Genetic characterization of infectious bronchitis viruses in Thailand, 2014–2016: identification of a novel recombinant variant

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ABSTRACT Infectious bronchitis (**IB**) causes severe economic losses to the poultry industry worldwide owing to frequent emergence of novel infectious bronchitis virus (**IBV**) variants, which potentially affect the effectiveness of the currently used IBV vaccine. Therefore, continuous monitoring of IBV genotypes and lineages recently circulating in chickens worldwide is essential. In this study, we characterized the complete S1 gene from 120 IBVs circulating in chickens in Thailand from 2014 to 2016. Phylogenetic analysis of the complete S1 gene of 120 Thai IBVs revealed that the 2014–2016 Thai IBVs were divided into 3 lineages (GI-1, GI-13, and GI-19) and a novel IBV variant. Our results also showed that GI-19 lineage has become the predominant lineage of IBV circulating in chicken flocks in Thailand from 2014 to 2016. It is interesting to note that a novel IBV variant, which was genetically different from the established IBV lineages, was identified in this study. The recombination analysis demonstrated that this novel IBV variant was a recombinant virus, which was originated from the GI-19 and GI-13 lineage viruses. In conclusion, our data demonstrate the circulation of different lineages of IBV and the presence of a novel recombinant IBV variant in chicken flocks in Thailand. This study highlights the high genetic diversity and continued evolution of IBVs in chickens in Thailand, and the importance of continued IBV surveillance for effective control and prevention of IB.

Key words: chicken, genetic characterization, infectious bronchitis virus, Thailand

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INTRODUCTION

Infectious bronchitis (**IB**), caused by infectious bronchitis virus (**IBV**), is a highly contagious viral disease in chickens, causing significant economic losses to the poultry industry worldwide. The disease is primarily characterized by an upper respiratory disease; however, some strains of IBV can cause clinical diseases in the urogenital, reproductive, and gastrointestinal tracts, resulting in nephritis, reduced egg production and quality in layers, enteritis, and significant mortality (Bande et al., 2017). Although the mortality rate of IBV infection is relatively low, the secondary infection or coinfection with other pathogens, including *Escherichia coli* and *Mycoplasma gallisepticum*, can increase the severity of IB, resulting in a high mortality rate (Jackwood, 2012; Jackwood et al., 2012).

IBV is an enveloped, positive-sense, single-stranded RNA virus in the genus *Gammacoronavirus* of the family *Coronaviridae*. IBV genome encodes 4 major structural proteins, including nucleocapsid (**N**), membrane (**M**), envelope (**E**), and spike (**S**) proteins (Jackwood et al., 2012; Bande et al., 2017). Among the IBV genes, the S1 gene has been shown to have the highest variability because it involves in host cell attachment and contains virus-neutralizing and serotype-specific epitopes (Kant et al., 1992). Like other coronaviruses, IBV has a high frequency of genetic mutation and recombination in its genome, especially in the S1 gene, leading to the emergence of several new IBV variants

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(Valastro et al., 2016; Bande et al., 2017; Xu et al., 2018). Therefore, the genotypes and lineages of IBV are usually classified based on the genetic variation of the S1 gene (Valastro et al., 2016). Recently, IBV has been genetically described into 35 distinct viral lineages (GI-1-29, GII-VII) and several interlineage recombinants by a new classification based on analysis of the complete S1 gene (Valastro et al., 2016; Chen et al., 2017; Jiang et al., 2017; Ma et al., 2019).

Currently, several new IBV variants have been continuously emerging worldwide, while little or no cross protection usually occurs between different IBV variants and vaccines, leading to outbreaks of IB in vaccinated chicken flocks (Jackwood et al., 2012). This finding emphasizes the importance of continuous monitoring of IBV genotypes and lineages in chickens worldwide for development of effective control and prevention strategies. Currently, different IBV genotypes and lineages are distributed worldwide, in which some of them circulate globally, while the others are restricted to particular countries or regions (Bande et al., 2017). In Thailand, GI-1 (Massachusetts), GI-13 (4/91), GI-19 (QX-like IBV), and IBV variants (a unique Thai IBV (THA50151) and THA001) were reported to circulate in chickens in some regions of Thailand during 2008–2013 (Pohuang et al., 2011; Promkuntod et al., 2015). Although the IBV vaccine is extensively used in Thailand, IB outbreaks have occurred frequently in Thai chicken flocks. However, the genetic characteristic of IBVs recently circulating in chickens in Thailand remains unknown and the information on IBV genotypes and lineages circulating in Thailand reported previously was limited to some regions of Thailand (Pohuang et al., 2011; Promkuntod et al., 2015). In this study, we investigated the genetic characteristic of 120 IBVs circulating in chickens in all chicken-raising regions of Thailand from 2014 to 2016 by analysis of the complete S1 gene.

MATERIALS AND METHODS

Viruses

One-hundred twenty IBVs isolated from pooled tissue samples (trachea, kidney, cecal tonsil, and oviduct) from individual broiler, layer, and breeder chickens showing clinical signs of IBV infection, including upper respiratory, reproductive, and/or urogenital disorders, were included in this study. These isolates were randomly selected on the basis of the representative of location, type of chicken raising, and year of collection for genetic characterization (Supplementary Table S1). Based on these selection criteria, 120 IBV isolates were selected from among IBV-positive samples from 98 broiler and layer chicken farms in 24 provinces located in all chicken-raising areas of Thailand, including the central, northern, eastern, north-eastern, western, and southern parts of Thailand, from July 2014 to December 2016 (Figure 1; Supplementary Table S1 and S2). All chickens were vaccinated with commercial live-attenuated H120 and 4/91 vaccine strains. All 120 IBVs were isolated in 10-day-old specific pathogen-free embryonated chicken eggs and tested positive for IBV by 5′-untranslated region–specific real-time RT-PCR (Callison et al., 2006). These isolates were also tested and confirmed to be negative for other common chicken viruses that may cause similar symptoms, including avian influenza virus and Newcastle disease virus (Liu et al., 2007; Suarez et al., 2007). The viruses were stored at −80°C until use.

Complete S1 Gene Sequencing

One-hundred twenty Thai IBVs were subjected to the complete S1 gene sequencing as previously described with minor modifications (Pohuang et al., 2011). Viral RNA extraction was performed using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Viral RNAs of 120 Thai IBVs were initially converted to cDNA using random hexamers and an Improm-II reverse transcription system (Promega, Wisconsin) following the manufacturer's instructions. The cDNA was used as a template for amplification of the complete S1 gene by PCR using a TopTaq Master Mix Kit (QIAGEN, Hilden, Germany). The amplicons were subsequently subjected to DNA sequencing (1st Base Laboratories Sdn Bhd, Malaysia). The nucleotide sequences of the S1 gene of Thai IBVs were assembled using SeqMan software v.5.03 (DNASTAR Inc., Wisconsin). The nucleotide sequences of Thai IBVs characterized in this study were submitted to the GenBank database under the accession number MG190958-MG191077.

Phylogenetic and Recombination Analyses of the S1 Gene

Phylogenetic analysis was performed by comparing the complete S1 gene sequences of 120 Thai IBVs with those of the previously reported Thai IBVs and 89 selected reference IBV strains, which were representative of 35 well-established lineages, 7 genotypes, and unique variants available at the GenBank database (Valastro et al., 2016). The nucleotide sequences were aligned in Muscle v.3.6 (Edgar, 2004). The phylogenetic trees were generated by MEGA v.6.0 using neighbor-joining and maximum-likelihood algorithms with 1,000 replications of bootstrap (Tamura et al., 2013). The nucleotide and amino acid sequences of complete S1 genes of Thai and selected reference IBV strains were aligned and compared using MegAlign software v.5.03 (DNASTAR Inc., Wisconsin).

To identify the recombinant events in the S1 gene sequences of Thai IBVs, the complete S1 gene sequences of 120 Thai IBVs were analyzed with the Recombinant Detection Program (RDP4, version 4.83) (Martin et al., 2015). Seven detection methods in RDP4 version 4.83, including RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq, were used to identify the recombination

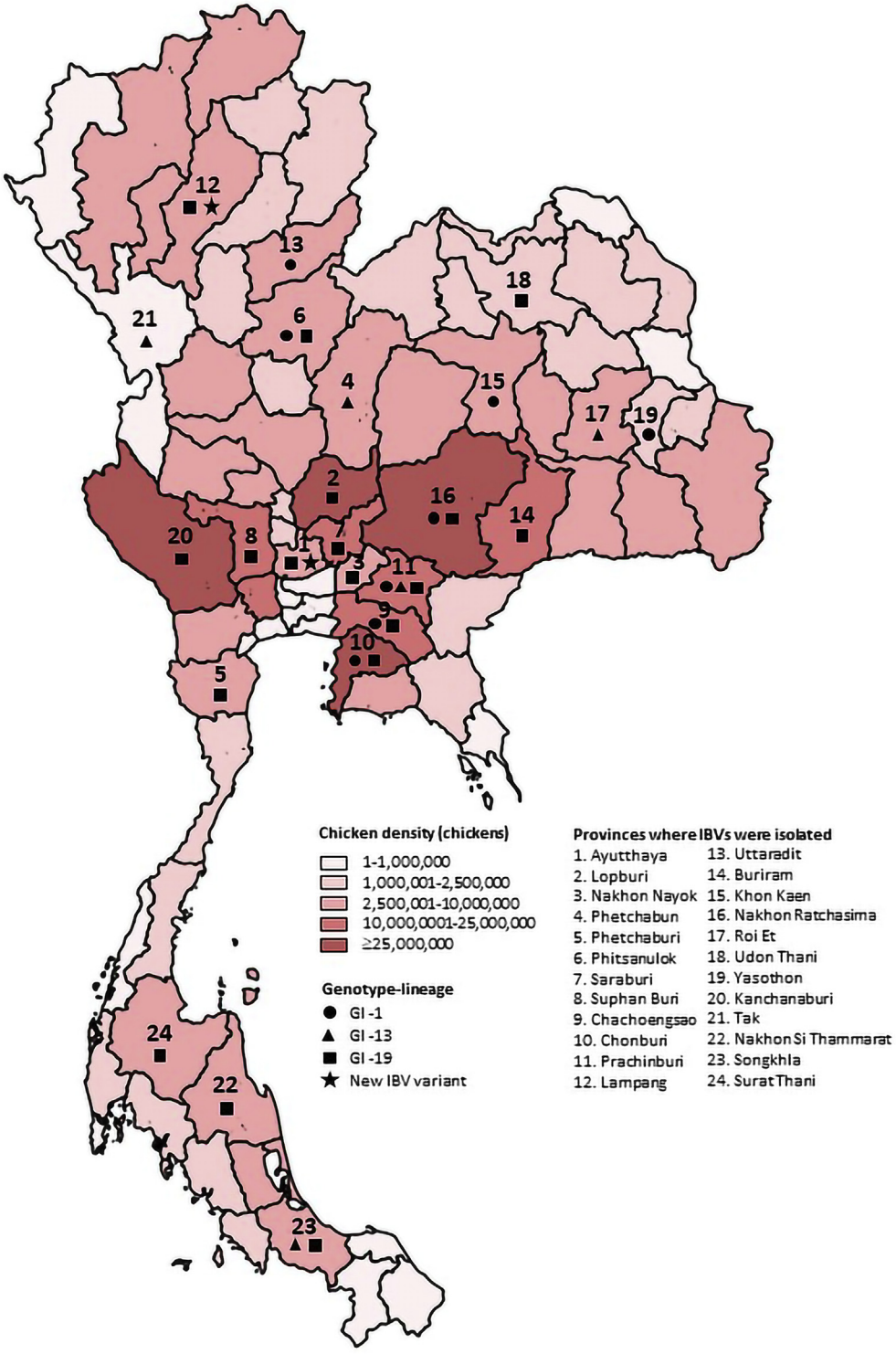

Figure 1. Geographic distribution of infectious bronchitis virus (IBV) in Thailand from 2014 to 2016.

events. A recombination event was accepted only when it was detected by 5 or more methods implemented in the program, with a *P*-value lower than 10^{-14} (Abozeid et al., 2017; Zhou et al., 2017). Recombination events and breakpoints were further confirmed by SimPlot version 3.5.1 software (http://sray.med.som.jhmi.edu/SCRoftware/simplot/). Nucleotide identity was performed by using the Kimura (2-parameter) method with a transition–transversion ratio of 2. The window width and step size were 200 and 20 bp, respectively. In addition, the phylogenetic analysis based on the different fragments of the S1 gene was performed to confirm the genetic recombination.

Ethical Considerations This study was conducted in accordance with the guidelines and approved by the Institutional Animal Care and Use Committee (**IACUC**) of Chulalongkorn University (approval number 1731017).

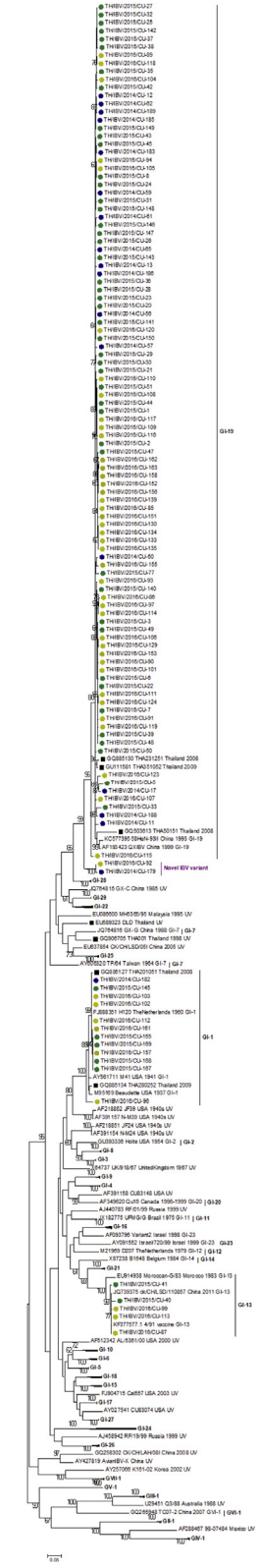

Figure 2. Phylogenetic tree of the complete S1 genes of the 2014–2016 Thai IBVs, the previously reported Thai IBVs, and 89 selected reference IBV strains from genotype I (GI-1-29), GII, GIII, GIV, GV, GVI, GVII, and unique variants (**UV**). A phylogenetic tree was constructed in MEGA v.6.0 using the maximum-likelihood algorithm (Tamura et al., 2013). A similar result was observed when applying the neighbor-joining algorithm. The countries, year of detection, genotypes, and lineages of IBVs are labeled behind the strain names. Dark blue, green, and yellow circles indicate the 2014, 2015, and 2016 Thai IBVs, respectively, whereas black squares indicate the previously reported Thai IBVs.

RESULTS

Phylogenetic Analysis of the S1 Gene

To genetically characterize the circulating Thai IBVs, 120 Thai IBVs isolated from 2014 to 2016 were used for the complete S1 gene sequencing (Figure 1; Supplementary Table S1). Phylogenetic analysis of the complete S1 gene using neighbor-joining and maximum-likelihood algorithms demonstrated that the 2014–2016 Thai IBVs were classified into 3 distinct IBV lineages, including GI-1 (n = 12; 10%), GI-13 (n = 5; 4.17%), and GI-19 (n = 101; 84.17%) lineages, and a novel IBV variant (n = 2; 1.67%) (Figure 2). Among these lineages, most of the 2014–2016 Thai IBVs (84.17%) grouped within the GI-19 lineage and were most closely related to the 2008–2009 Thai QX-like IBVs (94.8–98.5% nucleotide identity and 90.6–98% amino acid identity) (Figure 2). These viruses shared 94.1 to 100% nucleotide identity and 89.7 to 100% amino acid identity to each other, and 93.5 to 97.5% nucleotide identity and 90.1 to 97.1% amino acid identity to the G19 reference strains (QXIBV and 58HeN-93II). It should be noted that TH/IBV/2016/CU-115 formed a separate branch within the G19 lineage (Figure 2). This virus shared only 94.8 to 95.6% and 94.2 to 97.7% nucleotide identities, and 93.7 to 94.5% and 90.2 to 97.2% amino acid identities to the G19 reference strains and our other GI-19 viruses, respectively. In addition, our results also showed that 12 (10%) and 5 (4.17%) Thai IBVs characterized in this study clustered together within the GI-1 and GI-13 lineages, respectively (Figure 2). Our GI-1 and GI-13 viruses had 93.7 to 100% and 97.1 to 99.9% nucleotide identity, and 90.6 to 100% and 95.9 to 99.6% amino acid identity between each other in the same lineage. These GI-1 and GI-13 viruses were closely related to the live-attenuated IBV vaccine strains currently used in Thailand, including H120 (94.4–99.8% and 91.2–99.6% nucleotide and amino acid identities) and 4/91 (96.1–98.1% and 95–97.4% nucleotide and amino acid identities) vaccine strains, respectively. It should be noted that the cocirculation of 2 different IBV lineages, including GI-1 (TH/IBV/2016/CU-102) and GI-19 (TH/IBV/2016/CU-101) viruses, was observed in a broiler farm located in Prachinburi province in 2016. Interestingly, the phylogenetic analysis of the S1 genes also revealed that TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92 identified in this study were grouped together and formed a novel IBV variant, which was separated from the established lineages (Figure 2). These viruses shared 99.3% nucleotide identity and 98.9% amino acid identity to each other, but they shared only 58.7 to 91% nucleotide identity and 47.3 to 92.3% amino acid identity to the established GI-1-29 lineages.

S1 Gene Recombination Analysis

To examine the possible recombination events in the S1 genes of the 2014–2016 Thai IBVs, all 120 Thai IBVs were analyzed using RDP4 and SimPlot softwares. Among the 120 Thai IBVs, a total of 2 Thai IBV isolates

(TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92) were identified as a recombinant IBV variant. Recombination analysis demonstrated that this recombinant variant originated from TH/IBV/2016/CU-123 (GI-19 lineage virus) as a major parent and TH/IBV/2016/CU-99 (GI-13 lineage virus) as a minor parent (Table 1; Figure 3). The positions of the recombination break point in TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92 were located at 891 nt and 918 nt in the S1 gene, respectively (Table 1). The recombination events were further confirmed by SimPlot software, which also showed similar findings with RDP4 software (data not shown).

To further confirm the origins of each part of the S1 gene of this novel recombinant variant, the S1 gene sequence of TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92 was cut into 2 fragments according to the recombination analysis after which phylogenetic analysis was conducted. The phylogenetic analysis based on each recombination region of the S1 gene of a recombinant variant (TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92) demonstrated that the nucleotide positions at 1-891 of a TH/IBV/2014/CU-179 isolate were most closely related and clustered with TH/IBV/2016/CU-123 (GI-19 lineage) (96% identity), whereas the nucleotide positions at 892 to 1,608 shared high identity to TH/IBV/2016/CU-99 (GI-13 lineage) (100% identity) (Table 1). For a TH/IBV/2016/CU-92 isolate, the nucleotide positions at 1 to 918 appeared very similar to TH/IBV/2016/CU-123 (GI-19 lineage) (96.2% identity), whereas the nucleotide positions at 919 to 1,633 were grouped together with TH/IBV/2016/CU-99 (GI-13 lineage) (99.9% identity) (Table 1). Overall, the results collectively indicate that TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92 were a novel recombinant IBV variant, which possibly emerged from the recombination events between the GI-19 and GI-13 lineages.

DISCUSSION

IB causes significant economic losses to the poultry industry worldwide due to the frequent emergence of novel IBV variants, which can potentially lead to vaccination breaks (Jackwood, 2012; Han et al., 2016; Lounas et al., 2018). This highlights the importance of continuous monitoring of IBV genotypes and lineages in chickens worldwide for effective control and prevention of IB. In this study, we characterized the complete S1 gene from 120 IBVs circulating in chickens in Thailand from 2014 to 2016. Our results demonstrated that 3 IBV lineages, including GI-1, GI-13, and GI-19 lineages, and a novel IBV variant, were found to be circulated in chicken flocks in Thailand from 2014 to 2016. Among these lineages, GI-19 lineage was the predominant lineage of IBV circulating in chickens in Thailand. In addition, we also identified a novel IBV variant with evidence of recombination between viruses in the GI-19 and GI-13 lineages in chickens in Thailand.

Genetic analysis revealed that IBVs circulating in Thailand from 2014 to 2016 could be divided into 3 lineages, including GI-1, GI-13, and GI-19, and a novel IBV variant. It is interesting to note that a unique Thai IBV (THA50151) and THA001 reported previously in Thailand were not detected in chickens in this study from 2014 to 2016. This result indicates that the genetic characteristic of IBVs in Thailand has changed when compared to the previous reports (Pohuang et al., 2011; Promkuntod et al., 2015), suggesting the continuing evolution of IBV in Thailand. Our results also showed that the GI-19 lineage has become the predominant lineage of IBV recently circulating in chickens in Thailand, replacing unique Thai IBV (THA50151) and THA001 (Pohuang et al., 2011). This finding corresponded to several previous studies, which demonstrated that the GI-19 lineage is one of the most predominant IBV lineages extensively circulating in chicken flocks worldwide (Valastro et al., 2016; Bande et al., 2017; Yan et al., 2019). Our findings also demonstrated a high identity between our GI-1 and GI-13 viruses, and the live-attenuated IBV vaccine strains currently used in Thailand, including Massachusetts (H120) and 4/91, suggesting that these viruses might be vaccine-like strains. However, it should be noted that some of these GI-1 and GI-13 viruses were possibly mutated vaccine

Table 1. Recombination events in the S1 gene of a novel recombinant IBV variant (TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92) detected by RDP4 software.

Virus name	Breakpoints		Major parent[1] (similarity)	Minor parent[2] (similarity)	Detection methods (*P*-value)[3]
	Beginning	Ending			
TH/IBV/2014/CU-179	891	1,608	TH/IBV/2016/CU-123 (96%)	TH/IBV/2016/CU-99 (100%)	RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, 3Seq (7.668×10^{-44}, 8.756×10^{-37}, 1.422×10^{-35}, 2.063×10^{-23}, 1.880×10^{-23}, 1.216×10^{-29}, 1.004×10^{-57})
TH/IBV/2016/CU-92	918	1,633	TH/IBV/2016/CU-123 (96.2%)	TH/IBV/2016/CU-99 (99.9%)	RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, 3Seq (7.668×10^{-44}, 8.756×10^{-37}, 1.422×10^{-35}, 2.063×10^{-23}, 1.880×10^{-23}, 1.216×10^{-29}, 1.004×10^{-57})

[1]Major parent is the gene sequence of parent providing the larger part of the recombinant virus's sequence.
[2]Minor parent is the gene sequence of parent providing the smaller part of the recombinant virus's sequence.
[3]All recombination events were detected by all 7 detection methods and the recombination events were accepted only when they were detected by 5 or more methods implemented in the RDP4, with a *P*-value lower than 10^{-14}.

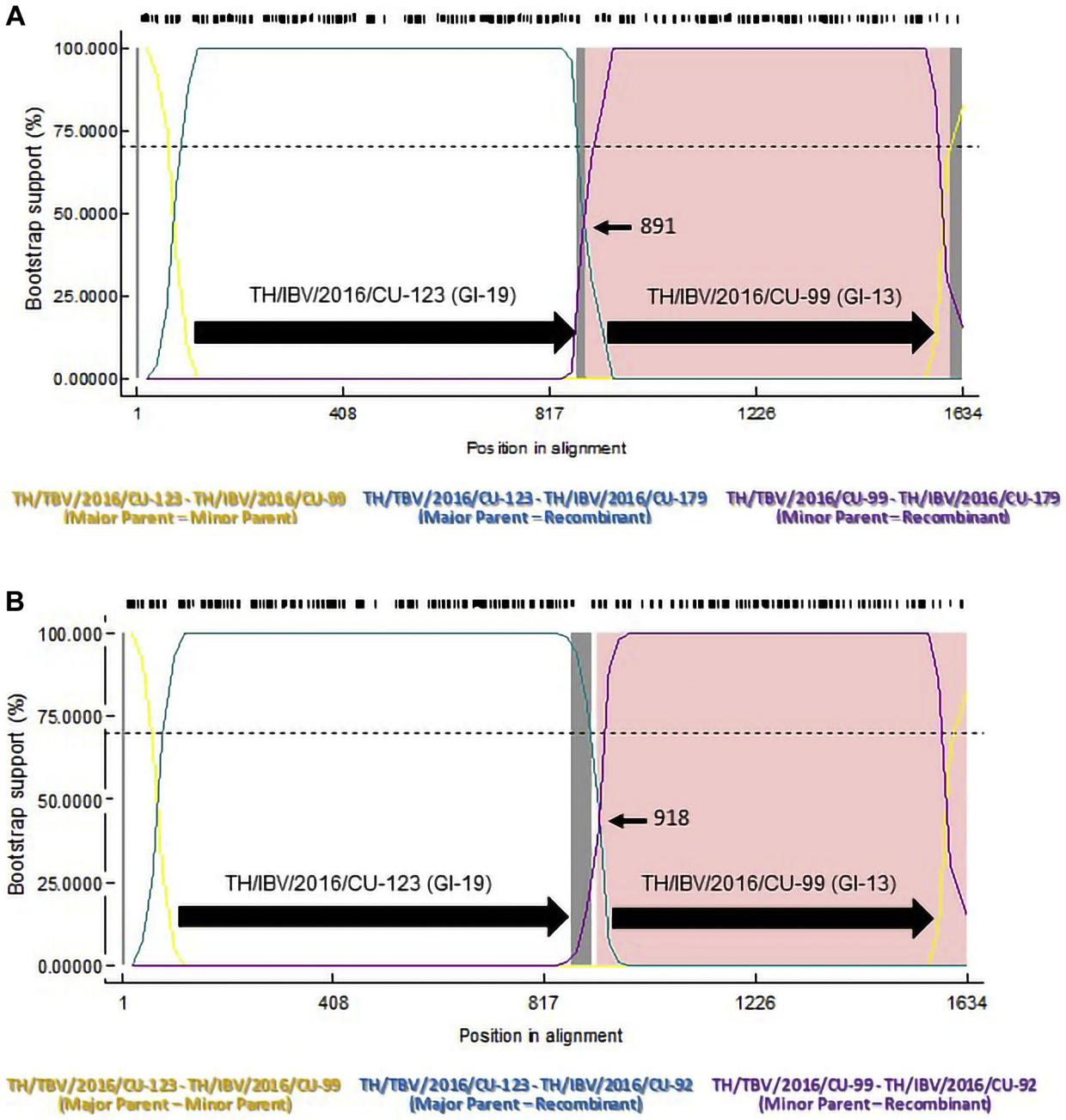

Figure 3. Recombination analysis of a novel recombinant IBV variant (TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92). The results from RDP4 showing the possible recombination events in TH/IBV/2014/CU-179 (A) and TH/IBV/2016/CU-92 (B). The positions of recombination break point are shown as black arrows.

strains because they shared only 91.2% and 95% amino acid identity to Massachusetts (H120) and 4/91 vaccine strains, respectively (Ganapathy et al., 2015). Whether these mutated vaccine strains can cause disease in chickens needs to be further investigated. Interestingly, a novel IBV variant was identified in this study. The S1 gene of this novel variant was genetically different from the established IBV lineages. It is well known that S1 protein plays an important role in protective immunity because it is involved in the induction of virus-neutralizing antibodies. Therefore, the amino acid mutations in S1 protein of novel IBV variants can potentially affect the host immune response against other IBV strains as well as the protective efficacy of classic IBV vaccines (Jackwood, 2012; Jackwood et al., 2012). Several previous studies demonstrated that no cross reactivity was detected between novel IBV variants containing the genetic changes in S1 gene and classic IBV vaccine strains (Yan et al., 2011; Chen et al., 2017; Ma et al., 2019). Based on our field observation, higher mortality rates were observed in farms where the novel IBV variant was isolated (5.56–16.8%) than other farms where commonly circulated GI-19 viruses (Thai QX-like IBVs) were isolated (1.48–4.5%), although the vaccination program used in these farms was similar. This indicates reduced protective efficacy of the currently

used IBV vaccines against novel IBV variant and the potential enhanced virulence of this variant in infected chickens. However, the characteristics of the antigenicity, pathogenicity, tissue tropism, and complete genome of this novel variant remain unknown and require further investigation. In addition, the cross-protective efficiency of the current IBV vaccines against this novel variant should also be evaluated for effective control and prevention of IB in Thailand.

The recombination analysis demonstrated that a novel IBV variant was a recombinant virus, which was originated from GI-19 (Thai QX-like IBV) and GI-13 (4/91 vaccine-like IBV) lineage viruses. Corresponding to this finding, the genetic recombination between field and vaccine strains was also frequently detected in several countries (Moreno et al., 2017; Xu et al., 2018). In this study, Thai QX-like field strains were isolated from Massachusetts- and 4/91-vaccinated diseased chickens. As a result, this novel IBV variant might have been generated by recombination resulting from cocirculation of Thai QX-like field strains and 4/91 vaccine-like strains. This indicates that vaccination with ineffective live-attenuated IBV vaccines may generate an environment where coinfection between circulating field and vaccine strains can increase the possibility of recombination. However, no evidence of recombination between field and Massachusetts vaccine-like strains was found in this study, although this vaccine has been extensively used to control IBV in Thailand. This may be related to the shorter persistence and shedding of Massachusetts vaccine in chickens, which might decrease the possibility of recombination between this vaccine and field strains (Bijlenga et al., 2004). Besides S1 gene, it is known that genetic recombination of IBV can occur throughout the entire genome of IBV (Thor et al., 2011). Further studies on recombination analysis based on other genes of this novel recombinant variant should be conducted to provide more information on the genetic characteristic of this variant.

In conclusion, our data collectively demonstrate the circulation of different lineages of IBV and the presence of a novel recombinant IBV variant originating from GI-19 and GI-13 lineage viruses in chicken flocks in Thailand. Our findings indicate the high genetic diversity and continued evolution of IBVs in chickens in Thailand, which possibly affect the effectiveness of the currently used IBV vaccines. This study highlights the necessity of continued IBV surveillance to monitor the genetic evolution of IBVs and update vaccination strategies for effective control and prevention of IB.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.psj.2019.11.044.

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