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RESEARCH ARTICLE

# Isolation and phylogenetic analysis of avian infectious bronchitis virus from an imported chicken meat product in Malaysia

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**ABSTRACT**

Avian infectious bronchitis (IB), a *Gammacoronavirus*, is a highly contagious upper respiratory disease, affecting chickens of all ages with a significant economic threat to the poultry industry. In February 2020, a specimen of imported chicken meat product was received and requested for coronavirus testing. The result was positive for the avian coronavirus, the IB virus (IBV) by molecular detection in the pre-screening test. Thus, this study aimed to isolate and characterize the IBV from the specimen. Virus isolation via egg inoculation was attempted and IBV was successfully isolated. The S1 subunit of the spike (S) gene of the IBV was amplified, sequenced, and the *Basic Local Alignment Search Tool* (BLAST) analysis showed that the IBV has 99% and 98% nucleotide similarity with the Malaysian and China IBVs, respectively. The phylogenetic analysis indicated that the virus belongs to the GI-19 lineage (also known as the QX strain) and is grouped with other IBVs from Malaysia and China. The GI-19 lineage is one of the primary IB strains that circulate in Malaysia. The recovery of the virus may be due to the persistence characteristic of the virus on meat; and the cold chain practices in the imported food product prolong the survival of this coronavirus. Though IBV is not identified as a hazard in chicken meat or meat products, raw food should be cooked thoroughly before being consumed. With the increase in international trade in poultry and poultry products, disease screening at the entry point and import risk analysis is crucial to ensure food safety and prevent the introduction of new viruses into Malaysia.

**Keywords**: Avian infectious bronchitis; GI-19 lineage; QX strain; imported meat product; Malaysia

## INTRODUCTION

Avian infectious bronchitis (IB) caused by the IB virus (IBV) is an acute, contagious upper respiratory tract disease in chickens (OIE, 2018). The infected chicks may show signs of gasping, coughing, tracheal rales, sneezing, nasal discharges, watery eyes; and swollen sinuses is occasionally seen (Jackwood & de Wit, 2020). IBV can cause infection of the oviduct leading to a decrease in egg production and egg quality in layers and breeders (OIE, 2018). Some IB strains are nephropathogenic resulting in acute nephritis, urolithiasis and mortality, especially in young chickens (OIE, 2018).

IBV belongs to the family *Coronaviridae*, subfamily *Coronavirinae* and genus *Gammacoronavirus* (OIE, 2018). The IBV genome is a single-stranded positive-sense RNA. The full-length genome (mRNA1) encodes the viral polymerase polyproteins (Jackwood & de Wit, 2020). The subgenomic mRNAs of the IBV encode the spike (S), 3a/3b/envelope (E), membrane (M), 5a/5b and nucleocapsid (N) protein (Jackwood & de Wit, 2020). The surface glycoprotein S is involved in virus attachment to the host, membrane fusion of the virion and host to release the viral genome into the host (Cavanagh, 2007; Jackwood & de Wit, 2020). The S protein can be cleaved into S1 and S2 subunits (Cavanagh, 2007). S1 is the major inducer of protective immunity where it contains the virus neutralizing epitopes including the serotype specific epitopes (Cavanagh *et al*., 1992; de Wit *et al*., 2011). The virus neutralizing epitopes determines the IB serotype (Wang *et al*., 1994; Cavanagh *et al*., 1997). Small changes of 2-3% of the amino acid (10-15 residues) in the S1 can lead to changes in serotypes as changes in virus neutralizing epitopes occur (Cavanagh, 2007; de Wit *et al*., 2011).

As S1 contains the most variable region in the IBV, phylogenetic analysis (Valastro *et al*., 2016) on this region is commonly used in the IBV genotyping (Jackwood & de Wit, 2020). Due to the genetic diversity of IBVs and lack of standard classification of the virus, Valastro and colleagues (2016) classified IBV into six genotypes (GI to GVI) consisting of 32 viral lineages globally based on the complete S1 gene. The GI comprised of 27 lineages while GII to GVI comprised one lineage each (Valastro *et al*., 2016). Subsequently, few researchers updated the Valastro's classification scheme by adding either new IBV genotype or lineage. To date, there are nine genotypes (GI to GIX) comprising of 39 lineages of IBV that has been identified worldwide (Chen *et al*., 2017; Jiang *et al*., 2017; Ma *et al*., 2019; Molenaar *et al*., 2020, Mendoza-González *et al*., 2022).

Meat is a very good protein source as it provides multiple vitamins, minerals and essential amino acids (Geiker *et al*., 2021). Nevertheless, poultry diseases have been occasionally reported in the meat product. For example, avian influenza H5N1 and Newcastle disease virus have been detected in imported avian meat or meat product (Tumpey *et al*., 2003; Mase *et al*., 2005; Leow *et al*., 2016).

Likewise, coronavirus has also been found in the food product as well. In August 2020, the virus that causes the pandemic COVID-19, the SARS-CoV-2 virus was detected in a sample collected from the surface of imported frozen chicken wing (Shenzhen Epidemic Prevention and Control Headquarters, 2020).

Different IB strains have been reported in the poultry in Malaysia. Study showed that there were five distinct groups of IBVs, the QX, 4/91, Malaysian strain MH5365/95, Massachusetts and Taiwanese IB strains that circulated in chickens in the country from year 2014 to 2016 (Leow *et al*., 2018). Clinical signs of the IBV-infected chickens that has been reported in Malaysia including nephritis and respiratory signs (Leow *et al*., 2018). Dull, no appetite, diarrhea, sudden death has also been seen in the chickens (Leow *et al*., 2018).

In February 2020, a homogenate filtrate of an imported chicken meat product was received at the Veterinary Research Institute (VRI) Ipoh from the regional veterinary laboratory, the *Makmal Veterinar Zon Tengah (Selangor)* requested for coronavirus screening. The aim of this study is to isolate, characterize and phylogenetically analysed the S1 gene of this IBV.

## MATERIALS AND METHODS

### Virus isolation

The homogenate filtrate received from the regional veterinary laboratory was subjected to avian coronavirus screening by molecular detection. Virus isolation via egg inoculation technique (OIE, 2018) was then attempted. Specific Pathogen Free (SPF) embryonated chicken eggs obtained from VRI's donor unit were used in the virus isolation. The specimen is inoculated into five 9 to 11 days old SPF eggs via the intra-allantoic route. The eggs were incubated at 37°C for seven days and candled daily with mortality within the first 24 hours are considered non-specific (OIE, 2018). Infected allantoic fluid was harvested for further molecular testing and the embryo was examined for IBV lesion. Non-inoculated SPF embryonated chicken egg was incubated for seven days and serves as control.

### Reverse transcription – polymerase chain reaction (RT-PCR) and gene sequencing

At first, the specimen received was screened for avian coronavirus, the IBV before the temptation of virus isolation. The viral ribonucleic acid (RNA) was extracted from the received specimen using the IndiSpin Pathogen Kit (Indical Bioscience, Germany) in accordance with the manufacturer's instructions. Primer sets UTR1- (5'-GCTCTAACTCTATACTAGCCTAT-3') and UTR2+ (5'-AAGGAAGATAGGCATGTAGCTT-3') (Adzhar *et al*., 1996) targeting the 3' untranslated region (UTR) of the IBV genome was used in the screening of IBV. The amplification is performed using the *SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen, USA)* in T100 Thermal Cycler (Bio-Rad, USA). In brief, the RT was carried out at 48°C for 30 min *(Invitrogen, USA)*. 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 48°C for 1 min, and extension at 68°C for 1 min with a final extension for 10 min at 68°C.

Subsequently, the infected allantoic fluid that harvested from the SPF embryonated chicken eggs was subjected to molecular identification for IBV as well. RNA extraction was performed by the same kit as mentioned above. The full-length S1 gene was amplified from the extracted RNA using the primer set, S1F- (5'-AAGACTGAACAAAAGACCGACT-3') and S1R- (5'-CAAAACCTGCCATAACTAACATA-3') (Ji *et al*., 2011). The thermal protocol is the same as mentioned previously except the annealing temperature is set at 51°C and the extension step in the 40 cycles is prolonged to 2 minutes.

Amplicons were loaded on 1.5% and 1.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, USA) respectively during the initial screening of IBV and amplification of S1 gene. The results were viewed under UV transilluminator. The amplified PCR product of the S1 gene was cut from the gel and sent for Sanger sequencing (Apical Scientifics (M) Sdn Bhd). Primers used in the Sanger sequencing are the same as those used in the RT-PCR amplification.

### Molecular characterization and phylogenetic analysis

The S1 nucleotide sequence of the isolate was assembled using the SeqMan Pro software (DNA Star Lasergene, USA). Analysis of local similarity regions between the sequence in the study with other published sequences in the GenBank was conducted through *Basic Local Alignment Search Tool* (*BLAST*) (NCBI Resource Coordinators, 2016). Sequence alignment and comparison of the S1 gene in the study and other IB strains representative of different genotypes and lineages was performed by BioEdit Sequence Alignment Editor version 7.1.9 (Hall, 1999). The phylogenetic analysis was constructed by Maximum Likelihood method based on Tamura-Nei model and bootstrapping 1000 replicates using Molecular Evolutionary Genetic Analysis (MEGA) version 11 (Tamura *et al.*, 2021). A total of 79 IB strains of different genotypes and lineages worldwide were used in the analysis (Table 1). The phylogram is used to analyze the genetic relationship of the virus in the study with other representative IB strains in the world.

**Table 1.** IB representative strains of different genotypes and lineages used in the S1 gene sequence alignment and phylogenetic analysis

| Strain name | Country | Lineage | GenBank accession number | Reference |
|---|---|---|---|---|
| Beaudette | USA | GI-1 | M95169 | Valastro *et al*., 2016 |
| Holte | USA | GI-2 | GU393336 | |
| Gray | USA | GI-3 | L14069 | |
| Holte | USA | GI-4 | L18988 | |
| N1/62 | Australia | GI-5 | U29522 | |
| VicS | Australia | GI-6 | U29519 | |
| TP/64 | Taiwan | GI-7 | AY606320 | |
| L165 | USA | GI-8 | JQ964061 | |
| ARK99 | USA | GI-9 | M99482 | |
| B | New Zealand | GI-10 | AF151954 | |
| UFMG/G | Brazil | GI-11 | JX182775 | |
| D3896 | The Netherlands | GI-12 | X52084 | |
| Moroccan-G/83 | Morocco | GI-13 | EU914938 | |
| B1648 | Belgium | GI-14 | X87238 | |
| B4 | Korea | GI-15 | FJ807932 | |

| | | | | |
|---|---|---|---|---|
| IZO 28/86 | Italy | GI-16 | KJ941019 | |
| CA/Machado/88 | USA | GI-17 | AF419315 | |
| JP8127 | Japan | GI-18 | AY296744 | |
| 58HeN-93II | China | GI-19 | KC577395 | |
| Qu_mv | Canada | GI-20 | AF349621 | |
| Spain/97/314 | Spain | GI-21 | DQ064806 | |
| 40GDGZ-97I | China | GI-22 | KC577382 | |
| Variant 2 | Israel | GI-23 | AF093796 | |
| V13 | India | GI-24 | KF757447 | |
| CA/1737/04 | USA | GI-25 | EU925393 | |
| NGA/B401/2006 | Nigeria | GI-26 | FN182243 | |
| GA08 | USA | GI-27 | GU301925 | |
| D1466 | The Netherlands | GII-1 | M21971 | |
| N1/88 | Australia | GIII-1 | U29450 | |
| DE/072/92 | USA | GIV-1 | U77298 | |
| N4/02 | Australia | GV-1 | DQ059618 | |
| TC07-2 | China | GVI-1 | GQ265948 | |
| H120 | The Netherlands | GI-1 | FJ888351 | Jiang *et al*., 2017 |
| SDW | China | GI-2 | DQ070840 | |
| BL-56 | Mexico | GI-3 | AF352831 | |
| GX2-98 | China | GI-4 | AY251816 | |
| V2-02 | Australia | GI-5 | DQ490215 | |
| J9 | China | GI-6 | DQ515802 | |
| SE 17 | USA | GI-8 | M99484 | |
| CAL99 | USA | GI-9 | DQ912831 | |
| T6 | New Zealand | GI-10 | AF151960 | |
| IBV/Brasil/351/1984 | Brazil | GI-11 | GU393339 | |
| D207 | The Netherlands | GI-12 | M21969 | |
| UK/4/91 | United Kingdom | GI-13 | KF377577 | |
| K210-02 | Korea | GI-15 | AY257068 | |
| Ck/CH/LDL/97I | China | GI-16 | EF030995 | |
| AL/6609/98 | USA | GI-17 | AF510656 | |
| 53XJ-99II | China | GI-18 | KC577391 | |
| QXIBV | China | GI-19 | AF193423 | |
| Qu16 | Canada | GI-20 | AF349620 | |
| Italy/02 | Italy | GI-21 | AJ457137 | |
| 66GD-98VI | China | GI-22 | KC577397 | |
| IS/1494/06 | Israel | GI-23 | EU780077 | |
| IBV506 | India | GI-24 | KF809796 | |
| GA/12274/2012 | USA | GI-25 | KP085595 | |
| NGA/N545/2006 | Nigeria | GI-26 | FN182270 | |
| GA/12341/2012 | USA | GI-27 | KM660634 | |
| ck/CH/LGX/111119 | China | GI-28 | KX640829 | |
| GX-NN-13 | China | GI-28 | JX291989 | |
| γCoV/ck/China/I0111/14 | China | GI-29 | KY407557 | |
| γCoV/ck/China/I0114/14 | China | GI-29 | KY407556 | |
| V1397 | The Netherlands | GII-1 | M21968 | |
| V18/91 | Australia | GIII-1 | U29521 | |
| AR/6386/97 | USA | GIV-1 | AF274436 | |
| 018 | Australia | GV-1 | JX018208 | |
| SDIB781/2012 | China | GVI-1 | KF007209 | |
| THA001 | Thailand | GI | GQ906705 | |
| MH5365/95 | Malaysia | GI | EU086600 | |
| CK/NL/D181/2018 | The Netherlands | GII-2 | MK840961 | Molenaar *et al*., 2020 |
| γCoV/ck/China/I0636/16 | China | GVII-1 | MH924835 | Ma *et al*., 2019 |
| GX-NN130021 | China | GVII-1 | KM365468 | |
| Mex-07-3 | Mexico | GI-30 | ON470388 | Mendoza-Gonzalez *et al*., 2022 |
| Mex-430 | Mexico | GI-30 | ON470390 | |
| Mex-12 | Mexico | GVIII-1 | ON470391 | |
| Mex-3009 | Mexico | GVIII-1 | ON470392 | |
| Mex-14P | Mexico | GIX-1 | ON470393 | |
| Mex-56-7 | Mexico | GIX-1 | ON470394 | |
| IBS130/2015 | Malaysia | GI-19 | MG738155 | GenBank |
| ck/CH/LSD/101223 | China | GI-19 | KX364299 | |

## RESULTS

### Virus isolation

All the five SPF embryonated chicken eggs were alive after seven days post inoculation and the embryos were examined for IBV lesions. The embryos were stunted (dwarfed) and curled compared to the control (Figure 1). The virus was successfully isolated at the first passage and designated as ck/Malaysia/993/2020.

### Molecular detection

In the initial molecular detection on the received specimen, an amplicon of 297bp was generated using the primer set UTR1-/UTR2+ (Figure 2) revealing that the specimen was positive for IBV. Later, virus isolation was attempted and further confirmed as IBV by RT-PCR targeting the S1 gene producing an amplicon of 1620bp (Figure 3). The full-length of the S1 gene was sequenced. BLAST analysis showed that the virus was highly similar to the IBVs from Malaysia and China with 99% and 98% nucleotide identity respectively (Table 2). The HRRRR motif was observed in the S1 protein cleavage recognition site. The S1 nucleotide sequence was deposited in the GenBank with the accession number OR433086.

### Phylogenetic analysis

In the phylogenetic analysis of the S1 gene, the virus was grouped into the GI-19 lineage together with viruses from Malaysia and China (Figure 4).


**Figure 1.** Comparison of normal 16-day-old embryo (left) with embryo infected by IBV ck/Malaysia/993/2020 of the same age (right). The infected embryo was curled and stunted (dwarfed) compared to the normal embryo.

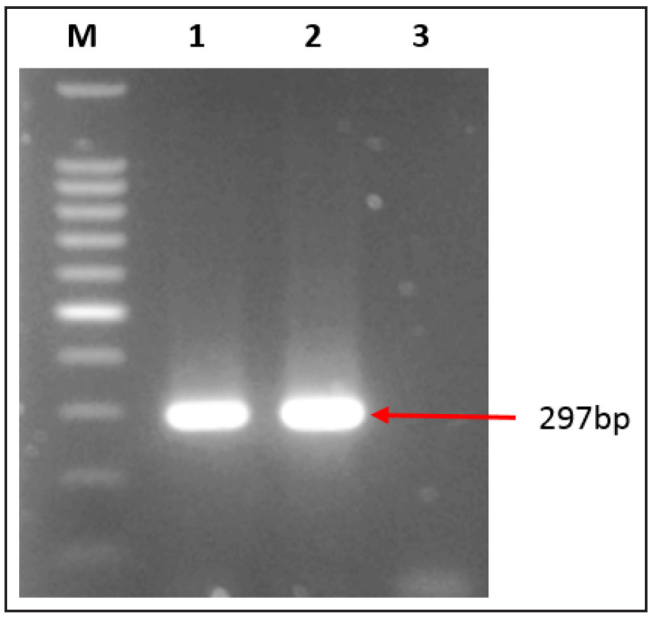


**Figure 2.** Avian coronavirus screening on the received specimen by targeting the UTR of IBV.
M: 100bp DNA ladder
1: ck/Malaysia/993/2020; 2: positive control; 3: non template control

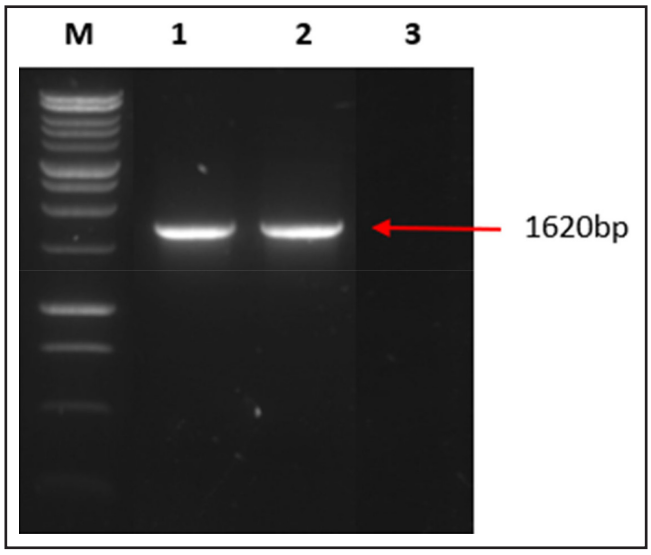


**Figure 3.** Amplification of the full-length S1 gene of the IBV ck/Malaysia/993/2020.
M: 1kb DNA ladder
1: ck/Malaysia/993/2020; 2: positive control; 3: non template control

**Table 2.** The nucleotide sequence identities between the IBV ck/Malaysia/993/2020 and the closest homologs in the GenBank database via BLAST search

| Virus isolate | GenBank accession number | Nucleotide sequence identity (%) | Country | Strain |
|---|---|---|---|---|
| IBV IBS130/2015 | MG738155 | 99.14 | Malaysia | QX-liked |
| IBV IBS138/2015 | KU949745 | 99.14 | Malaysia | QX-liked |
| IBV IBS126/2015 | KU949742 | 99.08 | Malaysia | QX-liked |
| IBV Ck/CH/LSD/101223 | KX364299 | 98.72 | China | LX4 |
| IBV Ck/CH/LJS/101111 | KX302862 | 98.72 | China | LX4 |

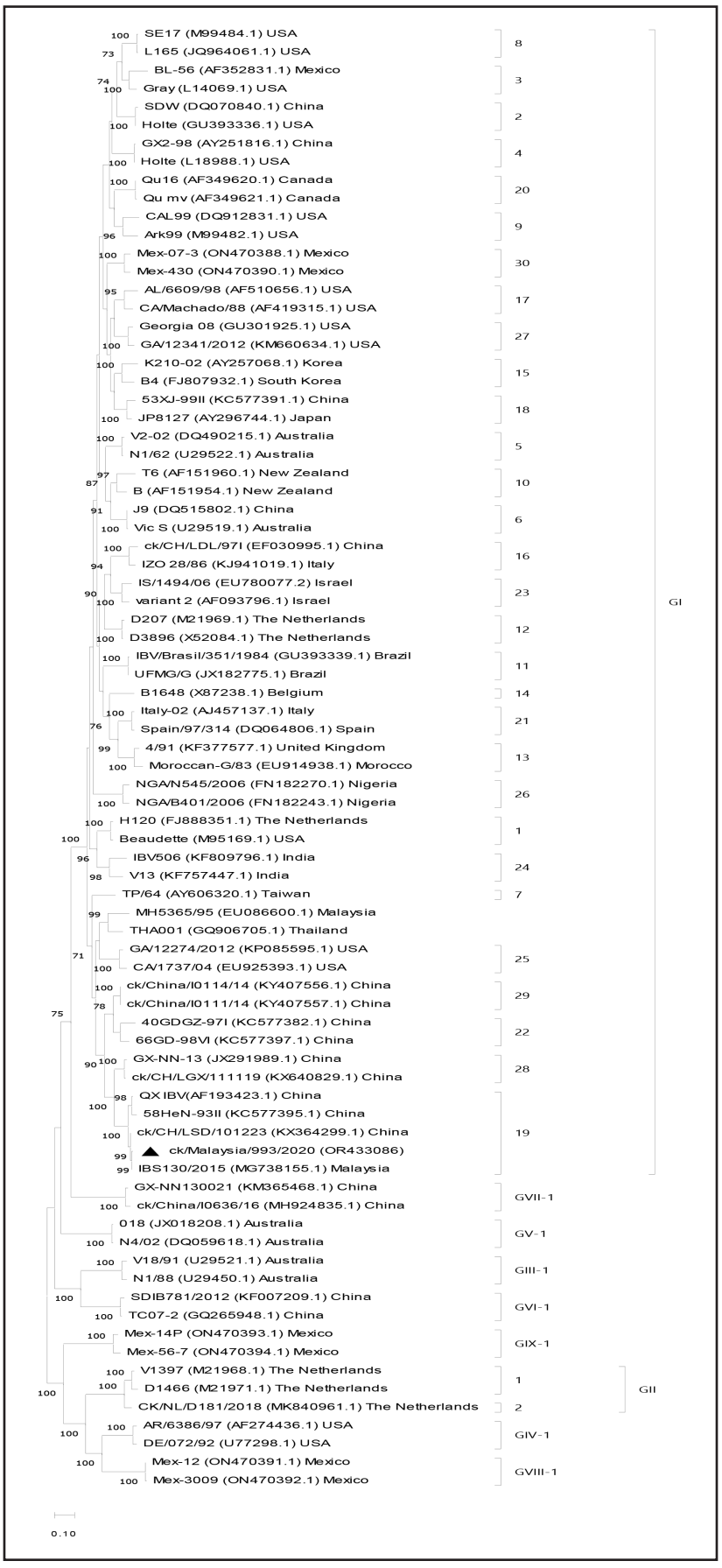


**Figure 4**. The phylogenetic tree was constructed by Maximum Likelihood method based on the Tamura-Nei model and setting bootstrap 1,000 replicates using MEGA version 11. Nodes with bootstrap values of more than 70% are shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The black triangles (▲) represent the IBV in the study. Total of 79 IBV strains worldwide were used in the analysis. The local strain name is suffixed by species/ country/ VRI disease investigation number/ year isolated/ GenBank accession number. The number in the phylogenetic tree indicates the lineages of the respective IBV genotype.

## DISCUSSION

The initial test requested by the regional veterinary laboratory on the imported meat product of this study is the coronavirus that caused the COVID-19 pandemic, the SARS-CoV-2 virus. However, due to the lacking and inadequate diagnostic methods for COVID-19 on animal /animal-origin specimens in the early phase of the pandemic (February 2020); and only the IBV diagnostic method is available in our laboratory, we attempted to pre-screen the specimen for IBV by molecular method as the specimen is of avian origin. Amplicons of 297bp amplified from the received specimen revealed the presence of the IBV. Subsequently, virus isolation was attempted. The observation of the characteristic lesions of IBV such as stunting (dwarfing) and curling of the infected embryo was in agreement with the positive molecular result for IBV in the pre-screening test. Generally, typical IBV field strain will cause embryo lesions including the stunting and curling of the embryo with feather dystrophy and deposition of urate in the mesonephros on the second to forth passage of virus isolation (OIE, 2018). Though IBV lesions was observed in the embryos, more studies such as pathogenicity test in chicken and whole genome sequencing have to be carried out to further confirm that the virus is of IB field strain.

BLAST search indicated that the nucleotide sequence of the IBV ck/Malaysia/993/2020 was 98% identical to the China strains which is in line with the country of origin for the imported chicken meat product. In the phylogenetic analysis, the IBV was grouped into the G1-19 lineage. According to Valastro's classification scheme (2016), the IB QX strain (also known as LX4 and A2); Korean-II (K-II) and Japanese-III (JP-III) viruses were classified as the GI-19 lineage viruses. The grouping of the IBV in the GI-19 lineage is also in agreement with the BLAST analysis that the closest homologs to this IBV was the QX/LX4 strain too (Table 2).

The GI-19 lineage virus was reported to be associated with proventriculitis (Li *et al*., 2010; Cook *et al*., 2012), renal pathology, (Li *et al*., 2010; Valastro *et al*., 2016) and false layer syndrome leads to decreased egg production and egg quality (Li *et al*., 2010). The viruses are now broadly spread and are the dominant IBV in other regions in Asia and some European countries (Li *et al*., 2010; Cook *et al*., 2012) causing huge economic damage to the both layer and broiler industry (Li *et al*., 2010). Similarly, the GI-19 lineage virus was one of the dominant IBV circulating in chickens in Malaysia (Leow *et al*., 2018).

The cleavage motif at the S1 gene of the IBV in the study was HRRRR which is in agreement with the observation of previous researchers that the HRRRR motif is unique to China strain and has not been detected in non-Chinese strains (Li *et al*., 2010; Feng *et al*., 2014; Leow *et al*., 2018). The cleavage in S1 protein in IBV is not required for viral and host cell fusion, but the cleaved S1 is more fusogenic than the uncleaved S1 protein (Jackwood *et al*., 2001). This is different from other viruses such as paramyxoviruses and orthomyxoviruses where the cleavage of the envelope glycoprotein is associated with host range and virus pathogenicity (Jackwood *et al*., 2001).

In general, low temperature can prolong the persistence of coronavirus in food (Han *et al*., 2021). The cold chain practice in the storage, transportation and distribution of food products provide a continuous low temperature condition which accommodate and prolong the survival of the virus (Han *et al*., 2021). IBV can survive up to 12 to 56 days in the cold storage (below 0°C) (Jackwood & de wit, 2020). Likewise, the SARS-CoV-2 virus has been reported to be survived on refrigerated and frozen meat samples and salmon beyond three weeks (Thomas, 2020) and manage to persist on the swine skin for 14 days at 4°C (Harbourt *et al*., 2020). Hence, it is not unusual that the IBV can be detected from the meat product in the study. As most of the poultry meats are kept frozen at the slaughter, there is a higher chance for the virus to retain in the infected carcass (Alexander, 1988). In this case, the retrieval of the IBV from the imported chicken meat product is likely due to the persistence characteristic of the virus.

Besides the import risk analysis, inspection of the foreign abattoir and food processing plants is crucial to ensure the food safety of the imported poultry and/or meat products into our country. A different designated area in the slaughtering facility comprising the live bird's section; slaughtering and defeathering; and evisceration area can reduce the risk of cross contamination (Silva, 2013). The application of engineering and strict hygiene measures helps in minimizing the contamination of poultry carcasses and/or meat products during the slaughtering and further processing. Alkaline and acidic cleaners which apply high and low pH action respectively; and the use of detergent in the cleaning of food processing operations can effectively decrease the virus population (Thippareddi *et al*., 2020). The implementation of antimicrobial intervention in slaughtering and meat processing operations using physical and chemical treatment can inactivate the coronavirus on fresh meat products (Thippareddi *et al*., 2020).

In general, imported poultry and poultry products are requested to be screened for highly contagious and economically important avian virus diseases such as avian influenza and Newcastle disease before they are released into Malaysia. Testing on other animal viral diseases is seldom ordered. Perhaps the finding of this study highlights the importance of screening for other avian virus diseases as well.

IBV is not a zoonotic disease and so far, there have been no human infections by IBV documented (OIE, 2018). Human or animal host are required for coronavirus multiplication (Han *et al*., 2021). The virus cannot multiply in food (Han *et al*., 2021) and can lose infectivity during storage (Thippareddi *et al*., 2020). IBV is not recognized as a hazard in chicken meat or meat products due to the lack of studies conclusively detecting the virus from chicken muscle tissues by virus isolation and molecular identification technique (Biosecurity New Zealand, 2022). IBV is heat sensitive and can be easily inactivated at the 56°C for 15 minutes (Jackwood & de wit, 2020). However, samples containing protein, for example meat products are recommended to be heated at 60°C for at least 30 minutes to thoroughly inactivate the virus (Jackwood & de wit, 2020). Nevertheless, it is advisable that the meat and meat products be cooked completely before consumption to avoid foodborne diseases.

## CONCLUSION

An IBV which belonged to the GI-19 lineage virus was detected from an imported chicken meat product. Although IBV in meat products is not considered as a threat to human health, thoroughly cooking before consumption is a must to prevent foodborne illness caused by other pathogens. With the increase in international trade in poultry and poultry products; import risk analysis, strict enforcement on poultry products importation, and disease screening at the entry point are important. It is not only to ensure food safety but to prevent the introduction of new strains of poultry disease from other countries into Malaysia.

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**Conflict of interest**

The author declares that they have no conflict of interest.

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