MOLECULAR CHARACTERISATION AND PATHOGENICITY OF CHICKEN ASTROVIRUS ISOLATED FROM COMMERCIAL BROILER CHICKENS IN MALAYSIA

By

RAJI ABDULLAHI ABDULLAHI

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

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DEDICATION

This thesis is dedicated to wonderful beloved parents, Alhaji Muhammad Raji Abdullah and Sayyidah Fatima Jibril for their prayers and love, May Allah's mercy and blessings continue to be with them in this world and here after.

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

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Chairman : Professor Abdul Rahman bin Omar, DVM, PhD
Institute : Bioscience

Chicken astrovirus (CAstV) is a ubiquitous enteric RNA virus that has been associated mainly with conditions including runting stunting syndrome, kidney and visceral gout, and white chick syndrome in broiler-type of chickens across the globe. In Malaysia, the detection of the virus amongst broiler flocks has not been studied. The description of CAstV in this chapter is based on an RT-PCR assay, serology, genome characteristics and pathogenicity of the virus in specific-pathogen-free (SPF) chickens. The viruses were detected from tissue broiler chickens suffering from kidney disease and poor performance. A total of 20 tissue samples obtained from different broiler flocks between 2017 and 2018 were confirmed positive for CAstV based on polymerase gene (*ORF1b*) specific RT-PCR detection. A serological study based on CAstV group B enzyme-linked immunosorbent assay (ELISA) revealed a high incidence of the virus amongst broiler-breeder flocks. The tissue samples were then used to isolate CAstV using 5-day-old SPF embryonated chicken eggs (ECE). After four passages, only three isolates, IBS503/2017, IBS543/2017 and UPM1019/2018 were isolated and considered for further studies.

Three pairs of overlapping primer sets were designed to amplify a nearly complete genome sequence of the three CAstV that were propagated in SPF-ECE. The amplicons were sequenced on the Illumina MiSeq platform. The generated raw sequencing data were transferred and *de novo* assembled in a genome assembly software for consensus generation and mapping to reference. The analysis produced a near-complete genome sequence of the three CAstV isolates IBS503/2017, IBS543/2017 and UPM1019/2018 with

the genome length of 7424bp, 7379bp and 7397bp, respectively. The genomic organisation of the three isolates exhibited three open reading frames, *ORF-1a*, *ORF-1b*, and *ORF-2*, that encode for trypsin-like serine protease, RNA-dependent RNA polymerase (RdRp) and capsid protein, respectively. A point mutation of guanine (G) to thymine (T) was observed in the spacer sequence between *ORF-1a* and *ORF*-1b. Additionally, a third stem-loop like motif (s2m) was observed at the 3'-end of the untranslated region (UTR). Genome analysis of the isolates at the nucleotide level with other CAstV genomes showed a similarity of 77% with group B CAstV from China, 87% with group B Indian strain, 88 to 89% with group B North American strains, and 74% similarity with group A CAstV from Poland. However, analysis based on the capsid gene sequences classified the isolated viruses as group B CAstV, showing a sequence similarity at the nucleotide level (91.96 to 93.78%) and amino acid (90.51 to 93.63%) with CAstV isolates in subgroup Bi, Biii and Biv. Sequence similarity of 76.18 to 90.09% and 86.02 to 89.97% at nucleotide and amino acid levels, respectively, were observed between the three Malaysian isolates and subgroup Bii. Interestingly, phylogenetic analysis indicated the three Malaysian isolates were clustered and formed a new subgroup, tentatively subgroup Bv.

Pathogenicity study of one of the isolates, UPM1019/2018 on one-day-old SPF chickens, produced clinical manifestations related to CAstV infection. However, no mortality was recorded throughout the study. Diarrhoea and somnolence were the most observed clinical signs accompanied by decreased feed intake in both the challenged and exposed sentinel groups. Dehydration, cachexia, ballooned intestines were observed on post-mortem examinations. Three birds (two from the challenged group and one from the exposed sentinel group) exhibiting enlarged kidneys and ureters with urate deposits and visceral gout were observed on days 6 and 9 post-inoculation. Microscopically, the observable lesions were mild lymphocytic aggregates in the duodenum, tubular degeneration and interstitial nephritis. Real-time RT-PCR assay detected CAstV RNA from the cloacal swabs of both the challenged and exposed sentinel groups throughout the study. The highest mean virus copy number ($\log_{10}$ 13.23) was on day 3 post-inoculation in the challenged group. In contrast, the exposed sentinel group has a peak mean virus copy number ($\log_{10}$ 9.04) on day 6 post-inoculation.

In conclusion, the isolated Malaysian CAstV is pathogenic in SPF chickens, causing lesions in the gut and kidneys in both the infected and exposed chickens. Although the studied CAstV are classified under group B, they are distinct from other CAstV strains forming a new subgroup Bv.

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

PENCIRIAN MOLEKUL DAN KEPATOGENAN ASTROVIRUS AYAM PENCILAN DARI AYAM PEDAGING KOMERSIAL DI MALAYSIA

Oleh

RAJI ABDULLAHI ABDULLAHI

April 2021

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Astrovirus ayam (CAstV) merupakan virus RNA yang kerap dikaitkan dengan beberapa keadaan termasuklah sindrom terbantut, gout ginjal dan viseral, dan sindrom anak ayam putih dikalangan ayam pedaging seluruh dunia. Di Malaysia, pengesanan virus ini di kalangan kelompok ayam pedaging masih belum pernah di dokumentasikan. Di sini, mengesan CAstV menggunakan kaedah RT-PCR, dan menghurai pencirian genome dan patogenisiti virus teresbut dalam ayam bebas-patogen-spesifik (SPF). Virus ini telah dikesan dari sampel tisu ayam pedaging komersial yang menghidap penyakit buah pinggang dan menunjukkan prestasi yang tidak memuaskan. Sejumlah 20 sampel tisu telah diambil daripada kelompok ayam pedaging yang berbeza antara tahun 2017 dan 2018, telah disahkan positif terhadap CAstV berdasarkan pengesanan RT-PCR spesifik gen polimerase (ORF1b). Pengkajian serum berasaskan CAstV kumpulan B asai imunoserap terangkai ensim (ELISA) menunjukkan kadar jangkitan virus ini dikalangan ayam pembiak baka adalah tinggi. Tisu sample kemudiannya digunakan untuk pencilan CAstV menggunakan telur ayam SPF berembrio (ECE) berusia lima hari. Selepas empat laluan dalam SPF-ECE, hanya tiga isolat, IBS503/2017, IBS543/2017 dan UPM1019/2018 terisolasi dan dipertimbangkan untuk kajian lanjutan.

Tiga pasang set primer yang bertindih direka bentuk untuk menghasilkan urutan genom yang hampir lengkap bagi ketiga-tiga CAstV yang digandakan di dalam SPF-ECE. Amplikon tersebut dijujukan menggunakan platform Illumina MiSeq. Data penjujukan mentah yang terhasil dipindahkan dan disusun secara *de novo* dalam perisian pemasangan genom untuk penjanaan konsensus dan pemetaan untuk

dijadikan rujukan. Analisis ini menghasilkan urutan genom yang hampir lengkap untuk isolat CAstV IBS503/2017, IBS543/2017 dan UPM1019/2018 dengan kepanjangan genom masing-masing 7424bp, 7379bp dan 7397bp. Susunan genom bagi ketiga-tiga isolat tersebut mempamerkan tiga rangka bacaan terbuka, ORF-1a, ORF-1b dan ORF-2 yang masing-masing mengekod protinase serin seumpama-tripsin, RNA polimerase bergantung RNA (RdRp) dan kapsid protin. Titik mutasi guanin (G) ke timin (T) dikesan dalam urutan jarak antara ORF-1a dan ORF-1b. Tambahan pula, motif seakan gelung batang ketiga (s2m) dilihat ada di penghujung 3' bahagian yang tidak diterjemah (UTR). Analisis genom isolat pada tahap nukleotida dengan genom CAstV lain menunjukkan kesamaan sebanyak 77% dengan CAstV kumpulan B daripada China, 87% dengan virus kumpulan B dari India, 88 hingga 89% dengan virus kumpulan B dari Amerika Syarikat dan 74% dengan virus kumpulan A dari Poland. Walau bagaimanapun, analisis berasaskan urutan gen kapsid mengklasifikasikan isolat virus tersebut sebagai CAstV kumpulan B, yang menunjukkan kesamaan urutan pada tahap nukleotida (91.96 hingga 93.78%) dan asid amino (90.51 hingga 93.63%) dengan isolat CAstV dalam subkumpulan Bi, Biii dan Biv. Kesamaan urutan sebanyak 76.18 hingga 90.09% dan 86.02 hingga 89.97% pada tahap nukleotida dan asid amino, masing-masing, diperhatikan antara ketiga-tiga isolat Malaysia dan subkumpulan Bii. Menariknya, analisis filogenetik menunjukkan ketiga-tiga isolat Malaysia berkelompok dan membentuk subkumpulan baharu, berkemungkinan subkumpulan Bv.

Kajian kepatogenan menggunakan salah satu isolat, UPM1019/2018 pada ayam SPF yang berusia satu hari menghasilkan manifestasi klinikal yang berkaitan dengan jangkitan CAstV. Walau bagaimanapun, tidak ada kematian yang dicatatkan sepanjang kajian. Cirit-birit dan mengantuk adalah tanda klinikal yang paling kerap dilihat diikuti dengan pengurangan pengambilan makanan oleh kedua-dua kumpulan tercabar dan sentinel yang terdedah. Dehidrasi, cachexia, usus mengembung dilihat berlaku pada pemeriksaan post-mortem. Tiga unggas (dua dari kumpulan yang dicabar dan satu dari kumpulan sentinel yang terdedah) memperlihatkan ginjal dan ureter yang membengkak dengan deposit hablur urate dan gout viseral pada hari ke-6 dan 9 pasca inokulasi. Secara mikroskopik, lesi yang dapat dilihat adalah agregat limfositik ringan dalam duodenum, degenerasi tubular dan nefritis interstitial. Ujian RT-PCR masa-nyata mengesan RNA CAstV dari calitan kloaka kedua-dua kumpulan sepanjang kajian. Bilangan salinan virus min tertinggi (log_{10} 13.23) adalah pada hari ke-3 pasca inokulasi di kalangan kumpulan yang dicabar. Sebaliknya, kumpulan sentinel yang terdedah mempunyai bilangan salinan virus min puncak (log_{10} 9.04) pada hari ke-6 pasca inokulasi.

Kesimpulannya, CAstV Malaysia yang dipencil adalah patogenik pada ayam SPF menyebabkan lesi pada usus dan ginjal pada ayam yang dijangkiti dan yang terdedah. Walaupun CAstV yang dikaji diklasifikasikan dalam kumpulan B, ia adalah berbeza dengan strain CAstV lain dan merupakan subkumpulan baru, Bv.

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

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LIST OF ABBREVIATIONS

Aa	Amino acid
µL	Microlitre
µm	Micrometre
AAstV	Avian astrovirus
Ab	Antibody
Abs	Antibodies
ANOVA	Analysis of variance
ANV	Avian nephritis virus
ARV	Avian rotavirus
AvRV	Avian reovirus
BAstV	Bovine astrovirus
BHK	Baby Hamster kidney
BLAST	Basic Local Alignment Search Tool
BLT	Bead Linked Transposome
bp	Base pair
℃	Degree Celsius
CAstV	Chicken astrovirus
cDNA	Complementary DNA
CDS	Coding sequences
ChPV	Chicken parvovirus
COMeT	Comparative Medicine and Technology Unit
cq	Quantification cycle
DEPC	Diethylpyrocarbonate

DNA	Deoxyribonucleic acid
DOC	Day old chick
dpi	Day(s)-post inoculation
dsDNA	Double-stranded DNA
ECE	Embryonated chicken egg
EID_{50}	Embryo infectious dose
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
FAdV	Fowl adenovirus
FAM	Fluorescein amidites
FAO	Food and Agricultural Organisation
FISH	Fluorescence in-situ hybridization
frgmnt	fragment
g	gram
gDNA	Genomic DNA
GFAstV	Guinea fowl astrovirus
GSPs	Gene-specific primers
H&E	Haematoxylin and eosin
H_2O	water
HAstV	Human astrovirus
HTS	High throughput sequencing
IACUC	Institutional Animal Care and Use Committee
IBS	Institute of Bioscience
IBV	Infectious bronchitis virus
ICTV	International Committee on Taxonomy of Virus

IF	Immunofluorescences
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IIF	Indirect immunofluorescent
IL	Interleukin
JTT	Johns-Taylor-Thornton
Kb	Kilobyte
KDA	Kilodalton
LIVES	Laboratory of Vaccine and Immunotherapeutic
LMH	Hepatocellular carcinoma cells
mAbs	Maternal antibodies
MAstV	Mammalian astrovirus
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram
MGB	Minor groove binder
mL	Millilitre
mM	Millimole
MVP	Malaysian Vaccine Pharmaceuticals
NA	Not Available
NAb	Neutralising antibody
NaOH	Sodium hydroxide
NC	Noncoding
NCBI	National Centre for Biotechnology Information
NDV	Newcastle disease virus

ng	Nano gram
NGS	Next-generation sequencing
NK	Natural killer
NLS	Nucleotide localisation signal
nM	Nano molar
NO	Nitric oxide
NSP	Non-structural protein
ntd	Nucleotide
OAstV	Ovine astrovirus
ORF-1a	Open reading frame-1a
ORF-1b	Open reading frame-1b
ORF-2	Open reading frame 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pM	Pico mole
pNPP	para-Nitrophenylphosphate
PTC	Programmed protocol
QC	Quality control
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RdRP	RNA-dependent RNA polymerase
Rfam	RNA family
RNA	Ribonucleic acid
RSB	Resuspension buffer
RSS	Runting stunting syndrome

RT-PCR	Reverse transcriptase Polymerase chain reaction
S/P	Sample to positive
sgRNA	Sub-genomic ribonucleic acid
SIAS	Sequence identity and similarity
SNP	Single nucleotide polymorphism
SPB	Sample purification beads
SPF	Specific-pathogen-free chicken
SRVs	Small round viruses
ssRNA	Single-stranded ribonucleic acid
TAG	Tagmentation
TB1	Tagment Buffer
TGF	Transforming growth factor
TSB	Tagment Stop Buffer
TWB	Tagment Wash Buffer
UK	United Kingdom
UPM	Universiti Putra Malaysia
US	United States
UTRs	Untranslated regions
UV	Ultraviolet
VP	Viral protein
VPg	Viral protein genome linked

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Astrovirus was first discovered in 1975 during an electron microscopic (EM) examination of diarrhoeic stool samples from young children of 2 years old and below suffering from gastroenteritis (Madeley & Cosgrove, 1975). It is now a ubiquitous enteric virus with a worldwide distribution and a leading cause of enteritis and diarrhoea in neonates, the immunocompromised and the aged. Shortly after its discovery, small round virus particles with astrovirus morphology were recorded in domestic animals; particularly calves and lambs with gastroenteritis (Snodgrass et al., 1979; Woode et al., 1985); and ducks with viral hepatitis in ducks in the early 1980s leading to acute mortality (Gough et al., 1985), and conceivably, served as earliest indication of extra-intestinal tropism of astrovirus (De Benedictis et al., 2011). Interestingly, in the 1960s, a similar report recognised astrovirus as the aetiologic agent of duck hepatitis (Asplin, 1965). Over the years, an extensively wide range of animal species are found susceptible to astroviral infections from synanthropic to domesticated, avian and mammalian species on the land and in water.

Astroviruses are broadly classified into two genera; the *Avastroviruses* and *Mamastroviruses* that are found to infect avian and mammalian species, respectively, and both belong to the *Astroviridae* family (Mendez & Arias, 2007). Presently, based on host species, the International Committee on the Taxonomy of Viruses (ICTV) recognises 19 species of mamastrovirus (MAstV-1–19) and three genogroups in the avastrovirus (Avastrovirus 1-3) with 14 new strains and four new strains awaiting grouping under mamastrovirus and avastrovirus, respectively. (Karlsson et al., 2015; Woo et al., 2015). Generally, *Astroviridae* are naked, positive-sense, single-stranded RNA viruses, typically 28–38 nm in diameter with an ultrastructural star shape under the EM (Monroe et al., 1993).

Baxendale and Mebatsion (2004) identified and characterised an emerging avastrovirus in chickens, the chicken astrovirus (CAstV) making it the recent member of the avastroviruses and most recent in the poultry birds. Chicken astrovirus shares a genomic organisation and familial characteristics with other known astroviruses. Typically, it is within the range of 28 to 30 nm in diameter, round, small, non-enveloped virus, positive sense, and single-stranded RNA, with a length of nearly 7.5 kb (Kang et al., 2012). The virus is made up of a missing 5'-end cap and

possesses a poly(A) tail at 3'-end. The genome is segmented into three sequentially arranged open reading frames (ORFs); classified as *ORF-1a*, *ORF-1b*, and *ORF-2*, and bound by untranslated regions (UTR) on both 5' and 3'-ends (Monroe et al., 1993). Variations observed in the *ORFs* are dependent on virulence factors of the astrovirus type and modification of the virus during propagation in either cell culture or specific-pathogen-free (SPF) embryonated chicken egg (ECE) (De Benedictis et al., 2011). The *ORFs* at the leader sequence of the virus genome (*ORF-1a* and *ORF-1b*), encode two non-structural polyproteins, a trypsin-like serine protease and an RNA-dependent RNA polymerase (RdRp), while *ORF-2* encodes a polyprotein, a viral capsid protein precursor. The enormous variety observed in the astroviruses genome is within the structural capsid protein region, a polyprotein synthesised from subgenomic RNA (sgRNA). However, the capsid protein has a highly conserved N-terminus and highly acidic variable C-terminus domains (Dong et al., 2011).

Chicken astrovirus infections occur early in life, within the first few days to a week. Some strains are transmitted vertically from naïve in-lay breeders to chicks which will, in turn, shed high levels of the CAstV infectious particle or via horizontal route where the virus transmission is predominately via the faecal-oral route (Smyth, 2017). Several studies on the diversity of CAstV grouped the viruses into Groups A and B, with a relatively low similarity share of 38-40% across the capsid protein precursor gene (*ORF-2*) that encode the hypervariable immunogenic and antigenic regions (Smyth, 2017). The two groups are comprised of intra-subgroups; Group A has three subgroups with a 77-82% shared similarity, while B group with four subgroups has a shared similarity of 84-98% (Smyth, 2017).

Clinical signs could remain subclinical and can mainly be detected by RT-PCR, or full-blown varying clinical signs relative to the strain of the CAstV, viral load and presence of maternal antibodies (mAbs) could limit clinical signs and disease development (Smyth, 2017). On the other hand, co-infection with other enteric and immunosuppressive viruses can exacerbate CAstV infection (Smyth, 2017). Alternatively, a flock may be potentially affected by multiple CAstV types simultaneously.

The clinical signs commonly shared by all the presently known strains are mild to moderate depression, diarrhoea by 3 to 4dpi and partly digested feed seen in the faeces (Baxendale & Mebatsion, 2004; Kang et al., 2012). Other clinical signs include growth retardation with chicks huddling for warmth, irregular feathering and leg weakness (Kouwenhoven et al., 1978). Recently, a strain that causes white chicks' hatchery disease or white chick syndrome has been identified in Scandinavian countries, Canada and Brazil (Sajewicz-Krukowska et al., 2016; Nuñez et al., 2020; Palomino-Tapia et al.,

2020). The strain causes early embryonic death, or chicks are hatched frail with pale feather, and a temporary but yet a substantial reduction in hatchability in breeder flocks (Smyth et al., 2013; Sajewicz-Krukowska et al., 2016; Nuñez et al., 2020).

Pathogenicity investigations in SPF chickens reveal the presence of the virus in the entire length of the intestine and the colorectal region with extra-intestinal affinity to other organs including bursa of Fabricius, thymus, kidneys, liver, spleen, lungs and the synovial fluid of the legs (Smyth et al., 2007; de Wit et al., 2011; Bulbule et al., 2013). Microscopically, depending on the strain type, severity of the strain and immune status of the bird, lesions are usually observed 3dpi in the intestinal tract. These lesions are mostly seen in the small intestine and include intestinal cysts, reduced villus size or altered villus shape (Baxendale and Mebatsion, 2004; Kang et al., 2012; Kang et al., 2018). Similarly, degeneration and necrosis of the epithelial cells lining of the proximal convoluted tubules with granulocytic infiltrates and interstitial lymphocytes, urate deposits are the lesions observed in the kidneys especially in cases of nephritis and visceral gout disease caused by CAstV (Bulbule et al., 2013).

With a worldwide coverage and diverse strain presence in all the continents, the standard detection method of CAstV is RT-PCR and enzyme-linked immunosorbent assay (ELISA), specifically for the B group of the virus (Smyth, 2017). Similarly, few strains of the virus from China, India, UK, US and Poland have been fully sequenced and deposited in the GenBank; however, several sequences of the capsid gene which is used in the grouping are equally available in the GenBank database.

Although sequencing the capsid protein gene (*ORF2*) of CAstV determines the strain variability and group, a complete sequence analysis, will identify possible recombination in the entire genome (Smyth et al., 2012). Additionally, progresses in next-generation sequencing (NGS) technologies has enabled the discovery of new genotypes or groups and enabling the classification of new virus strains (Cortez et al., 2017).

1.2 Problem statements

In Malaysia, the repeated identification of CAstV by RT-PCR from commercial broiler chickens with a history of poor performance call for a thorough investigation of the virus. And with a non-existing report on CAstV in the country, an in-depth study in addressing the emergence of the virus through a new protocol using Illumina Nextera DNA Flex™ library prep kit on the NGS platform for the determination of the complete

genome sequence is pertinent. Also, the pathogenicity of the Malaysian isolate of CAstV in a susceptible chicken model is not well defined.

1.3 Hypothesis

It is therefore, hypothesised that full genome sequence analysis of the newly isolated Malaysian CAstV isolates will determine the possible evolution and grouping of the virus. Secondly, inoculation of the isolated and propagated CAstV in SPF chickens will provide valuable information on the virulence and pathogenicity of the virus. Hence, based on the above hypothesis, the objectives of the study are.

1.4 Main objective

The main objective of this study is to detect, isolate and characterise Malaysian CAstV based on molecular and pathogenicity.

1.5 Specific objectives

1) To detect the presence of CAstV amongst commercial chickens based on polymerase chain reaction and serological detection.
2) To establish a next-generation sequencing protocol using the Nextera DNA Flex™ library prep kit for genome sequencing of the newly identified Malaysian isolate of CAstV.
3) To determine the molecular characteristics of the newly identified Malaysian isolate of CAstV.
4) To determine the pathogenicity of the newly identified Malaysian isolate of CAstV in SPF chickens.

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