

Molecular detection of chicken astrovirus in broiler chicken, Malaysia

Muhammad Redzwan Sidik*, Syamsiah Aini Shohaimi, Faizul Fikri Mohd Yusop, Leow Bee Leng, Mohd Khairil Azhar Md Haris, Ong Geok Huai, Maizatul Zaimi, Mohammad Jihan Redzuan

Veterinary Research Institute, 59, Jalan Sultan Azlan Shah, 31400 Ipoh, Perak, Malaysia

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✉*Corresponding author:
Muhammad Redzwan Sidik
Veterinary Research Institute, 59,
Jalan Sultan Azlan Shah, 31400
Ipoh, Perak, Malaysia
Email: redzwan@dvs.gov.my

Abstract

The emergence of avian diseases can cause major economic problems due to production losses and mortality in domestic poultry. Astrovirus is frequently associated with enteric diseases in poultry, being isolated from cases of runting-stunting syndrome (RSS) of broiler chickens, poult enteritis complex (PEC), and poult enteritis mortality syndrome (PEMS) of turkeys. Avian astrovirus can be detected in chickens from both healthy and poorly performing flocks. In Malaysia, information and reports regarding chicken astrovirus (CAstV) in poultry are limited. The objective of this study is to perform a phylogenetic study on the avian astrovirus isolated from a suspected case in 2019 and to determine the subgroups of avian astrovirus strains that existed in Malaysia. Reverse Transcription Polymerase chain reaction (RT-PCR) was performed based on the partial ORF1b gene and the nucleotide sequence was analyzed. Phylogenetic analysis showed that this isolate was clustered together with CAstV strains from several strain from USA, Malaysia and others. Furthermore, the isolate from broiler chicken showed 97.2% to 99.4% of its nucleotide identity with isolates from the American strains, compared to the previously CAstV Malaysia strain, which shared 94.8% to 95%. Therefore, the current study provides important information on the epidemiology of CAstV and highlights the importance of control strategies against CAstV-infected poultry in Malaysia.

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1. INTRODUCTION

High demand for chicken meat, egg production and an early marketing age has increased the popularity of broiler farms (Long *et al.*, 2017). The optimum performance of the chicken is largely dependent on factors including rearing management, feed quality and overall health, as well as conditions that affect the proper functioning of the gastrointestinal tract (Nunez *et al.*, 2016). Enteric disease in chickens causes significant economic losses and several enteric viruses are linked to them (Nunez *et al.*, 2015). Bacteria and parasites have long been thought to be the major causes of gastroenteritis in commercial poultry. However, many viral infections, including coronavirus, reoviruses, rotaviruses, adenoviruses and enteroviruses have been related to gastrointestinal illnesses in chickens (Mettifogo *et al.*, 2014). Chicken astrovirus (CAstV) has been associated with runting-stunting syndrome (RSS) in chickens, which is characterized by poor weight gain, lower feed conversion and mortality, resulting in economic losses (Donato and Vijaykrishna, 2017). Meanwhile "white chicks" disease is characterised by the increased embryo and chick mortality, weakness, and white plumage (Donato and Vijaykrishna, 2017).

Avian astroviruses are members of the Astroviridae family (Pantin-Jackwood *et al.*, 2012). Astroviruses are nonenveloped, icosahedral viruses with a positive-sense and single-stranded RNA genome of 6 – 8kb. The genomes consist of three open reading frames (ORFs) and are known as ORF1a, ORF1b, and ORF2 (Smyth, 2017). Both ORF1a and ORF1b encode viral nonstructural protein precursors whereas ORF2 encodes the viral structural protein precursor (Chen *et al.*, 2020; Bidokhti *et al.*, 2019). There are about five identified avian astrovirus species: two of turkey origin, the TAsTV 1 and TAsTV 2; two of chicken origin, avian nephritis virus (ANV) and chicken astrovirus (CAstV); and duck-origin astrovirus (DAsTV) (Day and Zsak, 2013).

However, in Malaysia, the study of avian astrovirus in poultry is currently inadequate. This is agreed by Palomini-Tapia *et al.* (2020), understanding of molecular and epidemiological studies in many countries regarding avian astrovirus remains limited. Therefore, based on case from a broiler farm, a poor growth or runting-stunting of chickens with symptoms of diarrhoea and white green faeces was suspected and diagnosed as CAstV. This study aimed to perform a phylogenetic study on the avian astrovirus isolated from a suspected case in 2019 and to determine the subgroups of avian astrovirus

strains that existed in Malaysia, as well as to compare them with other local and foreign isolates. This study is crucial for acquiring a better understanding of avian astrovirus in poultry and serves as preliminary data for future epidemiological studies and vaccine development.

2. MATERIALS AND METHODS

2.1. Sample preparation

A broiler chicken kidney, liver and intestine suspected of being infected with chicken astrovirus (CAstV) was sent to the Veterinary Research Institute, Ipoh, Malaysia for disease investigation. The organs tissue was homogenised PBS to a final concentration of 10% (w/v), and then the supernatant was collected into 1.5-ml microcentrifuge tubes by low-speed centrifugation at $2500 \times g$ for 5 min. Finally, the supernatant was stored at -80°C and the supernatant was used for virus isolation.

2.2. Viral isolation

Six-day-old White Leghorn chicken embryonated eggs (CEEs) that were specifically pathogen-free (SPF) were used. The filtered inoculum (0.2 mL) sample was inoculated into the yolk sac route of SPF embryonated chicken eggs and propagated for 5 days. The eggs were incubated at a temperature range of 37.5°C to 38°C for 5 days. The virus isolation protocol was followed in accordance with the protocols provided by Nunez *et al.* (2015).

2.3. RT-PCR

The viral RNA was extracted from the infected allantoic fluid by IndiSpin Pathogen Kit (Indical Bioscience, Germany). The SuperScript III One-Step RT-PCR System with Platinum Taq was used for the RT-PCR. The primer sets (Day *et al.*, 2007) were used in the partial ORF1b gene amplification. Amplification was carried out in a T100 Thermal Cycler (Bio-Rad, USA). In the amplification, the RT was carried out at 48°C for 30 min. The reaction mixture was then subjected to 94°C for 5 min for initial denaturation, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 68°C for 1 min with a final extension for 10 min at 68°C . After gel electrophoresis of the RT-PCR products, the amplicon was sent to First Base Laboratory (Apical Scientific Sdn Bhd, Malaysia) for sequencing.

2.4. Sequences analysis

The nucleotide sequences were assembled and edited using Seqman (DNASTar Lasergene, USA). The DNA sequences were aligned using ClustalW multiple alignments in Bioedit Sequence Alignment Software Version 7.1.9. Molecular Evolutionary Genetic Analysis (MEGA) Version 6.0 was used to construct a phylogenetic tree and perform sequence comparison using the neighbor-joining method with 1,000 bootstrap replicates. Seventeen (17) sequences were downloaded from Genbank for

comparison and phylogenetic analysis of the partial ORF1b gene.

3. RESULTS AND DISCUSSION

The allantoic fluid harvested was positive as CAstV using RT-PCR, which targets the ORF1b gene with a size of 362bp as shown in Figure 1. The reason for using this gene is that the majority of RT-PCR tests developed for detecting CAstV, TAsTV, ANV and DAsTV used primers located in the comparatively well-conserved RNA polymerase (ORF1b) gene (Pantin-Jackwood *et al.*, 2012). Besides that, the ORF1b gene is responsible for encoding nonstructural proteins (nsPs) that are involved in RNA transcription and replication of viruses (Bosch *et al.*, 2014). Furthermore, based on Pantin-Jackwood (2006a), molecular tests are more specific and sensitive in comparison with the electron microscope (EM) and enzyme-related immunosorbent assays in the identification of CAstV.

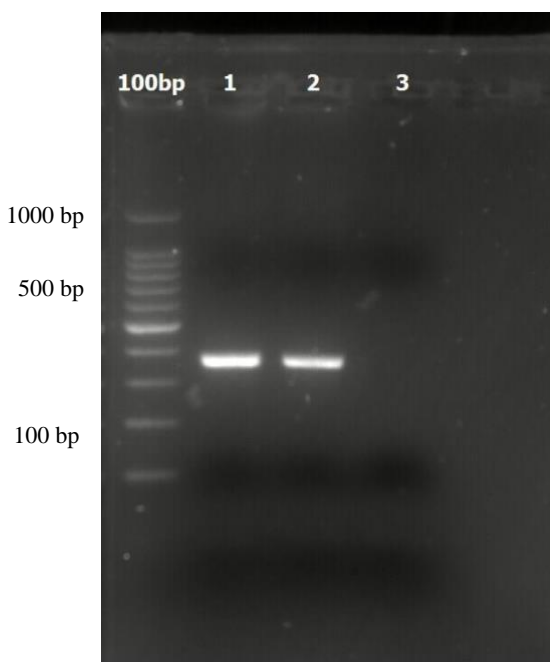


Figure 1: RT-PCR detection of an astrovirus in the samples. Visualise by using electrophoresis 1.5% gel showed a positive sample at 362bp. Ladder used 100bp. Line 1: sample, Line 2: positive control, Line 3: negative control.

A phylogenetic analysis of the ORF1b partial nucleotide sequence as shown in Figure 2 was performed to determine the relationship between CAstV/7239/Malaysia/2019 and other astrovirus genotypes. Based on the phylogenetic tree, the isolate in this study was clustered together with UPM1019/Malaysia/2018, IBS503/Malaysia/2017, CAst/4175/USA/2006, CAstV/GA2011/USA/2007, AAstV/Chicken/NJ1701/China/2017 and CAstV/HBLP717-1/China/2018. As a result, the isolate in this study was confirmed and identified as a CAstV, instead of other subgroups such as ANV and DAsTV. Besides

that, this isolate was grouped with Malaysian, USA and China strains. It's interesting to notice that CAstV/7239/Malaysia/2019 is more closely clustered with USA strains than previous local isolates. However, more investigation is needed to determine the reason for this problem. Furthermore, recent studies using developing sequences and phylogenetic analysis have demonstrated the genomic diversity of isolates, indicating that nucleotide sequence analysis is a sensitive and precise method for isolate differentiation (Day and Zsak, 2013; Sajewicz-Krukowska and Domanska-Blicharz, 2016). The sequencing data collected also enabled phylogenetic analysis to identify several genotypes of astroviruses such as CAstV, ANV, TAstV-2 and other genotypes based on specific genes (Day and Zsak, 2013; Pantin-Jackwood *et al.*, 2006a).

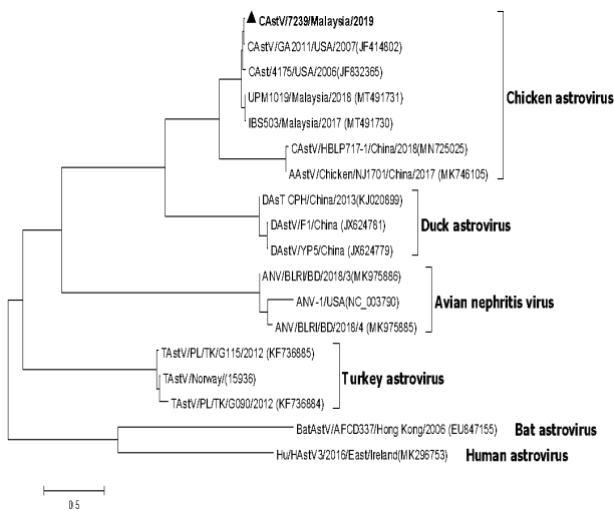


Figure 2: Phylogenetic tree of seventeen reference strains from GenBank database and isolate in this study (CAstV/7239/Malaysia/2019) were constructed based on 362 bp long nucleotide sequence of ORF1b gene. Accessions number of the sequence from GenBank are showed parenthesis. Tree was constructed using MEGA version 6.06 by Neighbour-Joining statistical method with model number of differences and setting bootstrap 1,000 replicates.

Based on nucleotide sequence using Bioedit software, CAstV/7239/Malaysia/2019 has 97.4% to 99.4% nucleotide identity to CAst/4175/USA/2006 and CAstV/GA2011/USA/2007 (Table 1). As compare to previous Malaysia strains, isolate in this study have 94.8% to 95.0% nucleotide identity to UPM1019/Malaysia/2018 and IBS503/Malaysia/2017. According to the results, CAstV/7239/Malaysia/2019 demonstrated a high nucleotide identity percentage to strains from other countries, including strains from the USA, as in comparison to previous Malaysia strains. The cause of this situation is undetermined. However, more studies should be performed on other genes such as the ORF2 gene (Bulbule *et al.*, 2013). The capsid precursor protein gene is the most variable astrovirus gene, with sequence variations that very probably represent antigenic and pathogenicity differences (Bulbule *et al.*, 2013). Pairwise analyses of the nucleotide and amino acid sequences of the ORF2 region of the virus can reveal significant variation across all

groups of avian astroviruses (Zhang *et al.*, 2018; Pantin-Jackwood *et al.*, 2006b).

Table 1: Comparison of the nucleotide of CAstV/7239/Malaysia/2019 isolate with selected representative astroviruses strains

Astrovirus strains	Percent identification (%) to CAstV/7239/Malaysia/2019	Description
UPM1019/Malaysia/2018	95.0	Chicken astrovirus (CAstV)
IBS503/Malaysia/2017	94.8	
CAst/4175/USA/2006	97.2	
CAstV/GA2011/USA/2007	99.4	
CAstV/HBLP717-1/China/2018	78.4	
AAstV/Chicken/NJ1701/China/2017	79.2	Avian nephritis virus (ANV)
ANV-1/USA	56.2	
ANV/BLRI/BD/2018/4	50.8	
ANV/BLRI/BD/2018/3	51.6	Duck astrovirus
DAst CPH/China /2013	75.4	
DAstV/F1/China	56.4	
DAstV/YP5/China	56.4	Turkey astrovirus
TAstV/Norway	55.8	
TAstV/PL/TK/G115/2012	56.6	Turkey astrovirus
TAstV/PL/TK/G090/2012	55.8	
BatAstV/AFCD337/Hong Kong/2006	48.2	Bat astrovirus
Hu/HASV3/2016/East/Ireland	47.4	Human astrovirus

Astrovirus in avian species has been reported in chickens, turkeys, and ducks (Smyth, 2017). In chickens, CAstV and ANV are the most prevalent astroviruses found. The ANV was discovered in chickens with intestinal nephritis and growth retardation (Donato and Vijaykrishna, 2017). Meanwhile, CAstV infections are common in broiler chickens and have been linked to young bird illnesses and hatchery diseases (Smyth, 2017). CAstV infections are frequently transmitted early, either horizontally via the faecal-oral pathway or vertically via naïve in-lay parent birds, with chicks shedding high amounts of CAstV. CAstV is more resistant to disinfection and cleaning than other viruses as it is a non-enveloped virus (Adebiyi *et al.*, 2019). The younger they are when they become infected, particularly vertical infections, the worse the outcome (Smyth, 2017). Meanwhile, the presence of CAstV in older birds indicates persistent infection and horizontal transmission. However, it depends on the specific CAstV strain, as pathogenicity varies significantly among viruses with RNA genomes (Smyth, 2017).

Currently, the control of this disease is difficult due to its wide geographical distribution, horizontal and likely vertical transmission, the environmental stability of the virus and CAstV disinfection resistance. The lack of

commercially-available vaccines may be in part since CAstV is difficult to grow at immunogenic titers and causes challenges for cost-effective commercial vaccine production (Palomino-Tapia *et al.*, 2020). Hence, strict containment is an effective strategy for disease prevention and management. Infected flocks need to be treated with the utmost concern for biosecurity, such as complete sanitation of all materials and restricted access to facilities by personnel is required to contain the outbreak (Oluwayelu and Todd, 2012). Therefore, such strategies are critical in preventing CAstV contamination in poultry.

4. CONCLUSION

Finally, based on the ORF1b gene, our data indicate the presence of CAstV the isolate, and this subgroup is present in Malaysia. This research gives useful information and a better knowledge of the CAstV prevalent poultry viruses in Malaysia. As a result, this data may be useful for epidemiological studies and vaccine development, which will contribute to the eradication of the disease.

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