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Research paper

Evolution of H9N2 low pathogenic avian influenza virus during passages in chickens

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ABSTRACT

The process of avian influenza virus (AIV) evolution in a new host was investigated in the experiment in which ten serial passages of a turkey-derived H9N2 AIV were carried out in specific pathogen free chickens (3 birds/group) inoculated by oculonasal route. Oropharyngeal swabs collected 3 days post infection were used for inoculation of birds in the next passage and subjected to analysis using deep sequencing. In total, eight mutations in the consensus sequence were found in the viral pool derived from the 10th passage: four mutations (2 in PB1 and 2 in HA) were present in the inoculum as minority variants while the other four (2 in NP, 1 in PA and 1 in HA) emerged during the passages in chickens. The detected fluctuations in the genetic heterogeneity of viral pools from consecutive passages were most likely attributed to the selective bottleneck. The genes known for bearing molecular determinants of the AIV host specificity (HA, PB2, PB1, PA) contributed most to the overall virus diversity. In some cases, a fast selection of the novel variant was noticed. For example, the amino-acid substitution N337K in the haemagglutinin (HA) cleavage site region detected in the 6th passage as low frequency variant had undergone rapid selection and became predominant in the 7th passage. Interestingly, detection of identical mutation in the field H9N2 isolates 1-year apart suggests that this substitution might provide the virus with a selective advantage. However, the role of specific mutations and their influence on the virus adaptation or fitness are mostly unknown and require further investigations.

1. Introduction

Influenza A virus is a highly diverse pathogen affecting a wide range of host species (Alexander, 2007). The virus genome consists of eight negative-sense RNA segments, each coding one or two proteins. The surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), are the basis of division of type A influenza viruses into subtypes (Swayne et al., 2013). There are 18 subtypes of HA and 11 subtypes of NA distinguished so far, and avian hosts harbour the highest diversity of these subtypes (H1-H16 and N1-N9) (Swayne et al., 2013). Based on the clinical outcome of infections with avian influenza virus (AIV) in chickens, two forms are distinguished: low pathogenic AIV (LPAIV) and highly pathogenic AIV (HPAIV). All AIV subtypes occur naturally in wild aquatic birds as LPAIV, whereas the H5 and H7 subtypes, when propagating in gallinaceous poultry, are capable of becoming highly pathogenic and cause severe disease (Swayne and Pantin-Jackwood, 2006). LPAI viruses show a high level of adaptation to their natural reservoir hosts enabling efficient virus replication with no or minimal detriment to the bird (Kuiken, 2013). Due to the low adaptation of wild-bird origin LPAIV to gallinaceous poultry, the emergence of adaptive mutations is a prerequisite for sustained circulation of virus in the poultry population. Mutations involved in the process of AIV's adaptation to poultry are located mainly in the HA and NA genes, and include changes in the receptor binding site (RBS), modifications of glycosylation profile in the HA, and deletion in the stalk region of NA (Munoz et al., 2016). The main virulence determinant for the H5 and H7 subtypes *i.e.* the polybasic cleavage site in the HA also emerges in gallinaceous poultry (Abdelwhab et al., 2013). The emergence of adaptive mutations is a combined effect of the activity of error-prone viral polymerase and the selection pressure in the host, with other factors involved such as transmission bottlenecks (Varble et al., 2014). Recent studies revealed higher mutation rate in the influenza virus than previously estimated, which translates into 2–3 mutations introduced into each newly synthetized viral genome (Pauly et al., 2017). The high mutation rate leads to the generation of heterogeneous virus population consisting of genetically and phenotypically different variants, termed

Abbreviations: SPF, specific pathogen free; AIV, avian influenza virus

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quasispecies (Andino and Domingo, 2015). Genetic diversity is highly important for the adaptability of AIV since more complex virus populations are more capable of surviving in novel environmental conditions. The rapid development of sequencing technologies in recent years has provided tools to investigate virus population structure in a variety of clinical and experimental specimens enabling more comprehensive studies on the dynamics of viral infections, virus diversity, evolution, pathogenicity and transmission (Debbink et al., 2017; Moncla et al., 2016; Sobel Leonard et al., 2016; Xiao et al., 2018). In the case of avian influenza virus, studies employing high-throughput sequencing enabled tracking the evolution of low pathogenic AIV into highly pathogenic forms (Dietze et al., 2018; Monne et al., 2014; Seekings et al., 2018), emergence of adaptive mutations in the field outbreaks (Croville et al., 2012), reconstruction of transmission network (Fusaro et al., 2016), and analysis of virus population diversity (Ramakrishnan et al., 2009; Milani et al., 2017).

Adaptation of LPAIV to poultry has been studied experimentally by passaging the virus followed by sequencing and comparison of pathogenicity of pre- and post-passage viruses (Ito et al., 2001; Sorrell and Perez, 2007; Hossain et al., 2008). However, limited studies have taken into account the composition of virus population and differentiated mutations that were present in the inoculum stock from those that arose independently during infection (Dlugolenski et al., 2011; Ridenour et al., 2015; Jegede et al., 2018). The diversity of virus population generated by random mutations is modified by non-random selection process that either promotes advantageous mutations or purifies the deleterious ones. It was shown that strong bottleneck acting on a virus population during infection of a novel host may result in a loss of genetic diversity, and thus impair the adaptation (Zaraket et al., 2015). Knowledge about the within-host genetic diversity of AIV in avian hosts and how it affects virus adaptation and long-term evolution is still limited. To study the changes in the composition of virus population over a series of transmissions, we performed serial passages of a turkey-origin H9N2 avian influenza virus in chickens and characterized the virus populations in the parental isolate and those derived from each passage to trace the emergence and selection of mutations and describe changes in the virus population complexity.

2. Material and methods

2.1. Virus

Low pathogenic avian influenza virus H9N2 subtype (A/turkey/Poland/14/2013) isolated from a clinical outbreak in turkeys in Poland in 2013 (Śmietanka et al., 2014) was used in the study. The virus was propagated in 9–11-day-old embryonated specific pathogen free (SPF) chicken eggs. The virus stock from the 2nd egg passage was titrated in eggs to determine the 50% embryo infectious dose (EID_{50}) and was used in the first inoculation cycle.

2.2. Birds and experimental infections

Experiments were performed in 2–3-week old SPF chickens. In the first round, three chickens were inoculated oculonasally with the virus at the dose of 10^6 EID_{50} in 0,1 ml. After three days, oropharyngeal swabs were collected from each bird using swabs with viral transport medium (Copan, Italy). The fluid from all swabs was pooled and used for inoculation of another group of three chickens (0,3 ml per bird). At 3 dpi swabs were collected and used to infect another three chickens. A total of ten passages were performed in this way. The animal experiments were approved by the Local Ethical Committee (decision no. 88/2015).

2.3. Evaluation of viral RNA quantity

Viral RNA was extracted from the allantoic fluid of virus stock used for inoculation, from pooled swabs from each passage and from each swab separately using Viral Mini Kit (Syngen, Poland) according to the manufacturer's protocol. The amount of viral RNA in each sample was tested in real time RT-PCR with primers and probe targeting the M gene of influenza virus (Nagy et al., 2010) using QuantiTect Probe RT-PCR Kit (Qiagen, Germany). The final concentration of each primer and probe was 0.6 µM and 0.2 µM, respectively. The tests were performed in 7500 Fast Real Time PCR System (Applied Biosystems, USA) with the following thermal protocol: 1 stage of 50 °C for 30 min, 1 stage of 95 °C for 15 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 10 s. The quantity of viral RNA in swabs was determined with the standard curve generated from *in vitro* transcribed RNA standards run in parallel with the tested samples and expressed as the copy number per 1 µl of RNA.

2.4. High-throughput sequencing protocol

Deep sequencing was performed for the virus stock used for inoculation (IN) and swab pool from each passage (P1-P10). The viral genome was amplified in RT-PCR according to Zhou et al. (2009) with modification by Watson et al. (2013) to increase the yield of long genome segments. Briefly, two RT-PCRs were performed for each sample, one with primers MBTuni-12 and MBTuni-13, and another with primers MBTuni-12G and MBTuni-13, using SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase (ThermoFisher Scientific). The products of both reactions were pooled and purified with QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's manual. Libraries were prepared using Nextera XT DNA Library Preparation Kit and Nextera XT Index Kit (Illumina) and sequenced in MiSeq (Illumina) with MiSeq Reagent Kit v3 enabling paired-end sequencing of 300 bp (Illumina). The raw fastq files are available in the BioProject database under accession number PRJNA529216.

2.5. Analysis of sequencing data

The quality of obtained reads was assessed in FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Fastq files were processed in Trimmomatic (Bolger et al., 2014) to remove adapter sequences, low-quality fragments (4-base-pair sliding window with Phred quality threshold set to 30) and reads shorter than 50 bases. Cleaned reads were aligned in BWA (Li and Durbin, 2009) with the consensus sequence of the virus inoculum used as the reference genome. The BAM files were filtered using Samtools (Li et al., 2009) to remove PCR duplicates. Then the reads were realigned in LoFreq (Wilm et al., 2012) to correct mapping errors. Variant calling was performed using LoFreq with the following parameters to minimize the erroneous calls: mean mapping quality of ≥30, mean Phred score of ≥35, coverage of ≥500 and minimum frequency of 2%. The analysis was repeated with the frequency threshold of 1% and any variant with frequency 1–2% that was present in other sample at > 2% was also considered reliable and included in the further analysis. Variant calling was repeated with VarScan (Koboldt et al., 2009) and the results were compared with those obtained with LoFreq. Consensus sequences and coverage date were generated with Samtools. To assess the complexity of virus population, Shannon entropy was calculated as previously described (Milani et al., 2017) using percentage values obtained with LoFreq.

3. Results

3.1. Quantity of virus RNA in swabs

The analysis viral RNA quantity in individual swabs showed efficient virus replication throughout the passages. All swabs from P1-P2 and P6-P10 were positive for viral RNA, but in P3, P4 and P5 only two birds shed the virus (Fig. 1).

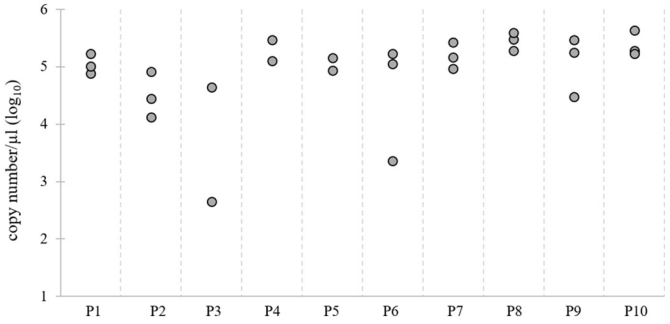

Fig. 1. Viral RNA load in individual oropharyngeal swabs from each passage.

3.2. Virus population diversity

The deep sequencing approach was used to study fluctuations in the complexity of virus population during passages. The complete genome sequences were obtained for each pooled sample. The average coverage depth for individual segments ranged from 1089 to > 30,000 with the highest values observed for the M and NS segments (Supplementary Table 1). The coverage plots are presented in the supplementary material. The analysis of the diversity of virus populations was based on the variant positions identified by both LoFreq and VarScan, except for six positions (PB1–201, NP-28 and 45, NA-47 and 240, NS-66) when variants were identified only in VarScan but were included in the analysis since they were found in consecutive passages (Supplementary Table 2).

The analysis of the polymorphic positions and the complexity of virus population showed high heterogeneity in the inoculum stock and in the first and second passage (Fig. 2). However, the high virus diversity in IN and P1 was caused mainly by the variation in the HA gene while in P2 the heterogeneity in polymerase complex genes (PB2, PB1, PA) contributed mostly to the overall virus population complexity. Twenty-four polymorphic positions were detected in the inoculum isolate with the variant frequency ranging from 1.0% to 41.3% (Supplementary Table 2). Of them, eleven were nonsynonymous mutations located mainly in the HA gene. Interestingly, the inoculum contained variants at neighbouring positions 433 and 434 in the HA gene which resulted in a mixture of three amino acid variants at position 145 of the HA protein (arginine – 57.7%, leucine – 41.3%, serine – 1.0%). The first passage resulted in the elimination of nine minority variants from the inoculum and emergence of additional 10 low-frequency polymorphisms. In P2 and P3 further selection of minority variants from the parental isolate led to fixation of four substitutions while the remaining were eliminated. This coincided with a decrease in virus diversity in P3, and in P4 highly homogeneous virus population was present, with the only variability detected in the M gene (Fig. 2). The virus complexity increased in P5 as novel variants emerged, and in subsequent passages remained at a similar level.

A total of 70 mutations arouse independently from the inoculum stock of which four were selected to the dominant level and maintained until the last passage.

Considering each gene separately, the highest diversity was identified for the HA gene in the inoculum and P1, and showed a severe drop in P2, when only one polymorphic position from the inoculum persisted, further selected for in the following passage (Fig. 2, Supplementary Table 2).

Table 1
Mutations in the consensus sequences of virus populations from each passage with the determination of the passage in which the mutation was first observed as a minority variant.

Gene	Passage (frequency)	Nucleotide variant	Amino acid change	First detection as a minority variant (frequency)
NP	P3 (90.4%)	G474A	–	P2 (18.7%)
	P7 (79.9%)	T891C	–	P6 (38.7%)
PB1	P3 (100%)	A623G	K208R	IN (5.0%)
	P3 (100%)	T1911C	–	IN (7.6%)
PA	P10 (90.7%)	G1172A	R391K	P8 (10.5%)
HA[a]	P1 (80.7%)	C1334T	T445I	IN (14.5%)
	P2 (78.1%)	C433A	R145S	IN (1.0%)
	P7 (87.9%)	T1011G	N337K	P6 (38.5%)

[a] H9 pre-HA0 numbering.

3.3. Mutations in the consensus sequences

A total of eight changes at the consensus level of virus sequence occurred during passages (Table 1). As previously mentioned, four mutations were already present in the inoculum virus as low-frequency variants while the other four emerged in the infected chickens. Three nonsynonymous mutations were detected in the HA gene, two of them were present in the inoculum and their frequency increased to the dominant level in the early passages. Of the three amino acid variants at position 145 in the HA protein only variant coding for serine survived and was selected during passages (Table 1). The mutation at position 1334 was also rapidly selected since in P1 it constituted for > 80%. In contrast, the mutation at position 1011 was first detected in P6 as a relatively high frequency variant (~40%) and accounted for almost 90% in the following passage. This mutation conferred a change in the HA cleavage site (HACS), from PAASNR*GLF to PAASKR*GLF motif.

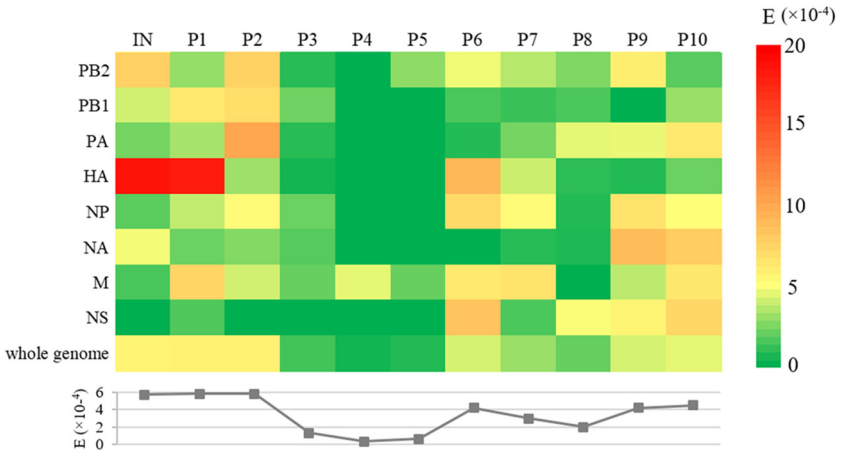

Fig. 2. Heatmap presenting virus population complexity in the inoculum stock and each passage at the level of individual genes and the whole genome. The colour reflects the value of Shannon entropy as indicated in the scale bar. The graph below shows fluctuations in the whole genome entropy values.

Interestingly, the same modification was observed in the field strains of AIV H9N2 isolated approximately 1 year later than the strain used in the experimental passages (Świętoń et al., 2018). Two mutations were identified in the PB1 gene, including one non-synonymous mutation. Both mutations were found in the inoculum material as variants constituting 5.0–7.6%, which dominated the virus population in P3. Two synonymous mutations were identified in the NP gene, both emerged in chickens. The nonsynonymous mutation in the PA gene also emerged during passages.

4. Discussion

The process of adaptation of AIV to new species is usually reproduced under experimental conditions by performing a series of virus passages in selected bird species. Passages of wild-bird origin AIVs in quail pointed to this species as an intermediate host supporting adaptation of virus to other species of gallinaceous poultry and even mammals (Sorrell and Perez, 2007; Hossain et al., 2008; Giannecchini et al., 2010; Yamada et al., 2012). Similar studies have contributed to the understanding of the importance of gallinaceous poultry in the generation of highly pathogenic viruses (Ito et al., 2001; Diederich et al., 2015). Multiple mutations located in different genome segments were reported to occur during passages, indicating that adaptation to a new host is a multifactorial process. However, substitutions in the HA receptor binding site and stalk deletion in the NA are the most important host-specificity determinants for gallinaceous poultry (Munoz et al., 2016). In the present study, the pre-passage virus was isolated from turkeys and already possessed molecular traits indicative of adaptation to gallinaceous birds (Świętoń et al., 2018). Additionally, the virus stock was prepared in SPF chicken embryos which could contribute to the high genetic diversity of the virus inoculum and some mutations may have emerged at that stage. However, it was shown that passaging of mallard-origin viruses in embryonated chicken eggs is not disadvantageous comparing to isolation in eggs from the homologous species (*i.e.* mallard), neither in terms of virus titre nor the number of mutations arising in the virus genome (Järhult et al., 2015). Nevertheless, passaging of the virus in chickens resulted in eight mutations, mostly found in the HA gene. The substitution in the HA cleavage site was the most noteworthy one since the identical trait was observed in the field isolates of AIV H9N2 from poultry in Europe (Świętoń et al., 2018). To exclude the possibility that this mutation was present in the viral pool of the parental isolate, deep sequencing and minority variant analysis was performed for each passage and the inoculum strain. This substitution was first detected in the sixth passage as a variant with relatively high frequency (almost 40%) and dominated the virus population in the following passage. Variant calling repeated without any frequency threshold confirmed its absence in all previous passages and in the inoculum stock which proves its independent emergence, while the rapid positive selection and presence in the field strains indicates it could confer an advantage in gallinaceous poultry. The previous study showed that a field isolate with the PAASKR*GLF needs supplementation with exogenous trypsin for efficient replication in chicken embryo fibroblasts and is low pathogenic for chickens as indicated by IVPI of 0.0 (Świętoń et al., 2018). Since there are no *in vivo* studies that would elucidate the significance of this mutation, neither in turkeys, nor in chickens in the present study, further experiments are needed to reveal if this mutation causes any changes in the virulence or replication efficiency of the virus. The remaining mutations in the HA protein were an effect of selection of variants from the inoculum stock. Three amino acids at position 145 of HA in the parental virus were identified and the variant possessing serine outgrew the virus population in early passages. Similarly, isoleucine at HA position 445 and both variants in PB1 were selected in initial passages. Several studies on the adaptation of avian influenza to novel host species also demonstrate the importance of discrimination between the mutations that emerged *de novo* and those selected from the parental virus population (Dlugolenski et al., 2011; Croville et al., 2012; Mancera Gracia et al., 2017; Ridenour et al., 2015). Passages of two H5 LPAI viruses of poultry and wild-bird origin in chickens resulted in several mutations in the HA and NA proteins. Most of the detected mutations were found in the initial passages, which led the authors to conclude that they could be present in the inoculum isolate as low-frequency variants, which was then confirmed by further experiments and deeper analysis of chromatograms (Dlugolenski et al., 2011). Similarly, early selection of virus subpopulations present in the pre-passage isolates was observed during passages of H5 LPAIVs in ducks (Ridenour et al., 2015). In another study with H9N2 virus showing heterogeneity at position 226 of the HA, glutamine was outcompeted by leucin during passages of the virus in chickens (Jegede et al., 2018). Mutations contributing to successful crossing the host species barrier may be already present in the virus population in the donor organism, as it was shown for the NA stalk deletion in AIV H6N1 outbreak in poultry (Croville et al., 2012).

The positive selection of minority variants from the inoculum in the early passages was accompanied by the decline in the virus population diversity. Simultaneous elimination of other minority variants and lack of newly emergent ones resulted in very low viral population complexity in P4. Initially, the virus population diversity was comparable to that in the inoculum and the most pronounced decrease in the entropy value was observed between P2 and P3, when a complete selection of most of the inoculum-origin mutations occurred. Studies on the within-host diversity of influenza virus in avian hosts are scarce and to our knowledge this is the first report on the fluctuations of virus heterogeneity during experimental passages in birds. Similar studies on influenza virus in mammals (ferrets, pigs, horses) showed that diversity of virus population in the recipient hosts is affected by transmission bottleneck (Murcia et al., 2010, 2012; Wilker et al., 2013; Moncla et al., 2016), and its stringency depends on the transmission route (Varble et al., 2014; Frise et al., 2016). In the present study, birds were directly inoculated with the virus suspension from the previous passage, so the decrease in the virus population complexity cannot be attributed to sequence-independent transmission bottleneck. The reduction in the virus diversity could result from the selective bottleneck acting on the virus population and especially on the HA protein in the early passages, but the lack of virus replication in one of the birds in P3-P5 could also contribute to this outcome. The bottleneck could occur due to positive selection, but the effect could be enhanced by negative selection of specific variants (*e.g.* at HA positions 73, 113, 434 and 1344). It was shown that both positive and negative selection might shape the evolution of a virus population in a single host (Illingworth et al., 2014). It is worth noting that some polymorphisms that arose during passages to a relatively high frequency were eliminated in subsequent passages (*e.g.* PA 1323, HA 1570, M 896). Since pooled swab samples were analysed, it is possible that these mutations emerged with the high frequency in a single bird and consequently were present in the pooled sample.

Studies investigating adaptation of LPAIV of different origin (wild birds or chickens) to chickens and ducks showed more extensive genetic changes in the strains passaged in hosts genetically distant from the species from which the virus was derived (Dlugolenski et al., 2011; Ridenour et al., 2015). In the present study, passages of the turkey-origin virus were performed in chickens. Both bird species are closely related at the order level (Galliforms) but different enough at the genetic and phenotypic level to explain why viruses highly adapted in turkeys may require additional modifications when passaged in chickens. Turkeys are generally more susceptible to AIV infection than chickens (Pillai et al., 2010) and these differences could result from the structure and distribution of AIV receptors (Pillai and Lee, 2010) or from distinct patterns of early immune response in the two species. These differences may account for the fact that minority variants present in the turkey-derived inoculum were subject to rapid selection during passages in chickens, apparently giving some adaptive advantages in the new host. However, it is also possible that some of these mutations emerged during passages of virus in chicken eggs. On the

other hand, the relationship between chickens and turkeys is close enough to explain the emergence and fast selection of mutations in the viral genome that increase virus fitness in both species. Previous studies showed that mutations such as deletion in the NA stalk, introduction of additional basic amino acids in HACS of H5 and H7 subtypes are advantageous in both chickens and turkeys (Li et al., 2011; Abdelwhab et al., 2013). A lack of experimental evidence of better adaptation or increased fitness of the passaged virus is a limitation of the present study. The significance of identified mutations needs to be verified in *in vivo* studies in which replication efficiency, transmission and tissue tropism of the pre- and post-passage isolates will be compared. Previous experimental studies using A/ty/PL/14/13 showed the low virulence of this strain for SPF chickens, but despite the lack of clinical symptoms, the virus replicated with high efficiency, allowing transmission to contact birds (Śmietanka et al., 2014). This suggests that chickens can be asymptomatic reservoirs and virus circulation in this species may result in the emergence of mutations that could be also important for adaptation to turkeys.

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Declaration of Competing Interest

The authors declare no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103979.

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