disease virus, apoptosis, avian, spleen, bursa of Fabricius, IgM+ cells.

Received: 17 July 2016 Accepted: 12 November 2016 Published: 28 December 2016

*Article based on a Thesis submitted by the senior author in partial fulfillment of requirements for the Master Degree.

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INTRODUCTION

Infectious bursal disease virus caused immosuppression in chickens, especially between the ages of 3 to 6 weeks [1]. This virus belongs to the *Avibirnavirus* genus in the *Birnaviridae* family with bi-segments genomes, namely segment A and B [6]. IBDV strains are classified based on their virulence and can be divided into mild (vaccine strain), classical and very virulent (vvIBDV) [4]. IBDV serotype 1 normally targeted the IgM bearing B cells in the chicken bursa of Fabricius. Infection resulted in depletion of B cells and subsequently destruction of the bursa which leaded to immunosuppression of the bird [1]. It was postulated that the depletion of cells in the bursa was due to apoptosis [1] and evidence showed apoptosis in lymphoid tissues of embryo and young chickens after being infected with vvIBDV strains [8,20]. Also, in vitro Vero cells were reported to be infected by vvIBDV and resulted in increasing apoptosis events [19].

Worldwide, the vvIBDV affected poultry industry and caused economic losses especially in commercial poultry industry [3,6]. The infection was reported to be endemic in worldwide including Malaysia and can be spread orally thru polluted fodder and water source [17]. It was well documented that IgM+ cells are the main target of the IBDV, but there was no detailed report about the comparison of IBDV infection on IgM+ cells from chicken spleen and bursa.

As the disease peaks between 2 to 5 days post infection [16], the apoptosis of enriched IgM+ cells from chicken spleen and bursa following infection of Malaysia isolates vvIBDV was investigated to understand the relationship between the enriched IgM+ cells death and IBDV viral replication.

MATERIALS AND METHODS

Experimental design

Malaysia isolated very virulent IBDV (vvIBDV) strain, known as UPM0081 was used in this study. A total of sixty eggs were obtained from Veterinary Research Institute (Ipoh, Malaysia). The eggs were incubated and hatched inside an isolator, then raised until the age of 3 weeks old. The birds were divided into two groups (n = 30), which one of them served as control while IBDV strain UPM0081 was used to infect another group of birds at the concentration of 10^3 ELD50. The birds were observed and sacrificed at day 2, 4 and 5 post infections. Spleen and bursa of Fabricius were harvested and subjected to further procedures.

Enrichment of IgM+ cells from chicken spleen and bursa of Fabricius.

Single cells were harvested from chicken spleen and bursa of Fabricius from 35 days old SPF chickens. The cells were then stained with 10 µg/µL of PE-labelled IgM[1] for 45 min and washed twice with PBS. Subsequent to that, the cells were incubated with 20 µL of anti-PE micobeads[2] for 30 min. After the incubation, the cells were washed twice with PBS to wash away the excessive microbeads. LS Column[2] was attached to the magnetic field and prepared by rinsing with 3 mL of PBS before the cell suspension was applied onto the column. The column was then washed with 3 mL of PBS buffer for three times. Lastly, the LS column was removed from the magnetic field and 3 mL of buffer was applied onto the column to flush out the enriched IgM-labeled B lymphocytes. The enriched B cells were then being washed twice again with PBS before cell counting was performed using haemacytometer.

IgM+ cell viability assessment using MTT

To determine the cell viability of enriched IgM+ cells, colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay [11[was carried out. Enriched IgM+ cell suspension was isolated from chicken spleen and bursa of Fabricius and plated in a 96-well microtitre plate with cell density of 4 X 10^4 cells/well in a total volume of 200 µL culture media supplied with 10% FBS and 1% FCS. Each of the well was then added with 20 µL of MTT[3] at 5 mg/mL concentration. The plate was then incubated at 37ºC in an incubator with 5% CO_2 and 90% humidity for 3 h. After the incubation period, the plate was spun at 300 x *g* for 5 min before 170 µL of the supernatant was aspirated out from each well. To solubilize the formazan crystals, 100 µL of DMSO[3] was added to each well followed by an incubation period for another 15 min. Lastly, the plate was read at absorbance 570 nm and 630 nm using µQuant ELISA Reader[4].

Cell cycle analysis of Enriched IgM+ cells

Cell cycle analysis was performed by using propidium iodide to stain the nucleic acid content of the cell. Enriched IgM+ cells were fixed in 80% ethanol overnight at temperature of -20ºC. The samples were

then re-pelleted and washed twice by using PBS-Sodium-Azide-EDTA buffer. Finally, the pellet was dissolved in 1 mL of PBS buffer containing 0.1% Triton X-100, 10 mM EDTA, 50 µg/mL RNase and 2 µg/mL SYTOX green in the dark, followed by incubation for at least 30 min on ice. The samples were then subjected to analysis by flow cytometry using FACSCalibur Cell Quest Pro software[5]. Triplicates were carried out using three different biological samples from each group for the experiment.

Flow cytometry assessment of IgM+ cells apoptotic event using Annexin V- FITC stain

Apart from cell viability assessment, the apoptosis event in enriched IgM+ cells after infected with IBDV was investigated using AnnexinV FITC staining. In brief, the cells were washed with PBS and stained with Annexin V-FITC[5] for 20 min prior to analysis using FACSCalibur machine with CellQuest Pro Software[5]. The assay was conducted in triplicates.

Microscopy assessment of IgM+ cells apoptotic event using Acridine Orange/ Propidium Iodide (AO/PI) Double Staining Assay

Along with apoptosis assay by using Annexin V, the apoptosis events in enriched IgM+ cell suspension was also evaluated using acridine orange and propidium iodide (AOPi) stains. In short, the cells were washed with PBS twice before 10 µL of the cells were put on a glass slide. Acridine orange (50 µg/mL) and propidium iodide (50 µg/mL) were mixed together at ration 1:1 to a total volume of 1 mL. After that, 10 µL of the mixture was added to the cells on the glass slide and resuspended carefully. The slide was then analyzed using fluorescent microscope within 30 min. This assay permitted clear differentiation of viable and nonviable cells using two fluorescent dyes. Through counting a total population of 200 cells, the percentage of viable, apoptotic and necrotic cells were determined. The experiment was repeated using 3 biological replicates for each group.

Viral load quantification in enriched IgM+ cells using real time PCR

After different time points of IBDV UPM0081 infection, total RNA from each spleen and bursa samples (enriched IgM+ cells) were extracted using TRIZOL[6]. The samples were suspended in 350 µL of PBS before 750 µL of TRIZOL solution was added in dark. After incubation for 5 min at room temperature, 200 µL of chloroform was added and the mixtures were vortex vigorously for 15 s. Then, the samples were incubated for another 15 min at room temperature before centrifuged at 12,000 x g for 20 min at 4°C. After the centrifugation, 500 µL of the top aqueous layer was carefully pipetted to a new tube and added with 800 µL of isopropanol. The samples were mixed well and incubated for 40 min at 4°C. After the incubation period, the samples were subjected to centrifugation at speed of 14,000 x *g* for 20 min at 4 °C. Then, the supernatants were discharged and the pellets were suspended in 75% ethanol before they were centrifuged at 7,500 x *g* for 20 min at 4°C. The step was repeated again using absolute ethanol before the samples were left to air dried for 20 min. The RNA pellets were then suspended in 20 µL of RNAse free water and subjected to reverse transcriptase PCR using iScript cDNA Kit[7] incubated in a thermal cycler for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C with the reaction component including 5X iScript Reverse Transcription Supermix, 100 ng RNA template and nuclease-free water. After cDNA was obtained, SYBR Green real time PCR[7] assay was performed with cycling program of 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 58.7°C for 30 s. Forward primer with the sequence of 5'-ATG CTC CAG ATG GGG TAC TTC-3' and reverse primer 5'-TTG GAC CCG GTG TTC ACG-3' were used for IBDV viral load determination. Log viral copy number was determined using the equation of:

$$Log\ Quantity = \frac{Ct - b}{m}$$

Ct = threshold cycle, b = y-intercept, m = slope

From the standard curve plotted, b = 36.7 and m= -3.155 (Appendix)

Statistical Analysis

GraphPad Prism 4.0 statistical software was used to analyze the data. One way ANOVA and post-hoc tests were used to test for significant differences at $P \leq 0.05$. All the results are presented as mean ± standard deviation from three replicates.

RESULTS

Enriched IgM+ population was isolated from chicken bursa and spleen at days 0, 2, 4 and 5 post infection to determine the response of chicken B cells towards the infection of very virulent IBDV. Cell viability of B lymphocytes was assessed using trypan blue and MTT assays. Table 1 showed viable

cell count of B lymphocytes in both chicken bursa and spleen upon infection with very virulent IBDV, whereas Figure 1 represents the cell viability using MTT method. In chicken bursa, both trypan blue and MTT results showed that IBDV induced increasing cell death events in B lymphocytes as the time course of infection increased too. The cell viability dropped drastically starting from day 4 after infection of the virus. On the other hand, B lymphocytes from chicken spleen also decreased in their cell viability starting from day 2 post infection.

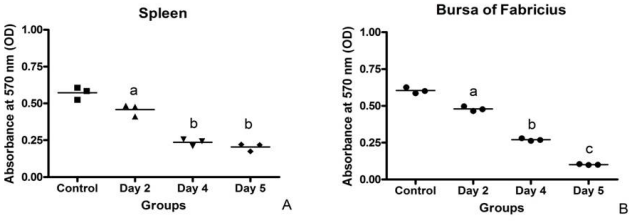

Figure 1. MTT cell viability of enriched IgM+ cells isolated from A) chicken spleen and B) bursa of Fabricius after IBDV UPM0081 post infection day 2, 4 and 5. The differences between the control group and infected groups were determined by one-way ANOVA. Groups labeled with different alphabets are significantly different ($P \leq 0.05$).

In the cell cycle analysis, different cell cycle phases of B lymphocytes from both chicken spleen and bursa of Fabricius were determined by staining the DNA content of the cells with Sytox Green dye and further analysed with flow cytometer. Figure 2 showed the distribution of different cell cycle phases in enriched IgM+ cells from chicken bursa and spleen after being infected with very virulent IBDV. It was noticeable that IBDV UPM0081 caused increment of Sub G0/G1 population in both chicken spleen and bursa B lymphocytes. However, result also indicated that bursal B cells suffered from higher degree of DNA fragmentation compared to B lymphocytes from chicken spleen. Depletion in G2M cell distribution among B lymphocytes from chicken bursa and spleen was also observed after the infection with IBDV UPM0081.

To determine the cell apoptosis events in chicken spleen and bursa B lymphocytes upon very virulent IBDV infection, FITC labeled Annexin V stain was applied. Exposure of phosphatidylserine at the cell surface is a hallmark of apoptosis, and this can be measured by Annexin V assay. Figure 3 shows the percentage of cells that were apoptotic by measuring the uptake of AnnexinV FitC. Upon virus infection, bursal B lymphocytes showed drastic increase of apoptotic population starting on day 2 post infection (11.59 ± 0.52%) from the initial of only 4.59 ± 0.57% and peaked on day 5 (68.81 ± 1.45%). In spleen, apoptosis rate grew from the initial of 1.73 ± 0.95% to the highest of 52.30 ± 1.72% on day 5 post infection.

Apart of determination of apoptosis using flow cytometry approach, the apoptotic rate of enriched IgM+ cell population was assessed using Acridine orange/ propidium iodide double stain to determine the morphological changes in the cells (Figures 4 & 5). Higher cell death events were reported in chicken bursa of Fabricius than chicken spleen when the birds are being infected with IBDV UPM0081 (Table 2).

SYBR Green based real time PCR was used to determine viral load in the chicken samples infected with very virulent IBDV UPM0081. Standard curves were plotted using viral RNA of IBDV UPM0081 (Supplementary data: Figure 1S), whilst non template control (NTC) served as negative control to confirm the PCR efficiency is good and the condition not contaminated. Results showed that both viruses can be detected in the samples starting day 2 post infection and the viral load increased on the following day (Table 3). From the result, it is shown that virus replicated in the cells and the increment of viral load is dependent of the time course of infection. Virus load was reported to be higher in the chicken bursa of Fabricius than in spleen, whereas when compared between crude cells and IgM+ cells, results showed that higher viral load in the IgM+ cells.

Table 1. Viable cell counts of enriched B lymphocytes population after infection of IBDV UPM0081 at different time points using trypan blue.

Group	Viability (%)	
	Bursa of Fabricius	Spleen
Control	90.65 ± 0.80^{a}	92.13 ± 0.54^{a}
Day 2	84.62 ± 0.51^{a}	63.64 ± 0.41^{b}
Day 4	36.67 ± 0.12^{b}	58.75 ± 0.43^{b}
Day 5	30.71 ± 0.53^{c}	41.52 ± 0.31^{c}

All results represent the mean ± standard deviation (n = 3). Within the same column, groups labelled with different superscript are significantly different ($P \leq 0.05$).

Table 2. AO/Pi Assay of Enriched B lymphocytes infected with IBDV UPM0081.

Group	Cell number		
Spleen	Viable cells	Apoptotic cells	Death cells
Control	82.42 ± 15.64^{ae}	6.18 ± 1.09^{a}	4.77 ± 4.05^{a}
Day 2	61.33 ± 1.42^{b}	20 ± 0.51^{ac}	18.66 ± 1.42^{a}
Day 4	45.93 ± 7.55^{c}	15.07 ± 6.14^{a}	38.98 ± 8.09^{b}
Day 5	18.49 ± 8.51^{d}	37.33 ± 5.73^{b}	44.18 ± 6.45^{b}
Bursa	Viable cells	Apoptotic cells	Death cells
Control	84.94± 10.70^{a}	12.93 ± 0.60^{a}	2.13 ± 3.51^{a}
Day 2	72.75 ± 3.22^{e}	19.42 ± 2.60^{a}	7.84 ± 2.16^{a}
Day 4	7.97 ± 0.63^{f}	28.90 ± 4.59^{bc}	62.12 ± 5.51^{c}
Day 5	0.621 ± 0.38^{f}	14.29 ± 2.51^{a}	85.09 ± 1.58^{d}

All results represent the mean ± standard deviation (n = 3). Within the same column, groups labeled with different superscript are significantly different ($P \leq 0.05$).

Table 3. SYBR Green real time PCR result of detection of IBDV UPM0081 virus in the cells population isolated from chicken spleen and bursa of Fabricius.

Sample	C(t) Mean ± S.E.M	Viral Copy Number
Control spleen IgM^{+}	Not detectable	Not detectable^{a}
Day 2 spleen IgM^{+}	29.67 ± 2.49	2.22^{b}
Day 4 spleen IgM^{+}	26.60 ± 2.68	3.20^{c}
Day 5 spleen IgM^{+}	22.70 ± 0.80	4.43^{d}
Control bursa IgM^{+}	Not detectable	Not detectable^{a}
Day 2 bursa IgM^{+}	30.21 ± 1.17	2.05^{b}
Day 4 bursa IgM^{+}	20.85 ± 1.34	5.02^{d}
Day 5 bursa IgM^{+}	19.88 ± 0.57	5.33^{d}

C(t)= Cycle threshold. All results represent the mean ± standard deviation (n = 3). Within the same column, groups labeled with different superscript are significantly different ($P \leq 0.05$).

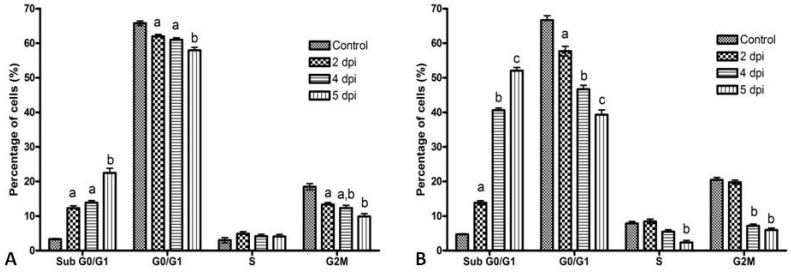

Figure 2. Perturbations of cell cycle phases of enriched IgM+ cells in A) chicken spleen and B) bursa of 35 days old chicken infected with IBDV UPM0081 at days 2, 4 and 5 post infection. The differences between the control group and infected groups in each cell cycle phase were determined by one-way ANOVA. Groups labeled with different alphabets are significantly different ($P \leq 0.05$).

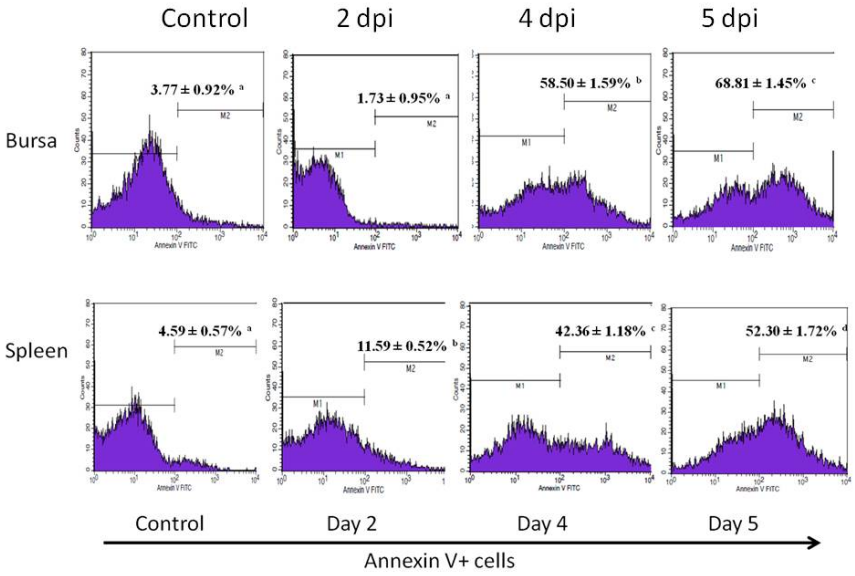

Figure 3. FACS analysis of AnnexinV-FITC binding of enriched IgM+ cells in chicken spleen and bursa of Fabricius of 35 days old chicken infected with IBDV UPM0081 at day 2, 4 and 5 post infection. M2 gate the percentage of Annexin V- FITC positive cells. The differences between the control group and infected group were determined by one-way ANOVA. Groups labeled with different superscripts are significantly different ($P \leq 0.05$).

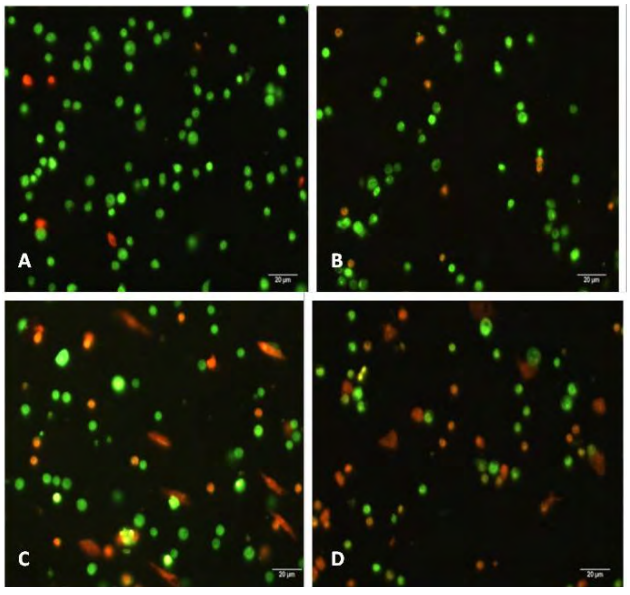

Figure 4. Representative figure of AO/PI assay showing chicken enriched IgM+ cells from chicken spleen after being infected with very virulent IBDV A) Control, B) 2 dpi, C4 dpi and D) 5 dpi. Magnification: x40, AO (excitation: 488 nm, emission: 545 nm), PI (excitation: 535 nm, emission: 617 nm).

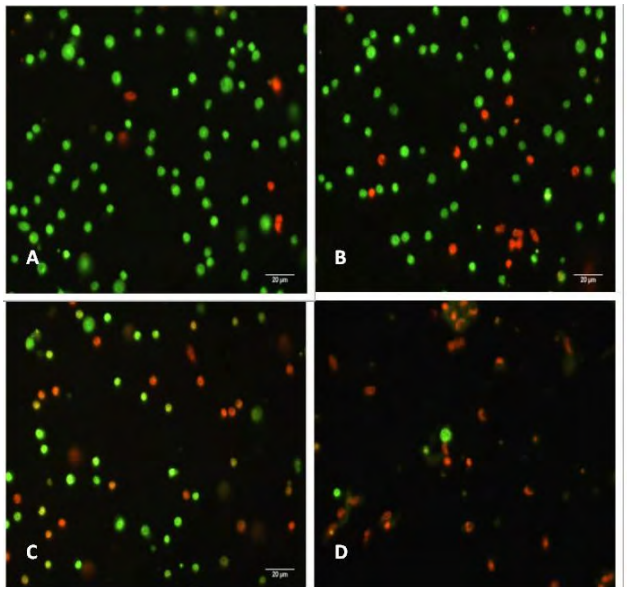

Figure 5. Representative figure of AO/PI assay showing chicken enriched IgM+ cells from chicken bursa of Fabricius after being infected with very virulent IBDV A) Control, B) 2 dpi, C4 dpi and D) 5 dpi. Magnification: x40, AOexcitation: 488 nm, emission: 545 nm), PI (excitation: 535 nm, emission: 617 nm).

DISCUSSION

Very virulent strains of IBDV caused not only immunosuppresion in chickens [10,15] but also variable mortality in chicken flock [12]. The innate responses against IBDV as the first line of defense can be avoided through the virus suppression of interferon and intrusion of the pathway [13,22]. Subsequent adaptive immunity responses are crucial to control the infection of the virus by neutralizing antibodies [2] as well as T cells dependent immunity [15]. To study the adaptive immunity against IBDV infection, the pathogenesis of very virulent IBDV strain isolated from Malaysia on its immunoregulatory effect on lymphocytes was investigated, especially the IgM+ cells from chicken spleen and also bursa of Fabricius. This study demonstrated varied immune responses between two IBDV targeted lymphoid organs which are the bursa of Fabricius and spleen after infected with Malaysia strain of vvIBDV UPM0081. From the results, it was shown that IBDV UPM0081 resulted in the reduction of IgM+ cells viabilty in both chicken spleen and bursa. Sharma and team reported that virulent IBDV caused extensive destruction of bursal follicles, which subsequently leaded to the depletion of B lymphocytes [17]. The bursal B lymphocytes cell viability was observed to be decreasing following the infection of very virulent IBDV. The cells were then investigated of their apoptotic rate and data showed that increasing apoptotic cells (early and late apoptosis) were observed in AO/PI double stain as well as increment of SubG0/G1 population in the cell cycle analysis and also increment of Annexin V FITC bound cells in the apoptosis study. It was deduced that the apoptotic population in the chicken bursa was characterized more likely to be the late apoptotic ones according to the morphological assessment using AO/PI double stains.

As for B lymphocytes from chicken spleen, the magnitude of damage caused by very virulent IBDV was not as severe as what being observed in the chicken bursa, with the cell viability drastically decreased on day 4 following IBDV infection. Interestingly, only at this time point the viability of B lymphocytes is lower in spleen compared to chicken bursa and the apoptotic events is higher. Looking at the IBDV viral load to the apoptosis events in B lymphocytes of the chicken spleen, no direct relationship between these two parameters were observed. Thus, it was postulated that the increasing event of cell death in B lymphocytes of chicken spleen at this time point could be caused by others factors, which included the elevation of NO and MDA level as oxidative stress and nitric oxide were reported to play a role in the apoptosis of immune cells [23]. Earlier expression of proinflammatory cytokines (IL-1β, IL-6, CCL4, CXCLi1) were detected in chicken spleen compared to bursa specimens [14] may also attributed to the trigger of apoptotic event in spleen isolated IgM+ cells.

Relating these results to the IBDV viral load detected in the chicken bursal enriched B lymphocytes population, it was observed that IBDV was detected in the chicken organ starting day 2 post infections and the copy number of the virus gene increased on the subsequent day. Thus a positive correlation between the virus replication in the B lymphocytes of chicken bursa and the induction of apoptosis can be concluded as the number of apoptotic B cells in the bursa of Fabricius increased with time and amount of viral RNA load. Similar finding was reported by Jungmann and team that apoptosis in cells was associated with the IBDV replication process [7].

CONCLUSION

In conclusion, the cell death events of IgM+ cells in chicken spleen and bursa of Fabricius may be accounted by different factors upon infection with Malaysia strain of IBDV UPM0081. It is obvious that IgM+ cells from chicken bursa suffered from apoptotic cell death in an increasing manner considerably with time of infection and RNA load detected in the cells, which supported by previous literature that IBDV induces host cells apoptosis, with both VP2 and VP5 playing a role in binding and apoptosis [2,9,21]. Meanwhile, the cell death events of B lymphocytes in chicken spleen was observed to be more relevant to other factors such as the oxidative stress or pro-inflammatory cytokines that caused by the virus infection rather than the viral RNA load.

Supplementary data. Supplementary data associated with this article can be found on the *Acta Scientiae Veterinariae*'s website.

Figure 1S. A linear relationship between quantification cycle (Cq) and 10-fold serial dilution RNA (from 1000 ng to 0.1 ng/ reaction). The standard curve was produced using RNA extracted from IBDV UPM0081.

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***Funding*.** This project was supported by Institute of Bioscience, Higher Institution Centre of Excellence (IBS HICoE) grant from the Ministry of Higher Education, Government of Malaysia. Kristeen Ye Wen Teo was funded by a Graduate Research Fellowship, Universiti Putra Malaysia.

***Ethical approval*.** The animal trials were carried out under approval by the Animal Care and Use Committee, at the Faculty of Veterinary Medicine, Universiti Putra Malaysia (reference number UPM/IACUC/AUP-R022/2014).

***Declaration of interest*.** All authors declare that they have no competing interests.

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