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Molecular Characterization of Avian Infectious Bronchitis Virus Isolated in Malaysia during 2014-2016

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Abstract. Avian Infectious Bronchitis (IB) is a highly contagious disease which can cause huge economic losses to the poultry industry. Forty five IB viruses (IBV) were isolated from poultry in Malaysia during 2014-2016. Phylogenetic analysis of the spike glycoprotein 1 (S1) gene revealed that all isolates were clustered into five distinct groups. The predominant type of IBV isolated was QX strains (47%), second was 4/91 type (27%), followed by Malaysian strain MH5365/95 (13%), Massachusetts type (11%) and finally Taiwanese strains (2%). Four types of S1 protein cleavage recognition motifs were found among the isolates which includes HRRRR, RRSRR, RRFRR and RRVRR. To our knowledge, this is the first report describing the motif RRVRR and are unique to Malaysian strains. Six IBVs were grouped in Malaysian MH5365/95 strains. Among these, one isolate was different from others where it only shared 82% identity with MH5365/95 and to others. It formed its own branch in the Malaysian cluster suggesting it may be a variant unique to Malaysia. Alignment analysis of the S1 amino acid sequences indicated that point mutations, insertions and deletions contribute to the divergence of IB variants. This study indicated at least five groups of IBV are circulating in Malaysia with most of the isolates belonged to QX strains. As new IBV variants continue to emerge, further study need to be carried out to determine whether the current available vaccine is able to give protection against the circulating virus.

INTRODUCTION

Infectious bronchitis (IB) is a highly contagious disease and has been one of the major causes of economic losses in the poultry industry. IB causes respiratory disease in young chickens and the infections can predispose poultry to secondary infections which may lead to mortality (Cavanagh, 2007; Cavanagh and Gelb, 2008). Some IB strain are pathogenic causing nephritis which can induce mortality up to approximately 25% in the flocks (Cavanagh, 2007; Cavanagh and Gelb, 2008; Cook *et al.*, 2012). The disease is caused by IB virus (IBV) which belongs to the order Nidovirales, family *Coronaviridae*, subfamily *Coronavirinae* and genus Gamma*coronavirus* (OIE Terrestrial Manual 2013). In layer and breeder chickens, IBV can infect oviduct causing reduction in egg production and poor egg quality (Cavanagh and Gelb, 2008; Cook *et al.*, 2012). The virus also replicates in the digestive tract and always present as subclinical infections (Cavanagh, 2007). IBV can persist in the intestinal tract and faeces up to few weeks or months and can shed through respiratory tract and faeces (de Wit *et al.*, 2011).

IBV is a positive sense, single-stranded RNA which encodes four structural proteins: the spike glycoprotein (S), membrane glycoprotein (M), nucleoprotein (N) and small membrane protein (E) (Cavanagh and Gelb, 2008). The S protein is involved in virus attachment and activation of fusion of viral and host cell membranes to release the viral genome (Cavanagh, 2007). The S protein which comprises of approximately 1160 amino acids is cleaved into two glycopolypeptides, the amino-terminal S1 and carboxyl-terminal S2 which consists of 520 and 625 amino acids respectively (Cavanagh *et al.*, 1988; Cavanagh, 2007). The S1 subunit forms the distal part of S protein and the S2 subunit anchors the S protein onto the membrane (Cavanagh *et al.*, 1988; Cavanagh, 2007). The S1 subunit which contains virus neutralizing and serotype specific epitopes, is the major inducer of protective immunity (Cavanagh *et al.*, 1992; Cavanagh, 2007; de Wit *et al.*, 2011). The virus neutralization epitopes in S1 determines the serotype of IB (Wang *et al.*, 1994; Cavanagh *et al.*, 1997). S1 also involved in infectivity and haemagglutinin activity (Cavanagh *et al.*, 1986; Moore *et al.*, 1998). Amino acid in S1 is far more variable than S2 subunit (Cavanagh *et al.*, 1992). Thus, S1 is widely used in genotyping and serotypic evolution of IB strains (Liu *et al.*, 2006; Li *et al.*, 2010; de Wit *et al.*, 2011).

Many IBV variants has been reported and at least 30 serotypes have been identified (Feng *et al.*, 2014). Some variants are generally distributed and some are restricted to a certain area. Nowadays, most countries seem to have their own native/ local IB strains as well (de Wit *et al.*, 2011). In Malaysia, first IBV was isolated in 1967 and variants was believed to be present since 1979 (de Wit *et al.*, 2011). Some Malaysian IBV were of Massachusetts (Mass) serotype which may be similar to the H120 vaccine strain and some were closely related to China and Taiwanese strains (de Wit *et al.*, 2011). In 1995, a nephropathogenic IB strain, MH5365/95 was isolated from vaccinated broiler chickens in Perak with high mortalities and severe renal lesions (Zulperi *et al.*, 2009). In 2004, a variant V9/04 which is unique to Malaysia was reported. This variant may have evolved from IB vaccine strain (Zulperi *et al.*, 2009). The aim of this study is to genetically characterize the S1 gene of IB strains that were isolated in Veterinary Research Institute (VRI) Ipoh from samples received from the whole country between year 2014 and 2016. The isolation of IBV seems to be increasing over the past three years (2014-2016) as the last isolation of IBV was in 2012 with only single IBV isolated.

MATERIALS AND METHOD

Virus isolation

From year 2014 to 2016, a total number of 329 cases comprised of 603 specimens from the suspected IBV-infected chickens were sent to VRI for diagnosis. Specimens comprising of tracheal, kidney, oviduct, intestine or pooled organ were sent for diagnosis. There is no complete history of the heath management of the chicken flock, status of vaccination, type of vaccine used, poultry management system and the related information for most of the cases. For virus isolation, specimens were homogenised and diluted 1:10 with tryptose phosphate buffer containing antibiotics prior to centrifugation at 2500 g for 5 minutes at 4°C. The supernatant was collected and filtered through a 0.22um syringe filter. Virus isolation was attempted for IBV by inoculating the filtrate sample into 9-11 days old embryonated specific pathogen free (SPF) chicken eggs via intra-allantoic route and incubated for three to five days at 37°C for four passages. A total of 45 isolates were obtained. Infected allantoic fluid and chorioallantoic membrane (CAM) were harvested and subjected for molecular detection and Agar Gel Precipitation Test (AGPT), respectively. The embryo was observed for abnormal changes such as curling, dwarfing or stunting of the embryo, feather clubbing and presence of urate deposits.

RNA extraction and Reverse Transcription - Polymerase Chain Reaction (RT-PCR) amplification

Only those samples that were positive IB by virus isolation were subjected to RT-PCR. RT-PCR was not attempted directly from the specimens to detect IBV prior to virus isolation. The viral RNA was extracted from the infected allantoic fluid by phenol chloroform method using TRIzol LS Reagent (Invitrogen) based on manufacturer's instruction. Briefly, 250ul of the infected allantoic fluid was incubated with 750ul of TRIzol LS Reagent. The viral RNA was then isolated with chloroform and precipitated with isopropanol. The RNA pellet was washed by ethanol and dried before being dissolved in nuclease free water. The total RNA was used as template in RT-PCR. RT-PCR was carried out using SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen). A primer set, S1F 5'-AAGACTGAACAAAAGACCGACT-3' and S1R: 5'-CAAAACCTGCCATAACTAACATA-3' (Ji *et al.*, 2011) was used to amplify approximately 1600 bp which covers the entire S1 gene of IBV. In brief, the RT was carried out at 48°C for 30 min. The reaction mix 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 51°C for 1 min and extension at 68°C for 2 min with a final extension for 10 min at 68°C. The amplicons were then analysed by electrophoresis on 1.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen).

Gene sequencing, nucleotide/amino acid homology analysis and phylogenetic analysis

The amplified PCR products were cut from gel and sent for sequencing (First Base Laboratories Sdn Bhd). The DNA products were subjected to direct nucleotide sequencing using BigDye® Terminator v3.1 cycle sequencing kit chemistry (Applied Biosystems). Nucleotide sequences were assembled using SeqMan Pro software (DNAStar Lasergene, USA). The nucleotide sequences of S1 gene from the start codon to the cleavage recognition site of 45 local isolates and published sequences downloaded from NCBI were aligned and compared using *BioEdit* Sequence Alignment Editor (Version 7.1.9). Homologies of S1 nucleotides and deduced amino acid among isolates and references strains were analysed. A phylogenetic tree was constructed using MEGA version 6.06 by Neighbor-Joining statistical method with model number of differences and setting bootstrap 1000 replicates.

RESULTS

Virus isolation

A total number of 45 IBVs were isolated from dead or diseased chickens suspected of IBV infection from different states in Malaysia (Perak, Selangor, Penang Island, Kelantan, Kedah, Pahang, Negeri Sembilan, Johor and Sabah) during the years 2014 to 2016 (Fig. 1). Seventeen IB strains were isolated from chickens showing respiratory sign, two strains showing nephritis while thirteen strains showed both respiratory and nephritis symptoms. One strain was isolated from chickens showing signs of nephritis, undeveloped ovaries and decrease in egg quality. On the other hand, two IBV strains were from chickens that presented with diarrhoeal symptoms; two more strains were isolated from chicken that presented with inappetence and dullness. Another two strains were isolated from chickens showing dullness followed by sudden death; and chickens with no reported clinical symptoms yielded another six strains. The details of the isolated IBV strains are shown in Table 1. All isolates were positive for IBV by AGPT using specific antiserum against IBV. All isolates generated PCR products of approximately 1620bp which amplified the entire S1 gene of IBV (data not shown).

Phylogenetic analysis of clusters of 45 local IBV strains into five groups

In order to understand the relationship between the local isolates and other IBV strains in the world, a phylogenetic tree was constructed. Forty nine published S1 gene representing the respective serotypes were included in the phylogenetic tree.

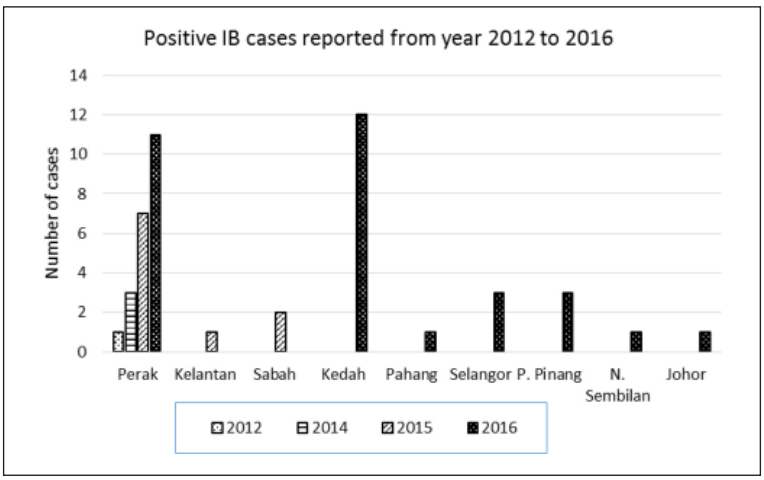

Figure 1. Positive IB cases reported in different states of Malaysia from year 2012 to 2016.

Phylogenetic analysis of the S1 gene showed all local isolates to be clustered into five distinct groups. The predominant type of IBV isolated was QX strains (47%), second was 4/91 type (27%), followed by Malaysian strain MH5365/95 (13%), Massachusetts type (11%) and finally Taiwanese strains (2%) (Fig. 2). Out of the 45 isolates, six were grouped in Malaysian MH5365/95 strain. Among these, one IBV (VRI 3929-2015) forms a single lineage within the MH5365/95 cluster.

Homologies of S1 nucleotides and deduced amino acids among isolates and reference strains

The S1 gene nucleotides of the local isolates were aligned and compared using BioEdit Sequence Alignment Editor. The nucleotide and deduced amino acid similarities of S1 gene among the 45 isolated IBV was ranging from 76.0% to 99.9% and 61.1% to 100% respectively (data not shown). When comparing with the 49 reference strains, homology analysis of total 94 IB strains revealed nucleotide and deduced amino acid similarities ranged from 56.7% to 100% and 41.3% to 100% respectively (data not shown). These results indicate high variation and low homology among the local isolates and reference strains in the S1 gene.

Among the 45 local isolates, it is worth to emphasize on two distinct isolates, the VRI 3929-2015 and VRI 7104-2016 strains. Homology analysis for both isolates and respective reference strains were carried out for better understanding of their relationship (Table S1 and Table S2). VRI 3929-2015 strain shared only 82% nucleotide similarities with both MH5365/95 and other five local IBVs respectively. Likewise, this isolate also showed 81% amino acid similarities with both MH5365/95 and other five IBVs respectively (Table S1). On the other hand, the other five local IBVs showed 92% nucleotide similarities with MH5365/95 and 100% similarities to each other; and 88-89% amino acid similarities with MH5365/95 and 99-100% similarities to each other. Isolate VRI 7104-2016 showed the highest nucleotide similarities (93%) with ck/CH/LSD/051 strain from China via BLAST search (data not shown). Homology analysis revealed that this isolate showed higher nucleotide (93%) and amino acid identities (91%) with ck/CH/LSD/051 strain compared to other Taiwanese strains (Table S2). Conversely, this IBV showed only 88% nucleotide and 87% amino acid similarities respectively with TW I; 87% and 83% respectively with TW II.

For better understanding of the genetic variations among the 45 isolates, these IBVs sequences were aligned and compared with reference strain H120. The alignment of deduced amino acid sequences demon-

Table 1. IBV strains isolated from chickens in different states of Malaysia from year 2014 to 2016

Isolates	State	S1 cleavage recognition site[a]	Genotype	Clinical signs	Passage number of positive IB[c]
VRI 5668-2014	Perak	HRRRR	QX	Dull, no appetite	P1
VRI 8450-2014	Perak	HRRRR	QX	Respiratory, nephritis	P2
VRI 13310-2014	Perak	RRSRR	Mass	Respiratory, nephritis	P2
VRI 438-2015	Kelantan	HRRRR	QX	NA[b]	P3
VRI 797-2015	Sabah	RRSRR	Mass	NA	P4
VRI 1881-2015	Perak	RRSRR	4/91	Respiratory	P1
VRI 3929-2015	Sabah	RRSRR	MH5365/95	Respiratory	P4
VRI 5677-2015	Perak	HRRRR	QX	Respiratory, nephritis	P4
VRI 10685-2015	Perak	RRVRR	MH5365/95	Respiratory, nephritis	P1
VRI 10839-2015	Perak	RRSRR	4/91	Nephritis, irregular shape egg increased, undeveloped ovaries	P1
VRI 11531-2015	Perak	RRVRR	MH5365/95	Respiratory, nephritis	P1
VRI 12940-2015	Perak	RRVRR	MH5365/95	Respiratory, nephritis	P3
VRI 13161-2015	Perak	RRSRR	4/91	Respiratory	P2
VRI 3912-2016	Kedah	RRSRR	4/91	NA	P3
VRI 3962-2016	Perak	HRRRR	QX	Respiratory, nephritis	P1
VRI 4256-2016	Perak	HRRRR	QX	Respiratory, nephritis	P3
VRI 4633-2016	Perak	RRFRR	Mass	Respiratory, nephritis	P1
VRI 4853-2016	Perak	RRVRR	MH5365/95	Respiratory	P1
VRI 5109-2016	Perak	HRRRR	QX	Dull, no appetite	P1
VRI 5233-2016	Kedah	HRRRR	QX	Respiratory	P1
VRI 5349-2016	Perak	RRSRR	4/91	Respiratory, nephritis	P2
VRI 5783-2016	Perak	HRRRR	QX	Respiratory, nephritis	P2
VRI 5785-2016	Perak	HRRRR	QX	Respiratory, nephritis	P3
VRI 5992-2016	Kedah	HRRRR	QX	Respiratory	P1
VRI 6224-2016	Kedah	RRFRR	Mass	Respiratory	P2
VRI 6778-2016	Pahang	HRRRR	QX	Diarrhoea, no appetite	P2
VRI 6798-2016	Selangor	RRSRR	4/91	NA	P1
VRI 6923-2016	P. Pinang	HRRRR	QX	Respiratory	P2
VRI 6924-2016	P. Pinang	HRRRR	QX	Respiratory	P1
VRI 7099-2016	Perak	HRRRR	QX	Nephritis	P1
VRI 7103-2016	Selangor	RRSRR	4/91	Respiratory	P1
VRI 7104-2016	Selangor	RRSRR	Taiwanese	Respiratory	P2
VRI 7626-2016	N. Sembilan	RRSRR	4/91	Nephritis	P2
VRI 7972-2016	Kedah	RRSRR	4/91	NA	P3
VRI 7973-2016	Kedah	RRSRR	Mass	NA	P4
VRI 8510-2016	Perak	HRRRR	QX	Respiratory	P1
VRI 8524-2016	Kedah	HRRRR	QX	Respiratory	P1
VRI 8525-2016	Kedah	HRRRR	QX	Respiratory	P2
VRI 8528-2016	Kedah	RRSRR	4/91	Dull, Sudden death	P2
VRI 8529-2016	Kedah	RRSRR	4/91	Dull, Sudden death	P2
VRI 8530-2016	Kedah	RRSRR	4/91	Respiratory	P2
VRI 8531-2016	P. Pinang	RRVRR	MH5365/95	Diarrhoea, pale	P2
VRI 8987-2016	Kedah	HRRRR	QX	Respiratory	P1
VRI 9322-2016	Johor	HRRRR	QX	Respiratory	P2
VRI 9693-2016	Perak	HRRRR	QX	Respiratory, nephritis	P4

[a]Cleavage recognition site of S1 glycoprotein
HRRRR: His-Arg-Arg-Arg-Arg (21/45)
RRSRR: Arg-Arg-Ser-Arg-Arg (17/45)
RRVRR: Arg-Arg-Val-Arg-Arg (5/45)
RRFRR: Arg-Arg-Phe-Arg-Arg (2/45)
[b]NA: Not available
[c]The passage number where the isolate confirm as IB by AGPT.

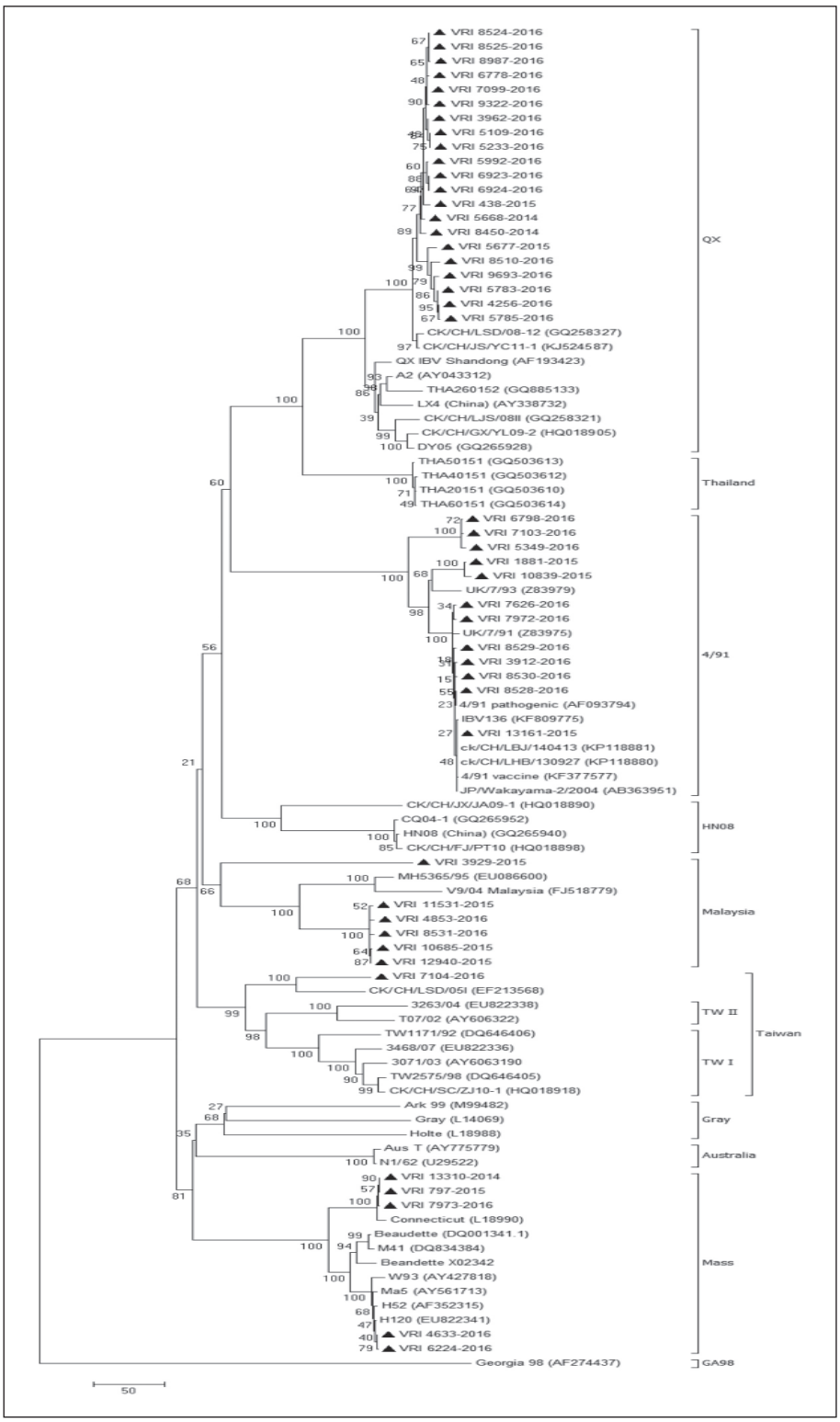

Figure 2. Phylogenetic tree of 45 isolates and 49 reference strains based on S1 gene of IBV. Tree was constructed using MEGA version 6.06 by Neighbour-Joining statistical method with model number of differences and setting bootstrap 1000 replicates. GenBank accession numbers was shown in parenthesis.

Supplementary Table S1. Nucleotide and amino acid similarities of the S1 gene among MH5365/95-like local isolates and Malaysia reference strain (MH5365/95)

	1	2	3	4	5	6	7	
Amino acid identity (%)								
1 MH5365/95		80.7	88.6	88.6	88.6	88.3	88.3	1
2 VRI 3929-2015	81.8		80.7	80.7	80.7	80.7	80.5	2
3 VRI 10685-2015	91.7	82.2		99.8	100	99.2	99.6	3
4 VRI 11531-2015	91.8	82.3	99.7		99.8	99.4	99.4	4
5 VRI 12940-2015	91.7	82.3	99.9	99.8		99.2	99.6	5
6 VRI 4853-2016	91.6	82.2	99.6	99.6	99.7		98.8	6
7 VRI 8531-2016	91.5	82.1	99.6	99.5	99.7	99.5		7
Nucleotide identity (%)								

Supplementary Table S2. Nucleotide and amino acid similarities of the S1 protein gene among Taiwanese-like local isolates and Taiwanese reference strains

	1	2	3	4	
Amino acid identity (%)					
1 VRI 7104-2016		91.1	86.4	82.5	1
2 CK/CH/LSD/05I	92.6		85.5	82.2	2
3 TW1171/92 (TW I)	87.9	86.6		83.3	3
4 T07/02 (TW II)	86.8	86.0	87.2		4
Nucleotide identity (%)					

strated deletion, insertion and many point mutations in the S1 gene (Fig. 3).

Various S1 protein cleavage recognition site sequences

Four S1 protein cleavage recognition site sequences were observed among the local isolates. The motifs were HRRRR (47%), RRSRR (38%), RRVRR (11%) and RRFRR (4%) (Table 1).

DISCUSSION

From year 2014 to 2016, 45 IBVs were isolated from the specimens sent from various states in Malaysia (Perak, Selangor, Penang island, Kelantan, Kedah, Pahang, Negeri Sembilan, Johor and Sabah). The positive cases of IB seems increasing over the few years. From single IBV isolated in year 2012, no positive cases detected in 2013, three positive IB isolates in 2014, 10 isolates in 2015 and to a sharp increase in 2016 of 32 positive cases indicating the rise of IB incidence in the country (Fig. 1). There was no reported IB cases from other states such as Perlis, Sarawak, Melaka and Terengganu during these few years. However, we cannot conclude that there is no IB incidence or outbreak in these states as the IB cases maybe under-reported due to farmers sending the specimens to private laboratories for diagnosis.

Phylogenetic analysis of the local IBVs isolated during the years 2014 to 2016 revealed that there were five groups of IBV strains circulating in Malaysia. The wide range of the nucleotide similarities (76.0% to 99.9%) of the 45 IBVs indicates high variation and low homology in the S1 gene among these local isolates. The predominant type was QX strain (47%). QX strain was first isolated in China in 1996 with the history of proven tricultitis in chickens (Li *et al.*, 2010; Feng *et al.*, 2014). The QX-like strain prevalent in China is mainly associated with nephritis and false layer syndrome (Feng *et al.*, 2014). Whereas, in Malaysia, chickens that were infected with the QX strain mainly

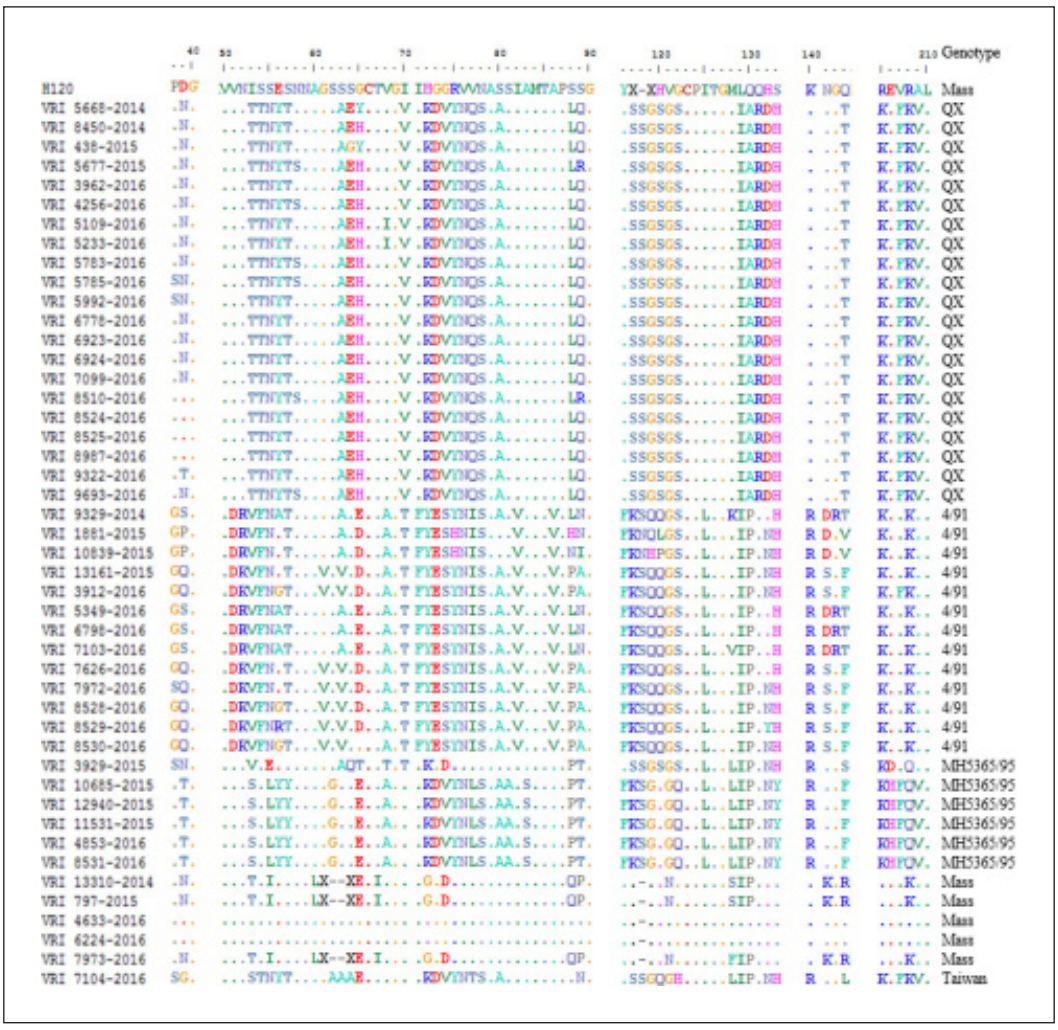

Figure 3. Alignments of S1 amino acid sequences of isolated IBVs compare with H120 reference strain. A *dot* indicates an amino acid identical to that of H120 strain. A *dash* indicates an amino acid deletion compare with H120 strain. The position of amino acids start with methionine that encodes the start codon of S1.

manifested with respiratory and/or nephritis symptoms (Table 1). This strain is now widely spread and become the dominant type of IB strain in other areas in Asia and some European countries (Li *et al.*, 2010; Cook *et al.*, 2012; Feng *et al.*, 2014). Thus, it is not surprising that the QX strain has become the primary genotype of IBV circulating in Malaysia. Moreover, the finding is also in agreement with de Wit *et al.* (2011) that some Malaysian IBVs were similar to the China and Taiwanese strains.

Twelve IBVs belonged to 4/91 type in the phylogenetic tree. 4/91 type which is also known as 793B and CR88 strain was first reported in the UK and France and now is detected worldwide except the USA (Cook *et al.*, 2012). It is associated with muscle myopathy, mortality in breeding hens and scouring in broiler (Cook *et al.*, 1996; Cook *et al.*, 2012). The antigenicity of this variant does not seem to change much. The first vaccine that was developed is still able to give protection to poultry today (Cook *et al.*, 2012). Since this variant has been spreading worldwide, it is common to find the presence of this virus in Malaysia. However, possibility of re-isolation of

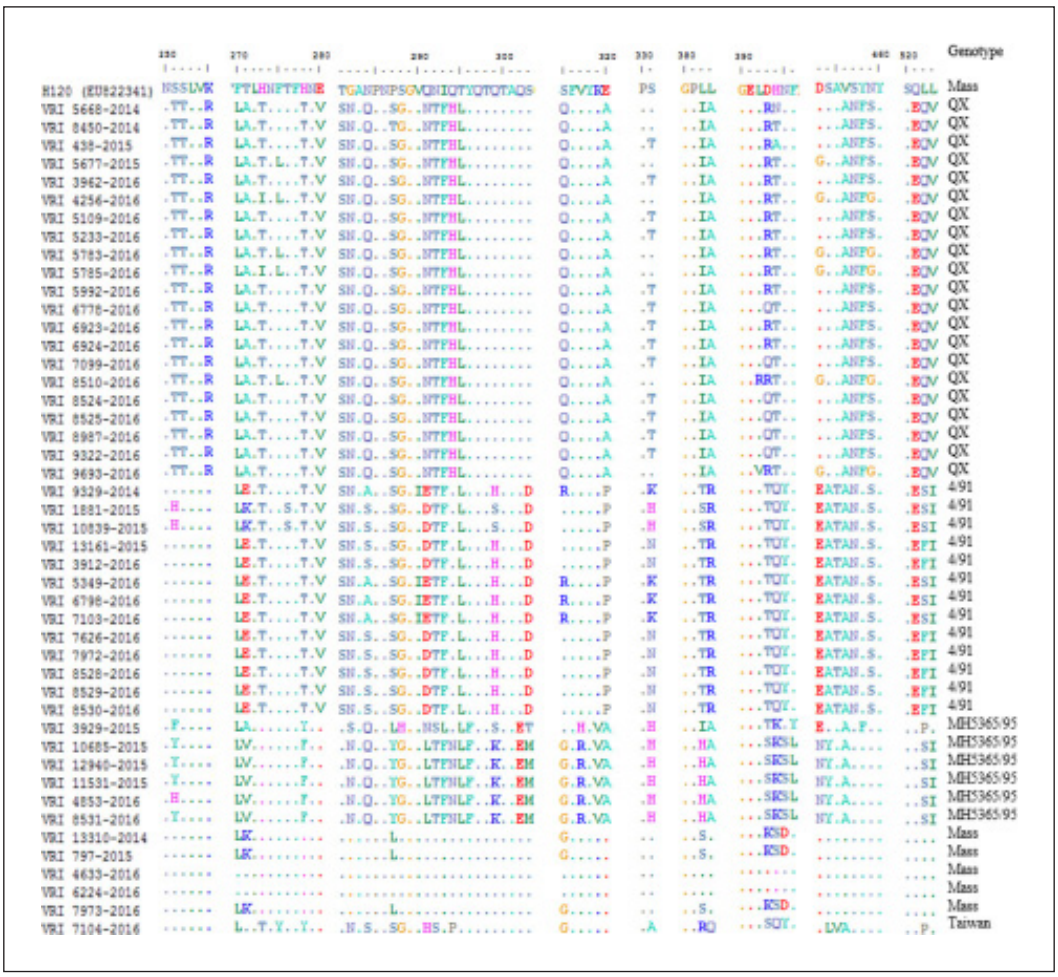

Figure 3 (Cont'd). Alignments of S1 amino acid sequences of isolated IBVs compare with H120 reference strain. A *dot* indicates an amino acid identical to that of H120 strain. A *dash* indicates an amino acid deletion compare with H120 strain. The position of amino acids start with methionine that encodes the start codon of S1.

vaccine strains used in the field cannot be ruled out as 4/91 type IB vaccine which includes 4/91, CR88 and IBV 1/96 strain is an authorized vaccine in the country (Department of Veterinary Services Malaysia).

The third major type of IBVs circulating in the country was MH5365/95 strain, a non-Mass variant that was unique to Malaysia (Zulperi *et al.*, 2009). In the phylogenetic tree, six local IBVs belonged to this strain. Isolation of this strain is not uncommon in Malaysia as Maizan in year 2000 reported the isolation of MH5365/95 strain from local field isolates. So far, MH5365/95 strain has only been detected in Malaysia and this was in agreement with de Wit *et al.* (2011) who mentioned that IB variant seems to be correlated with geographical distribution. Though some common serotypes may be present in many countries, IBV in one particular area is always unique and distinct (Liu *et al.*, 2006). From the phylogenetic analysis, it is noteworthy that one isolate (VRI 3929-2015) formed its own distinct branch in this group while the other five IBVs were grouped into one cluster (Fig. 2). VRI 3929-2015 was isolated from 43 days old vaccinated broiler in Sabah. The chickens showed loss of appetite and respiratory clinical signs. The morbidity rate was 15%, yet mortality record was not provided. In

BLAST search, this isolate showed 97% query cover and 83% sequence identity with MH5365/95 (data not shown). As mentioned earlier, MH5365/95 is a nephropathogenic IB strain that causes high mortalities in vaccinated broilers (Zulperi *et al.*, 2009). In contrast, VRI 3929-2015 was isolated from chickens with respiratory symptoms. This isolate shared a low nucleotide (82%) and amino acid similarities (81%) with both MH5365/95 and other five IBVs (Table S1). While the other five IBVs showed 100% nucleotide and 99-100% amino acid similarities to each other. The low homology of the nucleotide and amino acid similarities of this isolate compared to others made it form its own lineage in the MH5365/95 group. Emergence of new IB variant is not new in Malaysia as presence of variants in Malaysia was as early as 1979 (de Wit *et al.*, 2011). In this case, the emergence of this IBV may be due to the recombination of genetic material of the vaccine strain used and circulating IBV such as MH5365/95 strain. Another possibility is that this virus may be a totally new variant which is distinct from other local IBVs. However, the real situation is not known as vaccination, flock healthy history from where the IBV was isolated was unclear. In RNA replication, recombination can occur through template switching mechanism (Han *et al.*, 2011; Feng *et al.*, 2014) where the polymerase switches from one template to another when the host is infected by more than one IB strain (Feng *et al.*, 2014). Moreover, evidence of IBV recombination in field has also been reported (Kusters *et al.*, 1990; Wang *et al.*, 1993; Jia *et al.*, 1995). Hence, recombination analysis and perhaps whole genome sequencing have to be carried out for this isolate to better understand its genetic variability.

In phylogenetic tree, five local isolates were falls into the branch containing Mass virus. Out of five isolates, three were grouped with H120 cluster whereas two were grouped within Connecticut lineage. The first isolate of IBV was named as Mass serotype (Cavanagh *et al.*, 1992) and it has been isolated in Europe and Asia since 1940s (Canavagh and Gelb, 2008). Connecticut strain isolated in 1951 was generally believed to be the first variant of IBV (de Wit *et al.*, 2011). However, a study conducted in 2002 by Jia and colleague demonstrated that non-Mass IBVs has been in existence as early as 1940s. The isolation of H120 strains was corresponding to de Wit *et al.* (2011) who reported that some IBVs isolated in Malaysia were probably more identical to H120 vaccine. Similarly, in year 2000, Maizan identified Mass type IB from local isolates in Malaysia. Commercial vaccine based on Mass type such as H120 are commonly used worldwide (Canavagh and Gelb, 2008; Li *et al.*, 2010; Ji *et al.*, 2011). In Malaysia, the authorized vaccines for IB are of Mass type which includes H120, H52, M41, Ma5 and 4/91 type. Since IB vaccine is widely used in poultry industry to combat the disease, re-isolation of vaccine strain is possible. It has been reported in countries such as China, Spain, Argentina and Jordan (Dolz *et al.*, 2008; Rimondi *et al.*, 2009; Roussan *et al.*, 2009; Han *et al.*, 2011; Ma *et al.*, 2012) that IB Mass type is frequently isolated even in those vaccinated with H120 strain or other Mass type vaccine. Canavagh and Gelb (2008) pointed out that if the virus is retrieved from a Mass vaccinated flock with respiratory signs, it can be suggested that it is a re-isolation of the vaccine strain and other IB serotypes is the cause of the disease. However, they further explained that this may not always be the scenario as virulent Mass type IB virus is still present in many countries. As the vaccination status of most of the cases in this study were unknown, therefore the Mass type IBV isolated here cannot be distinguished from field isolates or re-isolation of the vaccine strain.

Among the 45 local isolates, one IBV (VRI 7104-2016) was clustered in Taiwanese strains. Isolation of Taiwanese strain is not unusual in the country as mentioned earlier that de Wit *et al.* (2011) reported some IBVs in Malaysia were similar to China and Taiwanese strains. VRI 7104-2016 was isolated from broiler with history of respiratory distress and the vaccination status of the flock was unclear. This isolate showed the highest nucleotide similarities

(93%) with ck/CH/LSD/051 strain via BLAST search (data not shown) and formed a distinct branch with this China strain in the Taiwanese group in the phylogenetic tree. ck/CH/LSD/051 strain was isolated from H120 vaccinated broiler from Shandong, China in 2005 (Liu *et al*., 2008). Liu and colleagues demonstrated through an experiment that although this virus was initially isolated from the kidney of a diseased chick, it may not be nephropathogenic but conversely, it has a high affinity for the respiratory tract (Liu *et al*., 2008). The respiratory distress exhibited by chicken infected by VRI 7104-2016 strain was in corresponding to the finding of Liu and colleagues. In order to understand better, the nucleotide and amino acid similarities of VRI-7104-2016, ck/CH/LSD/051 and Taiwanese strains were compared. Homology analysis revealed that this local isolate showed higher nucleotide (93%) and amino acid identities (91%) with ck/CH/LSD/051 strain compared to other Taiwanese strains (Table S2). Although VRI 7104-2016 formed a single lineage with ck/CH/LSD/051 strain, which was different from existing Taiwanese virus, the TW I and TW II subgroup in the phylogenetic analysis; however, both shared the common ancestor of the Taiwanese virus (Fig. 2). Though the nucleotide similarities were high and exhibited the same clinical signs as ck/CH/LSD/051 strain; however, the cleavage site of the S1 gene of VRI 7104-2016 was RRSRR which is different from RRFRR found in the former strain.

The S1 protein cleavage recognition site contains five amino acid and is recognized by host cell serine protease (Jackwood *et al*., 2001). For IBV, cleavage is not necessary for fusion of viral and host cell membranes, but cleaved S protein is more fusogenic than uncleaved S protein (Jackwood *et al*., 2001). In this study, four cleavage site motifs were found among 45 local isolates. The most common cleavage site found was HRRRR and is found only in the China virus in this study. This observation is in agreement with Liu *et al*. (2006), Bing *et al*. (2007) and Li *et al*. (2010) which stated that this motif is unique to China virus and has not been reported in non-Chinese strains. The second most common site was RRSRR. This motif was observed in all IBVs of serotype 4/91, Connecticut strains of Mass serotype and one MH5365/95-like isolate (VRI 3929-2015). Presence of the motif RRSRR in different IB serotypes is corresponding to the finding of Jackwood *et al*. (2001) that the cleavage recognition site does not correlate with serotype. On the other hand, there were five IBVs contain RRVRR motif. To the best of our knowledge, this is the first study to describe the cleavage site sequence RRVRR. This motif was only seen in MH5365/95 strains suggesting that it is unique to Malaysia strains. Formerly, Jackwood *et al*. (2001) reported that the cleavage site motif is associated with virus in different geographic regions. Again, he mentioned that serotypically and genotypically distinct viruses have unique cleavage motif at S1 gene. Another interesting point is though VRI-3929-2015 is grouped within MH5365/95 cluster, it's cleavage motif (RRSRR) is different from RRVRR which is unique to MH5365/95 strains. The dissimilar motif and the distinct lineage in the phylogenetic tree formed by VRI-3929-2015 give an idea that this isolate may be a variant unique to Malaysia. Only two IBVs had the cleavage motif of RRFRR and this motif has been found in many countries (Jackwood *et al*., 2001; Liu *et al*., 2006; Li *et al*., 2010; Ji *et al*., 2011; Pohuang *et al*., 2011). As demonstrated in the phylogenetic tree, though five local IBVs belonged to Mass type, two different cleavage motifs were found. Three isolates that clustered within the Connecticut lineage had the motif of RRSRR while the other two that clustered within H120 strains contained RRFRR motif. This again complements the report of Jackwood *et al*. (2001) that the cleavage recognition site does not correlate with serotype. By comparing the cleavage site motif with the clinical signs of the 45 local isolates, it showed that the cleavage site sequence is not associated with the pathogenicity as demonstrated by Jackwood *et al*. (2001).

The emergence of new variants in IB evolution are believed to be caused by insertions, deletions, point mutation and genetic recombination (Lee *et al*., 2000). The large genome of IBV (27.6kb) tends to have higher mutation rates in the genome transcription due to lacking of proof reading function in the RNA polymerase (Wang *et al.*, 1994; Lee *et al*., 2001). The genetic variation of the 45 local IBVs resulting from insertion, deletion and point mutations are featured in Figure 3. Point mutations were observed throughout the S1 gene despite the difference in genotypes. The high frequency of point mutations contributes to the high genetic variability of the S1 gene. The hypervariable region (HVR) were similar to the previous studies as reported by Moore *et al*. (1997), Liu *et al*. (2006) and Feng *et al*. (2014). Moreover, sequence variation also seen in region 393-397, 453-60, 521-523 (position based on S1 sequence of H120 strain). For local Mass strains, three isolates (VRI-13310-2014, VRI-797-2015 and VRI-7973-2016) that were clustered within Connecticut strain had the same pattern of genetic variation except at position 128 where VRI-13310-2014 and VRI-797-2015 strains contained phenylalanine (F) while VRI-7973-2016 strain comprised of serine (S). On the other hand, those local isolates that belonged to H120 strains had different pattern of variation compared to the former three isolates (Figure 3). It is noteworthy that VRI-3929-2015 strain has a wide range of genetic variation compared with other local MH5365/95 strains. The divergence of this strain results in generating its own lineage within the MH5365/95 group. A small 2-3% changes in S1 amino acid (10-15 residues) can change the serotype (Canavagh *et al*., 1992; Canavagh, 2007) as a result of changes in the virus neutralizing epitopes. As serotype prevalent in a certain region changes with time (Cook *et al.*, 1996) and IBV tends to change by genetic recombination and spontaneous mutation (Cavanagh and Gelb, 2008), it is not uncommon that the virus may have evolved and arose as a new variant. Therefore, the emergence of this VRI-3929-2015 strain is suggestive of a new variant which is unique to the country.

As QX strain is the predominant type of IB in the country, control strategy needs to be taken to prevent future outbreak. Besides good poultry management system such as strict biosecurity, good hygiene practice, one-age system; vaccination is an effective way to control the disease. A study showed that Mass type (H120 strain) vaccine gave poor protection against QX strain (Sun *et al.*, (2011). However, two vaccination of Mass and 4/91 type strain has been proven to offer good protection in chickens against challenges with QX strain (Terregino *et al.*, 2008; Awad *et al.*, 2016). Study on efficacy of QX-like vaccine against QX challenge has been carried out in Thailand. Sarueng and colleagues (2014) conducted an experiment to verify the effectiveness of four different vaccine programme against QX strain including single vaccination of QX-like vaccine; vaccination and booster with QX-like vaccine respectively at 14 days interval; vaccination with H120 strain and booster with QX-like vaccine; vaccination with H120 strain and booster with 4/91 type vaccine. They found that all vaccinated chicken was 100% protected in the flocks that were vaccinated with a combination of H120 and 4/91 type and they showed the highest protection score in the ciliastasis test of tracheal at 7 days post challenge with QX strain. The same flock also had the lowest histological lesion scores of tracheal, lungs and kidneys at 14 days' post challenge compare to other flocks. Sarueng *et al.* (2014) proven that protection against heterologous IB serotype challenge can be improved by re-vaccinating the flocks with different IB serotypes. Currently, there is no QX based vaccine available in Malaysia as the authorized IB vaccine approved by government are of Mass and 4/91 type. Referring to the work done by the previous scientists, and the comment by Cook *et al.* (1999) that the development of new vaccines to control the newly emerging IB strain is not a necessity as he successfully demonstrated that vaccination using two antigenitically distinct strains give broader

protection against challenges of different IB serotypes. Therefore the development of local QX-like vaccine is not a need and usage of two different serotype of IB vaccine is recommended in order to combat the disease caused by QX strain in the country. However, more studies need to be carried out to verify the effectiveness of the available vaccines against local QX strain in Malaysia.

CONCLUSION

In conclusion, the present study demonstrated that five different groups of IBV were circulating in Malaysia from the year 2014 to 2016 with most of the isolates belonging to the QX strains. The high degree of genetic variation in the S1 gene contributes to the genetic diversity of IB strains in the country. One isolate belonged to MH5365/95 strain formed its own branch in the MH5365/95 group in phylogenetic tree and the high degree of genetic variation suggested that it may be a variant unique to Malaysia. However, recombination analysis and perhaps whole genome sequencing has to be carried out for better understanding of its genetic variability. This is the first report that describes the S1 protein cleavage recognitions site RRVRR which is unique to Malaysian strains. As new IBV variants continue to emerge, further study needs to be carried out to determine whether the current available vaccine is able to give protection against the circulating virus.

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