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Review

Avian influenza in the Greater Mekong Subregion, 2003–2018

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ABSTRACT

The persistent circulation of avian influenza viruses (AIVs) is an ongoing problem for many countries in South East Asia, causing large economic losses to both the agricultural and health sectors. This review analyses AIV diversity, evolution and the risk of AIV emergence in humans in countries of the Greater Mekong Subregion (GMS): Cambodia, Laos, Myanmar, Thailand and Vietnam (excluding China). The analysis was based on AIV sequencing data, serological studies, published journal articles and AIV outbreak reports available from January 2003 to December 2018. All countries of the GMS have suffered losses due repeated outbreaks of highly pathogenic (HP) H5N1 that has also caused human cases in all GMS countries. In Laos, Myanmar and Vietnam AIV outbreaks in domestic poultry have also been caused by clade 2.3.4.4 H5N6. A diverse range of low pathogenic AIVs (H1-H12) have been detected in poultry and wild bird species, though surveillance for and characterization of these subtypes is limited. Subtype H3, H4, H6 and H11 viruses have been detected over prolonged periods; whilst H1, H2, H7, H8, H10 and H12 viruses have only been detected transiently. H9 AIVs circulate endemically in Cambodia and Vietnam with seroprevalence data indicating human exposure to H9 AIVs in Cambodia, Thailand and Vietnam. As surveillance studies focus heavily on the detection of H5 AIVs in domestic poultry further research is needed to understand the true level of AIV diversity and the risk AIVs pose to humans in the GMS.

1. Introduction

Avian influenza viruses (AIVs) are negative-sense RNA viruses from the Family *Orthomyxoviridae* and the genus Influenza virus A. They are composed of eight genomic segments encoding for at least twelve viral proteins, including: hemagglutinin (HA), neuraminidase (NA), polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), PB1-F2, polymerase acidic protein (PA), PA-X, nucleoprotein (NP), matrix protein 1 (M1), matrix protein 2 (M2), nonstructural protein 1 (NS1) and non-structural protein 2 (NS2). Viruses are subtyped based on combinations of the two surface glycoproteins, HA and NA. Sixteen HA (H1-H16) and nine NA (N1-N9) genes have been identified in wild aquatic birds, the natural reservoir (Hinshaw et al., 1980; Rohm et al., 1996; Fouchier et al., 2005). AIVs are sporadically transmitted from waterfowl to susceptible hosts such as other avian and mammalian species. This has resulted in the establishment of a number of stable AIV lineages in domestic poultry; and, to a lesser extent, mammalian species such as humans, pigs, horses and dogs.

In domestic poultry species, most AIVs typically exhibit low pathogenicity, causing little or no apparent illness. Sometimes these viruses mutate to form highly pathogenic (HP) variants capable of causing high mortality in poultry flocks. On a molecular level, HPAIVs are defined by the insertion of multiple basic amino acids into the HA cleavage site and only two subtypes, H5 and H7, are currently known to be capable of developing high pathogenicity. Overall, the establishment of low pathogenic (LP) and HPAIVs in domestic poultry species, increases risk for human infection with AIVs, especially in individuals with close contact to poultry (Claas et al., 1998; Mounts et al., 1999; Zhou et al., 2017).

A number of AIV subtypes have been detected in humans, including: H5N1, H5N6, H6N1, H7N2, H7N3, H7N4, H7N7, H7N9, H9N2, H10N7 and H10N8 (Koopmans et al., 2004; Arzey et al., 2012; Gao et al., 2013; Shi et al., 2013; Qi et al., 2014; Belser et al., 2017; Uyeki et al., 2017; Tong et al., 2018). Clinical symptoms range in severity from

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asymptomatic infections and mild presentations such as conjunctivitis and influenza-like illness (ILI; fever, sore throat, and cough) to severe acute respiratory illness (SARI), pneumonia, and even respiratory or multiple-organ failure and, in some cases, death (FAO, 2019; WHO, 2018). Currently, no direct evidence suggests stable human-to-human transmissibility for AIVs without specific adaptations to mammalian hosts. Indeed, one concerning hallmark of AIVs is their ability to evolve rapidly, with genomic changes occurring through two main mechanisms: (1) the accumulation of point mutations in viral quasi-species; and, (2) reassortment of AIVs during co-infections. In the past century, both mechanisms have contributed to the emergence of four pandemic AIVs. The most recent being the 2009 swine influenza pandemic that was caused by a triple reassortant virus containing genes from swine, avian and human viral lineages (Garten et al., 2009).

Asia is a hotspot of endemic and emerging infectious diseases and has been considered a hub for the emergence of novel AIVs with pandemic potential. Risk factors for emergence of AIVs in humans include: the high density of human and poultry populations, suboptimal biosafety and biosecurity in poultry rearing and trading practices, and the relatively high diversity of AIVs that circulate in wild and domestic avian species (Shortridge and Stuart-Harris, 1982). Popular throughout Asia, traditional backyard farming systems and live bird markets (LBMs) are major sources of AIV infections in domestic poultry and they significantly impede disease control efforts. In backyard farms, poultry are typically raised as free-range scavengers with minimal to no biosecurity measures in place. These backyard poultry frequently interact with wild bird species, increasing the chance of AIV transmission across the wild bird-poultry interface. Concurrently, LBMs may promote AIV circulation in domestic poultry due to the high density and diversity of avian species. Humans working in close contact with poultry in backyard farms and at LBMs are at high risk of becoming infected with AIVs through zoonotic transmission (Bridges et al., 2002; Zhou et al., 2017). The importance of Asia, especially China, in the ecology of AIVs is well established and numerous surveillance studies monitoring viral evolution have been conducted (Ni et al., 2015; Chen et al., 2017; Luo et al., 2017). Several AIVs with zoonotic potential have emerged from China in recent years, including subtypes H5N1, H5N6, H7N4, H7N9, H9N2 and H10N8 (Claas et al., 1998; Peiris et al., 1999; Gao et al., 2013; Qi et al., 2014; Pan et al., 2016; Tong et al., 2018). However, less attention has been given to neighbouring countries in the Greater Mekong Subregion (GMS) despite sharing borders with China and presenting similar risk factors for AIV geographical spread in avian species and emergence in humans.

The GMS includes a region of six countries that share the Mekong River: Cambodia, China (Yunnan Province and Guangxi Zhuang Autonomous Region), Lao People's Democratic Republic (Laos), Myanmar, Thailand and Vietnam (Fig. 1). Combined, the GMS encompasses an area of approximately 2.6 million km^2 with a population over 300 million people. Countries within the GMS share similarities in their landscapes, climates and certain cultural practices; however, they differ with regards to stages of economic development, population size and population density. Due to the high dependence on agriculture for livelihood and food supply, AIVs can have a devastating effect on the economy of the region. AIV establishment and spread in the GMS occurs mainly through cross-border poultry trade (Wang et al., 2008; Buchy et al., 2009; Van Kerkhove et al., 2009; Meyer et al., 2018), widespread backyard farming, the popularity of LBMs and, to a lesser extent, through the movement of free grazing ducks (Meyer et al., 2017) and/or wild bird migration (Gilbert et al., 2010; Keawcharoen et al., 2011). A number of AIV lineages have been established in domestic poultry in the region. As a result, numerous AIV outbreaks in poultry as well as infections in humans have occurred sporadically throughout the region over the past 15 years.

While numerous reviews have covered circulation of H5N1 HPAIVs in the GMS (Gutierrez et al., 2009; Eagles et al., 2009; Pfeiffer et al., 2013), the diversity and impact of LPAIVs has not received equal attention. Therefore, this review seeks to examine the availability of data from GMS countries, focusing on what is currently known about the diversity of AIVs circulating in avian species in the region and to evaluate the risk of AIV emergence in humans.

1.1. Search strategy and data collection

The circulation of AIVs in the GMS countries of Cambodia, Laos, Myanmar, Thailand and Vietnam was analysed using published journal articles (including serological and molecular data), AIV outbreak reports from the World Organisation for Animal Health (OIE) and Food and Agriculture Organization of the United Nations (FAO) (OIE, 2019), outbreak reports from the EMPRES Global Animal Disease Information System (EMPRES-i) database (empres-i.fao.org/) and available sequencing data. The Chinese provinces that are part of the GMS were not included in this review as there are many articles that focus on AIV circulation in China. Relevant journal articles were identified by searching PubMed, using MeSH terms and Boolean operators, for all reports related to AIV circulation in countries of the GMS.

Sequencing data was downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and GISAID (https://www.gisaid.org/). This included data on all AIVs from the GMS with sequencing available for one or more genes and HA subtyping information available. Data was limited to AIVs detected in avian species from January 2003 to December 2018 (Supplementary Tables 1a-b). Sequence information from each database was collated and curated to identify and remove duplicate viruses based on viral designations (Supplementary Table 1c).

2. Molecular analyses

To investigate molecular indicators of viral pathogenicity the HA cleavage sites for all GMS AIVs that had sequencing data available were collated (Supplementary Table 2). Cleavage sites were determined to be multibasic or monobasic based on the presence of basic amino acids at critical sites, as described by the OFFLU OIE/FAO network guidelines (OFFLU OIE/FAO Network, 2018). Additionally, HA molecular markers associated with changing the receptor binding preference of AIVs from avian-type α2,3 sialic acid receptors to human-type α2,6 receptors were investigated, including: E190D, G225D, Q226L and G228S (H3 numbering; Supplementary Table 2) (Matrosovich et al., 1997; Glaser et al., 2005; van Riel et al., 2010). A change in AIV receptor binding capacity from α2,3 to α2,6 receptors increases the transmissibility of AIVs to humans and their pandemic potential.

3. Phylogenetic analyses

Maximum likelihood phylogenetic trees were produced for all HA AIV subtypes identified to investigate the evolution of AIVs in the GMS. Sequences were aligned using MAFFT v7.308 and curated (Katoh and Standley, 2013). Model testing was performed using IQ-Tree v1.6.3 (Nguyen et al., 2015a,b; Kalyaanamoorthy et al., 2017) and models were chosen based on the Akaike information criterion. Phylogeny was inferred using IQ-Tree with the general time reversible (GTR) model, including four gamma rate categories with or without a proportion of invariant sites, GTR + I + G4 or GTR + G4 respectively. The phylogeny for HA subtypes H1, H5, H6 and H11 was produced using GTR + I + G4. Trees for subtypes H2, H3, H4, H7, H8, H9, H10 and H12 were produced using GTR + G4. Branch support was assessed by running 1,000 ultrafast bootstrap replicates (Hoang et al., 2018). Trees were visualised with Figtree v1.4.3 (Rambaut, 2016).

3.1. Availability of data on AIVs from countries of the GMS

Overall, 2,546 AIVs had sequencing data available for one or more genomic segments with HA subtyping information listed (Supplementary Table 1c). The amount of available data varies greatly

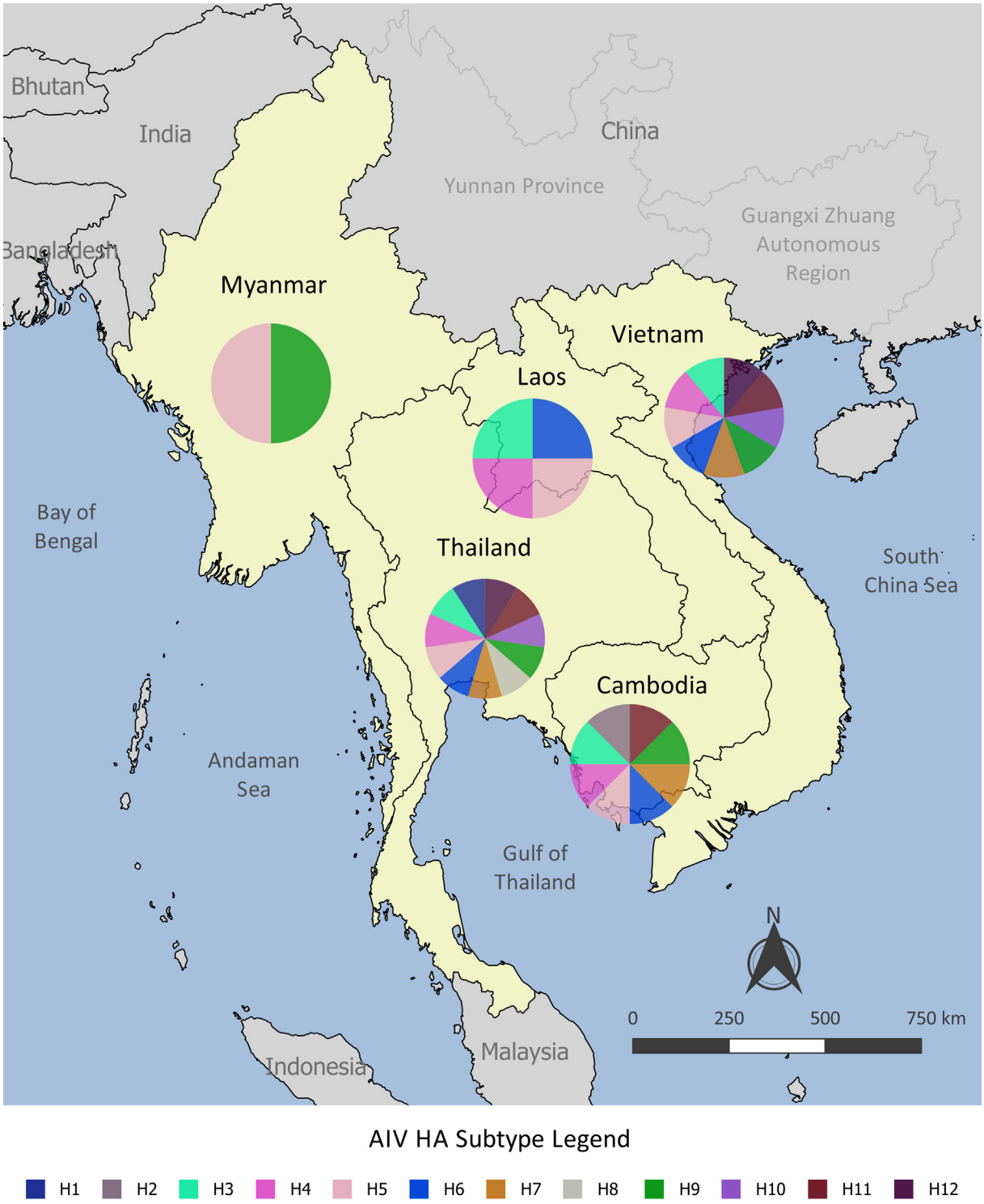

Fig. 1. Map of the Greater Mekong Subregion (GMS) showing the diversity of HA AIV subtypes detected in the region. Countries of the GMS that are the focus of this review (Cambodia, Laos, Myanmar, Thailand and Vietnam) are shown in yellow. The Yunnan province and Guangxi Zhuang Autonomous region indicated in China are part of the GMS but have not been included in this review. Pie charts for each country display subtypes detected from sequencing data and serological studies. The divisions within the pie charts do not signify the proportions of subtypes detected.

from country-to-country, for each AIV subtype, for individual host species (Fig. 2) and temporally (Supplementary Fig. 1). Peaks in sequencing data coincide with major AIV outbreaks in poultry or years of more intensive surveillance. Vietnam has consistently released the most sequencing data in the region since 2007, accounting for 72.0% (n = 1,833) of the total number of viruses sequenced; followed by Thailand (17.2%, n = 438), Cambodia (7.5%, n = 190), Laos (2.7%, n = 69) and Myanmar (0.6%, n = 16) (Fig. 2a). Thus, the effort of surveillance groups and their reporting habits are both major biases that affect analyses of AIV diversity and prevalence in wild and domestic avian species in the GMS. Data variability between countries in the GMS may also be a result of differences in political climate as well as economic resources and laboratory infrastructure available for AIV surveillance and research.

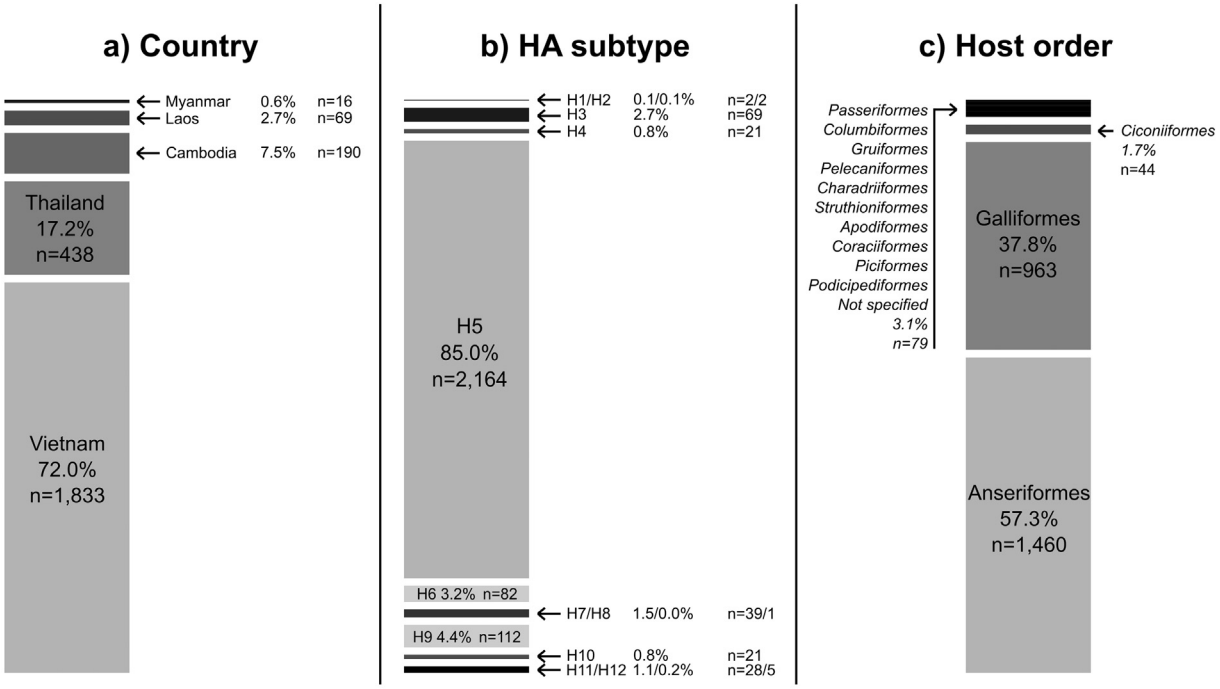

Fig. 2. Availability of AIV sequencing data from January 2003 to December 2018 in the GMS, separated by a) country b) HA subtype and c) host. Percentages shown are relative to the number of viruses with sequencing available for one or more genomic segments and subtyping information listed (n = 2,546).

Based on the data available, AIV sequence information in the GMS is heavily biased towards H5 AIVs, representing approximately 85.0% (n = 2,164) of AIVs with sequencing data available (Fig. 2b). Assessing the available cleavage site motifs, 77.0% (n = 1666) of H5 viruses had multibasic cleavage sites, 0.1% (n = 3) were monobasic, and 22.9% (n = 495) could not be categorized (Supplementary Table 2). The high proportion of AIV subtype H5 is unsurprising considering many studies only screened samples for H5 viruses without testing for other LPAIVs. However, a plethora of other AIV subtypes have been identified in the region (Table 1, Fig. 2b), including: H9 (4.4%, n = 112), H6 (3.2%, n = 82), H3 (2.7%, n = 69), H7 (1.5%, n = 39), H11 (1.1%, n = 28), H4/H10 (each 0.8%, n = 21), H12 (0.2%, n = 5), H1/H2 (each 0.08%, n = 2) and H8 (0.0%, n = 1). All AIV subtypes, excluding H5, which had HA cleavage sites sequencing available had monobasic cleavage sites, indicative of LPAIVs (Supplementary Table 2).

Due to the nutritional and economic dependence on domestic poultry in the GMS, previous studies focus heavily on the detection of AIVs in *Galliformes* (chicken, fowl, partridge, pheasant, quail) and *Anseriformes* (duck, geese). Chickens and ducks are listed as the host species for 34.5% (n = 879) and 57.0% (n = 1,452) of viruses that have sequencing data available, respectively (Supplementary Table 1c). There have been comparatively fewer studies on AIV circulation in wild birds in the GMS. Reports are available from Cambodia (captive wild birds) (Desvaux et al., 2009; Theary et al., 2012), Thailand (Uchida et al., 2008; Siengsanan et al., 2009; Keawcharoen et al., 2011; Siengsanan-Lamont et al., 2011; Ratanakorn et al., 2012; Wongphatcharachai et al., 2012; Poltep et al., 2018) and Vietnam (Thinh et al., 2012; Takakuwa et al., 2013). Collectively, studies focusing on AIV surveillance in wild or captive wild birds have tested birds representing 22 taxonomic orders and approximately 148 different avian species (though not all reports specify species tested; Supplementary Table 3). AIVs were detected in 15 of these 22 orders. Sequencing data was available from AIVs infecting various avian hosts, including: *Apodiformes* (swiftlet), *Charadriiformes* (gull, woodcock), *Ciconiiformes* (stork), *Columbiformes* (dove, pigeon), *Coraciiformes* (rollers), *Gruiformes* (crane, moorhen, watercock), *Passeriformes* (Chinese hwamei, crow, myna, stonechat, sparrow, swallow), *Pelecaniformes* (egret, heron), *Piciformes* (barbet), *Podicipediformes* (grebe), *Struthioniformes* (ostrich) and in some cases unspecified avian species (Fig. 2c, Supplementary Table 1c). Very few studies have investigated the role migratory birds play in AIV transmission in the region. Therefore, under-representation of subtypes other than H5 and H9 could also be explained by a high proportion of studies that only sampled domestic poultry, whereas the largest range of LPAIVs circulate in wild waterfowl.

4. Limitations

Current limitations of analysing AIV diversity in the GMS is the reliance on reporting, published articles and data from areas where limited studies have been performed on AIV circulation. Publication is often delayed or incomplete and, excepting subtype H5 and H7 AIVs, influenza subtypes are not required to be reported to international groups or databases. Surveillance programs in the GMS are not typically designed to identify AIV diversity and estimate AIV prevalence in avian species. They are largely conducted as part of outbreak response systems or target sampling of domestic poultry in known problematic areas such as LBMs. This creates a heavy bias towards the detection and characterisation of H5 AIVs, with minimal data available for LPAIVs.

This review focuses on sequencing data and serological data to provide an overview of AIV diversity and the risk AIVs in the GMS pose to humans. It is important to note sequencing data does not accurate reflect AIV prevalence or diversity in the GMS as only a subset of AIVs are sequenced, data is not always released to public databases and serological detection is still employed to determine viral subtype. It is also likely that AIV diversity in the region is greater than currently indicated as wild bird populations have not been extensively sampled and AIV surveillance systems have not always screened samples for all AIV subtypes in circulation. With regards to the molecular analysis of AIV risk to humans, this focuses on available sequencing data for HA genes. The regions of interest, such as the HA cleavage site motifs, are not always sequenced. Additionally, the NA and internal genes of AIVs

Table 1
Summary of AIV subtypes identified in poultry from the GMS, January 2003 – December 2018

HA	Subtype[a]	Country	Host	Year/s[b]	Reference[c]
H1	H1N3	Thailand	Duck	2011	Chaiyawong et al., 2016
	H1N9	Thailand	Duck	2011	
H2	H2N2	Cambodia	Duck	2013	Horm et al., 2016
	H2N5	Cambodia	Duck	2013	
H3	H3N2	Cambodia	Duck	2013	Horm et al., 2016
		Thailand	Duck	2008	Bunpapong et al., 2014
		Vietnam	Duck	2009-2014	Nomura et al., 2012; Kim et al., 2013; Okamatsu et al., 2013; Chu et al., 2016
	H3N6	Cambodia	Chicken, duck	2012-2013	Horm et al., 2016; Osbjer et al., 2017
		Thailand	Duck	2011	Sequence DB
		Vietnam	Duck	2011-2014	Kim et al., 2013; Chu et al., 2016
	H3N8	Cambodia	Chicken, duck	2011-2013	Horm et al., 2016; Osbjer et al., 2017
		Laos	Chicken	2007	Boltz et al., 2010
		Thailand	Duck	2010-2011	Boonyapisitsopa et al., 2016
		Vietnam	Chicken, Duck	2006-2014	Nomura et al., 2012; Kim et al., 2013; Takakuwa et al., 2013
H4	H4	Laos	Duck	2010	Sonnberg et al., 2012
	H4N2	Vietnam	Duck	2011	Okamatsu et al., 2013
	H4N6	Cambodia	Chicken	2011	Osbjer et al., 2017
		Thailand	Duck	2009-2012	Wisedchanwet et al., 2011a; Wisedchanwet et al., 2011b; Boonyapisitsopa et al., 2016
		Vietnam	Duck	2009-2014	Nomura et al., 2012; Kim et al., 2013; Okamatsu et al., 2013; Chu et al., 2016
	H4N9	Thailand	Duck	2009	Wisedchanwet et al., 2011a; Wisedchanwet et al., 2011b
		Vietnam	Duck	2013	Sequence DB

(*continued on next page*)

Table 1 (*continued*)

HA	Subtype[a]	Country	Host	Year/s[b]	Reference[c]
H5	H5	Myanmar	Chicken, duck, fowl, quail	2006-2010	Saito et al., 2008; Cristalli et al., 2018
	H5N1	Cambodia	Chicken, duck, goose, heron, stork	2004-2013	Horm et al., 2013; Horm et al., 2016; Horwood et al., 2018
		Laos	Chicken, duck, goose, pigeon	2004-2010	Boltz et al., 2006; Boltz et al., 2010; Sonnberg et al., 2012
		Myanmar	Chicken, quail, fowl	2006-2016	Saito et al., 2008; Mon et al., 2012; Tun Win et al., 2017
		Thailand	Barbet, chicken, crane, crow, dove, duck, fowl, goose, grebe, gull, moorhen, myna, ostrich, partridge, pheasant, pigeon, quail, rollers, sparrow, stork, watercock	2004-2010	Viseshakul et al., 2004; Puthavathana et al., 2005; Amonsin et al., 2006; Songserm et al., 2006; Buranathai et al., 2007; Chutinimitkul et al., 2007; Poovorawan, 2007; Amonsin et al., 2008; Uchida et al., 2008; Naksupan et al., 2008; Bai et al., 2009; Chaichoune et al., 2009; Saito et al., 2009; Siengsanan et al., 2009; Suwannakarn et al., 2009; Amonsin et al., 2010; Gilbert et al., 2010; Siengsanan-Lamont et al., 2011; Poltep et al., 2018;
		Vietnam	Chicken, dove, duck, goose, ostrich, pheasant, quail, swallow, swiftlet	2004-2017	Nguyen et al., 2005; Muramoto et al., 2006; Smith et al., 2006; Le et al., 2008; Nguyen et al., 2008; Wan et al., 2008; Jadhao et al., 2009; Nguyen et al., 2009; Davis et al., 2010; Nguyen et al., 2010; Takakuwa et al., 2010; Henning et al., 2011; Long et al., 2011; Trevennec et al., 2011; Takakuwa et al., 2012; Zhao et al., 2012; Creanga et al., 2013; Nguyen et al., 2013; Okamatsu et al., 2013; Nguyen et al., 2014; Lee et al., 2015; Nguyen et al., 2015a, 2015b; Chu et al., 2016; Cuong et al., 2016; Nguyen et al., 2017;
	H5N2	Vietnam	Chicken, duck, woodcock	2007-2013	Takakuwa et al., 2013; Nishi et al., 2014
	H5N6	Laos	Chicken, duck	2014	Wong et al., 2015
		Myanmar	Chicken. duck		Tun Win et al., 2017
		Vietnam	Chicken, dove, duck, egret, goose, heron, moorhen, pheasant, quail	2013-2018	Chu et al., 2016; Thanh et al., 2018
	H5N8	Thailand	Duck	2012	Sequence DB
		Vietnam	Duck	2013	Sequence DB
H6	H6	Laos	Avian (unspecified)	2010	Sonnberg et al., 2012
	H6N1	Thailand	Duck	2005	Sequence DB
		Vietnam	Chicken, duck	2009-2015	Hotta et al., 2012
	H6N2	Cambodia	Chicken, duck	2011-2013	Horm et al., 2013; Horm et al., 2016; Osbjer et al., 2017
		Vietnam	Duck	2007-2014	Takakuwa et al., 2012; Kim et al., 2013; Chu et al., 2016
	H6N6	Vietnam	Chicken, duck	2010-2015	Kim et al., 2013; Okamatsu et al., 2013; Chu et al., 2016
	H6N8	Cambodia	Duck	2013	Horm et al., 2016
		Vietnam	Duck	2013	Sequence DB
	H6N9	Vietnam	Duck	2011	Okamatsu et al., 2013
H7	H7N1	Thailand	Quail	2009-2010	Wongphatcharachai et al., 2014
		Vietnam	Duck	2012	Okamatsu et al., 2013
	H7N3	Cambodia	Duck	2017	Suttie et al., 2018a
		Vietnam	Duck	2009	Kim et al., 2013
	H7N4	Thailand	Duck	2010-2011	Sequence DB
	H7N6	Thailand	Duck	2010	Lebarbenchon et al., 2013; Jairak et al., 2016
H8	H8N4	Thailand	Duck	2007	Sequence DB

(*continued on next page*)

Table 1 (*continued*)

HA	Subtype[a]	Country	Host	Year/s[b]	Reference[c]
H9	H9	Cambodia	Chicken, duck	2013	Horm et al., 2016
		Laos	Duck	2010	Boltz et al., 2006; Sonnberg et al., 2012
	H9N2	Cambodia	Chicken	2013	Horm et al., 2016; Horwood et al., 2018
		Myanmar	Chicken	2015	Lin et al., 2017; Tun Win et al., 2017
		Vietnam	Chicken, duck, Chinese hwamei, pigeon, quail, stonechat	2006-2017	Hotta et al., 2012; Nomura et al., 2012; Kim et al., 2013; Okamatsu et al., 2013; Chu et al., 2016; Thuy et al., 2016; Hoa et al., 2017; Trevennec et al., 2011; Takakuwa et al., 2013
	H9N6	Vietnam	Duck	2010-2014	Nomura et al., 2012; Chu et al., 2016
	H9N7	Thailand	Duck	2010	Sequence DB
	H9N8	Vietnam	Duck	2012	Okamatsu et al., 2013
H10	H10N2	Vietnam	Duck	2012	Sequence DB
	H10N3	Thailand	Duck	2009	Wisedchanwet et al., 2011a
	H10N7	Vietnam	Duck	2012	Okamatsu et al., 2013
H11	H11	Cambodia	Duck	2013	Horm et al., 2016
	H11N3	Thailand	Duck	2009-2012	Sequence DB
		Vietnam	Duck	2009-2012	Nomura et al., 2012; Okamatsu et al., 2013
	H11N5	Vietnam	Duck	2011	Okamatsu et al., 2013
	H11N6	Thailand	Duck	2012	Sequence DB
		Vietnam	Avian (unspecified)	2014	Chu et al., 2016
	H11N7	Vietnam	Duck	2014	Chu et al., 2016
	H11N9	Cambodia	Duck	2013	Horm et al., 2016
		Thailand	Duck	2012-2013	Sequence DB
		Vietnam	Duck	2008-2012	Nomura et al., 2012; Takakuwa et al., 2012; Okamatsu et al., 2013;
H12	H12N1	Thailand	Duck, watercock	2009-2010	Wongphatcharachai et al., 2012
	H12N5	Vietnam	Duck	2009	Takakuwa et al., 2012

[a] Subtypes identified are based on reports of AIVs in journal articles and includes both serological and molecular data.
[b] When a range of years is listed this indicates the initial year of detection and most recent year of detection. This does not necessarily mean the virus was continuously circulating during this period.
[c] Sequence DB – indicates no associated publication could be identified and the sequence was found from either the GenBank or GISAID Database.

also contribute to viral virulence and transmissibility. Therefore, this analysis provides a simplified overview of the risk AIVs pose to humans.

4.1. The risk of AIV emergence in humans in the GMS

For the purpose of this review, AIVs have been broadly classified into high, moderate and low risk groups based on their perceived threat to humans. Risk assessment takes into account AIV geographical distribution, host range, prevalence and pathogenicity. It is important to note that this categorisation may be overly simplistic as the risk posed by AIVs changes within individual subtypes and even within specific viral clades over time. Tools that evaluate the risk of individual AIVs are available, such as the Influenza Risk Assessment Tool (IRAT) and the Tool for Influenza Pandemic Risk Assessment (TIPRA) developed by the CDC and WHO, respectively (Cox et al., 2014; WHO, 2016). Both systems have created weighted categories for a number of factors associated with an increase in the pandemic potential of AIVs to compute a risk score. Computationally assigning risk scores to individual viruses is beyond the depth of this review. Particularly as this assessment requires information that is not always available for viruses detected in the GMS including: receptor binding properties, transmission in animal models, susceptibility to antiviral treatment and geographic distribution in animals.

4.2. High risk avian influenza subtypes in the GMS

Historically, AIVs with the highest risk of causing zoonotic infections in humans are subtypes H5, H7 and H9. The global distribution, broad host range and ability of these AIVs to sporadically transmit from poultry to humans means that they pose a “high risk” to humans. They are also the most frequently reported subtypes of AIVs (i.e. non-seasonal influenza) infecting humans in recent years.

4.2.1. H5 Viruses

HA subtype H5 has been detected with all nine known avian NA subtypes with viruses classified from H5N1 to H5N9, with HP variants detected in all subtypes, excluding H5N4 and H5N7. A HPAIV H5N1 outbreak in domestic geese occurred in Guangdong, China in 1996 establishing the Goose/Guandong (Gs/Gd) lineage of H5 AIVs (Xu et al., 1999). Outbreaks of Gs/Gd lineage reassortant viruses were again detected in 2002 and 2005 in Hong Kong and Qinghai Lake, China, respectively (Ellis et al., 2004). These two outbreaks preceded the global expansion of the Gs/Gd lineage from China to the rest of Asia, Europe, the Middle East and Africa, resulting in numerous AIV outbreaks in poultry and sporadic transmission to humans (WHO, FAO, OIE, 2014). Thus, HPAIV H5N1 has been a dominant subtype over the last 10 to 15 years and the focus of numerous AIV surveillance programs worldwide.

Countries of the GMS were amongst the first affected when H5N1 began to cause outbreaks and spread beyond China in December of 2003. By January 2004, highly pathogenic H5N1 outbreaks in poultry were reported in Cambodia, Laos, Thailand and Vietnam and the virus reached Myanmar in March, 2006 (WHO, FAO, OIE, 2014). From 2003 to 2018 over 4,000 H5 outbreaks in poultry were reported in the GMS resulting in death or culling of over 100 million birds (Table 2) (Tiensin et al., 2005; OIE, 2019). All GMS countries employ “stamping out” as the main method to control AIV circulation and Vietnam is the only country in the GMS to implement a mass subsidised vaccination campaign to control H5 in poultry. The campaign was initiated by the Vietnamese government in September 2005, halted briefly mid-2012 due to vaccine ineffectiveness, and has been reinitiated and continues to run to date (Moh, 2011; Cuong et al., 2016). Despite active control measures, sporadic H5 outbreaks in poultry continue to be reported to the OIE/FAO in all GMS countries except Thailand (OIE, 2019). Indeed, Thailand has not reported an H5N1 outbreak in poultry since 2008 (OIE, 2019), likely due to changes in agricultural practices and tightened biosecurity in response to significant financial losses due to H5N1 HPAIVs from 2004 to 2005 in their highly commercialised poultry production system (Tiensin et al., 2007).

Gs/Gd lineage H5 viruses have evolved rapidly and the nomenclature has been consistently updated by the WHO/FAO/OIE H5N1 Evolution Working Group. Currently, H5 HA genes are classified into ten clades (0-9) with subclades reaching the fourth order (WHO/OIE/FAO H5N1 Evolution Working Group, 2014; Smith and Donis, 2015). In the GMS, clades 0, 1, 2, 3, 5 and 7 have been detected in circulation with the largest clade diversity reported from Vietnam (Fig. 3). Only clade 1 and 2 viruses have circulated for prolonged periods (Wan et al., 2008). Clade 1, H5 viruses originating from southern China, were detected in all countries of the GMS, excluding Myanmar (WHO Global Influenza Program Surveillance Network, 2005). This was the most frequently detected H5 clade from 2003 to 2005 in the GMS. Between 2010 and 2014, the continual circulation of clade 1 viruses between Vietnam and Cambodian resulted in the evolution of clades 1.1, 1.1.1 and 1.1.2 (Fig. 3). These three subclades have only ever been detected in Cambodia and Vietnam. There have been no reports of clade 1 viruses in the GMS since April, 2014 (Nguyen et al., 2017).

Clade 2 H5 viruses (subclades 2.3.2 and 2.3.4) initially became established in China prior to spreading to other countries such as: Myanmar, Thailand, Vietnam and Laos from 2005 to 2007 (Lee et al., 2017; Nguyen et al., 2008). At the present time, clade 2 viruses have eclipsed clade 1 in the GMS. Clade 2.3.4.4 H5Nx viruses are more frequently detected/reported than any other H5 clade in northern and central Vietnam (Nguyen et al., 2017) and this clade has also been intermittently detected in Laos and Myanmar (Wong et al., 2015; OIE, 2019). In contrast, clade 2.3.2.1c (exclusively subtype H5N1) is the main clade detected in southern Vietnam and Cambodia (Nguyen et al., 2017). Co-circulation of AIVs comprised of HA gene segments assigned to a diversity of H5 clades in the GMS has resulted in reassortment events between HPAIVs (Wan et al., 2008; Creanga et al., 2013; Rith et al., 2014; Nguyen et al., 2017). There is also evidence of reassortment between H5 HPAIVs with subtype H9N2 LPAIVs occurring in the GMS (Nishi et al., 2014; Suttie et al., 2018b). H5 clade shifts in the GMS have been the result of repeated introduction of viruses into the region, particularly from China, or evolution of endemic viruses, as seen with clade 1 subgroup AIVs.

Globally, there have been 860 confirmed human H5N1 infections resulting in 454 deaths (case fatality rate; CFR: 53% [April 2019]) (WHO, 2019). Cases have been reported from sixteen countries, including all countries in the GMS. The primary risk factor for infection is close contact to poultry. Sustained human-to-human transmission has not been documented. The number of reported cases and CFRs differ considerably between countries in the GMS (Table 2). Vietnam has reported the third highest H5N1 case numbers worldwide (127 cases, 64 fatalities, CFR: 50%) and Cambodia the fourth highest (56 cases, 37 fatalities, CFR: 66%) (WHO, 2019), including an unusually high number of cases reported from Cambodia in 2013. This peak coincided with the emergence of a Cambodian H5N1 reassortant virus containing HA and NA genes from clade 1.1.2, genotype Z viruses and internal genes from clade 2.3.2.1a viruses (Rith et al., 2014). The precise reasons for the increase in human cases remain unclear. Thailand has a human H5N1 CFR of 63%, Myanmar of 0% and Laos of 100%. The CFR for Myanmar and Laos cannot be confidently interpreted due to low case numbers ($n = 1$ and $n = 2$, respectively; Table 2). No human H5N1 cases have been reported in the GMS since 2014 and no cases have been reported globally since April, 2019 (WHO, 2019).

In recent years, circulating H5N1 HPAIVs have reassorted with LPAIVs resulting in HP H5Nx viruses of numerous combined subtypes, including: H5N2, H5N5, H5N6 and H5N8 (for review see Lee et al., 2017). As of December 2018, only H5N6 has infected humans (19 cases, 6 deaths, CFR: 32%) and only in China (WHO WPRO, 2018). In the GMS, H5N6 clade 2.3.4.4 viruses have caused outbreaks in poultry in Myanmar, Laos, and Vietnam (Table 2). The rapid dissemination of Gs/Gd HPAIVs, the propensity of H5 clade 2.3.4.4 viruses to reassort with

Table 2
H5 AIVs reported in poultry outbreaks and humans from countries of the GMS from January 2003 to December 2018.

	Cambodia	Laos	Myanmar	Thailand	Vietnam
H5N1 in poultry					
First reported outbreak	January, 2004	January, 2004	March, 2006	January, 2004	December, 2003
Last reported outbreak[a]	August, 2018	October, 2018	July, 2017	November, 2008	December, 2018
Total no. outbreak events [b]	68	53	121	1,968	4,754
H5N6 in poultry					
First reported outbreak	N/A	March, 2014	March, 2016	N/A	March, 2014
Last reported outbreak[a]	N/A	October, 2015	March, 2016	N/A	December, 2018
Total no. outbreak events[c]	0	2	1	0	67
H5N1 in humans					
First reported case	January, 2005	February, 2007	November, 2007	January, 2004	December, 2003
Last reported case [a]	March, 2014	February, 2007	November, 2007	August, 2006	January, 2014
Total no. cases	56	2	1	27	127
Total no. deaths	37	2	0	17	64
Case Fatality Rate (CFR)	66%	100%	0%	63%	50%

[a] As of December, 2018
[b] Based on confirmed outbreak reports from the EMPRES Global Animal Disease Information System (EMPRES-i) database (empres-i.fao.org/)
[c] Based on the OIE immediate notifications and follow up reports of highly pathogenic avian influenza (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2019/)

LPAIVs and the zoonotic potential of Gs/Gd HPAIVs demonstrates the elevated risk H5 HPAIVs pose to public health. For these reasons, monitoring the evolution of H5 viruses remains a priority for surveillance systems in the region.

4.2.2. H7 viruses

H7 AIVs have been detected in avian species worldwide, in addition to a number of mammalian species including cats, horses, seals, pigs and humans (Sovinova et al., 1958; Geraci et al., 1982; Kwon et al., 2011; Belser et al., 2017). Similar to H5, H7 viruses circulate predominantly in wild birds and are intermittently introduced into domestic poultry flocks (for reviews see Belser et al., 2009; Abdelwhab et al., 2014). Unlike H5 viruses, H7 viruses are not widely endemic in domestic poultry. The majority of these viruses are asymptomatic or mildly symptomatic LPAIVs; however, there have been multiple instances where H7 LPAIVs have mutated to form HPAIVs capable of causing high mortality in poultry flocks (Capua et al., 2000; Bowes et al., 2004; Suarez et al., 2004). For instance, H7N9 AIVs that emerged in Chinese poultry in 2013 were initially LP, circulating silently in poultry and mutating over time to form HP variants (FAO, 2019). H7N9 is attributed the most likely AIV to cause the next human pandemic (CDC, 2018). A total of 1,568 human H7N9 cases (615 deaths, CFR: 39%) have been documented, all occurring in China or individuals who travelled within the country (FAO, 2019). Efforts to control H7N9 in poultry, and consequently decrease human cases, have been very successful, though there are still concerns the virus will spread to surrounding countries. A number of other H7 subtypes have also caused human infections including: H7N2, H7N3, H7N4 and H7N7 (Koopmans et al., 2004; Tweed et al., 2004; Gao et al., 2013; Belser et al., 2017; WHO, 2018).

H7 AIVs are infrequently detected in the GMS. H7 viruses have been identified in Cambodia (H7Nx and H7N3), Thailand (H7N1, H7N4 and H7N6) and Vietnam (H7N1, H7N3) (see Table 1) (Okamatsu et al., 2013; Jairak et al., 2016; Suttie et al., 2018a). Currently, there are sequences available for 39 H7 AIVs from the GMS. The majority of these (35/38) are Thai viruses identified from 2009 to 2011. Available data shows the viruses are all LP with no major HA markers of viral adaptation to mammalian species (Supplementary Table 2). Phylogenetic analysis of the HA gene segment for GMS H7 viruses shows that they are all of the Eurasian lineage and do not cluster closely together (Fig. 4), indicating that H7 AIVs have been repeatedly introduced into the GMS region. The majority of HA gene segments of GMS H7 viruses show a high degree of sequence similarity to viruses isolated from ducks in other Asian countries such as China, Japan, and Korea. However, the Vietnamese HA gene segments of H7N1 and H7N3 viruses have a low sequence identity (87-88%) to other H7 AIVs and the phylogenetic analysis shows these viruses form a separate clade, indicating the progenitor viruses have been circulating undetected for a long period (Fig. 4). None of the HA gene segments of GMS H7 AIVs cluster within the Chinese H7N9 lineage. The risk of H7N9 emerging from China, the propensity for H7 viruses to infect humans, and the ability of H7 LPAIVs to evolve into HPAIVs, all indicate that H7 AIV pose a substantial risk to public health.

4.2.3. H9 viruses

Since the turn of the century, H9 AIVs have been detected in wild birds and domestic poultry worldwide with occasional reports of H9 AIVs infecting swine and humans in Bangladesh, Egypt and China (Yu et al., 2008; WHO, 2017). H9 subtype AIVs, particularly H9N2, are endemic in domestic poultry throughout Asia. H9 viruses are LP in nature and do not typically cause severe disease in poultry. However, H9 viruses still contribute a large burden on the poultry industry as infection is associated with a decrease in egg production and renders poultry more susceptible to secondary infections (Nili and Asasi, 2002; Pantin-Jackwood et al., 2012). A number of non-H9 AIVs (H5N1, H5N6, H7N9 and H10N8) that have repeatedly infected humans contain internal genes donated by H9 viruses (Guan et al., 1999; Qi et al., 2014; Pu et al., 2015). Therefore, H9 AIVs pose a risk on multiple fronts as they are capable of directly transmitting to humans and their internal gene cassettes increase the zoonotic potential of other AIVs. Even though H9 AIVs pose a threat to humans, countries are not obligated to report the circulation of H9 viruses.

All countries of the GMS have detected H9 AIVs in poultry; however, reports characterising these viruses are sparse. H9 AIVs are endemic in domestic poultry in Vietnam and Cambodia (Horm et al., 2016; Thuy et al., 2016; Horwood et al., 2018). The majority of data on H9 circulation comes from Vietnam. Detected since 2001 (Nguyen et al., 2005), H9 has been the most prevalent AIV subtype identified in Vietnamese chickens since 2009 (Nomura et al., 2012; Okamatsu et al., 2013; Thuy et al., 2016), similar to trends from LBMs in China and Bangladesh (Choi et al., 2004; Turner et al., 2017). The recent Vietnamese H9 AIVs are genotype S viruses (Thuy et al., 2016), generally equivalent to genotype 57 that has dominated circulation in China since its emergence in 2010 (Pu et al., 2015). In Vietnam, surveillance has also identified rare subtypes H9N3, H9N6 and H9N8 (Nguyen et al., 2005; Jadhao et al., 2009; Nomura et al., 2012). In Cambodia, H9 AIVs have been detected in circulation since 2013, the first year that Cambodian poultry were screened for this subtype (Horm et al., 2016).

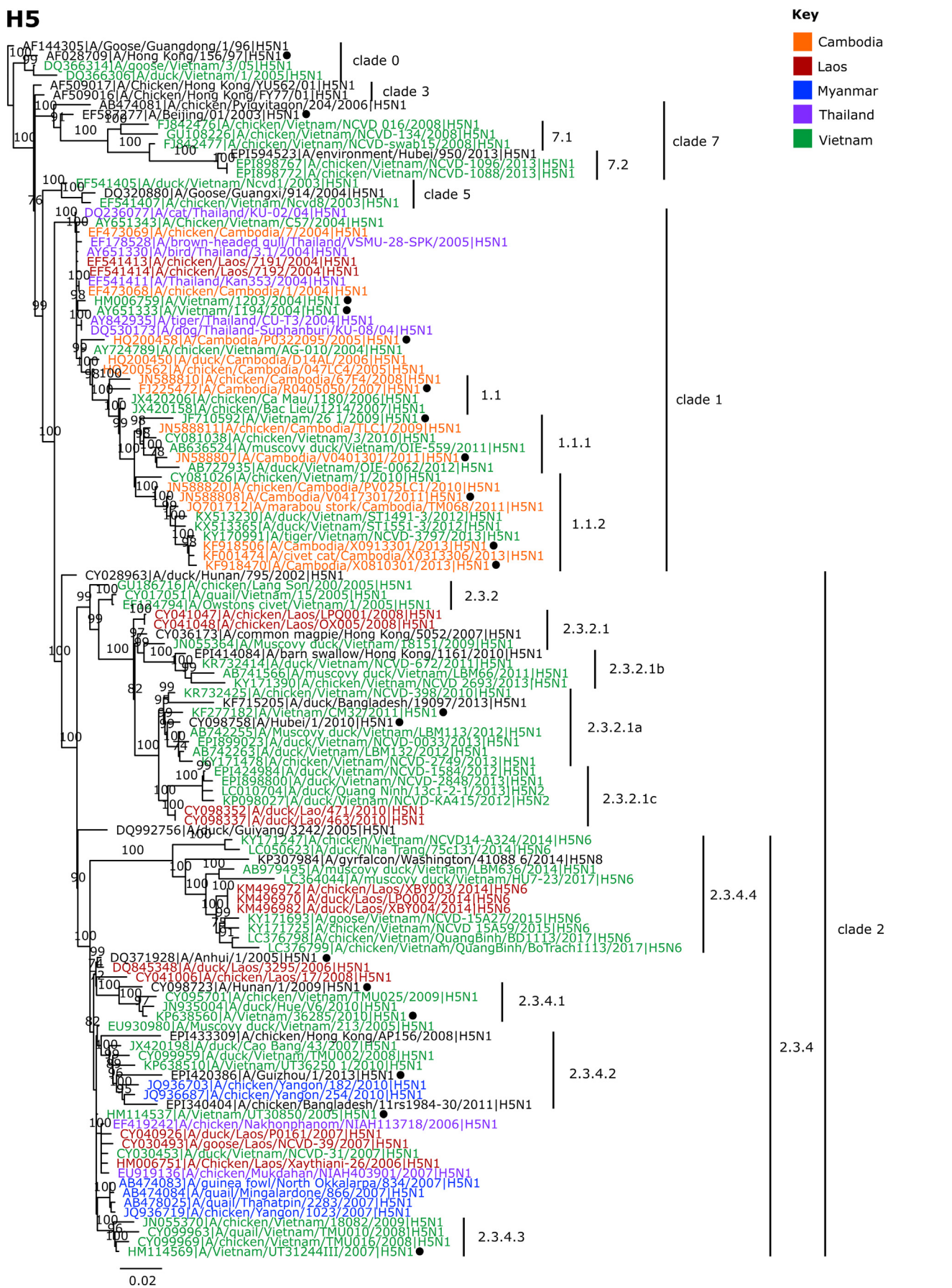

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Fig. 3. Maximum likelihood phylogenetic analysis of the H5 HA gene of viruses detected in the GMS produced with IQ-Tree using GTR + I + G4. Viruses from countries of the GMS have been shown in colour; those from Cambodia are in orange, Laos in red, Myanmar in blue, Thailand in purple and Vietnam in green. Viruses identified in humans have been indicated by a black circle. Viral lineages are shown on the right of the tree. Bootstrap values (n = 1,000) of 70 or greater are displayed on branches and scale bars indicate the number of nucleotide substitutions per site.

Longitudinal surveillance studies performed at Cambodian LBMs in 2013 and 2015 have shown that, in this setting, H9 AIVs are the second most prevalent subtype in Cambodian poultry following H5 (Horm et al., 2016; Horwood et al., 2018). Three major H9 phylogenetic lineages (Y439-like, BJ/94 and G1) have been detected in Vietnam and Cambodia (Fig. 5) (Thuy et al., 2016; Nomura et al., 2012; Kim et al., 2013).

Comparatively less H9 AIV information is available from Laos, Myanmar and Thailand. No sequencing data is available for H9 viruses from Laos; however, a 2010 study found antibodies against H9 viruses in Laotian ducks (G1-like and BJ/94-like lineages) (Sonnberg et al., 2012). In Myanmar, H9N2 and H9Nx AIVs were detected during surveillance efforts between 2014 and 2016 (Lin et al., 2017; Tun Win et al., 2017). Characterisation of the samples isolated in Myanmar LBMs from 2015 show that all viruses clustered in the BJ/94-like lineage (Lin et al., 2017). In Thailand, surveillance efforts at LBMs over many years have not detected H9 AIVs in circulation in poultry, possibly because surveillance measures have focused heavily on detecting H5 viruses. There is sequencing data available for one Thai H9N7 strain from 2010, but no associated report has been published. Further surveillance and reporting in Laos, Myanmar and Thailand would be useful to determine the extent of H9 AIV circulation in these countries.

Despite the high prevalence of H9 AIVs in GMS countries, H9 subtypes account for only 4.4% (n = 112) of the total AIVs with sequencing data available (Fig. 2a). Phylogenetic analysis of the H9 GMS AIVs shows a number of distinct clusters based on country of detection in the GMS and in each instance the closest related ancestral viruses are from China (Fig. 5). However, there are also clusters with closely related H9 AIVs detected in multiple GMS countries, such as h9-3.3.4 and h9-4.2.4 in Vietnam/Cambodia; possibly indicating movement of H9 AIVs between countries of the GMS. Interestingly, there are three separate instances where GMS H9N2 AIVs cluster very closely with H9N2 viruses detected in humans (Fig. 5). Analysis of molecular data shows that out of the 82 GMS H9 viruses that have sequencing available at position 226, 87.8% (n = 72) have the Q226L mutation associated with a change in HA binding preference from avian-type to human-type sialic acid receptors on the surface of host cells (Supplementary Table 2) (Wan and Perez, 2007). Prior to 2014, H9 viruses from the GMS were detected with either 226Q or 226L residues, but recent reports from Vietnam and Myanmar have only identified Q226L variants (Thuy et al., 2016; Lin et al., 2017).

Overall, the high prevalence of Q226L HA substitution in GMS H9 viruses provides evidence of zoonotic potential of viruses of this HA subtype; however, no active H9N2 human infections have been reported in the region to date. As human infections are usually subclinical or only cause mild respiratory illness, it is possible that a number of human H9 cases go undetected. Subclinical human exposure to H9 AIVs is investigated using serological studies (reviewed in Pusch and Suarez, 2018) and these studies have been performed in Cambodia, Thailand and Vietnam (Khuntirat et al., 2011; Blair et al., 2013; Uyeki et al., 2012; Krueger et al., 2013; Horm et al., 2016; Hoa et al., 2017). In Cambodia, H9 seroprevalence in poultry workers from 2013 was 1.8% (Horm et al., 2016) and seropositivity was also reported in rural villagers (Blair et al., 2013; Gray et al., 2014). In Thailand, H9 seroprevalence was 4.7% in a cohort of rural villagers in 2008 (Khuntirat et al., 2011), contradictory to the lack of reports of H9 AIVs in Thai poultry. In a cohort of rural famers from northern Vietnam, 3.5% of individuals had elevated serum antibody titres against H9 AIVs in 2013 and 2015 (Uyeki et al., 2012; Hoa et al., 2017). The zoonotic potential of H9 AIVs, their high prevalence in domestic poultry, their propensity to reassort with other AIVs and the increased prevalence of molecular markers associated with a shift to human-type receptor binding heighten the human pandemic risk of H9 AIVs, warranting further surveillance in the GMS.

4.3. Moderate risk avian influenza subtypes

Influenza subtypes that pose a “moderate risk” to humans can be broadly classified into two main groups: (1) subtypes that have established sustained circulation amongst humans (i.e. H1, H2 and H3 subtypes); and, (2) viruses that have caused rare cases of human infection or are known to infect other mammalian species, including: H4, H6, H10 and H11. H1 and H2 AIVs also infect swine and AIV H3 viruses have been detected in dogs, horses, swine and seals (Webster, 1993; Callan et al., 1995; Karasin et al., 2004; Ma et al., 2007; Song et al., 2008; Jeoung et al., 2013). As all other subtypes have only transiently infected humans without becoming established some people hypothesise that only H1, H2 and H3 AIVs pose a substantial threat to humans (Jones et al., 2014). All moderate risk viruses are widely distributed in avian species globally.

4.3.1. H1, H2 and H3 avian influenza viruses

H1, H2 and H3 are the only influenza HA subtypes that are known to have established stable viral lineages in humans. In the past 100 years, these viruses have caused four known influenza pandemics: the 1918 Spanish influenza (H1N1), 1957 Asian influenza (H2N2), 1968 Hong Kong influenza (H3N2), and 2009 Swine influenza (H1N1pdm09) pandemics. Genetically, these pandemic viruses were either wholly of avian origin or reassortants containing a combination of genes from avian, human and/or swine influenza viruses (Taubenberger et al., 1997; Reid et al., 1999; Reid et al., 2000). At the time of emergence, these viruses ran rampant through the immunologically naïve population. After 2–3 years, increased immunity to the pandemic strains developed and they adopted a seasonal circulation pattern. The human and avian H1-H3 HA lineages are phylogenetically distinct. In Asia avian lineage H1 and H2 viruses have been detected infrequently in birds, whereas H3 AIVs have been identified more regularly.

In the GMS, H1 and H2 subtypes have been detected very rarely in avian species. H1 subtype viruses (H1N3 and H1N9) were detected in Thai ducks in 2011 (Chaiyawong et al., 2016). In 2013, H1 was also detected in Cambodian LBM environmental samples, however the virus was not detected in poultry species (Horm et al., 2016). During the same Cambodian study, H2N2 and H2N5 were identified in ducks (Horm et al., 2016). H2 AIVs have not been detected in any other GMS country. Phylogenetic analysis shows the GMS H1 and H2 AIVs cluster with Eurasian avian lineage viruses. The Thai H1 viruses group together and are closely related to H1 AIVs isolated from ducks and wild waterfowl in China, Japan, Korea and Mongolia (Supplementary Fig. 2). The phylogeny of the Cambodian H2 viruses shows they form a distinct clade with long branch lengths connecting ancestral viruses, likely indicating the progenitor viruses have circulated undetected for a prolonged period of time (Supplementary Fig. 2b).

Comparatively, there is more data available for H3 AIVs with sequencing data available for 69 AIVs from all countries of the GMS, excluding Myanmar (Boltz et al., 2010; Boonyapisitsopa et al., 2016; Horm et al., 2016). H3 subtypes identified include: H3N2, H3N6 and H3N8, detected from 2006 to 2014. The detection of H3N8 in particular is interesting as this subtype has a broad host range. H3N8 viruses have become established in equine species and have been intermittently detected in dogs, swine and seals. Phylogenetic analysis of GMS H3 HA

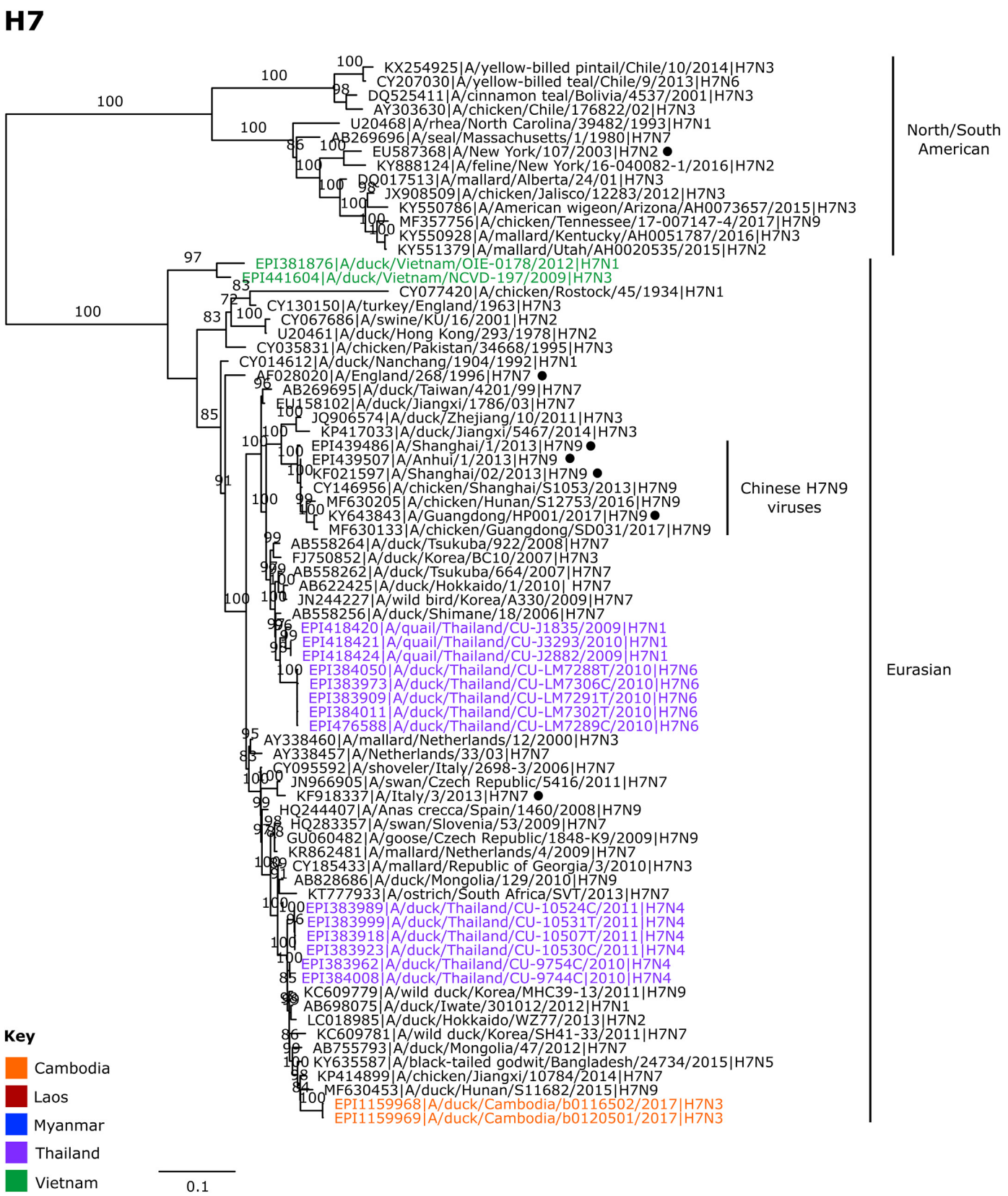

Fig. 4. Maximum likelihood phylogenetic analysis of H7 HA gene of viruses identified in the GMS produced with IQ-Tree using GTR + G4. Viruses from countries of the GMS have been shown in colour; those from Cambodia are in orange, Laos in red, Myanmar in blue, Thailand in purple and Vietnam in green. Viruses identified in humans have been indicated by a black circle. Viral lineages are shown on the right of the tree. Bootstrap values (n = 1,000) of 70 or greater are displayed on branches and scale bars indicate the number of nucleotide substitutions per site.

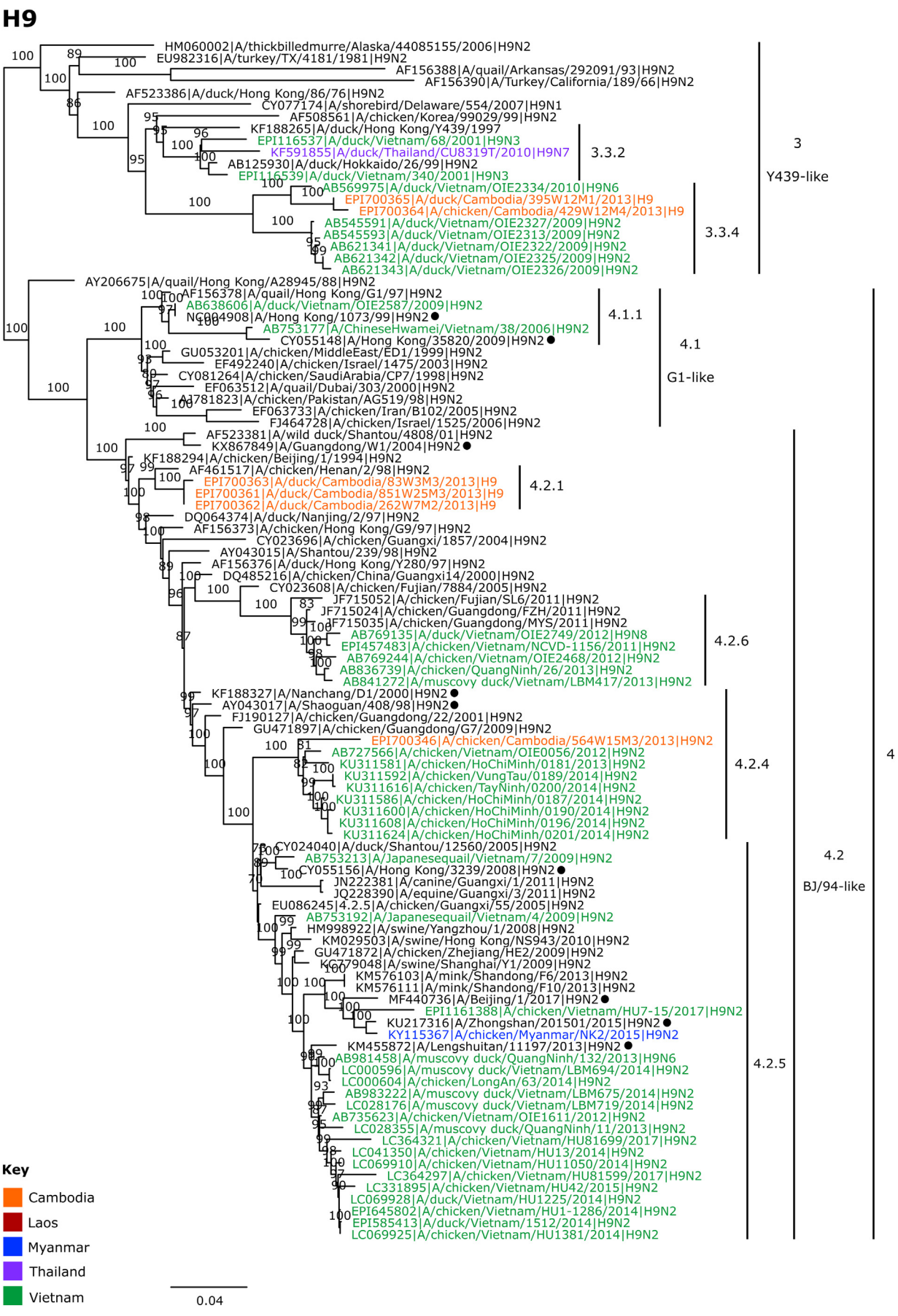

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Fig. 5. Maximum likelihood phylogenetic analysis of H9 HA gene of viruses identified in the GMS produced with IQ-Tree using GTR + G4. Viruses from countries of the GMS have been shown in colour; viruses detected in Cambodia are shown in orange, Laos in red, Myanmar in blue, Thailand in purple and Vietnam in green. Viruses identified in humans have been indicated by a black circle. Viral lineages are shown on the right of the tree. Bootstrap values (n = 1,000) of 70 or greater are displayed on branches and scale bars indicate the number of nucleotide substitutions per site.

genes shows they are all of the Eurasian lineage and that they do not always cluster together based on time or country of detection (Supplementary Fig. 2c). Indicating there have been multiple introductions of H3 AIVs into Cambodia, Thailand and Vietnam. Similarly to the H1 GMS AIVs, the H3 GMS viruses frequently cluster with viruses identified in China, Japan and Mongolia. None of the H1, H2 or H3 AIV HA segments have major markers of viral adaptation to mammals (Supplementary Table 2).

4.3.2. H4, H6, H10 and H11 avian influenza viruses

AIV subtypes H4, H6, H10 and H11 have been identified in mammalian species such as dogs, seals and swine (Donis et al., 1989; Bao et al., 2008; Zhang et al., 2011; Krog et al., 2015; Lin et al., 2015). Of these subtypes, human infections have been caused by H6N1, H10N7 and H10N8 viruses (Arzey et al., 2012; Wei et al., 2013; Qi et al., 2014). These viruses were genetically similar to those circulating in poultry around the same time. There have been no confirmed cases of human infection with H4 or H11 AIVs, but serological data suggests human exposure to these viruses in occupational settings (Gill et al., 2006; Kayali et al., 2010; Kayali et al., 2011). Although limited, these reports show that these AIVs are capable of infecting mammalian species and may pose a risk to humans.

The subtypes H4, H6, H10 and H11 have all been identified in the GMS predominantly from 2009 onwards, possibly reflecting adoption of broader AIV surveillance strategies. All of these subtypes have been identified in Cambodia, Thailand and Vietnam (Wisedchanwet et al., 2011a,b; Takakuwa et al., 2012; Okamatsu et al., 2013; Horm et al., 2016). Although, H10 AIVs were only identified in LBM environmental samples for Cambodia, not in avian species (Horm et al., 2016). In Laos, only H4 and H6 viruses have been detected (Sonnberg et al., 2012). There is sequencing data available for: 21 H4 viruses (H4N2, H4N6, H4N9); 82 H6 viruses (H6N1, H6N2, H6N6, H6N8, H6N9); 21 H10 viruses (H10N2, H10N3, H10N7) and 28 H11 viruses (H11N2, H11N3, H11N5-H11N7, H11N9) (Shu and McCauley, 2017).

Phylogenetic analyses show there have been multiple introductions of Eurasian lineage H4, H6, H10 and H11 AIVs into the GMS. In a number of cases this appears to be the result of AIV introduction from Chinese poultry, however there is also evidence of AIV transmission between countries of the GMS. The H6 AIVs detected belong to two major lineages: HN573-like and ST2853-like (Supplementary Fig. 2e). Of interest, the H11 HA phylogeny shows a monophyletic cluster of GMS viruses, including a single H11 AIV detected in China. This HA clade contains AIVs of various subtypes: H11N2, H11N3, H11N5, H11N7 and H11N9 detected from 2009 to 2014 (Supplementary Fig. 2h). The distance between this cluster and other H11 HA genes means the origin of this clade cannot be deduced. Similarly, a distinct H10 HA Vietnamese lineage exists, including H10N6 and H10N7 AIVs detected in 2012 (Supplementary Fig. 2g). Other H10 AIVs detected in the region, H10N2 and H10N3, are closely related to viruses identified in ducks from China. Based off the available data H10 AIVs do not circulate persistently in the GMS. Whereas, certain HA lineages of the H4, H6 and H11 subtype viruses have been detected over subsequent years. Molecular analysis of the H4, H6, H10 and H11 AIVs shows these viruses contain no major HA molecular markers indicative of AIV adaptation to mammalian species (Supplementary Fig. 3).

4.4. Low risk avian influenza subtypes

AIV subtypes characterised as “low risk” include those with no reports to suggest transmission to mammals has occurred. These subtypes also display limited geographical range and/or species distribution. Based on these factors, it is unlikely that these viruses will be transmitted to humans. Six subtypes fit these parameters: H8 and H12-H16. These subtypes circulate predominantly in gulls, shorebirds and waterfowl but can also be transmitted to domestic poultry. Although H8 and H12-H16 viruses represent a low risk to humans, having a large number of influenza subtypes circulating increases AIV genomic diversity and the possibility of viral reassortment occurring in the region.

In the GMS, there are very few reports of low risk AIV subtypes. Sequencing data is available for a single H8N4 virus identified in a Thai duck in 2007. NCBI blast analysis shows this virus is 99% identical to an H8N4 virus detected in a duck from Japan the same year. Additionally, the Thai H8N4 clusters with H8N2, H8N4, H8N6 and H8N8 viruses identified in ducks from Alaska, Russian, Mongolia and Japan from 2007 to 2016 (Supplementary Fig. 2f). There is also data available for four Thai H12N1 AIVs identified between 2009 and 2010 (Wongphatcharachai et al., 2012) and one Vietnamese H12N5 virus identified in 2009 (Takakuwa et al., 2012). The H12 phylogeny shows the Thai and Vietnamese viruses are closely related as they cluster in a monophyletic clade and share a sequence identity of approximately 98% (Supplementary Fig. 2i). These viruses are also closely related to H12N1 viruses detected in Japan in 2012 and Chinese H12Nx viruses identified in 2011. Neither the H8 nor H12 GMS AIVs have HA molecular markers indicative of AIV adaptation to mammalian species. A better indication of LPAIV diversity would be obtained by more extensive sampling of wild avian species, particularly waterfowl.

5. Conclusions and future directions

Overall, the risk of AIV emergence in humans in the GMS is high. Humans, pigs and poultry live in close contact and at high densities, increasing the chance of zoonotic transmission of AIVs. Poor biosafety and biosecurity in poultry rearing and trade practices concentrated around LBMs can increase zoonotic risk. These LBMs act as hubs for viral evolution and provide an optimal interface for transmission of AIVs between domestic poultry and to humans. In addition, a diverse range of AIVs (H1-H12) have been detected in the region and high risk subtypes circulate endemically in domestic poultry in multiple GMS countries. Detection of subtype H7 viruses, as well as moderate and low risk AIVs, has continually increased since 2009. This increase may reflect changes to surveillance strategies that historically focused on detection of H5 AIVs, AIV reporting or advances in technology and laboratory infrastructure. An increase in detection may also indicate elevated prevalence of LPAIVs, though the likelihood of this is unclear due to the lack of longitudinal data on LPAIV circulation in the GMS.

Moving forward there is much that can be done to further develop AIV surveillance systems in the GMS. Surveillance systems in the region are typically not designed to detected AIV diversity. This could be improved by expanding surveillance effort to encompass all AIV subtypes in circulation in domestic poultry and by sampling more wild bird species. Realistically, a number of countries in the GMS do not have the infrastructure or funding available to deal with the demands of such research. In this case, the first priority should be to continue to improve surveillance measures in place to detect and control the circulation of H5 and H7 AIVs. This should move beyond passive surveillance efforts that only investigate sudden increases in the number of sick or dead poultry. An improvement that is particularly important due to the potential spread of H7N9 out of China as these viruses can circulate silently in poultry and have repeatedly infected humans. It is impossible to predict when or where the next pandemic will emerge, therefore it is

crucial to have strong surveillance systems in place to closely monitor viral evolution, especially with the large pool of AIVs that are currently circulating in the GMS.

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