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Analysis of the S1 gene of the avian infectious bronchitis virus (IBV) reveals changes in the IBV genetic groups circulating in southern Thailand

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ABSTRACT

The new variants of the avian infectious bronchitis virus (IBV) produce a range of symptoms and cause global economic losses to the poultry industry. We investigated the S1 glycoprotein of 24 recent IBV isolates from chickens and demonstrated that two predominant genetic groups were circulating in southern Thailand between 2008 and 2013. Seven IBV variants, isolated from 2008 to 2009, were clustered in the Thailand THA001 group I while 15 IBV variants, isolated from 2009 to 2013, were classified into the QX-like group II. Moreover, a single isolate from a broiler was categorized into the Massachusetts-type, and an isolate from a layer belonged to the 4/91 type virus. Interestingly, both the IBV groups I and II were isolated from native chickens (62.5%) and caused a range of symptoms. Our results indicate that the QX-like viruses were predominant after 2009, replacing the THA001 type viruses. Furthermore, native chickens may contribute to the epidemiology of IB.

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Infectious bronchitis (IB), caused by the gammacoronavirus avian infectious bronchitis virus (IBV), is a major and highly contagious disease of the poultry industry. IB is frequently involved in upper respiratory disease, nephritis, and enteritis. Furthermore, IB frequently results in poor fertility and low egg production in layers. Recently, it appears that IB broadly affects other tissues, for example, the proventriculus (reviewed in Cook et al., 2012; de Wit et al., 2011). Moreover, super infection and other notable bacterial co-infections can lead in an increase in the severity of IB (Bakaletz, 1995).

IBV is a member of a genus Gammacoronavirus. IBV genome consists of a single-stranded RNA that encodes four structural proteins: envelope (E) glycoprotein, membrane (M) glycoprotein, nucleocapsid (N) protein, and spike (S) glycoprotein. The S glycoprotein of IBV, which forms large club-shaped projections, is a dimer or trimer (Cavanagh, 2007). The S protein is formed by two non-covalently bound polypeptides, S1 and S2 (Stern and Sefton, 1982). The function of the S1 protein is to attach to host cell receptor molecules. The S1 protein includes epitopes and determinants for virus neutralizing antibodies that provide protective immunity. Thus, S1 gene analysis has been widely used to differentiate IBV genotypes and serotypes (Cavanagh, 2007; Ignjatovic and Galli, 1994; Ladman et al.,

2006). It is well known that two major forces drive coronavirus evolution: mutations due to the high error rates of the viral RNA polymerase, and recombination of the S1 sequences. Thereby, these generate new strains or genotypes of IBV worldwide (Jackwood et al., 2012; Yu et al., 2001).

The first IB outbreak in Thailand was reported between 1953 and 1954 (Chindavanig, 1962). Despite several available commercial vaccines, for example, M41, H120, Ma5, Connecticut, 4/91 and the locally produced DLD vaccine, which have been widely used for many years, IB outbreaks continue to occur and seem to be endemic. The commonly circulating IBV strains in Thailand can be clustered into the following: an indigenous Thailand THA001 group, an unnamed unique group, a QX-like group, and a Massachusetts group (Antarasena et al., 1990, 2008; Pohuang et al., 2009, 2011).

In this study we investigated the situation and distribution of IBV strains in southern Thailand by collecting isolates from different bird species, with confirmed infections, from different regions between 2008 and 2013. We then differentiated them using sequence analysis and determined their relationships with vaccine and reference strains.

Suspected IB cases from chicken flocks, raised in the southern area of Thailand, were submitted to an authorized laboratory between 2008 and 2013. Most birds were village chickens exhibiting mild to moderate symptoms associated with respiratory, enteric, and kidney signs and depression with low morbidity and mortality. Of the total cases submitted, 24 samples were IBV positive. Co-infections or secondary bacterial infections were not found in any diseased birds. Virus isolation and propagation were achieved by

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inoculating 0.2 ml of 10% (w/v) pooled and homogenized suspected tissues into the allantoic cavity of 9- to 11-day old antibody-free embryonated chicken eggs (ECE), which came from healthy flocks screened for key poultry diseases (Guy, 2008). Forty-eight to 72 hours after inoculation, the allantoic fluid and chorioallantoic membrane (CAM) were harvested. Following three to five blind passages, indirect fluorescent antibody test (IFA) was used to identify the causative virus. Briefly, frozen infected CAMs were acetone-fixed, following this the slides were incubated with a nucleocapsid protein-specific mouse monoclonal antibody (clone Ch/IBV 48.4; Prionics, The Netherlands), for 30 min at 37 °C. After washing three times with PBS, the slides were incubated with FITC-conjugated rabbit anti-mouse immunoglobulin (Dako, Denmark) for 30 min at 37 °C to detect antibody binding. Next, the sections were washed in a similar manner and examined using epifluorescence microscopy at 100–200× magnifications.

The allantoic fluids harvested from the inoculated ECE that were IBV positive, during IFA testing on CAM, were kept at –80 °C until use. Viral genomic RNA was extracted from the allantoic fluid using a viral RNA extraction kit (Omega Bio-tek, USA). A single-step RT-PCR was performed using a commercial kit (Qiagen, USA), according to the manufacturer's protocol to amplify a 298-bp cDNA fragment of the 3′UTR gene that confirmed positive IBV strains (UTR 1$^-$ 5′-GCTCTAACTCTATACTAGCCTAT-3′ and UTR 2$^+$ 5′-AAGGAAG ATAGGCATGTAGCTT-3′) (Adzhar et al., 1996). Next, the viral RNA extracts were phylogenetically characterized using the specific S1 glycoprotein gene primer set, XCE 1$^+$ 5′-CACTGGTAATTTTTCAGATGG-3′ and XCE 3$^-$ 5′-CAGATTGCTTACAACCACC-3′, to amplify a 385 bp fragment of the S1 gene between nucleotides 729 and 1111 (amino acids 243–370) (Adzhar et al., 1996; Cavanagh et al., 2002). The amplified RT-PCR products were purified using a QIAquick PCR Purification kit (Qiagen, USA). The purified products were sequenced using a commercial sequencing service (SolGent ASSA service, Korea).

The partial S1 nucleotide and deduced amino acid sequences obtained from the Thai IBV isolates were compared with IBV sequences deposited in the GenBank database using the National Center of Biotechnology Information (NCBI) BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/), and included the reference IBV vaccine strains commonly used in Thailand. Sequence identities by BLAST were included in the alignment and phylogenetic construction. BioEdit version 7.0.5.2 (www.mbio.ncsu.edu) was used to perform multiple sequence alignments and determine the nucleotide and amino acid identities. Aligned sequences were used for phylogenetic analysis using the neighbor-joining method available in MEGA software version 6.0 (Tamura et al., 2013). The bootstrap values were determined from 1000 replicates of the original data to designate confidence levels to branches.

The phylogenetic analysis of 24 purified PCR products revealed that the local isolates could be clustered into two predominant groups. Group I comprised seven isolates, correlating to the previously published THA001. Group II, hosting a larger branch, consisted of 15 isolates. This group belonged to QX-like viruses, correlating to the previous isolate reported by Pohuang et al. (2011). In addition, two single isolates were detected, one of which was related to the Massachusetts-type vaccine, while the other was related to the 4/91 strain (Fig. 1). The group I viruses were isolated in 2008–2009, and were geographically disseminated in the southernmost areas and southwestern coast. The isolates in group II, found in 2009–2013, were distributed mostly in the central part of the southern region, ranging from the west to the east coast. A total of 62.5% of the viruses in group II were isolated from native chickens associated with a range of symptoms. Moreover, single isolates of the Massachusetts-like (Mass-like) and the 4/91-like viruses were isolated in 2012 and 2013, respectively (Fig. 2). This indicates that the IBV infection is endemic and has regularly circulated within this region. Furthermore, occasional co-circulation was present in some areas.

The nucleotide and amino acid identities were then determined within and between phylogenetic groups. The isolates in group I were highly related at the nucleotide and amino acid levels with 92.2–98% and 89.2–97.5% identities, respectively. The isolates of the QX-like group II shared 89.3–97.7% nucleotide identity and 85–95.8% amino acid identity to each other. Finally, we determined the phylogenetic relationships of the local isolates both with vaccine strains commercially used in Thailand and with the IBV sequences available in GenBank from other geographical origins. During this analysis, we found that the single isolates B/Sk3589/55 and L/Nk3251/56 fell within the Mass-type (Ma5 vaccine) and the 4/91 groups, respectively (Fig. 1).

IBV is a major pathogen in both vaccinated and unvaccinated flocks of commercial broilers and layers, as well as in village/free range birds. The reasons for this are flock management system, mismatched vaccination, and emergence and reemergence of new variant strains. Extensive antigenic variation, caused by genetic mutation in the hypervariable region of the S1 glycoprotein, is a feature of IBV and new variants thereof (Jackwood et al., 2012). Moreover, the S1 protein is associated with virus-neutralizing epitopes, thereby inducing a strain-specific neutralizing antibody (de Wit et al., 2011; Hofstad, 1981). In Thailand, commercially available IBV vaccines are applied in intensive poultry farming. Aside from commercial poultry, a limited number of backyard chickens are vaccinated. This is, at least in part, why IBVs are still prevalent. A better understanding of the genetic diversity of the IBV S1 gene from diverse geographies is essential for effective control and prevention of IB. Selection of proper vaccines is an ultimate goal for minimizing economic loss.

As previously mentioned, IBV has been reported to circulate in southern Thailand. A major outbreak occurred in 2007, in which diseases were mainly associated with respiratory and kidney symptoms (Antarasena et al., 2008). Our data indicated that the QX-like viruses appear to be the predominant viruses currently circulating in southern Thailand, replacing the THA001-type viruses.

In this study, in addition to the two predominant IBV groups detected, only the single isolates were related to vaccine strains. To determine whether they are directly vaccine-derived or mutated vaccine viruses that have been circulating for some time in birds, full-length gene sequence data, and the analyses of mutation rate and recombination events are required.

We also observed a range of subclinical signs in the infected village-birds diagnosed with THA001 and QX-like IBV infections. Birds showed mild respiratory symptoms and depression. These clinical signs differed from the nephropathogenic form of IBV-infected chickens previously reported by Antarasena et al. (2008). They were also different from the clinical forms detected in chickens infected with the novel QX IBV that caused severe proventriculitis, nephritis, and decreased egg production (Yudong et al., 1998). However, most native chickens are not vaccinated or they are raised in poor farming condition. Therefore, they most likely play an important role in the spread of IBV.

In summary, isolation and typing of the IBV field isolates is necessary for studying IB epidemiology. Although the partial S1 sequence can be used to obtain preliminary genotype information of the isolates and determine whether available vaccines are expected to be protective, future studies must incorporate the entire S1 sequences and, preferably, whole genome sequences to further investigate evolution and recombination of the virus.
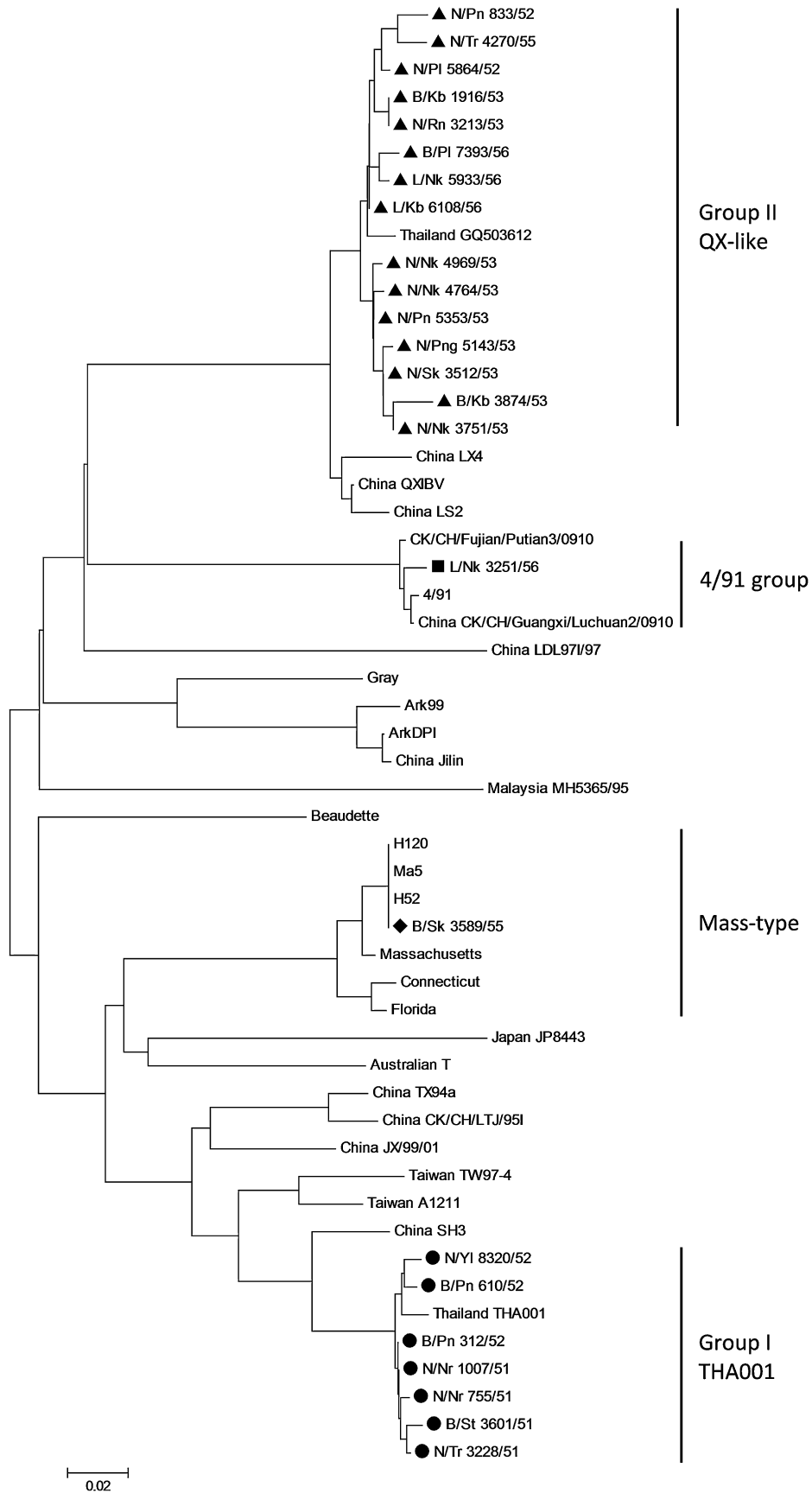
Acknowledgement

Fig. 1. Phylogenetic relationship of local isolates, vaccine and global IBV strains. Analysis was based on partial S1 nucleotide sequences determined using MEGA 6.0 with the Clustal W method. The isolates sequenced in this study are illustrated with black triangles, black squares, black diamonds and black circles.
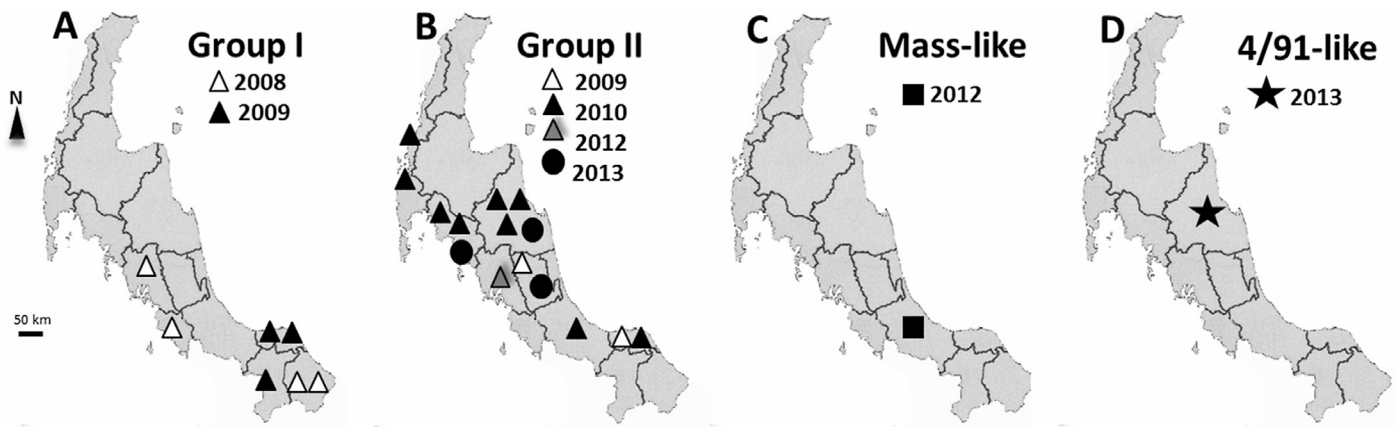
Fig. 2. Geographic distribution of field isolates of IBV in southern Thailand between 2008 and 2013. (A) Group I, the THA001 isolates clustered between 2008 and 2009. (B) Group II, the QX-like field isolates circulated from 2009 to 2013, (C) the Mass-like isolated in 2012, and (D) the 4/91-like recovered in 2013.

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