
**UNIVERSITI PUTRA MALAYSIA**

# *ADAPTATION, ATTENUATION AND MOLECULAR CHARACTERISATION OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS FOR DEVELOPMENT OF TISSUE CULTURE-BASED ATTENUATED AND INACTIVATED VACCINES*

**NAFI'U LAWAL**

**FPV 2018 10**

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By

**NAFI'U LAWAL**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

**March 2018**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

# ADAPTATION, ATTENUATION AND MOLECULAR CHARACTERISATION OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS FOR DEVELOPMENT OF TISSUE CULTURE-BASED ATTENUATED AND INACTIVATED VACCINES

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**NAFI'U LAWAL**

**March 2018**

**Chairman : Professor Mohd Hair-Bejo, PhD**
**Faculty : Veterinary Medicine**

Infectious bursal disease (IBD) is an important viral disease of chickens worldwide that causes high mortality, immunosuppression and serious economic loss in poultry industry that can only be prevented by proper vaccination and biosecurity programmes. The disease is endemic in Malaysia. Although IBD vaccines have been produced successfully using chicken eggs, but high cost, limited supply of specific pathogen free (SPF) eggs and possible contamination by viruses of avian origin limits the use of eggs as a tool for vaccine production. Currently, IBD vaccines mostly produced using classical (ca) and variant (va) IBDV as seed virus are SPF egg based. However, the high level of maternal antibody in chicks induced by the vaccine can be neutralized by vvIBDV. The vvIBDV strain also was reported to be difficult to adapt to tissue cultures in vitro

It was the objectives of this study to determine suitable tissue cultures for the adaptation, propagation and attenuation of vvIBDV and produce a suitable live attenuated and inactivated IBDV isolates for vaccine development using bioreactor technology. Two Malaysian vvIBDV strains UPM0081 (also known as B00/81) and UPM190 (also known as UPM04/190) isolated from local IBD outbreaks were serially passaged in SPF embryonated chicken eggs via the chorioallantoic membrane (CAM) route for up to 12 passages. The isolates were then further adapted and propagated into chicken embryo fibroblast (CEF), Vero and BGM-70 cell lines for 10, 10 and 20 consecutive passages, respectively. The molecular characteristics of the vvIBDV at different passages were identified, analyzed and phylogenetic trees were constructed. Selected isolates passaged either in SPF embryonated chicken eggs or cell lines were further propagated in BGM-70 cell line using bioreactor. The pathogenicity,

immunogenicity and efficacy of attenuated and inactivated of these vvIBDV isolates were determined in chickens.

The study showed that 75% cytopathic effect (CPE) was developed in CEF following propagation of the UPM190 vvIBDV at passage 5 (P5) with the virus titre of $10^{6.28}$TCID$_{50}$/mL. The UPM0081 vvIBDV was similarly adapted at P5 with 75% CPE and a titer of $10^{5.5}$TCID$_{50}$/mL. In the Vero cells, 75% CPE was recorded at P5 and became 100% by P10 within 7days pi with virus titer of $10^{5.5}$ TCID$_{50}$/mL for UPM190. The UPM0081 similarly adapted at P1with the same CPE pattern, and by P5 the CPE was 75% with a virus titer of $10^{4.62}$ TCID$_{50}$/mL. Adaptation of UPM190 in BGM-70 cell line was achieved as early as P1 and was more prominent at P5 with a virus titer of $10^{9.5}$ TCID$_{50}$/mL. Based on the titers obtained, BGM-70 cell line was selected for further propagation until the CPE became subtle at P18 and P19 for UPM0081 and UPM190, respectively. The serial passaging was finally stopped at P20. The CPE pattern was observed in BGM-70 cells inoculated with UPM190 isolate started at 50% CPE and gradually increased up to 100% at P9 and fall to 25% by P20 with a virus titer of $10^{9.5}$ TCID$_{50}$/mL at P5. The UPM0081 exhibited similar adaptation and CPE pattern with UPM190 except that the titer was $10^{9.2}$ TCID$_{50}$/mL at P5 and the CPE was 25% at P1 and fell to 25% by P20. The CPE pattern was similar in all the cell lines characterized by cell rounding, cytoplasmic vacoulation and granulation as well as detachment from flask.

The UPM0081 inoculated and adapted in SPF embryonated chicken eggs resulted in hyperemia, ecchymotic hemorrhages in the thigh and breast muscles, greenish to yellowish liver and intracranial hemorrhages. The UPM190 showed abdominal distension, subcutaneous edema, intracranial hemorrhages and mottled liver of the infected embryo.

Both CAM and cell culture samples at various passages were positive for vvIBDV by RT-PCR and sequencing. Sequence analysis using MEGA v.7 and BioEdit v.7 bioinformatics software that UPM190 vvIBDV propagated in SPF embryonated chicken eggs resulted in amino acid changes at A279D position as early as P8. Other amino acid changes observed were N212, E249, M264, A270 and N279 at EP12 which were maintained for up to P16. However, the UPM0081 had no amino acid changes. The amino acids observed in CEF adapted viruses at P1, P5 and P10 were E249, M264, A270 and D279 except for P10 where there was a change at 279 from D to N. Similarly, the Vero adapted viruses were showing similar molecular changes with the CEF adapted strains and only P10 was showing N279 mutation. In BGM-70 cell line, UPM190 showed that P1 and P5 viruses possessed E249, M264, A270 and N279 amino acids; Q249, I264, E270 and A279 amino acids at P10 to P20. The E249Q, I264 and A270E appeared to be maintained from P10 up to P20 in the UPM190 isolates. The UPM0081 also possessed Q249, I264 and E270 amino acids at P10 to P20 which appeared to be unique. The phylogenetically, all the isolates were in the vvIBDV clade as they cluster with other reported vvIBDV sequences deposited in GeneBank.

The vvIBDV UPM190 and UPM0081 propagated in BGM-70 at P15 were selected and successfully propagated in a bioreactor system with high viral yield and the bioreactor passaged viruses demonstrated the A270E amino acid changes. However, UPM190EP8 and UPM0081EP8 isolates propagated once in BGM-70 cell line and or bioreactor were lacking the E270 amino acid changes that was seen in the BGM-70 P15 or its bioreactor propagated isolate.

The study showed that UPM0081 and UPM190 isolates at BGM-70 P5 and P8 when inoculated in SPF chickens did not cause any clinical signs, gross or histopathological lesions at day 7 post inoculation (pi). However, IBDV were detected in the bursa of Fabricius using RT-PCR technique. The IBDV was not detected in the bursa of Fabricius when chickens were inoculated with BGM-70 P15 IBDV isolate. In contrast, clinical signs, gross and histological lesions were observed when UPM0081 and UPM190 at EP8 were inoculated in chickens. Similarly, when the isolates EP8 isolates were propagated in BGM-70 at P1 in flask and P1 in bioreactor, the IBDVs were detected in the bursa of Fabricius by RT-PCR technique. It appeared the UPM0081 and UPM190 IBDV isolates loss their pathogenicity when passaged in BGM-70 at P15. The EP8 (EP8BGMP1) isolates propagated in bioreactor were inactivated for the development of killed IBDV vaccine.

Furthermore, the study showed that day old commercial broiler chickens when inoculated with either the inactivated UPM190 (group A), inactivated UPM0081 (group B), attenuated UPM190BGMP10 (group C), attenuated UPM0081BGMP10 (group D), attenuated and inactivated UPM190 (group E) or attenuated and inactivated UPM0081 (group F) as well as the non-inoculated control group G did not cause any clinical abnormalities throughout 28 days of the trial. Grossly, bursa atrophy was recorded in the IBDV inoculated groups A to F when compared to the control group G. Histologically, overall bursa lesion scoring ranging from mild (scoring of 1) to mild to moderate (scoring of 2) were recorded in chickens from groups A to F when compared to the control group G with lesion scoring of 1 (mild) throughout the trial. The IBD antibody titre in the IBDV inoculated groups A (283±40), B (244±18) and E (253±94) were not significantly different ($P>0.05$) when compared to the control group (253±97) at day 28 post inoculation (pi). In contrast, the titre was significantly higher ($P<0.05$) in groups C (505±91), D (412±146) and F (642±187). The study also showed that no abnormal clinical signs were recorded in chickens inoculated with IBDV in all groups (A to F) at 7 days post challenged (pc) when they were challenged with vvIBDV at 21 days pi. However, in the control group F severe depression and ruffled feathers were recorded at days 3 to 6 pc, but it recovered at day 7 pc. Grossly, bursa atrophy was recorded in the IBDV inoculated groups A to F at day 7 pc. Severe bursal atrophy with hemorrhages, congestion and yellowish to red exudates in the bursal mucosa were recorded in the control group F. Histologically, overall bursa lesion scoring ranging from mild to moderate (scoring of 2) to moderate (scoring of 3) were recorded in chickens from groups A to F when compared to the control group G with lesion scoring of 4 (moderate to severe) at day 7 pc. The IBD antibody titre in the IBDV inoculated groups A (5782±1517), B (5151±1479), C (4670±787), D (4644±1359), E (7315±1838) and F (6235±1655) were significantly higher ($P>0.05$)

when compared to the control group G (2475 ±991) at day 7 pc. This demonstrated that the inactivated and attenuated combination of UPM190 isolate (group E) offered the best protection against vvIBDV challenged followed in descending order by inactivated and attenuated UPM0081 combination (group F), inactivated UPM190 (group A), inactivated UPM0081 (group B), attenuated UPM190 (group C) and lastly attenuated UPM0081 (group D), suggesting that UPM190 isolate is more immunogenic when compared to UPM0081 isolate.

It was concluded that the UPM190 and UPM0081 vvIBDV isolates were successfully adapted in various cell lines with high suitability to BGM-70. The vvIBDVs were attenuated following serial passaging with changes in amino acids composition at VP2 region resulting lost in pathogenicity and immunogenicity in the higher passage isolates. The vvIBDVs were successfully propagated in a bioreactor system with high titer yield for the development of attenuated and inactivated IBD tissue culture based vaccines. The chicken trials conducted showed that the bioreactor propagated IBD viruses were good immunogens that induced the production of high levels of IBD neutralizing antibodies which protected the chickens against severe bursa of Fabricius damage when challenged with vvIBDV isolate.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**ADAPTASI, ATENUASI DAN MOLEKUL PENCIRIAN VIRUS PENYAKIT BURSA BERJANGKIT YANG AMAT VIRULEN UNTUK PEMBANGUNAN VAKSIN ATENUASI DAN TIDAK AKTIF BERASASKAN TISU KULTUR DI MASA HADAPAN**

Oleh

**NAFI'U LAWAL**

**Mac 2018**

**Pengerusi : Profesor Mohd Hair-Bejo, PhD**
**Fakulti : Perubatan Veterinar**

Penyakit bursal berjangkit (IBD) adalah penyakit virus ayam yang penting di seluruh dunia yang menyebabkan kematian yang tinggi, melemahkan daya imunisasi dan kerugian ekonomi yang serius dalam industri ayam. Penyakit ini hanya boleh dicegah dan dikawal dengan program vaksinasi dan biosekuriti yang sesuai. Penyakit ini adalah endemik di Malaysia. Walaupun vaksin IBD telah dihasilkan dengan jayanya menggunakan telur ayam, tetapi kos yang tinggi, bekalan terhad telur bebas patogen spesifik (SPF) dan kemungkinan pencemaran oleh virus ungas yang lain menghadkan penggunaan telur sebagai alat untuk pengeluaran vaksin. Pada masa ini, vaksin IBD kebanyakannya dihasilkan menggunakan virus IBD (IBDV) klasik (ca) dan varian (va) sebagai benih virus dengan menggunakan telur ayam SPF. Walaubagaimanapun, tahap antibodi ibu yang tinggi pada ayam yang dihasilkan oleh vaksin ini dapat dinutralkan oleh vvIBDV. Strain vvIBDV juga dilaporkan sukar untuk pensesuaian dalam tisu kultur secara in vitro.

Adalah menjadi objektif kajian ini bagi menentukan tisu kultur yang sesuai untuk mengadaptasi, menyebar dan mengatenuasi IBDV yang amat virulen (vvIBDV) dan menghasilkan isolat IBDV yang diatenuasi dan tidak aktif untuk pembangunan vaksin dengan menggunakan teknologi bioreaktor. Dua strain vvIBDV Malaysia UPM0081 (dikenali juga sebagai B00/81) dan UPM190 (dikenali juga sebagai UPM04/190) yang diasingkan daripada wabak IBD tempatan telah dipasag secara bersiri dalam telur ayam berembrio patogen SPF melalui membran korioalantoik (CAM) sehingga 12 pasag. Isolat tersebut kemudiannya diadapatasi and disebarkan dalam tisu kultur fibroblas embrio ayam (CEF), sel Vero dan BGM-70 masing-masing sebanyak 10, 10 dan 20 pasag berturut-turut. Ciri molekul vvIBDV pada pasag yang berbeza telah

dikenalpasti, dianalisis dan pokok filoginetik telah dibina. Isolat yang terpilih samaada yang telah dipasag dalam telur ayam SPF atau sel BGM-70 kemudianya disebarkan dengan menggunakan bioreaktor. Patogenisiti, imunogenisiti dan keberkesanan isolat vvIBDV yang telah diatenuasi dan tidak aktif ditentukan dalam ayam SPF dan ayam pedaging komersial.

Kajian menunjukkan bahawa 75% kesan sitopatik (CPE) telah terbentuk di CEF berikutan penyebaran UPM190 vvIBDV pada pasag 5 (P5) dengan titre virus $10^{6.28}$TCID$_{50}$ / mL. UPM0081 vvIBDV juga mengalami adaptasi pada P5 dengan 75% CPE dan titer $10^{5.5}$TCID$_{50}$ / mL. Dalam sel Vero, 75% CPE direkodkan pada P5 dan menjadi 100% pada P10 dalam tempoh 7 hari pasca inokulasi (pi) dengan titer virus $10^{5.5}$ TCID$_{50}$ / mL untuk UPM190. UPM0081 juga mengalami adaptasi yang sama di P1 dengan corak CPE yang sama, dan pada P5 CPE adalah 75% dengan titer virus $10^{4.62}$ TCID$_{50}$ / mL. Adaptasi UPM190 dalam sel BGM-70 telah dicapai seawal P1 dan lebih menonjol pada P5 dengan titer virus $10^{9.5}$ TCID$_{50}$ / mL. Berdasarkan titer yang diperoleh, sel BGM-70 telah dipilih untuk penyebaran lanjut sehingga CPE menjadi kurang kelihatan masing-masing di P18 dan P19 untuk UPM0081 dan UPM190. Siri pasag akhirnya dihentikan pada P20. Corak CPE diperhatikan dalam sel BGM-70 yang di inokulasi dengan isolat UPM190 bermula pada CPE 50% dan secara beransur meningkat sehingga 100% pada P9 dan jatuh ke 25% pada P20 dengan titer virus $10^{9.5}$ TCID$_{50}$ / mL pada P5. UPM0081 mempamerkan penyesuaian dan corak CPE yang sama dengan UPM190 kecuali titer adalah $10^{9.2}$ TCID$_{50}$ / mL pada P5 dan CPE adalah 25% pada P1 dan jatuh kepada 25% pada P20. Corak CPE adalah serupa terdiri daripada sel bulat kecil dan biasan, granulasi dan vakuola sitoplasma dengan sel terlepas dari kelalang.

UPM0081 yang disuntik dan diadaptasi dalam telur ayam berembrio SPF menyebabkan hiperemia, pendarahan ekimotic di paha dan otot dada, kehijauan hingga kekuningan pada hati dan pendarahan intrakranial. UPM190 menunjukkan lesi seperti menyebabkan pembengkakan abdomen, edema subkutaneus, pendarahan intrakranial dan hati berbintik keatas embrio yang dijangkiti.

Sampel CAM dan sel kultur pada pelbagai pasag adalah positif untuk vvIBDV dikesan dengan RT-PCR. Analisis urutan dijalankan menggunakan perisian MEGA v.7 dan BioEdit v.7 perisian bioinformatik. UPM190 vvIBDV yang dipasag dalam telur ayam SPF berembrio menyebabkan perubahan asid amino pada kedudukan A279D seawal P8. Perubahan asid amino lain yang dilihat adalah N212, E249, M264, A270 dan N279 di EP12 yang dikekalkan sehingga P16. Walaubagaimanapun, UPM0081 stabil dari P1 hingga P16 tanpa perubahan asid amino. Perubahan asid amino dapat dilihat pada virus yang diadaptasi dalam CEF pada P1, P5 dan P10 adalah E249, M264, A270 dan D279, kecuali P10 di mana terdapat perubahan pada 279 dari D ke N. Begitu juga, virus yang diadaptasi dalam sel Vero telah menunjukkan perubahan molekul yang serupa dengan CEF adaptasi strain dan hanya P10 menunjukkan mutasi N279. Dalam BGM-70, UPM190 vvIBDV menunjukkan bahawa virus P1 dan P5 mempunyai asid amino E249, M264, A270 dan N279; Q249,

I264, E270 dan A279 asid amino pada P10 hingga P20. E249Q, I264 dan A270E kelihatan dikekalkan dari P10 hingga P20 dalam isolat UPM190. UPM0081 juga mempunyai asid amino Q249, I264 dan E270 pada P10 hingga P20. Strain virus ini juga mengekalkan perubahan A270E dari P10 hingga P20, tetapi tidak mempunyai perubahan E249Q yang dilihat di UPM190. Perubahan A270E kelihatan unik. Pokok filogenetik yang dibina menunjukkan bahawa kedua-dua isolat adalah dalam kluster vvIBDV bersama dengan urutan vvIBDV yang lain yang dilaporkan dalam simpanan GeneBank.

UPM190 dan UPM0081 vvIBDV yang disebarkan dalam BGM-70 pada P15 telah dipilih dan berjaya disebarkan dalam sistem bioreaktor dengan hasil virus yang tinggi dan virus yang dipasag dalam bioreaktor menunjukkan perubahan asid amino A270E. Walaubagaimanapun, isolat UPM190EP8 yang disebarkan sekali lagi dalam sel BGM-70 menggunakan kelalang tisu kultur dan sekali dalam BGM-70 menggunakan bioreaktor menunjukan ketiadaan perubahan asid amino E270 yang dilihat dalam BGM-70 P15 atau isolat yang disebarkan dalam bioreactor.

Kajian menunjukan isolat UPM0081 dan UPM190 yang diadptasi di BGM-70 pada P5 dan P8 apabila diinokulasi pada ayam SPF tidak menyebabkan tanda klinikal, lesi matakasar dan histologi pada hari ke 7 pi. Walaubagaimanapun, IBDV dikesan di bursa Fabricius menggunakan teknik RT-PCR. IBDV tidak dikesan di Bursa Fabricius apabila ayam disuntik dengan isolat BGM-70 P15 IBDV. Sebaliknya, tanda klinikal, lesi matakasar dan histologi diperhatikan apabila UPM0081 dan UPM190 di EP8 disuntikkan dalam ayam. Begitu juga, apabila isolat EP8 isolat disebarkan dalam BGM-70 pada P1 dalam kelalang tisu dan P1 dalam bioreaktor, IBDV dikesan di bursa Fabricius dengan teknik RT-PCR. Ini menunjukan bahawa UPM0081 dan UPM190 IBDV hilang patogenisiti apabila dipasag dalam BGM-70 pada P15. Isolat EP8 (EP8BGMP1) yang disebarkan dalam bioreaktor telah tidak diaktifkan untuk pembangunan vaksin IBDV terbunuh.

Selain itu, kajian ini menunjukkan ayam pedaging komersial berumur satu hari apabila disuntik samada dengan UPM190 yang tidak aktif (kumpulan A), UPM0081 yang tidak aktif (kumpulan B), UPM190BGMP10 yang diatenuasi (kumpulan C), UPM0081BGMP10 yang diatenuasi (kumpulan D), UPM190 yang diatenuasi dan tidak aktif (kumpulan E) atau UPM0081 yang diatenuasi dan tidak aktif (kumpulan F) serta kumpulan kawalan G yang tidak disuntik, tidak menyebabkan keabnormalan klinikal sepanjang 28 hari kajian. Secara matakasar, atrofi bursa direkodkan dalam kumpulan A hingga F yang disuntik dengan IBDV berbanding dengan kumpulan kawalan G. Secara mikroskopi, keseluruhan skor lesi bursa berkisar dari ringan (skor 1) kepada ringan hingga sederhana (skor 2) dicatatkan dalam ayam dari kumpulan A hingga F apabila dibandingkan dengan kumpulan kawalan G dengan skor lesi 1 (ringan) sepanjang kajian. Titer antibodi IBD dalam kumpulan yang disuntik IBDV, kumpulan A (283 $\pm$ 40), B (244 $\pm$ 18) dan E (253 $\pm$ 94) tidak berbeza ($P > 0.05$) berbanding dengan kumpulan kawalan (253 $\pm$97) pada hari ke 28 pasca inokulasi (pi). Sebaliknya, titer adalah lebih tinggi ($P < 0.05$) dalam kumpulan C (505 $\pm$91), D (412

± 146) dan F (642 ± 187). Kajian ini juga menunjukkan bahawa tiada tanda klinikal yang tidak normal dicatatkan dalam ayam yang disuntik dengan IBDV dalam semua kumpulan (A hingga F) pada 7 hari pasca dicabar (pc) apabila mereka dicabar dengan vvIBDV pada 21 hari pi. Walaubagaimanapun, dalam kumpulan kawalan F kemurungan yang teruk dan bulu yang tidak terurus dicatatkan pada hari 3 hingga 6 pc, tetapi ia pulih pada hari 7 pc. Secara matakasar, bursa atrofi direkodkan dalam kumpulan yang disuntik IBDV (kumpulan A hingga F) pada hari 7 pc. Atrofi bursal yang teruk dengan pendarahan, kongesi dan kekuningan hingga ke merahan eksudat dalam mukosa bursal dicatatkan dalam kumpulan kawalan F. Secara mikroskopi, keseluruhan skor lesi bursa berkisar dari ringan hingga sederhana (skor 2) hingga sederhana (skor 3) dicatatkan dalam ayam dari kumpulan A hingga F apabila dibandingkan dengan kumpulan kawalan G dengan skor lesi 4 (sederhana hingga teruk) pada hari 7 pc. Titer antibodi IBD dalam kumpulan yang disuntik IBDV, kumpulan A (5782 ± 1517), B (5151 ± 1479), C (4670 ± 787), D (4644 ± 1359), E (7315 ± 1838) dan F (6235 ± 1655) adalah lebih tinggi (P> 0.05) apabila dibandingkan dengan kumpulan kawalan G (2475 ± 991) pada hari 7 pc. Ini menunjukkan bahawa gabungan UPM190 yang diatenuasi dan tidak diaktifkan (kumpulan E) menawarkan perlindungan yang terbaik terhadap cabaran vvIBDV diikuti dalam urutan menurun oleh gabungan UPM0081 yang diatenuasi dan tidak diaktifkan (kumpulan F), UPM190 yang tidak diaktifkan (kumpulan A), UPM0081 yang tidak diaktifkan (kumpulan B), UPM190 yang diatenuasi (kumpulan C) dan akhirnya UPM0081 yang diatenuasi (kumpulan D), mencadangkan bahawa UPM190 isolat lebih imunogenik apabila dibandingkan dengan UPM0081 isolat.

Disimpulkan bahawa isolat UPM190 dan UPM0081 vvIBDV telah berjaya diadaptasi di pelbagai sel kultur dengan kesesuaian tinggi di BGM-70. vvIBDV telah diatenuasi dengan perubahan dalam komposisi asid amino pada kawasan VP2 semasa pasag bersiri yang mengakibatkan patogenisiti dan imunogenisiti isolat hilang dalam pasag yang lebih tinggi. vvIBDV telah berjaya disebarkan dalam sistem bioreaktor dengan hasil titer yang tinggi untuk pembangunan vaksin atenuasi dan tidak diaktifkan berasaskan tisu kultur. Kajian dalam ayam yang dilakukan menunjukkan bahawa IBDV yang disebarkan dalam bioreactor adalah immunogen yang baik yang dapat menghasilkan IBD antibodi titer yang tinggi dan dapat melindungi ayam terhadap kerosakan bursa Fabricus yang teruk ketika dicabar dengan vvIBDV isolat.

## ACKNOWLEDGEMENTS

All praise is for Allaah, we thank Him, seek His help and His forgiveness and we seek refuge with Allaah from the evil of our own souls and from our bad deeds. Whomsoever Allaah guides will never be led astray, and whomsoever Allaah leaves astray, no one can guide. I bear witness that there is no god but Allaah, alone and without any associate and I bear witness that Muhammad is His slave and Messenger.

"O you who have believed! fear Allaah as He should be feared and do not die except as Muslims [in total submission to Him]" (Aal 'Imraan 3:102). "O mankind, fear your Lord, who created you from one soul and created from it its mate and dispersed from both of them many men and women. And fear Allaah, through whom you ask one another, and the wombs. Indeed Allaah is ever, over you, an Observer". (An-Nisaa' 4:1). "O you who have believed! fear Allaah and speak words of appropriate justice. He will [then] amend for you your deeds and forgive you your sins. And whoever obeys Allaah and His Messenger has certainly attained a great attainment" (al-Ahzaab 33:70-71).

The prophet Muhammad (SallaLahu Alaihi Wasallam) said "He has not thanked Allaah who has not thanked people" (Ahmad, Abu Dawood). It is therefore, a moral obligation on me to thank my Creator, Allaah (The Exalted, Most High) for His unlimited blessings and mercies and for giving me the opportunity to successfully complete this study. I would like to express my sincere gratitude and heartfelt appreciation to my great mentor Professor Dr. Mohd Hair Bejo, my academic father and supervisor, for his kind guidance, astute comments, painstaking supervision, continuous encouragement, and kind assistance and patience throughout my research journey in this study. I am also grateful and indebted to the support and mentoring of my co-supervisors, Professor Dr. Abdul Rahman Omar, for his unscrupulous supports, guidance and mentorship; I really appreciate the knowledge transfer. My special thanks and appreciation goes to Professor Dr. Siti Suri Arshad who was always kind and supportive with constructive criticisms and for your motherly guidance. I also want to express my sincere appreciation and thanks to Professor Datin Paduka Dr. Aini Ideris, my mother, for her encouragements, professional support and guidance without which, this study will not come to reality.

Words alone cannot express my gratitude and sincere appreciation to my wonderful mother Fatima, who have supported my study journey every step of the way. Your support, prayers and foresight fuelled my drive and strengthened my determination, an essential ingredient for the success of this work. Thank you for being there and I love you. I want to thank my brothers Naziru, Umar, Aliyu (Abba), Nura and Najib, my sisters Sumayya, Labiba and Ummulkhair (Ameera) for their love, support and prayers, I really appreciate.

My lovely wife Samira has been a source of support, prayers and encouragements throughout this research journey. She has tolerated my late nights and incessant absence as I battle my way through. Words cannot express my appreciation for the understanding and patience you have shown and for the wise and sound advises you rendered to me my love. May Allah continue to strengthen our marital bonds with love, mercy and understanding till eternity (Jannatul Firdaus), I love you. Equally important is my lovely wife Dr. Aisha, whom I have caused an unmeasurable pain as I treaded on this path. Words cannot express how sorry I am for the pain caused and the time lost, ours is a strange love that is hard to fathom. May Allah reward you abundantly, grant us an everlasting peace and love and strengthen our marital bond till eternity (Jannatul Firdaus), I love you.

To my little angels Amina and Aisha (daughters) and Muhammad Auwal (Ameer-Hajji, son) and your yet to be born siblings, who constitute my happiness and perfect my little world, I appreciate your understanding and prayers and the patience you have shown for my absence when you needed my fatherly attention and love the most, a big thanks to you all.

To my friends Drs Jamilu, Bashir Tambuwal, Faruku Bande, Yusuf Abba, Hussain Abdallah, Nur Fitriah, Ashwak, Abdullahi, Bashir Garba, Mohammad Talba, Aliyu, Isah, Alhassan Sharif, Hassan Yahya, Sirajo Bello, Muhammad Sanusi, Abdullahi Gindi, Rabiu, Adamu Abdul, Abdulkarim, Aliyu Sa'adu, Abdullahi Raji, Chidozie Ugwu, Bello Jambako, Malams Rayyan, Mukhtar (Bola), Sunusi, Muhammad Waziri, Sagir Ahmad, Sufyanu Maru, Nasir Funtua and many more too numerous to mention, your contributions both in kind and any other way towards the success of this work is sincerely acknowledged, thank you for all you have done.

I would also like to acknowledge and thank the entire staff and students of Laboratories of Serology, Virology, Bacteriology, Parasitology, Histopathology, Central Sterilization Room and Animal Research Facility of the Faculty of Veterinary Medicine, UPM as well as Laboratory of Vaccine and Immunotherapeutics of the Institute of Bioscience, UPM for allowing me to use their facilities thereby making this study possible.

Finally, I offer my regards and prayers to all of those who supported me in any way during this journey but I did not specifically mentionned in person, the kindness was well acknowledged.

Lastly, I would like to humbly dedicate this thesis to my late father, Alh. Lawal Muhammad. May Allaah in His infinite mercy forgive your shortcomings, have mercy on you, and grant you Jannatul Firdaus as your final abode. You are surely missed!

*"Glory is to You, O Allaah, and praise is to You. I bear witness that there is none worthy of worship but You. I seek Your forgiveness and repent to You"* [Abu Dawood, At-Tirmidhim Ibn Majah and An-Nasai].

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

**Mohd Hair Bejo, PhD**
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

**Abdul Rahman Omar, PhD**
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

**Siti Suri Arshad, PhD**
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

**Datin Paduka Aini Ideris, PhD**
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

_____
**ROBIAH BINTI YUNUS, PhD**
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature: _____
Name of Chairman of Supervisory Committee: Professor Dr. Mohd Hair Bejo

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Abdul Rahman Omar

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Siti Suri Arshad

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Datin Paduka Aini Ideris

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | Amino acid |
| AGPT | Agar gel precipitation test |
| atIBDV | Attenuated infectious bursal disease virus |
| ATV | Antibiotic-trypsin-versine |
| BEI | Binary ethylenimine |
| BF | Bursa of Fabricius |
| BGM-70 | Baby grivet monkey-70 |
| Bp | Base pair |
| CALT | Conjunctiva associated lymphoid tissue |
| CAM | Chorioallantoic membrane |
| caIBDV | Classical infectious bursal disease virus |
| cDNA | Complementary deaoxyribonucleic acid |
| CEB | Chicken embryo bursa |
| CEF | Chicken embryo fibroblast |
| CEK | Chicken embryo kidney |
| CMI | Cell-mediated immunity |
| $ddH_2O$ | Deionized double distilled water |
| DAB | Diaminobenzidine tetrahydrochloride |
| DF-1 | Douglas Foster-1 |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleoside triphosphate |
| dsDNA | Double-stranded DNA |

| | |
|---|---|
| DXV | Drosophila Melanogaster X virus |
| ELL | East lansing line |
| EID50 | Embryo effective dose fifty |
| ELISA | Enzyme-linked immunosorbent assay |
| FAPP | Filtered air positive pressure |
| FBS | Fetal bovine serum |
| FMD | Food and mouth disease |
| FP | Fowlpox |
| GALT | Gut associated lymphoid tissue |
| GDP | Gross domestic product |
| HALT | Head associated lymphoid tissue |
| H&E | Haematoxylin-and-eosin |
| HPAI | Highly pathogenic avian influenza |
| HPVR | Hypervariable region |
| HSV | Herpes simplex virus |
| IBD | Infectious bursal disease |
| IBDV | Infectious bursal disease virus |
| IB | Infectious bronchitis |
| IFT | Immunofluorescence technique |
| IgM | Immunoglobulin M |
| ILT | Infectious laryngotracheitis |
| IPNV | Infectious pancreatic necrosis virus |
| IPS | Immunoperoxidase staining technique |
| kB | Kilobase pair |

| | |
|---|---|
| kD | Kilo Dalton |
| LL | Lymphoid leucosis |
| LSCC-BK3 | Leucosis sarcoma chicken cell line |
| MA-104 | Microbiological Associates-104 cell line |
| MD | Marek's disease |
| Min | Minute |
| MRC-5 | Medical Research Council-5 cell strain |
| mRNA | Messenger RNA |
| MT | Metric tone |
| NaCl | Sodium chloride |
| NAP | National agro-food policy |
| ND | Newcastle disease |
| Nt | Nucleotide |
| OD | Optical density |
| OIE | Office international des epizooties |
| OK | Ovine kidney |
| ORF | Open reading frame |
| $P_{BC}$ | Minor hydrophilic region I |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| $P_{HI}$ | Minor hydrophilic region II |
| pH | Hydrogen ion exponent |
| Pi | Post infection |
| % | Percentage |

| | |
|---|---|
| Ps | Post seeding |
| RdRp | RNA dependent RNA polymerase |
| RK-13 | Rabbit kidney-13 |
| RNA | Ribonucleic acid |
| Rpm | Revolution per minute |
| RSV | Respiratory syncytial virus |
| RT-PCR | Reverse transcriptase-polymerase chain reaction |
| RT-PCR/RFLP | Reverse transcriptase-polymerase chain reaction/restriction fragment length polymorphism |
| RT | Room temperature |
| SC-1 | Cellosaurus mouse embryo fibroblast |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SPF | Specific-pathogen-free |
| TAE | Tris-acetate-EDTA |
| TV | Tequila virus |
| UPM | Universiti Putra Malaysia |
| USA | United State of America |
| vaIBDV | Variant strain of infectious bursal disease virus |
| Vero | Green monkey kidney |
| VP1 | Viral protein 1 |
| VP2 | Viral protein 2 |
| VP3 | Viral protein 3 |
| VP4 | Viral protein 4 |
| VP5 | Viral protein 5 |

| | |
|---|---|
| vaIBDV | Variant infectious bursal disease virus |
| vvIBDV | Very virulent infectious bursal disease virus |
| w/v | Weight per volume |
| µg | Microgram |
| µl | Microliter |
| µm | Micrometer |
| A | Alanine (Ala) |
| R | Arginine (Arg) |
| N | Asparagine (Asn) |
| D | Aspartic Acid (Asp) |
| Q | Glutamine (Gln) |
| E | Glutamic Acid (Glu) |
| G | Glycine (Gly) |
| I | Isoleucine (Ile) |
| L | Leucine (Leu) |
| K | Lycine (Lys) |
| M | Methionine (Met) |
| F | Phenylalanine (Phe) |
| P | Proline (Pro) |
| S | Serine (Ser) |
| T | Threonine (Thr) |
| W | Thyptophan (Trp) |
| V | Valine (Val) |

# CHAPTER 1

## INTRODUCTION

Commercial poultry production is one of the backbones of Agricultural economy in Malaysia. The industry has witnessed rapid advances within the last six decades similar to the advancement recorded in developed countries contributing 62.9% to the Malaysian livestock GDP (Shanmugavelu, 2014). With the rapid increase in population and economic growth in the country, there is a reciprocal increase in the demand of poultry and livestock products for consumption (Nor Amna A'liah & Mohammad, 2015). Based on the Malaysian National Agro-food Policy 2011-2020 (NAP), the demand for meat production is expected to increase with a 2.4% annual growth from 1.4 million metric tonnes (MT) in 2010 to 1.8 million MT in 2020 while meat production rate is estimated to rise at an annual 2.7% growth rate from 1.6 million MT to 2.1 million MT respectively within the same time frame (Nor Amna A'liah & Mohammad, 2015). The rapid growth of the industry can be due to favorable government policies, introduction of breeds of good quality (high vigor), shift from the traditional free range system of rearing to intensive management system, formulation of high quality feed, technological advancement and development of effective indigenous vaccines using local isolates. However, despite this high economic growth, the industry is faced with challenges of high production costs due to diseases and its management especially the reemerging ones (Hair-Bejo, 2010). Interestingly, almost all the major known poultry diseases such as Infectious Bronchitis (IB), Infectious Bursal Disease (IBD), Newcastle Disease (ND), Marek's Disease (MD), Lymphoid leukosis (LL), Fowl Pox (FP), Infectious Laryngotracheitis (ILT), Highly pathogenic Avian Influenza (HPAI), Salmonellosis, Fowl Cholera, Mycoplasmosis, and Coccidiosis have been reported in Malaysia (Hair-Bejo, 1992). This could be attributed to the global movement of migratory birds that respect no international boundary as well as movement of breeding parent stock, biologics and pet birds among other things (Hair-Bejo, 2010). This further necessitates the need for adequate and effective high level biosecurity, a feat that is difficult to maintain at all times.

It is a well known fact that, successful poultry production is determined by the health status of the birds which is in turn dependent upon high quality feed intake and prevention of diseases occurrence among the flock. Immunosuppression is the sequellae of most poultry diseases especially IBD, a feature that increases the susceptibility of infected birds to other pathogens (Kibenge *et al*., 1988a).

The disease, IBD or avian nephrosis (Edgar, 1966), was first described by Cosgrove (Cosgrove, 1962) after an initial outbreak of 1957 in a broiler flock near a town called Gumboro, Delaware, USA; hence the synonym Gumboro disease. There were a lot of speculations about the right classification for the virus based on its morphology; some considered it a reovirus (Lukert & Davis, 1974) or an adenovirus (Almeida & Morris, 1973). In 1979, observations by Dobos *et al.* (1979) and Muller *et al.* (1979) revealed

the bisegmented double stranded nature of its RNA genome leading to the adoption of the name birnavirus. Their conclusion was based on the viral morphology (58 nm to 60 nm icosahedron), unique biophysical properties and possession of bisegmented, double stranded ribonucleic acid (2 dsRNA). The disease became a global problem within the span of four (4) decades spreading from USA to Europe, Middle East, Africa, Asia and Australia (Lasher & Shane, 1994; Lasher & Davis, 1997; Berg, 2000). It was first reported in Malaysia in 1991 (Hair-Bejo, 1992).

The virus (IBDV) together with infectious pancreatic necrosis virus (IPNV) of fish, tellina virus (TV), oyster virus (OV), blotched snakehead virus (BSVN) and crab virus of bivalve molluscs belonging to Aquabirnavirus and drosophila X virus (DXV) of the fruit fly Drosophila melanogaster belong to the *Birnaviridae* family, genus *Avibirnavirus,* members of which contain a double-stranded RNA (dsRNA) genome with two large and small segments, designated A and B segments, enclosed within a naked (non enveloped) single-shelled icosahedral capsid of approximately 60 nm in diameter (Dobos *et al*., 1979; Muller *et al*., 1979). The smaller B segment (approximately 2.8 kb to 2.9 kb) encodes viral protein 1 (VP1 of size of 95 kDa), an RNA-dependent RNA polymerase (RdRp) (Azad *et al*., 1985; Spies *et al*., 1987; Bruenn, 1991; Macreadie & Azad, 1993). The VP1 is present in the virion both as VPg, a genome-linked protein attached to the 5′ end of the positive strands of the two genomic segments and as a free protein (Spies & Muller, 1990; Dobos, 1993). Segment A (approximately 3.3 kb to 3.4 kb) has two partially overlapping open reading frames (ORFs) (Tacken *et al*., 2003). The first, smaller ORF encodes nonstructural viral protein VP5 (17 KDa), a protein not vital for *in vitro* viral replication but essential for virus-induced pathogenicity (Mundt *et al*., 1995; Mundt *et al*., 1997; Yao *et al*., 1998). The larger ORF encodes a 110-kDa polyprotein which by automatic cleavage, give rise to three polypeptides: pVP2 (48 kDa), VP3 (32 kDa), and VP4 (28 kDa). This self processing is mediated by VP4 (Kibenge *et al*., 1988; Sanchez and Rodriguez, 1999; Lejal *et al*., 2000), a serine-lysine protease (Birghan *et al*., 2000). It is a soluble protein mainly associated with type II tubules of 24 nm in diameter (Granzow *et al*., 1997). Further processing of pVP2 at its carboxy-terminus leads to VP2 (40 kDa) (Lejal *et al*., 2000; Da Costa *et al*., 2002). Incomplete virus particles were reported to interfere with the replication of the normal IBDV and they have been found to contain high amounts of pVP2 and other defective polypeptides (Müller *et al*., 1986). The VP2 and VP3 are the structural proteins forming the outer and inner layers of the virion, and constitute 51% and 40% of the total viral proteins respectively (Bottcher *et al*., 1997; Caston *et al*., 2001).

There are two reported serotypes of IBDV, serotype 1 and serotype 2. Serotype 1 causes clinical disease in chicken, while serotype 2 infects both chicken and turkeys without any overt clinical signs (Kibenge *et al*., 1988a). Serotype 1 viruses have been divided further into three groups namely; classical (ca), variant (va) and very virulent (vv) strains (Winterfield & Thacker, 1978). The vvIBDV was recognized in 1986 as an isolate of serotype 1 with enhanced virulence resulting in vaccination failures (Jackwood & Saif, 1987; Chettle *et al*., 1989).The virus is reported to be stable and persist in the environment by withstanding high temperature, low pH of 3 and

treatment with many disinfectants and organic solvents such as ether or chloroform (Murphy *et* al., 1999).

The disease is controlled by vaccination and strict adherence to biosecurity (Jackwood & Sommer, 2002; Hair-Bejo, 2010). Effective vaccination is dependent on certain factors such as vaccine type, vaccination time, level of chick's maternally derived antibody and wild IBDV strain (Hair-Bejo, 2010). Some researchers reported some degree of bursal atrophy associated with lymphocyte depletion and immunosuppression in chickens vaccinated with live attenuated IBD vaccines (Tsukamoto *et al*., 1999; Jackwood *et al*., 2008a). Passage in chicken embryonated egg and or tissue culture is the technique used to attenuate IBDV for live attenuated vaccine production (Jackwood *et al*., 2008a; Hair-Bejo, 2010). The first live unattenuated "bursal-derived" IBD vaccine was developed by Edgar using bursal homogenate obtained from chickens with active infection (Edgar & Cho, 1973). The search for more effective vaccine lead Lasher and Gelenczei to develop chicken embryo vaccine and adapted three field isolates in chicken and duck embryo fibroblast by blind passage (Lasher & Davis, 1997), and later in the early 1970s, Lukert, Leonard and Davies successfully propagated the virus in continuous cell line using kidney cells passage first and then culturing onto Vero cells (Lasher & Davis, 1997).

The vaccine virus replicate in the bursa of the chicken and induced protective immunity. Unfortunately, reversion of live attenuated vaccine strains to wild pathogenic strains has been well documented (Muskett *et al*., 1985; Jackwood *et al*., 2008a). This necessitates the need for a safer vaccine with no reversion ability. Killed vaccines were developed, but with lesser potency compared to the live vaccines as they do not replicate in the target organ, hence they induce low immunity (Berg, 2000). Adjuvants such as oil and plant extracts were later incorporated in to killed vaccines to enhance the immune response (Jackwood *et al*., 2008b).

In recent years, expression of structural viral proteins of many viruses for use as a vaccine (subunit vaccine) has been explored using recombinant DNA technology. Scientist developed three types of vaccines using this technology; live genetically modified vaccines, recombinant inactivated vaccines and genetic vaccines (Jackwood *et al*., 2008c). This technology was adopted for IBDV and the viral protein VP2 with immunogenic properties was used as the vaccine candidate (Hair-Bejo *et al*., 2010).

Because RNA viruses genetically have high mutation rates, there is a potential risk that a poorly attenuated IBDV will revert to a more virulent state when replicating in poultry after several replication cycles within the bursa of Fabricius and this was observed in IBDV partially attenuated by site-directed mutation (Raue *et al*., 2004). Reverse genetic studies have shown that amino acids at positions 253 and 284 in VP2 are involved in virulence and cell tropism of IBDV (Mundt, 1999; Boot *et al*., 2000; van Loon *et al*., 2002; Liu & Vakharia, 2004). In another study, the amino acids 253, 279 and 284 were reported to control virulence and cell tropism (Brandt *et al*., 2001). Furthermore, it was shown that segment B which encodes the viral RNA dependent

RNA polymerase was important for efficient viral replication and virulence *in vivo* (Liu & Vakharia, 2004). The discovery of a natural reassortant of IBDV also demonstrated that segment B may be important for the pathogenicity of vvIBDV strains (Le Nouen *et al*., 2005), a proof that VP2 is not the sole determinant of virulence in vvIBDV (Boot *et al*., 2000). Together, these studies suggest that more than one molecular determinant contributes to the virulence of IBDV.

Chicken embryo fibroblasts (CEF) cells are used generally for the propagation and attenuation of IBDV and the production of IBD vaccine. Unfortunately, CEF being non continuous cells have several disadvantages such as limited *in vitro* life span, high cost and laborious preparation for continuous supply. There are two spontaneously immortalized CEF cell lines namely; DF-1 and SC-1 in existence. DF-1 (named after Douglas Foster, its founder) has been reported to be better than SC-1 cell line due to lower growth rate and lack of uniform cell morphology (Christman *et al*., 2005)., have an enhanced growth potential compared to secondary embryo fibroblasts and support the growth of different avian viruses (Himly *et al*., 1998; Maas *et al*., 2006; Tiwari *et al*., 2006; Ciota *et al*., 2007; Jia *et al*., 2007; Khatri and Sharma, 2007; Lin *et al*., 2007; Lee *et al*., 2008). Additionally, it was shown that the cytopathic effects of IBDV in both CEF and DF-1 cells were similar but, higher viral titers were detected in the DF-1 cell line when evaluated using real time RT-PCR (Wang *et al*., 2009).

Many mammalian continuous cell lines such as RK-13 from rabbit kidney (Petek *et al*., 1973), Vero cells from adult African green monkey kidney (Jackwood *et al*., 1987; Kibenge *et al*., 1988; Marquis, 1997; Fang, 2007; Donis *et al*., 2014), BGM-70 from baby grivet monkey kidney (Jackwood *et al*., 1987; Hassan & Saif, 1996), MA-104 from foetal rhesus monkey (Jackwood *et al*., 1987) and OK from ovine kidney (Kibenge *et al*., 1992) and LSCC-BK3 cells (Spies *et al*., 1987) were reported to be susceptible to IBDV. Mammalian cell lines are easy to handle and maintain and are free from vertically transmitted extraneous viruses of avian origin (Hassan *et al*., 1996a). The ability of continuous cell lines to yield higher viral titers is another valuable characteristic that make growing virus in them advantageous over primary cell cultures. Due to limited SPF eggs and SPF chickens supply, it will be useful for research laboratories especially those engaged in vaccine development, to propagate viruses of interest in continuous cell lines because large viral titers are required to rapidly manufacture large quantity of vaccines to meet the demand against the ever present poultry diseases. The high viral titer generated by the continuous cell lines can range from 6.85 to 8.35 $\log_{10}$ $TCID_{50}$ / ml (Kibenge *et al*., 1988a; Simoni *et al*., 1999; Hussain & Rasool, 2005) better than the primary CEF cells that generate 5.35 to 6.10 $\log_{10}$ $TCID_{50}$ /ml (Kibenge *et al*., 1988a). This means that mammalian cell lines are valuable medium for IBDV propagation when large quantity of the cultivated virus is needed especially when using bioreactor technology.

Although conventional vaccines against IBD have been produced successfully using SPF egg passage, but vaccine failures have been frequently reported in some vaccinated flocks especially when the challenging field strain is the vvIBDV since the strain can cross the maternally derived antibody (MDA) (Rasoli *et al*., 2015). The

vaccines are usually produced using caIBDV or vaIBDV and are effective in controlling infection with these strains as the field challenge, but vvIBDV strain was found to cause severe infection in vaccinated flocks and even breaking through high level of maternally derived antibody raised against caIBDV and vaIBDV vaccines (Jackwood & Saif, 1987). In Malaysia, there are vvIBDV based commercial vaccines such as MyVac UPM93 and MyHatch UPM93 are used for IBD control, but they have been produced using SPF eggs as medium of production which is costly and labor intensive (Hair-Bejo, 2010) and egg based production system cannot adopt the use of bioreactor technology. Although adaptation of vvIBDV to tissue culture was reported to be difficult, but when achieved, it will provide the cheapest and fastest means of generating high virus titer required for vaccine production. Furthermore, IBDV cell culture adaptation will allow the use of bioreactor system that will allow more system control of vital growth parameters such as pH and temperature, low bacterial and fungal contamination of seed virus and exponential increase in virus titer up to 50 fold compared to the conventional flask cultures. So there is a need to identify a cell line that can adapt the virus faster in conventional flask and allow its propagation using bioreactor technology to produce higher yields of infectious virus required for vaccine production purposes.

It was the hypothesis of this study that the vvIBDV isolates can be adapted and propagated in different cell lines; the adapted viruses will mutate in the amino acid composition of their VP2 hypervariable region following adaptation in the cell lines; the adapted viruses will propagate well in bioreactor; and the BGM-70 adapted and bioreactor propagated vvIBDV will be excellent candidates for attenuated and inactivated IBD vaccines with high immunogenicity in commercial chickens.

The main objectives of this study were to identify a suitable tissue culture type for the adaptation, propagation and attenuation of vvIBDV and produce a suitable live attenuated and inactivated IBDV isolates for vaccine development.

The specific objectives of the study were:

1. to adapt, propagate and attenuate UPM0081 and UPM190 vvIBDV isolates in embryonated SPF chicken eggs and tissue cultures.
2. to determine the molecular characteristics of the propagated vvIBDV isolates.
3. to propagate the vvIBDV isolates in bioreactor.
4. to determine the pathogenicity, immunogenicity and efficacy of the live attenuated and inactivated vvIBDV isolates in chickens.

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