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Detection of Avian Influenza A Virus in Malaysian Birds.

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ABSTRACT

A study was conducted to assess the presence of avian influenza A virus (AIV A) especially in wild forest birds of Malaysia. The annual north-south movements of migratory birds have been implicated as a factor that contributes to the spread of AIV A throughout the world. Mixing of local forest birds and migratory birds has been reported to occur during migrational stops of the migratory birds. It has been postulated that infected wild birds can pass on the virus to poultry, especially at areas that are considered as interface between wild birds and the domesticated poultry reared at the forest fringes. The possible widespread of AIV A in Malaysia and reports of outbreaks of H5N1 strain during the early 2003 to 2006, have led to the interest in finding support for this postulate. Occurrence of AIV A was postulated with possible implications with the ecological parameters of the areas. Transmission of viruses had been speculated to occur in certain seasons which give rise to the idea that some climatic factors such as temperature and rainfall plays their role in the transmission. Seventeen study areas from 12 geographical locations in Malaysia were studied and grouped into seven habitat types given as primary forest, secondary forest, urban, monoculture, mixed forest of lowland and limestone, beach forest and mixed forest of secondary growth and orchard to reflect a portion of the vast tropical habitat. The average temperature for all the habitats ranges from 23°C to 28°C while rainfall measure range from 0.7 mm to 9.6 mm. There are no significant differences in the temperature and rainfall measures that could be used to discriminate them into different habitats accordingly. The diversity indices were calculated based on Shannon's diversity index (H') and Simpson's diversity index (1−D) using Multivariate Statistical Package (MVSP) version 3.0. Both indices reflect the highest bird diversity in mixed forest of the limestone and lowland habitat (H'= 3.698, 1−D = 0.969). A total of 2199 throat washing and blood samples were collected from 1132 birds. The samples were screened for the presence of viral RNA genome. Out of the total samples, 1797 were analysed using reverse transcriptase polymerase chain reaction (RT-PCR) and 402 samples were analysed by the immunocapture RT-PCR. Detection by RT PCR was carried out using H5, H6, H7, H9 and nucleoprotein (NP) primers while immunocapture to the microplate was conducted using serowell plates pre-coated with H5, H6, H7 and H9 antigens. The results of the detection were negative for all isolates. This probably suggests that birds in Malaysia are free from avian influenza A virus. Optimisation of the detection based on the two techniques had been carried out using positive control. Amplification and detection of viral RNA were optimised at the temperature of 70°C with the use of H5 gene. However, the potential hosts, preferred habitats as well as climatic factor (rainfall and temperature) that were associated to the transmission and infection of the AIV could not be discriminated with reference to the negative detection. Although all isolates were tested negative, the infection of avian influenza virus is still a major concern because the fact that birds are free moving organisms suggests that higher chances of interaction between the birds and other fauna including human is plausible.

Keywords: assessment, avian influenza A virus, transmission, forest birds, Malaysia

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INTRODUCTION

Zoonotic diseases refer to diseases that are transmitted from animals to human and vice versa that will feature risk when there are contacts between animals and human under natural condition (Mandal *et al*., 2004). There are several forms of agent in zoonotic diseases including viruses, bacteria and other pathogens. The occurrence of the viruses and pathogens are associated with underlying causal factors (Morse, 1994). The underlying factors provide chance of interactions between the zoonotic pathogen's host and wildlife. These are usually dependent on the changes in the ecology of the hosts, pathogens and their vectors (Schrag and Weiner, 1995). The involvement of wild animals in the epidemiology of most zoonotic diseases has been reviewed (Kruse *et al*., 2004). Despite the clear implications and the important causes these diseases play in the epidemiology of some diseases that are important in human health and livestock, information on the pathogens of wildlife are relatively not well defined. In the light of exposure to human population and transmissibility of the pathogens to human, this inadequacy of information represents the zoonotic risks which need to be resolved.

Transmissibility of the pathogens to human forms the route of many zoonoses cases. The zoonotic agents are typically consisting of bacteria, viruses and parasites which can be carried by wild animals, serve as the major reservoirs for microbial transmission to both human and domestic animals (Acha and Szyfres, 2003). Wild birds have been known to be the natural reservoirs that shed zoonotic disease agents in their natural habitat as reviewed by various authors (Tripathy *et al*., 2000; Boon *et al*., 2007; Peiris *et al*., 2007; Pfeiffer, 2007; Boyce *et al*., 2009). Wild birds have been introduced as the reservoirs for a few zoonotic diseases including pox disease, avian malaria, neurotropic velogenic Newcastle disease as well as avian influenza A. Those diseases are transmitted with the help of pathogens such as *Avipoxvirus* (Tripathy *et al*., 2000), plasmodium species, Newcastle disease virus and influenza virus (Daszak *et al*., 2000). Among these diseases, the most known agents for the transmission of zoonotic diseases are the viruses which have been known to cause reemerging diseases (Desselberger, 2000) which later caused pandemic cases. This is specifically mentioned with the example of the migratory aquatic birds that shed avian influenza virus and caused pandemic threat to the recent human community (Pfeiffer *et al*., 2006).

The exact host of influenza A has not been clearly defined. According to Daszak *et al*. (2000), migratory wild birds are the reservoir hosts of influenza A. Perkin and Swayne (2003), in contrast, suggests the domestic fowl as the reservoir host for avian influenza A viruses. However, Peiris *et al*. (2007) argued to the statement mentioning that if the domestic fowls are the reservoir hosts, then eradicating the fowls would control the transmission of the viruses in the environment. Reality have shown that culling of infected fowls could not truncate the spread of influenza A viruses and therefore suggests that there are other reservoir hosts of the virus instead. Due to these matters, an assumption of the modified transmission cycle model has been portrayed.

The transmission may begin from shedding of viruses from the migratory birds to the wild forest birds and domestic fowls (Olsen *et al*., 2006; Boyce *et al*., 2009). This may happen due to global travelling of the migratory birds during their migration period which later provide contact between the infected migratory birds to the local wild birds. Contact and transmission of the viruses may also occur in the interphase with reason to urbanisation process, human encroachment into the wild birds' habitat and ecological manipulation. This interphase and transmission cycle of AIV is essential in describing the overview of the viruses being transmitted into a new environment. The interphase includes transmission of the viruses from one known host to another within the habitats they occupy. It is known that the tropical region act as a major migration route of the migratory water fowl (Myers, 2009). During migration, the birds will have few migrational stops in the tropical region to rest before they continue on (Phillipps and Phillipps, 2009). This enhances the chance of the infected birds to shed the virus collected from areas of infestation into the new environment that they use to nest and forage if they are the carriers of these viruses. Hence, there are chances of interaction with the local inhabitants and later spread the viruses to local population. The interphase where there are chances of interactions between the infected birds with the local population plays an important role in the transmission and spread of the virulent strains to newer hosts and habitats. There have been studies highlighting on the role of environmental ecology in the growth and transmission of the viruses (Sturm-Ramirez *et al*., 2005). Exploited habitats may also increase the chance of contact between animals and human. The habitat that is likely to suggest possible chance in transmission and spread of the viruses provided by the frequent interactions between animals and human occur in recreational areas or also known as urban habitat. As has been known

globally, the avian influenza viruses may be transmitted via faecal-oral route. Infection following the ingestion of virus shed in faeces will result in virus-contaminated ground (Webster *et al*., 1992). This will further provide an infectious site for any other bird species that dwell in the area and make possible for any birds to be infected with the virus.

Generally, avian influenza A viruses do not spread efficiently in mammals, and infections remain limited to individual animals or small groups (Reid and Taubenberger, 2003; Acha and Szyfres, 2003). However, some viruses could adapt to a new species and cause outbreaks with respect to either epidemics or pandemics (Hinshaw *et al*., 1984). Transmission between birds and human has been reported only with the H5, H7 and H9 subtype's strain (Olsen *et al*., 2002). Avian influenza viruses mainly infect birds, but some strains were reported to cause disease in mammals (Enserink and Kaiser, 2002; Keawcharoen *et al*., 2004). Waterfowls and shorebirds that tend to carry these viruses asymptomatically appear to be the natural reservoir hosts (Beard, 1998). However, serious diseases have been reported in gulls, wood ducks, farmed ostriches, emus, passerine birds and other avian species (Perkins and Swayne, 2003; Brown *et al*., 2006). Poultry can develop serious or mild diseases, depending on the subtypes and strains of viruses as well. The known occurrence of influenza A in domestic fowl namely chicken has arisen the idea that the influenza A could infect other avian species. This is later supported by Ellis *et al*. (2004), Kilpatrick *et al*. (2006) and Peiris *et al*. (2007) where there are evidence of spread of H5N1 in non-migratory wild birds from order Falconiformes (eagles), Strigiformes (owls), Passeriformes (crows, magpies and mynas), Psittaciformes (parrots), Ciconiiformes (herons and egrets) and Gruiformes (coots). Therefore, this study aims to detect influenza viruses from the local bird population in different classes of forest.

Besides, avian influenza has received much attention from the public because the viruses can be transmitted to human (Shortridge, 1982; Chen *et al*., 2006). In this connection, many studies have been conducted to observe the patterns of the influenza viruses in the environment and its host with the transmission mode of the viruses from the poultries such as chickens, ducks, quails and also pheasants (Mounts *et al*., 1999; Shortridge, 1999). However, most studies focused on the transmission and the host of the influenza viruses and were conducted elsewhere rather than in Malaysia (Clark and Hall, 2006; Ezenwa *et al*., 2006; Swaddle and Calos, 2008). There has been very little study conducted to observe the ecology of the area in relation to the influenza viruses detected in a specified area and yet none in Malaysia.

However, no other case of influenza A has been detected in Malaysia since 2006 (WHO, 2010). This has aroused speculation that the spread of these viruses depends on seasons and perhaps some other climatic factors. As influenza A viruses are known to occur in the neighbouring countries such as Indonesia and Thailand, it is not impossible for the virus to be re-introduced to Malaysia. A study by Ramji (2008) adhered to the following result as well. According to WHO (2010), there were no recent infections occurred in Malaysia. There were only a few infection detected in Malaysia, with the first occurrence in Kota Bahru, Kelantan and a later case in Setapak, Kuala Lumpur. The contributor to the virus spread in Malaysia was the import of domestic fowl from the neighbouring countries as well as tourism activities. Kalimantan, Indonesia has reported serious cases of H5N1 infection (WHO, 2007). Yet, no case has been detected in Sarawak and Sabah although they are the close neighbours to Kalimantan and the situation remains calm.

Another extensive study conducted by Ramiah (2008) on epidemiology of influenza A viruses in the avian (domestic fowl: chickens and ducks) and swine population in Peninsular Malaysia had not detect any avian influenza virus either. The five years sample collection was accumulated based on the surveillance conducted by the Department of Veterinary Service from 2000 till 2005. All samples were processed and subjected to RRT PCR using HA primer. Yet, neither a single strain of the highly pathogenic nor the low pathogenic avian influenza A was detected. However, the strain shows seropositive results towards the H1N1 and H3N2 subtypes isolated from the swine with high percentage. Though the mentioned study used a different detection approach, the infection of influenza was still negative.

Objectives

With the known possibilities of transmission of viruses among forest birds had led to this documentation. Thus, detection of avian influenza A virus was carried out to distinguish the possible factors that may have contribute to the transmission of the virus in the ecosystem.

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MATERIALS AND METHODS

Table 1.0: List of surveyed areas in Sarawak, Sabah and Peninsular Malaysia.

No.	Survey Area	GPS Coordinates	Date of Sampling
1.	Bukit Lima Forest Park, Sibu, Sarawak	N 2° 16′ 59.46″ E 111° 51′ 59.85″	4th – 8th June 2008
2.	Bukit Aup Recreational Park, Sibu, Sarawak	N 2° 24′ 45.66″ E 111° 44′ 52.04″	5th – 9th June 2008
3.	Ta Ann Naman Plantation, Sibu, Sarawak	N 2° 10′ 03.01″ E 111° 51′ 52.75″	9th – 13th June 2008
4.	Lanjak Entimau Wildlife Sanctuary, Sarawak	N 1° 23′ 05.28″ E 112° 15′ 49.76″	16th – 28th June 2008
5.	Balambangan Island, Sabah	N 07° 13' 02.4" E 116° 53' 00.8".	29th April – 3rd May 2009
6.	Tasek Bera Ramsar Site, Pahang	N 3° 07′ 31.58″ E 102° 42′ 42.16″	21th – 25th May 2009
7.	Nanga Merit, Kapit, Sarawak	N 2° 48′ 09.60″ E 113° 51′ 04.53″	16th – 22th June 2009
8.	Fraser's Hill Wildlife Reserves, Selangor	N 3° 47′ 06.88″ E 101° 41′ 35.14″	27th – 30th July 2009
9.	Sungai Dusun Wildlife Reserves, Selangor	N 3° 49′ 47.30″ E 101° 31′ 01.86″	27th – 30th October 2009
10.	Gunong Benom, Krau Wildlife Reserves, Pahang	N 3° 43′ 23.07″ E 101° 57′ 14.96″	12th -14th November 2009
11.	Batang Ai National Park, Sri Aman, Sarawak	N 1° 36′ 55.26″ E 112° 14′ 31.90″	5th – 10th January 2010
12.	Kampung Buntal, Kuching, Sarawak	N 1° 23′ 05.28″ E 109° 55′ 10.53″	13th – 18th February 2010
13.	Sg Asap, Belaga, Sarawak	N 3° 23′ 22.90″ E 114° 07′ 16.14″	6th – 10th June 2010
14.	Gunung Mulu National Park, Sarawak	N 3° 55′ 23.48″ E 114° 44′ 52.04″	10th – 16th July 2010

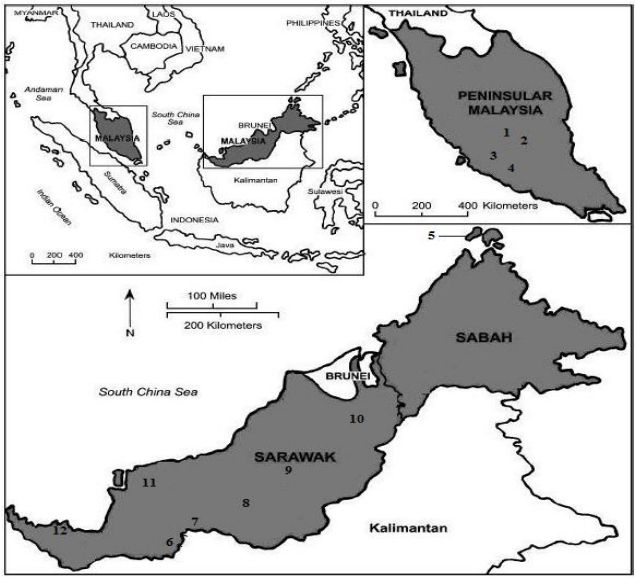

Fig. 1.0: Map of the study sites showing the major location. The numbering indicates the area as further listed. 1-Tasek Bera Ramsar Site; 2-Krau Wildlife Reserves; 3-Fraser's Hill Wildlife Reserves; 4-Sg Dusun Wildlife Reserves; 5-Balambangan Island; 6-Batang Ai National Park; 7-Lanjak Entimau Wildlife Sanctuary, Sg Menyarin & Sg Bloh; 8-Kapit, forest Nanga Merit & orchard Nanga Merit; 9-Sg Asap Belaga, strip 1 & strip 2; 10-Gunung Mulu National Park; 11-Sibu, Bukit Lima Forest Park, Bukit Aup Recreational Park & Ta Ann Naman Plantation; 12-Buntal.

The assessment of birds' diversity and isolation of AIV had been conducted in 17 study areas within 12 geographical locations in Malaysia. In Sarawak, the study areas included the northern Sarawak (Rejang Basin) to the southern region of Sarawak (Kuching-Sri Aman). The selected areas included all dryland, wetland and forest fringes that were near to human dwellings. Four geographical locations in peninsular Malaysia and one area in Sabah were also surveyed to provide better overview of the virus detection. The list of surveyed areas is shown in Table 1.0 and the map is presented in Figure 1.0.

Field Methods

Nets were set at all the study areas as plotted in Fig. 1.0. Four shelves mist-nets measuring 12 m x 2.5 m with mesh size of 36 mm were optimally used to capture birds (Rahman and Abdullah, 2002; Rahman *et al.*, 2002a, 2002b; Pardieck and Waide, 1992). Ten to twenty mist-nets were set up at the possible flyways for birds. Mist-nets were erected in the forest understorey, 20 m apart and 1 m above the ground. However, a few nets were also lowered to the ground whenever necessary to enhance the chances of capturing the forest floor dwellers. Nets were also erected higher above the ground or specifically on trees to capture the canopy birds. Mist-nets were generally set up at a given site for about three to five days. This is because it is typical to observe a dramatic increased in the number of recaptures after the third day (Okia, 1976; Wilson and Moriarty, 1976; Greig-smith, 1980; Wong, 1986; Meyers and Pardieck, 1993).

The nets were repositioned every two days to maximize the effort. The nets were checked at every two hours interval from 0600 hr till 1800 hr for bird collection. All specimens were placed in the cloth bags before they are identified, measured and processed. All birds identification were aided by 'A Field Guide to the Birds of Borneo, Sumatera, Java and Bali' by MacKinnon and Phillip (1993), 'Birds of Borneo' by Smythies (1999), 'Birds of Southeast Asia' by Robson (2007), 'Phillipps' Field Guide To The Birds of Borneo' by Phillipp and Phillipp (2009) and 'Birds of Borneo' by Myers (2009). The environmental parameters observed and recorded include; rainfall, temperature, vegetation type, forest type and also on the patterns of land use. Climate data (rainfall and temperature) were mainly obtained from the Malaysia Meteorological Department.

Sample collection

The nasal, tracheal and cloacal swabs were collected from the birds before they were released. A study by Sturm-Ramiraz *et al*. (2004) and Hulse-Post *et al*. (2005) results in the theory of the influenza H5N1 subtype could replicate to higher levels at longer period in the respiratory tract as compared to those in the gastrointestinal tract. The swabs were collected using dacron or rayon-tipped swabs as suggested by FAO (2006), and the swabs were placed in the viral transport medium or in the phosphate buffered saline (PBS) buffer. As for throat wash method, the ultrapure water or molecular grade RNAse free water was used by inserting them into the bird's esophagus through the bill. This is to ensure that any possible traits of the virus within the birds could be sampled. The wash products were then ejected into empty 2.0 ml cryogenic vials and kept in liquid nitrogen tank. All samples were later stored in -80°C upon arrival at the laboratory. Blood samples were collected from the blood veins located in the inner part of the wings. Blood samples were collected using the capillary tubes or insulin syringe and were left to dry on the sterilized (UV) filter paper (Whatman) and later sealed to ensure no contaminations occur. About 50 µl of the blood collected were preserved either in empty vial or PBS buffer. It was important to press the venipuncture site with cotton bud to stop the blood from flowing and also inhibit blood clotting that might affect the movement of the birds wing (FAO, 2006). The blood collected was kept in liquid nitrogen tank or stored at 4°C before being transported back to the laboratory.

Sample processing

The RNA extraction from wash and blood samples were conducted using TRIzol-invitrogen protocol which was performed based on extraction guidelines from Avian Virology Laboratory, Veterinary Research Institute, Malaysia. The PCR amplifications were consequently done using Biometra Tpersonal Thermoblock (Germany) for single annealed amplification, Biometra Tgradient Thermoblock (Germany) for gradient-regulated amplification and also BIORAD Thermal Cycler (USA). Laboratory exertions were conducted mostly at Molecular Ecology Laboratory (MEL), Department of Zoology, and partially at Virology Laboratory, Department of Molecular Biology, Universiti Malaysia Sarawak (UNIMAS).

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The mixture protocol is simplified as in Table 2.0 and Table 3.0. The tube was subjected to quick spin (about 10 seconds) before the PCR reaction started. All reagents and reaction mixture for RT PCR as well as PCR was performed using GoTaq® Flexi DNA polymerase PCR kit, Promega (Madison, Wisconsin, USA) except for M-MuLV reverse transcriptase.

Table 2.0: cDNA synthesis based on reverse transcriptase protocol from viral RNA.

Reactant	Volume (µl)
ddH_2O/depc treated water	0.5
5x RT Buffer (250 mM Tris-HCL, 250 mM KCl, 50 mM $MgCl_2$, 2.5 mM spermidine and 50 mM DTT)	2.0
dNTPs (10mM)	0.5
M-MuLV reverse transcriptase (100 U)	0.5
Forward primer (20 pmol)	1.5
Extracted RNA	5.0

Table 3.0: cDNA amplification mixture using PCR method.

Reactant	Volume (µl)
Cdna	5.0
5x RT buffer (10 mM Tris-HCL pH 9, 50 mM KCl, 0.1% Triton X-100)	5.0
$MgCl_2$ (25 mM)	3.0
dNTPs (10mM)	1.5
ddH_2O/depc treated water	32.0
Forward primer (20 pmol)	1.5
Taq DNA polymerase (5 U)	0.5

The PCR profile is shown in Table 4.0. The profile was similar to all subtypes except for annealing temperature. The annealing temperature varied depending to the melting temperature of the various primer sets.

Table 4.0: The PCR amplification profile

	Temperature (°C)	Duration (mins)	Cycle(s)
Pre-denaturation	94	3	1
Denaturation	94	0.5	1
Annealing	NP (61-65°C); H5 (65-70°C); H6 (60-68°C); H7 (60-65°C); H9 (61-68°C)	0.5	(NP, H5, H7) 35; (H6, H9) 30
Extension	72	5	
Soak	4°C	∞	1

There were five primers used in the study. All the primers were synthesised by 1st BASE Sdn. Bhd. (Selangor, Malaysia). The primers are listed in Table 5.0. The positive control was generously provided by Institute of Health and Community Medicine, Unimas.

Table 5.0: List and sequences of the primers used in the study (Lee *et al.*, 2001).

Primer Id	Gene	Sequences
NP 1200	Nucleoprotein	5' cag rta ctg ggc hat aag rac 3'
H5-155	H5	5' aca cat gcy car gac ata ct 3'
H7-12	H7	5' ggg ata caa aat gaa yac tc 3'
H9-151	H9	5' cty gac aca gar cac aat gg 3'
H6-661	H6	5' agc atg aat ttt gcc aag ag 3'

The immunocapture PCR was conducted based on four mixture protocols with annealing step carried out at seven different temperatures. The various modifications of parameters are listed in Table 6.0.

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Table 6.0: Immunocapture PCR protocol with four different mixtures.

Reactants	Volume			
	1x reaction (µl)			
	Mix 1	Mix 2	Mix 3	Mix 4
5x RT buffer	2.0	2.0	3.0	3.0
DEPC treated water	3.0	3.0	3.0	1.5
dNTP mix	2.0	2.0	2.5	2.0
Forward primer (20 pmol)	1.0	1.0	1.0	1.0
RNAsin	0.25	0.5	0.5	0.5
$MgCl_2$	1.5	1.0	1.5	1.5
Reverse transcriptase (M-MLV)	0.5	0.5	0.5	0.5

All amplified products were visualised on a 2 % pre-stained agarose gel. The 4.0 µl PCR product was executed together with 1.0 µl of 6x loading dye. The products were run at 90 volt. Product size was determined based on GeneRuler™ 100 bp DNA ladder plus (Fermentas, Canada) and GeneRuler™ 50 bp DNA ladder (Promega, USA).

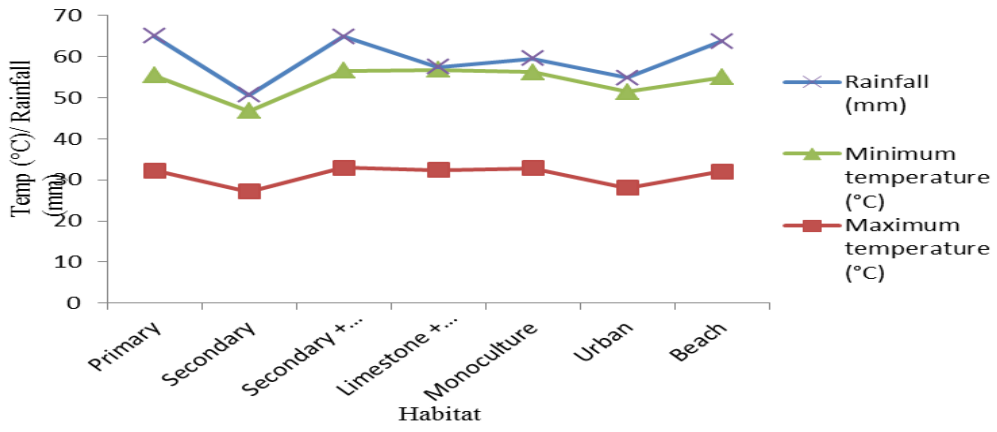
RESULTS

Distribution of Birds in Contrasting Ecological Habitats

A taxonomic list of 1132 individuals of birds consisting of 34 families, 78 genera and 134 species sampled from 17 locations using mist-netting technique had been recorded. The species list was computed based on the cumulative of 15840 trapping days' effort in total. The recorded species from the study areas in Sarawak and Sabah represented 18.4 % of the Borneo birds (Phillipp and Phillipp, 2009). The diversity of birds in their respective geographical areas was pooled into their respective habitat types.

The number of pooled species within the named habitat is highlighted in Table 7.0 where the most diverse species could be observed occupying the secondary forest with 66 species. This was followed by monoculture and mixed habitat of secondary and orchard with 57 species. The least richness is shown in beach forest with nine species. The bird list was dominated highly by the family Pycnonotidae and Timaliidae followed by the Muscicapidae, Alcedinidae and Nectariniidae.

As shown in Table 8.0, the average maximum temperature was 31.1°C while the average minimum temperature was 22.9°C. The average rainfall was about 5.4 mm. Rate of rainfall did not affect the diversity rate in primary forest and mixed forest of secondary and orchard forest. The temperature recorded did not show any extreme changes for all the habitats.



Figs. 2.0: The contrasting ecological habitats against temperatures and rainfall.

The graph showing the comparison between habitats against temperatures and rainfall is as shown in Figure 2.0. From figs. 2.0, it was clearly seen that the maximum temperatures for the habitats were shown by

secondary forest followed by urban habitats. However, the minimum temperature was quite consistent at all habitats with slight decrease in the secondary forest. The most rainfall detected was in the primary forest while the least was in limestone and lowland forest.

Table 7.0: Description on the forest type surveyed and the species of birds sampled for AIV A.

Forest type	Sampling location	Area description	Species of birds collected
Urban	Bukit Lima, Sibu, Sarawak Bukit Aup, Sibu, Sarawak	Logged forest developed into recreational ground (Marzluff et al., 1998). Some flowering trees and big trees were planted in the area with some modification.	32
Secondary forest	forest Nanga Merit, Kapit, Sarawak Sg Dusun Wildlife Reserves, Selangor Fraser's Hill Widlife Reserves, Selangor Tasek Bera Ramsar Site, Pahang	Forest that has been logged or cleared (Marzluff and Ewing, 2001). Secondary forest will evolve when shifting cultivation occurs. The forest structure shows many canopy gaps. Not many tall trees could be seen since the initial forest has been felled. According to Mohamad (2004), pioneer secondary species such as *Macaranga* sp. could be observed to dominate the area. This forest will re-form after maturation in many years interval with different forest compositions.	66
Primary forest	Sg Menyarin, Lanjak Entimau Wildlife Sanctuary, Sarawak Sg Bloh, Lanjak Entimau Wildlife Sanctuary, Sarawak Lata Bujang, Krau Wildlife Reserves, Pahang	An initial forest structure (Whitmore, 1990). This forest may have not experienced any intrusion such as logging or land clearing. Though, there may have been some minimal activity in the forest that would not disrupt the forest composition. Many tall trees and less secondary shrubs could be seen in the forest.	44
Monoculture	Ta Ann Naman plantation, Sibu, Sarawak Strip1 and 2, Sg Asap, Belaga, Sarawak	Large scale agriculture plantations. The area provides an open environment which could be used by some fauna that favours the open environment.	57
Secondary & Orchard	Orchard Nanga Merit, Kapit, Sarawak Batang Ai National Park, Sri Aman, Sarawak	A mixture of secondary forest and orchard plants is formed when the forest is cleared and planted with vegetable and fruit trees. This area later is overgrown by fruit trees and eliminates many forest plants species which is substituted with the newly planted trees. This mix forest also provides sufficient food sources and essential living environment for the fauna.	57
Lowland & Limestone	Mulu National Park, Miri, Sarawak Balambangan Island, Sabah	These two areas are mostly composed of various lowland plant species that occupy the three layer canopy. The trees found in the lowland forest usually grow up to 45 m high and at the elevation of 300 m above the ground (Whitmore, 1990). The lowest layer is mostly made of the shrubs and small herb trees while the second layer is composed of higher and bigger deciduous trees. The upper layer usually forms the canopy top the forest with tall trees having large buttresses. Limestone features are shown with the karsts formation within the two sites. The mineral content of the karsts containing calcium carbonate attracts many birds' species to occupy the area as well as harbouring high diversity of plants species (Tjia, 2004). The limestone is also a major swiftlet nesting area.	50
Beach	Buntal, Kuching, Sarawak	Dominated by sandy and mud flats. The area also features many of the *Casuarina equisetifolia*. Buntal area is not mainly made up of beach forest as minor area is also dominated by the mangrove plants. The sandy beach provides food sources to small crustaceans. The crustaceans are fed upon the shorebirds.	9

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Table 8.0: Comparison of the temperature and rainfall in the contrasting ecological habitats.

	Temperature (°C)		Rainfall
Habitats	Maximum	Minimum	(mm)
Primary	32.3	23.1	9.6
Secondary	27.1	19.7	3.9
Secondary + orchard	33.0	23.6	8.3
Limestone + lowland	32.4	24.4	0.7
Monoculture	32.8	23.4	3.4
Urban	28.1	23.4	3.4
Beach	32.1	22.9	8.8

Detection and optimisation of avian influenza A viruses using RT-PCR

From the 1132 individuals of birds collected, a total of 2199 wash and blood samples preserved in different methodology had been isolated and analysed via RT PCR and immunocapture RT PCR amplification. The number of swabs and throat wash that were isolated from all the study areas are summarised in Table 9.0.

Table 9.0: Summary of the samples collected with respect to the areas surveyed and the type of collection.

Study Area	Throat Wash	Blood	I.c wash	I.c blood
Kapit 1	33	18	0	0
Kapit 2	126	100	0	0
Tasek Bera	39	39	0	0
Fraser' Hill H5	71	71	71	30
Fraser' Hill H6	71	71	71	28
Fraser' Hill H7	71	71	71	30
Fraser' Hill H9	71	71	71	30
Sungai Dusun WR	27	27	0	0
Krau WR	33	0	0	0
Buntal	16	12	0	0
Santubong	12	0	0	0
Batang Ai NP	56	48	0	0
Bukit Aup	39	35	0	0
Bukit Lima	103	33	0	0
Ta Ann Naman	120	114	0	0
Balambangan Island	16	10	0	0
Mulu National Park Hq	37	37	0	0
Sg. Asap Belaga	74	0	0	0
Lanjak Entimau	12	13	0	0
Total samples screened	1040	757	284	118

Note: I.c refers to immunocapture

Detection of AIV based on Immunocapture PCR

From the total of 402 isolates of immunocapture coated with H5, H6, H7 and H9 antibodies, none were screened to be positive to avian influenza A viruses. The annealing temperatures were set at the range between 48 – 54°C. Each sample was amplified under the four different RT mixtures and optimised based on the given annealed temperature. The modifications of the mixtures were expected to prevent the degradation of viral RNA and further increase the chance of detection. Although vigorous modification of immunocapture PCR mixture had been carried out, all the modification did not result in positive detection of all the 402 isolates. The result of immunocapture isolates detection based on PCR techniques is as shown in Figs. 3.0 below.

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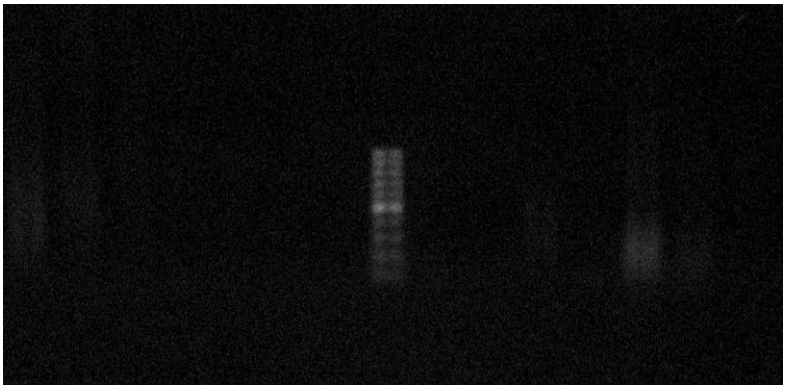
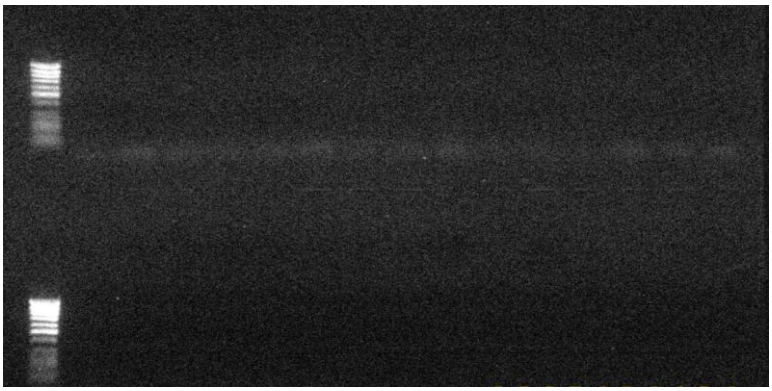


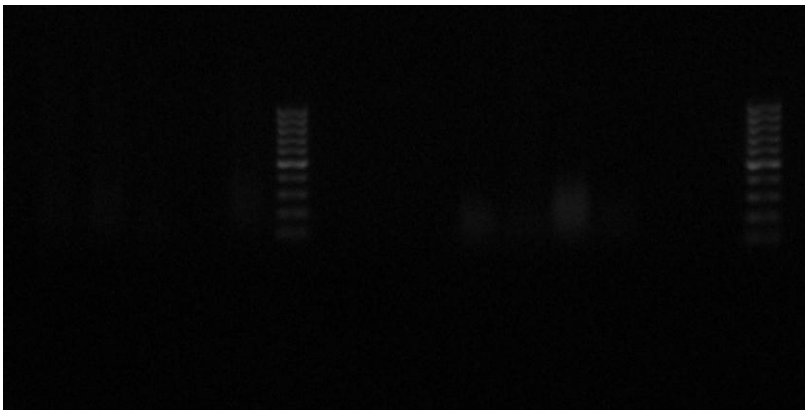
Figs. 3.0: The negative result of immunocapture isolates shown for selected samples annealed using H5 primer with the temperature ranging from 60-70°C. The marker shown was GeneRuler™ 50 bp DNA ladder (Promega, USA).

Detection of avian influenza viruses through RT PCR

All 1797 isolates from throat was and blood were tested with H5, H6, H7, H9 and NP primers and run accordingly. All samples were tested negative with all the set of primers. No bands were observed and results are shown on gel electrophoresis in Figs. 4.0 and Figs. 5.0. Although vigorous detection techniques and optimisation in the detection had been carried out, the isolated samples still showed negative results despite several attempts. In relation to the study, the negative results obtained actually convey a good indicator towards the scenario of avian influenza in Malaysia. The findings from the current survey thus, support the idea that Malaysia is free from avian influenza (WHO, 2010).



Figs. 4.0: The negative result shown for selected samples annealed using NP primer with the temperature ranging from 60-70°C. The marker shown was GeneRuler™ 50 bp DNA ladder (Promega, USA).



Figs. 5.0: The negative result shown for selected samples annealed using H5 primer with the temperature ranging from 65-70°C while H7 primer annealed at 60-65°C. The marker shown was GeneRuler™ 50 bp DNA ladder (Promega, USA). The figure is presented as, from right: ladder continued by H7 annealed sample, ladder, H5 annealed sample.

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DISCUSSION

The negative detection of influenza A viruses from all 2199 isolates although tested vigorously using RT PCR and immunocapture RT PCR techniques have brought few implications. The failure of using RT PCR technique to detect the presence of the various strains of avian influenza such as H5 and H7 could be explained as follows. Low concentration of viral RNA in the isolates that may not be detectable based on the RT PCR technique is one of the reasons behind the negative finding (Chaharaein *et al*., 2006). In this study, the virus samples were not inoculated into embryonated eggs because of preservation method used. All samples were preserved in either ribavirin solution or TRIzol invitrogen solution which will both make the virus within the samples collected to be non-replicating if ever they are present. This is due to lack of facilities for the best safety precautions in handling the viruses. The influenza viruses should always be handled in a BSL 4 lab which is not available as suggested by Eshaghi *et al*. (2004). Therefore, to avoid any contaminations and possible threats from the viruses isolated, the samples were not inoculated. This further contributed to lower titre of the virus in the samples isolated. If the virus were present, the concentration of viral RNA in the isolates may be too low that led to less sensitivity during detection. Inoculation process may help to breed the virus in a surrogate host, usually inserted into the allantoic fluid of the egg. When the virus breed, the titre of the virus will also increase and thus may generate bigger chance of detection.

All possible enhancements in detecting the virus had been carried out given the known possibility of lacking in sensitivity of RT PCR in detecting the small strains. The immunocapture technique was carried out to enhance the detection ability by coating the plate with antibodies (H5, H6, H7 and H9) that may attract the targeted antigen to bind to the bottom of the plate. In addition, possible modifications to the mixture protocols to increase the chance of detecting the viral RNA strains had been conducted as shown in Table 6.0. However, the modifications also gave negative results for the detection. Thus, the degradation of viral RNA probably is not causing failure in the virus detection.

The current study only focuses on the four HA subtypes which were H5, H6, H7 and H9. The choice was based on the subtypes that have been previously reported to cause pandemic infections worldwide (Beare and Webster, 1991; Perdue and Swayne, 2005; Gill *et al.,* 2006). As mentioned by Webster *et al*. (1992) primer selection is essential to obtain the targeted product crucially and as in this case, the H5, H6, H7 and H9 primer did not seem to occur in the samples. Hence, to observe the overall presence and absence of the influenza viruses, more trials facilitated by different primers should be conducted. Nevertheless, the study is only concentrating on the success of the provided primers.

There had been many studies on the detection of avian influenza viruses in wild birds carried out by various authors (Garamszegi and Moller, 2007; Chen *et al.*, 2008; Ramiah, 2008; Ramji, 2008; Munster and Fouchier, 2009). Thus far, none were conducted on the free flying birds of Malaysia. The study typically highlights birds that are previously infected with virulent strains either in the soil or water. By far, the current study is not selective in those terms as the nets were set at any possible passage ways that birds could feasibly be captured. For that reason, there are chances that the birds sampled were highly active and healthy as it could still fly from one point to another probably searching for food, partner or simply running its routine activities. Additionally, all the birds sampled did not show any clinical sign of infection as observed personally. Still, the low pathogenic avian influenza strains may not caused the clinical effects in birds (Kwon *et al*., 2005). They could occur healthy but they carry the disease within their genes because the virus is kept dormant in the carrier (Hulse-Post *et al*., 2005; Chen *et al*., 2006). Due to the ability of the birds in becoming carrier for influenza A, it is relevant to screen the birds (not showing clinical signs) to detect all possible hosts.

With reference to the results presented, it was proven that the samples collected at the given study areas were tested to be negative towards avian influenza virus. The finding proves supportively towards the idea of avian influenza viruses are not transmitted in Borneo, specifically Malaysia.

Thus, no assumption could be made in term of their association with hosts. On the whole, the result of this study with respect to the research carried out by Ramiah (2008), Ramji (2008) and the preliminary study by Rahim *et al*. (2010), suggest that the wild birds that were sampled shows no infection of avian influenza A viruses and could also signified the idea that Malaysia is free from avian influenza A viruses.

The occurrence of these viruses in the environment can be rooted back to the transmission model. If the infected birds migrated to another area and dwell in the newly occupied area, this suggests bigger possibilities for viruses' transmission that will later take place in larger scale when given an option to keep on replicating and infecting the local communities. However, based on the detection of AIV in the isolated samples, it is known that no virus strains were detected. There are many possibilities that could highlight the negative detection. Due to this matter, four assumptions had been made. Firstly, the migrated birds are all healthy and free from any virus infection. This is because only healthy and fit birds could travel that far within the migration route. Though it is still possible for the infected birds to be healthy before the viruses feature their risk within the birds' physical fitness, the chance of the birds to survive the long journey is quite low. This will reduce the risk of transmission and infection to the migrated area and resulting in no positive detection of AIV. Based on the surveillance result conducted over the years, most of the wild birds sampled did not show any infectious strains (Munster *et al*., 2006; Olsen *et al*., 2006). Most of the HPAI H5N1 virus was isolated from sick or dead birds (Boyce *et al*., 2009). Although some reports highlight the wild migratory birds were known to have high susceptibility towards the highly pathogenic H5N1 avian influenza virus as mentioned by Gilbert *et al*. (2006), the ability for these birds to migrate long distances after being infected has been controversial until now (Anna, 2007). Hence, the possibility for the sick bird to migrate and transmit viruses to Malaysia is relatively low.

Another assumption is viruses strains will die off in an introduced environment which is not compatible to their biology. Avian influenza virus only occurs at the exact temperature, pH, salinity and many other physical environmental parameters. At some point, the sites surveyed may not be the area prone to the transmission of the viruses. A study by Lang *et al*. (2008) suggests that the viruses were present in the area by the time that they experienced high humidity level. Although later, the humidity lowers as the lake became frozen, the viruses are kept dormant in the lake and transmission of the virus could still occurs. Temperature also plays important role in maintaining the viability of the viruses. Aside from the specified temperature, the viruses will die off and make it impossible to detect the strains in the new environment if the ecological condition is not similar or suitable to the AIV biology. An earlier study by Lowen *et al*. (2007) indicated that influenza virus transmission at a specified relative humidity level and temperature. Using guinea pig as the organism model, the experiment was conducted by injecting influenza strain into the guinea pig and placed it in a chamber. Later, another guinea pig was introduced into the same chamber. From that experiment, it was found that transmission of influenza viruses was at greater frequency at relative low humidity of about 20 % to 35 % and was fully blocked at 80% relative humidity. It was also mentioned that transmission was greater when the studied organism was kept in 5°C as compared to 20°C while no transmission was observed when placed in 30°C chamber. This finding suggested that virus transmission occur at specified temperature and relative humidity and transmission is suspended at temperature 30°C. As observed in Table 8.0, the average temperature for all the surveyed habitats ranged from 25°C to 28°C which may be too extreme for virus transmission to occur in all the habitats.

The third assumption refers to the ecology of AIV which is host specific. It is known that avian influenza A strains are host adapted and easily transmitted to another host. The transmission occurs mostly easy within rather than between host species (Perdue and Swayne, 2005). Host restriction in influenza A shows limited gene flow among viral strains isolated from different host species and population in diverse geographical areas (Boyce *et al*., 2009). Avian influenza virus sampled in Eurasia and North America had shown high genetic variants not only for strains isolated from different host species but also from the same host species collected from different flyways within the same continents that showed the genetic difference increases with geographic distances (Ito *et al*., 2001). Conversely, once they were introduced into new host, these viruses recombined and mutated into newer strain. Therefore, transmission of the viruses to introduced host while maintaining the same strain is not taking place frequently.

The last assumption is that interaction between the local birds and the infected migratory birds does not occur due to the widespread of the birds' species within their own ecological niche although they share the same resources. The potential of the migratory wild birds to spread AIV in different geographic areas has been mostly debated by previous researchers (Liu *et al*., 2005; Kilpatrick *et al*., 2006; Olsen *et al*., 2006). However, the effect of the virus when transmitted to the new geographic area is not known. Sustained transmission to new host in new area must take place to secure the development of viruses in the geographic range (Boyce *et al*., 2009). This is because absence of potential carrier will limit the chance of spreading the virus in the new area. Avian influenza A viruses will experience cessation in the transmission three weeks after

the viruses being shed from infected birds (Hulse-Post *et al*., 2005). If there are no potential carriers, the opportunity for cross-species transmission is doubt. The vast tropical rainforest in Malaysia provides enough resources for bird species to avoid competition for living needs. Therefore, wild forest birds have less contact to the infected migratory birds and their ecological niche. This phenomenon suggests less or no interaction between them that could make the transmission of the viruses possible.

The effectiveness of the contingency plan carried out by the local authorities to halt the transmission of the virus further, although there have been reports on the infection of the virus in domestic fowl and poultry in Kuala Lumpur and Kelantan (2003 – 2006), had shown with the negative detection of AIV in the country. Eradication of the infected fowl and the seizure of the farm had been seen to be the appropriate approach to exterminate the spread of the viruses in Malaysia. After the critical period, no current case has been reported in Malaysia which suggests that there is no risk of transmission between the local bird populations. Since the bird to bird and bird to human transmission is low, it is expected that all the avian viruses introduced in Malaysia originated from the migratory birds or through trading activities on both the domestic poultry and game bird. Thus, eradicating the introduced birds help to seize the transmission of influenza A virus in Malaysia.

Kuo *et al*. (2009) had introduced a spatiotemporal statistical model to calculate the risk of transmission of the virus in an introduced environment. When there is a human infection of H5N1 detected in the area, it will raise the chance of transmission by 22 %. Therefore, this model suggests that it is essential for continuous assessment of the influenza A virus to be conducted to control and maintain the healthy environment. This will avoid many losses including economics, country development and reduce many health impacts.

CONCLUSION

In summary, many speculate that wild birds transmit the influenza viruses worldwide. Reviews by various authors indicate that the avian influenza A viruses were spread by the migratory birds. As Malaysia is known to be one of the migration routes for most wild migratory birds, this matter is given very much attention. The current study reflected that all isolates of wild forest birds and a few shore birds were free from AIV after eliminating all possible errors and misdetection with various modification of parameters had been conducted based on both the RT PCR and immunocapture RT PCR techniques. This could further justify the report by the Malaysia Health Department and WHO which stated that Malaysia is free from avian influenza disease although their geographical location adjacent to Kalimantan, Indonesia and Thailand (known occurrence of avian influenza). However, no potential hosts and habitats that are prone to the virus transmission could be elucidated as no positive strains were detected from the isolates. Thus, the biology and ecology of avian influenza A viruses, biology of birds and its community as well as understanding the ecological role and impacts will be the key factor in determining the role of birds in the emergence and transmission of influenza A diseases worldwide.

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