Characterization of Fowlpox virus in chickens and bird-biting mosquitoes: a molecular approach to investigating Avipoxvirus transmission

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Abstract

Avian pox is a highly contagious avian disease, yet relatively little is known about the epidemiology and transmission of Avipoxviruses. Using a molecular approach, we report evidence for a potential link between birds and field-caught mosquitoes in the transmission of Fowlpox virus (FWPV) in Singapore. Comparison of fpv167 (P4b), fpv126 (VLTF-1), fpv175–176 (A11R-A12L) and fpv140 (H3L) gene sequences revealed close relatedness between FWPV strains obtained from cutaneous lesions of a chicken and four pools of Culex pseudovishnui, Culex spp. (vishnui group) and Coquillettidia crassipes caught in the vicinity of the study site. Chicken-derived viruses characterized during two separate infections two years later were also identical to those detected in the first event, suggesting repeated transmission of closely related FWPV strains in the locality. Since the study location is home to resident and migratory birds, we postulated that wild birds could be the source of FWPV and that bird-biting mosquitoes could act as bridging mechanical vectors. Therefore, we determined whether the FWPV-positive mosquito pools (n=4) were positive for avian DNA using a polymerase chain reaction-sequencing assay. Our findings confirmed the presence of avian host DNA in all mosquito pools, suggesting a role for Cx. pseudovishnui, Culex spp. (vishnui group) and Cq. crassipes mosquitoes in FWPV transmission. Our study exemplifies the utilization of molecular tools to understand transmission networks of pathogens affecting avian populations, which has important implications for the design of effective control measures to minimize disease burden and economic loss.

INTRODUCTION

Avian pox is a highly contagious viral disease caused by avipoxviruses (family Poxviridae, subfamily Chordopoxvirinae) that has been documented in more than 230 species of birds worldwide [1]. To date, ten species of avipoxviruses have been classified within the genus Avipoxvirus by the International Committee on Taxonomy of Viruses (ICTV) [2]. Avipoxvirus infection is often slow to develop and manifests in both cutaneous and diphtheritic form [3, 4]. The most common cutaneous form is usually self-limiting and is characterized by wart-like nodules that occur around the eyes, beak and non-feathered skin. The diphtheritic form, also known as ‘wet’ pox, results in the formation of lesions on the mucosal membranes of the mouth and respiratory tract (throat, trachea and lungs) of infected birds, causing impaired breathing and difficulty in feeding. The size and number of growths are dependent on the stage and severity

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Abbreviations: CAMs, chorioallantoic membranes; COI, cytochrome c oxidase I; EHI, Environmental Health Institute; FWPV, Fowlpox virus; IACUC, Institutional Animal Care and Committee; ICTV, International Committee on Taxonomy of Viruses; JEV, Japanese Encephalitis Virus; NEA, National Environmental Agency; ML, maximum likelihood; NParks, National Parks Singapore; VACV, vaccinia virus; WNV, West Nile virus.

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The GenBank accession numbers for the fpv167 (P4b) gene sequences of Fowlpox virus are KT372859-KT372862 (mosquito-derived), KT372863, KY464130 and KY644131 (chicken-derived), fpv126 (VLTF-1) and fpv175-176 (A11R-A12L) gene sequences are represented by KT372864-KT372867 (mosquito-derived), KT372868, KY464134 and KY644135 (chicken-derived) and KT372854-KT372857 (mosquito-derived), KT372858, KY464132 and KY464133 (chicken-derived) respectively.

Two supplementary tables are available with the online version of this article.
of the infection. Pathogenicity and clinical presentations tend to vary among birds infected with even the same Avipoxvirus strain [5, 6].

Avipoxviruses are large, double-stranded DNA viruses (~300 kb genome) that replicate in the cytoplasm of infected cells [7]. Conventional methods for the detection of Avipoxvirus infections include histopathological examination, electron microscopy and virus isolation on the chorioallantoic membranes (CAMs) of embryonated chicken eggs [8]. Avipoxvirus diagnosis has lately been facilitated by polymerase chain reaction (PCR) that facilitates the rapid, specific and highly sensitive detection of viral DNA [9–11]. Phylogenetic analyses have revealed three major clades of Avipoxvirus, namely clade A (FWPV-like), clade B (canarypox virus) and clade C (psittacinepox virus) [12–14]. These clades have further been classified into subclades [14]. To date, only FWPV (NCBI Accession no. AF198100) and canarypox virus (NCBI Accession no. AY318871) genomes have been fully sequenced. These share an amino acid identity of 64.2% [15–18]. FWPV is the most studied Avipoxvirus because of the substantial economic loss incurred through infections in the poultry industry.

Avipoxviruses are transmitted from an infected to a susceptible host either by direct contact of broken skin or indirectly. A common indirect mode of transmission is through mosquitoes, especially of Culex and Aedes spp., which act as mechanical vectors of avipoxviruses [19–25]. After the insects have fed on the lesions of an infected bird, the virus is able to remain localized on the proboscis for at least 14 days [24], with no evidence of further replication [26]. During this period, virions are mechanically transmitted to another susceptible bird [26]. Besides mosquitoes, biting midges and mites have also been reported as mechanical vectors of Avipoxvirus [27, 28]. Indirect transmission of Avipoxviruses also occurs via the ingestion of food and water sources contaminated with virus-containing scabs shed by infected birds. To date, avipoxviruses are not known to complete their replication cycle in non-avian species and bird-to-human transmission has not been documented.

Given the substantial economic loss caused by avipoxviruses and the complex nature of their transmission, it is imperative to understand the potential sources, routes and networks of Avipoxvirus transmission before designing effective control measures. This is especially applicable in tropical settings inhabited by mosquitoes that prefer avian hosts. In such settings where Avipoxvirus transmission has been documented, evidence supporting a potential role of mosquitoes in Avipoxvirus transmission must first be gathered. This can be accomplished by screening field-caught mosquitoes for Avipoxvirus and characterizing the extent of genetic similarity of virus strains obtained from infected hosts and mosquitoes. Molecular approaches, such as PCR and sequence-based typing, have been used to study the potential transmission links of Avipoxvirus and other vector-borne diseases [28, 29]. Moreover, it is important to determine whether the mosquito species potentially associated with Avipoxvirus transmission prefer feeding on avian hosts. Early investigations on the host feeding patterns of mosquitoes used immunological methods such as the capillary precipitin test and ELISA to identify the host origin of blood meals [30–35]. However, their utilization has been hindered by the limited availability of the species-specific antisera required to overcome antibody cross-reactivity between closely related host species [36, 37]. MALDI-TOF mass spectrometry is an alternative method, but has its own limitations [38]. In recent years, PCR and sequencing-based techniques have become increasingly popular because of their high specificity and sensitivity in identifying host DNA in insect blood meals [37, 39–42].

In the present study, avipoxviruses obtained from cutaneous lesions in three chickens inseparate infections were genetically characterized using a multi-locus sequencing approach. To the best of our knowledge, the study describes the first documentation of FWPV infection among chickens in Singapore. We present the evidence for a potential role of bird-biting mosquitoes in the transmission of FWPV, by comparing the genomic sequences of chicken- and mosquito-derived FWPV strains, as well as by analysing FWPV-positive mosquito pools for avian DNA.

RESULTS

Avipoxvirus infection detected in three chickens by virus isolation and PCR

We confirmed Avipoxvirus infection on three separate occasions, in January 2014, February 2016 and July 2016. Infections were detected in sentinel chickens (n=3) stationed in a wetland reserve for the purpose of zoonotic arbovirus surveillance in Singapore. All affected birds had multifocal, raised, grey/tan, crusty, verrucous irregular nodules around the eyes and comb, which were highly suspicious of avian pox (Fig. 1). Histopathological examination of formalin-fixed sections of cutaneous lesions showed multifocal, moderate to marked serocellular crusting and hyperkeratosis. Bacterial colonies were also present in the lesions. Even though virus isolation was attempted on tissue material obtained from cutaneous lesions of all three chickens, we isolated avipoxvirus from only two chickens, in January 2014 and February 2016. In these chickens, white pock lesions were detected in sentinel chickens (n=3) stationed in a wetland reserve for the purpose of zoonotic arbovirus surveillance in Singapore. We present the evidence for a potential role of bird-biting mosquitoes in the transmission of FWPV, by comparing the genomic sequences of chicken- and mosquito-derived FWPV strains, as well as by analysing FWPV-positive mosquito pools for avian DNA.

Avipoxvirus detected in adult mosquitoes caught in the vicinity of chicken cage

We caught 423 adult mosquitoes using BG Sentinel and CDC light traps stationed in the vicinity of chickens affected during the first episode of infection, in January 2014. Ten species of mosquitoes, belonging to the genera Anopheles,
**Aedes, Coquillettidia, Culex, Mansonia and Verrillina** were identified to their species level using taxonomic keys [43, 44]. Morphological identification was further confirmed by cytochrome-c oxidase subunit I (COI) gene-based barcoding [45–50]. Among 154 pools of mosquitoes (1–3 adults per pool) screened, four were confirmed as positive for Avipoxvirus DNA using a PCR assay targeting the fpv167 (P4b) gene (578 bp) [9]. Those Avipoxvirus-positive mosquito pools included Culex pseudovishnui, Culex spp. (vishnui group) and Coquillettidia crassipes (Fig. 2). Because avipoxviruses primarily infect avian hosts, we investigated whether mosquitoes in positive pools had fed on birds using a PCR-sequencing based assay [51]. The results confirmed avian DNA in all four positive pools (Table 1).

**Phylogenetic analysis confirmed that infected chickens and field-caught mosquitoes were positive for the subclade A1 of Avipoxvirus (Fowlpox virus)**

Avipoxviruses are classified into different subclades. Of these, subclade A1 includes FWPV that is known to infect chickens. Therefore, we characterized the subclade of avipoxviruses confirmed in cutaneous lesions of chickens (n=3) and field-caught mosquitoes (n=4 pools) using a multi-locus sequencing approach [52] targeting genes fpv167 (P4b, 578 bp), fpv126 (VLTF-1, 700 bp) and fpv175–176 (A11R-A12L, 700 bp).

The phylogenetic analyses of sequences obtained for respective gene regions revealed that viruses detected in chickens and mosquitoes clustered in subclade A1 of avipoxviruses (Figs 3, 4 and 5). We also included a segment of gene fpv140 (H3L), which encodes for the virion envelope protein p35. This gene segment is a newly identified locus that is able to differentiate clades A and B of avipoxviruses based on size polymorphism [12]. However, the sequencing was successful in only two isolates obtained from infected chickens. Our analyses, therefore, confirmed that the avipoxviruses detected in infected chickens and field-caught mosquitoes belonged to subclade A1, which is also known as FWPV.

**Infected chickens and field-caught mosquitoes harboured closely related Fowlpox virus strains**

Comparison of sequences revealed 100 % nucleotide identity for gene segments fpv167 (P4b), fpv126 (VLTF-1) and fpv175–176 (A11R-A12L) between chicken- and mosquito-derived FWPV. Moreover, the fpv140 (H3L) gene segment was also identical in size (1800 bp) and sequence between the two strains isolated from chickens.

**DISCUSSION**

In the present study, we report three separate events (January 2014 and February and July 2016) of FWPV infection in sentinel chickens stationed in the north-western part of Singapore. Initial clinical diagnoses suggested Avipoxvirus infection, which was confirmed by virus isolation (two events) and PCR (all events). PCR targeted a 578 bp region of gene fpv167 (P4b), which has so far been the most widely targeted gene region used for PCR confirmation of Avipoxvirus [12, 17, 18, 53, 54]. We further classified viruses involved in those infections into subclades using a multi-locus sequencing approach. Our panel included four loci, namely fpv167 (P4b), fpv126 (VLTF-1), fpv175–176 (A11R-A12L) and fpv140 (H3L). Of these, fpv167 (P4b), fpv126 (VLTF-1) and fpv175–176 (A11R-A12L) are commonly used for the subclade classification of avipoxviruses [9, 13, 52]. On the other hand, the fpv140 (H3L) locus is a less commonly used locus that can distinguish between clades A (especially subclade A2) and B based on size polymorphism [12, 13, 55]. Phylogeny-based analyses of sequences fpv167 (P4b), fpv126 (VLTF-1) and fpv175–176 (A11R-A12L) confirmed that avipoxviruses detected in the affected chickens clustered in subclade A1, also known as FWPV.

Since FWPV infection has never been documented in Singapore, we investigated whether there was any transmission link between those infections that potentially implies sustained transmission of FWPV in the area. As the three events were independent and did not occur in a chain, we postulated that the direct mode of transmission due to close contact was less likely. However, mechanical transmission through mosquitoes was a possibility. To determine whether mosquitoes had played a role in the transmission network, we employed several molecular approaches during the first event, in early 2014. First, we screened adult mosquitoes caught in the close vicinity of affected chickens for the presence of Avipoxvirus using fpv167 PCR. Of 154 pools screened, four were positive. Further characterization of mosquito-derived viruses confirmed these as FWPV. Comparison of fpv167 (P4b), fpv126 (VLTF-1), fpv175–176 (A11R-A12L) and fpv140 (H3L) revealed identical sequences between chicken- and mosquito-derived viruses. The evidence, therefore, confirmed that adult mosquitoes in the...
vicinity of the first infection carried a FWPV strain that was closely related to the strain infecting chicken. Chicken-derived viruses characterized during the second and third events, approximately two years later, were also identical to those detected in chicken and mosquitoes during the first event, suggesting repeated transmission of closely related FWPV strains in the locality. However, we could not confirm whether the viruses detected in three events were the same strain because the loci sequenced do not provide resolution to distinguish different FWPV strains [9, 16, 52]. This is primarily because fpv167 (P4b), fpv126 (VLTF-1), fpv175–176 (A11R-A12L) and fpv140 (H3L) are highly

Fig. 2. Phylogenetic analysis of COI gene sequences of mosquito species. Avipoxvirus-carrying mosquito species identified in the present study are highlighted in blue. A trimmed alignment of COI gene sequences (337 bp) was used to construct the neighbour-joining tree in MEGA 6.06 software [76], based on the Kimura-2 parameter substitution model with gamma-distributed rates. Robustness of clustering was determined by bootstrapping for 1000 replicates. Reference sequences were obtained from GenBank. All sequences are named with accession number, mosquito species, origin and reported year.
conserved gene regions. fpv167 (P4b) encodes for a virion core protein, 4b, which is a highly conserved 75.2 kilo-Dalton (kDa) protein [9]. Similarly, the fpv126 (VLTF-1) gene encodes for a late transcription factor (VLTF-1), which is highly conserved amongst all poxviruses. It is the protein most conserved between FWPV and canarypox virus, with 95% amino acid identity [16]. The fpv175–176 (A11R-A12L) gene region encodes for a non-structural protein involved in virion formation [56] and morphogenesis [57]. Moreover, at the time of writing, fpv140 (H3L) sequences obtained from two FWPV strains in the present study showed 100% nucleotide identity to 18 subclade A1 sequences available in GenBank, suggesting that fpv140 (H3L) is also a highly conserved gene. In a previous study, comparison of complete genomes revealed 118 mutations between the European Fowlpox virus (FP9) and the pathogenic US strain (FPVUS) [58].Jarmin et al. also described the potential of utilizing a polymorphic gene region, fpv241 (H9 locus), to distinguish between field strains and vaccine strains of FWPV [59]. These findings suggest that FWPV populations could be heterogeneous and that known markers based on conserved genes have limitations in distinguishing FWPV strains. Whole-genome sequencing is not feasible for all FWPV isolates because of its large genome (~289 kb). Our findings, therefore, highlight the need for other, reliable polymorphic markers designed based on sequence information from a wide geographical scale to facilitate the understanding of Avipoxvirus diversity and transmission network in a particular area.

Having found closely related FWPV strains between the affected chicken and mosquitoes in the vicinity, we were intrigued to determine whether mosquito species harbouring FWPV were known mechanical vectors. Previous studies have shown that mosquito species, including Cx. pipiens, Cx. quinquefasciatus and Aedes aegypti, act as mechanical vectors of Avipoxvirus transmission [19–21]. Taxonomic and COI-based DNA barcoding confirmed that four pools of FWPV-positive mosquitoes were Cx. pseudovishnui, Culex spp. (vishnui group) and Cq. Crassipes. Culex spp. and Coquellitidea spp. mosquitoes are known to bite birds, including chicken [24, 60–62]. Previous studies have shown that birds can be infected with different strains of Avipoxviruses, with variable pathogenicity [6, 63, 64]. Consequently, bird-biting mosquitoes are at greater risk of acquiring these viruses than those that prefer to feed on non-avian hosts. Therefore, we determined whether the FWPV-positive mosquito pools (n=4) were positive for avian blood using a PCR-sequencing assay. Results showed that all pools were positive for avian DNA and none was positive for mammalian DNA (Table 1). Taken together, these observations suggested a role for Cx. pseudovishnui, Culex spp. (vishnui group) and Cq. crassipes mosquitoes as mechanical vectors of FWPV transmission. Because the FWPV infections described in this study were three separate events that occurred over a 2-year period with no evidence of interim transmission, one probable source of infection was other infected birds in the vicinity. In fact, the study location is home to numerous resident birds and is an annual stop-over site along the East Asian–Australasian Flyway for migratory birds. In this context, it is plausible to assume that wild birds could be the source of FWPV and that bird-biting mosquitoes could act as bridging mechanical vectors.

Even though molecular tools, such as PCR-sequencing assays, have been widely used to screen for avipoxviruses and feeding host DNA [37, 39, 41] in mosquitoes, reports in the literature on the detection of virus and host DNA in the same pools of mosquitoes are sparse. This is mainly because viral DNA persists longer than host DNA, making it difficult to decipher feeding host species by analysis of mosquito blood meals. It is evident that mosquitoes harbour avipoxviruses for more than one month [65, 66]. In contrast, host DNA degrades faster due to blood meal digestion so that the sensitivity of PCR sequencing-based assays drops drastically more than 3 days after a blood meal and amplification is less likely beyond 7 days [67, 68]. The blood meal assay used in the present study has sufficient sensitivity to amplify feeding host DNA up to 3 days after feeding (limit of detection of 0.2 ng µl⁻¹; 0.8 ng per reaction) [51], which is comparable to assays reported elsewhere [37, 69]. Unlike universal primers that tend to co-amplify mosquito genomic material, resulting in unusable sequence data for host identification [39], our assay primers were designed to minimize non-specific amplification with the mosquito and its microbiome DNA [51].

**Table 1.** Molecular analysis of feeding host DNA in four FWPV-positive pools of mosquitoes

<table>
<thead>
<tr>
<th>Mosquito species* (no. in each pool)</th>
<th>Feeding avian host species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coquellitidea crassipes (n=1)</td>
<td>Otus semitorques</td>
</tr>
<tr>
<td>Culex pseudovishnui (n=1)</td>
<td>Corvus macrorhynchos</td>
</tr>
<tr>
<td>Culex pseudovishnui (n=3)</td>
<td>Corvus macrorhynchos</td>
</tr>
<tr>
<td>Culex spp. (vishnui group) (n=3)</td>
<td>Corvus macrorhynchos</td>
</tr>
</tbody>
</table>

*Mosquito species were identified by using taxonomic keys and DNA barcoding of COI gene. Each pool consisted of mosquito specimens of the same species.
February and July 2016, despite being vaccinated against FWPV with freeze-dried live vaccine (Diftosec CT, DCEP25 modified strain; Merial, Lyon, France). According to the manufacturer’s instructions, it is recommended that chicks receive booster doses three months after the first dose and every subsequent year (http://www.vetvac.org/find.php?ID=2201). None of our sentinel chickens received booster doses, highlighting the importance of administering booster doses as a preventive measure against Avipoxvirus, especially in areas with persistent virus transmission. In addition, the protection of chicken cages with mosquito-proof sieves is highly recommended because it has been shown to decrease morbidity due to avian pox, from 81.7 to 25.8 % in peafowl chicks [72].

In conclusion, the present study exemplifies the utilization of molecular tools to study transmission networks of pathogens affecting avian populations. The study also highlights
the potential role of mosquitoes in sustaining Avipoxvirus transmission, especially in bird sanctuaries and surrounding areas. To the best of our knowledge, this is the first report of FWPV infection among chickens in Singapore. The understanding of vector–host interactions has been proved crucial in predicting transmission patterns of avian pox, and has important implications for the development of efficient vector control policies to protect poultry farming from Avipoxvirus outbreaks.

**METHODS**

**Study area and subjects**

The study was conducted in the north-western part of Singapore. Since the study location is an annual stop-over site along the East Asian–Australasian Flyway for migratory birds and is home to numerous resident birds, the location was used as a sentinel site for the surveillance of Japanese encephalitis virus (JEV) and West Nile virus (WNV). For
this purpose, we have stationed 3–5 chickens (aged 3–4 weeks) in a metal cage at the same site since 2013. These sentinel chickens were obtained from flocks maintained at the Singapore Science Centre and the Agri-Food and Veterinary Authority of Singapore, and were certified free from major poultry diseases, including fowl pox. Blood samples and nasopharyngeal swabs were taken by trained veterinarians from each chicken once per month for the screening of JEV and WNV.

Sample collection from infected chickens

Three chickens stationed at the study location developed raised nodular skin lesions around the eyes and comb on three separate occasions, once in January 2014, followed by two subsequent events in February and July 2016. Skin lesions were suggestive of the cutaneous form of Avipoxvirus infection. All three chickens were euthanized as required by the Institutional Animal Care and Committee (IACUC) protocol. Samples from skin lesions were collected during
the post-mortem examination of infected birds for further analyses.

**Virus isolation from skin lesions of infected birds and the extraction of viral DNA**

Avipoxvirus isolation was carried out on tissue material obtained from skin lesions by inoculation into SPF embryonated chicken eggs via CAMs. Tissues were minced to make a suspension of approximately 10% (v/v) in the viral transport media. The suspension was further treated with an antibiotic cocktail for 1 h and centrifuged at 800 g for 10 min at 4°C. The supernatant was then inoculated into 10–12-day-old developing chicken embryos via the CAM route. Eggs were incubated at 37°C for 7 days, and were examined for the presence of pock lesions or a generalized thickening of the CAM. Viral DNA was extracted from CAMs using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions.

**Detection of Avipoxvirus in infected chickens using PCR assay**

Avipoxvirus-specific PCR assay was performed using a primer pair adopted from Huw Lee and Hwa Lee [9] based on the fpv167 (P4b) sequence of FWPV strain HP444 (forward primer: 5′-CAGCAAGGTGCTAAACACAA-3′ and reverse primer: 5′-CGTGATCTAAAACCGCGAATA-3′ [9]. Three additional gene loci, namely fpv126 (VLTF-1), fpv175–176 (A11R-A12L) and fpv140 (H3L), were amplified using primers described elsewhere [12, 52].

Samples (n=3) were assayed in a 20 µl reaction mixture containing 10 µl of Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA), 0.25 µmol of each primer and 4 µl of template DNA extracted from CAMs. The thermal profile for the fpv167 (P4b) and fpv175–176 (A11R-A12L) gene-specific PCRs consisted of an initial denaturation at 98°C for 10 s, followed by 35 cycles of denaturation at 98°C for 5 s, annealing at 56°C for 8 s, extension at 72°C for 10 s and a final extension step at 72°C for 60 s. The same profile was used for the amplification of gene fpv126 (VLTF-1), except for the annealing at 58°C. For amplification of the fpv140 (H3L) gene region, primers were annealed at 58°C and the cycle extension time was extended to 1 min at 72°C, with a 2 min final extension at 72°C. DNA extracted from the FWPV vaccine strain (DCEP25 modified strain) was used as the positive control and nuclease-free water was used as the negative control. All PCR products were sequenced to characterize the clade/subcline of avipoxviruses by phylogenetic analyses as described below.

**Mosquito trapping and taxonomic identification**

Mosquito trapping was conducted one month after the post-mortem examination of the first infected chicken diagnosed in January 2014. The 2-day trapping session comprised one overnight trapping (16 h, including 2 h of dusk and 2 h of dawn) at two sites within a 50 m radius of the chicken cage. Centre for Disease Control (CDC) light traps baited with dry ice were used for night trapping. Daytime trapping was conducted using BG Sentinel traps (BioGents AG, Germany). Mosquitoes were transported to the laboratory on ice. All mosquitoes were identified to the species level whenever possible, and to the species group level for morphologically similar species by using established taxonomic keys [43, 44]. Damaged specimens were identified to the genus level. Samples of the same species from the same trap site were pooled (1–3 mosquitoes per pool) and were kept at −80°C until further analysis.

**Extraction of DNA from mosquito pools**

Each mosquito pool was homogenized using a stainless steel bead in 500 µl of universal transport media (Copan Diagnostics, USA) in the Mixer Mill MM 400 (Retsch Technology GmbH, Germany) at 30 Hertz for 30 cycles. Each homogenate was centrifuged at 4000 g for 1 min. DNA was extracted from mosquito homogenates using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNA was stored at −20°C until further use.

**Screening of mosquito pools for Avipoxvirus**

All mosquito pools (n=154) were screened for the presence of Avipoxvirus using PCR as described above for the detection of the virus in infected chickens. DNA extracted from each mosquito pool was used as the template. All PCR products of positive pools were sequenced to characterize the virus clade/subcline by phylogenetic analyses as described below.

**DNA barcoding of mosquitoes in Avipoxvirus-positive pools**

Besides taxonomy-based identification, mosquitoes in Avipoxvirus-positive pools (n=4) were subjected to DNA barcoding for further species confirmation. A 735 base pair (bp) region flanking the mitochondrial COI gene was PCR amplified using a published primer pair, 5′-GGA TTGGAAATTTGATTAGTTCCCTT-3′ and 5′-AAAAA TTTTAAATTCCGTTGGAACG-3′ [45, 46]. Samples were assayed in a 50 µl reaction mixture consisting of 1.5 mM MgCl2, 0.2 mM dNTPs, 10 µl of GoTaq Flexi buffer (Promega Corporation, USA), 1.25 µl (0.3 µM) of forward and reverse primers, 1.5 U Taq DNA polymerase and 5 µl of template DNA, using a thermal profile described previously [46]. Mosquito species identity was determined by similarity search and phylogenetic analysis of COI sequences as described below. Results were compared to taxonomical identification for further confirmation.

**Amplification of avian and mammalian DNA in Avipoxvirus-positive mosquito pools**

PCR sequencing-based assay was used for the identification of avian and mammalian DNA in blood meals taken by mosquitoes in Avipoxvirus-positive pools [51]. Two pairs of primers (Table S1, available in the online version of this article) were used to amplify avian and mammalian DNA as described elsewhere [51]. These primers were originally designed to target regions of 16S and 12S rRNA genes.
conserved among non-avian and avian hosts, respectively, but variable in mosquitoes, to minimize primer cross-reactivity with mosquito DNA. Avian and mammalian DNA from an in-house reference panel were used as the positive controls, and nuclease-free water was used as the negative control. Amplicons were sequenced to determine the species/genus identity of host DNA by a similarity search in the Basic Local Alignment Search Tool (BLAST) as described below.

Validation of primer pair used for the detection of avian DNA in Avipoxvirus-positive mosquito pools

In addition to assay performance data available on 17 avian and non-avian species [51], we further validated the avian primer pair using a panel of 22 bird specimens (Table S2). Bird carcasses were collected as part of the zoonotic eco-epidemiology programme, which is a multi-agency collaboration between the National Environment Agency (NEA) and Singapore and National Parks Singapore (NParks). These were identified based on external morphological features. DNA was extracted from avian liver tissues using the DNeasy Blood and Tissue Kit (Qiagen, Germany) kit according to the manufacturer’s instructions. DNA in each sample was quantified using the Qubit dsDNA BR Assay Kit in Qubit 2.0 fluorometer (Thermo Fisher Scientific, USA). The level of detection of the assay was determined using a tenfold serial dilution, ranging from 20 to 0.02 ng µl⁻¹ of DNA obtained from ten specimens that represented different types of avian carcase available (Table S2).

Sequencing of amplified products

All PCR products generated from Avipoxvirus, mosquito and feeding host DNA were visualized in 1.5 % agarose gels stained with GelRed (Biotium Inc., USA). The desired fragments were gel-excised and purified using a QIAquick gel extraction kit (Qiagen, Germany) according to the manufacturer’s protocol. Purified products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) at a commercial facility. Consensus sequences were obtained by assembling raw sequencing data in the Lasergene 8.0 software suite (DNASTAR Inc., USA).

Determining species/genus identity of feeding host DNA in Avipoxvirus-positive mosquito pools

Consensus sequences obtained from amplified products of blood meal PCR assay were searched against the GenBank database using the BLASTn application in BLAST (www.ncbi.nlm.nih.gov) [73]. BLASTn hits with the lowest expected (E) values were taken as the closest genus/species identity. It is noteworthy that, at the time of writing, species/genus identification was dependent on the extent of genus/species coverage provided by sequence data available in the GenBank database.

Comparison and phylogenetic analyses of Avipoxvirus sequences derived from chicken and mosquito pools

Consensus sequences of gene regions fpv167 (P4b), fpv126 (VLTF-1), fpv175–176 (A11R-A12L) and fpv140 (H3L) were aligned using the Clustal W algorithm [74] implemented in BioEdit v7.0.5 software [75]. The percentage similarity of gene sequences fpv176 (P4b), fpv126 (VLTF-1), fpv175–176 (A11R-A12L) and fpv140 (H3L) between chicken- and mosquito-derived avipoxviruses was calculated using BioEdit v7.0.5 software [75]. Phylogenetic trees were constructed separately for gene regions fpv167 (P4b), fpv126 (VLTF-1) and fpv175–176 (A11R-A12L) using the maximum likelihood method as implemented in the MEGA 6.06 software suite [76]. fpv140 (H3L) was not included in the phylogenetic analysis because it had been used as a size polymorphic marker for clade classification. The most suitable substitution model was determined using the jModelTest [77, 78]. Accordingly, the Hasegawa–Kishino–Yano (fpy167; P4b) model with gamma-distribution (HKY+G), the Tamura Nei model (fpv126; VLTF-1) with gamma-distribution (TrN+G) and the Tamura Nei model (fpv175–176; A11R-A12L) with invariant sites distribution (TrN+I) were used for the construction of trees. Trees were formatted in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

Analysis of cytochrome oxidase subunit I gene-based DNA barcodes for the identification of mosquitoes in Avipoxvirus-positive pools

Based on taxonomic identification, COI gene sequences of the same species as each mosquito pool were retrieved from the NCBI database. COI sequences generated from Avipoxvirus-positive mosquito pools (n=4) were aligned against retrieved sequences using the Clustal W algorithm [74] implemented in BioEdit v7.0.5 software [75]. The alignment was trimmed (337 bp) to provide a uniform length for each sequence. Pairwise distance was calculated using the Kimura-2 parameter in the MEGA 6.06 software suite [76] to ensure that the intra-species barcode gap remained below 3 % [79]. To determine intra-species clustering, a neighbour-joining tree was constructed using the Kimura-2 parameter substitution model with gamma-distributed rates and the nearest neighbour interchange heuristic search method as implemented in the MEGA 6.06 software suite [76]. Reliability of the internal nodes of trees was assessed by the bootstrap method based on 1000 replicates.

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Author contributions

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Use of chickens for the sentinel surveillance of JEV and WNv, and the protocol for euthanization of chickens for post-mortem examination, were approved by the Institutional Animal Care and Committee (IACUC) of EHI, NEA, Singapore (IACUC005).

References


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