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Comparison of nucleic acid extraction methods for next-generation sequencing of avian influenza A virus from ferret respiratory samples

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ABSTRACT

Influenza A virus is a negative-sense RNA virus with a segmented genome consisting of eight RNA segments. Avian influenza A virus (AIV) primarily infects avian hosts and sporadically infects mammals, which can lead to adaptation to new species. Next-generation sequencing (NGS) of emerging AIV genomes extracted from respiratory samples collected on sequential days from animal models and clinical patients enables analysis of the emergence of evolutionary variants within the virus population over time. However, obtaining codon complete AIV genome at a sufficient coverage depth for nucleotide variant calling remains a challenge, especially from post-inoculation respiratory samples collected at late time points that have low viral titers. In this study, nasal wash samples from ferrets inoculated with different subtypes of AIV were collected on various days post-inoculation. Each nasal wash sample was aliquoted and extracted using five commercially available nucleic acid extraction methods. Extracted influenza virus RNA was amplified and NGS conducted using Illumina Mi-Seq. For each nasal wash sample, completeness of AIV genome segments and coverage depth were compared among five extraction methods. Nucleic acids extracted by MagNA pure compact RNA isolation consistently yielded codon complete sequences for all eight genome segments at the required coverage depth at each time point sampled. The study revealed that DNase treatment was critical to the amplification of influenza genome segments and the downstream success of codon complete NGS from nasal wash samples. The findings from this study can be applied to improve NGS of influenza and other RNA viruses that infect the respiratory tract and are collected from respiratory samples.

1. Introduction

Influenza A virus is an enveloped, negative-sense RNA virus with a segmented genome. It has a global distribution and infects a wide range of species (Short et al., 2015). Avian influenza A virus (AIV) circulates in wild birds and poultry, but can also infect mammalian hosts including humans. Infection of AIV in humans can cause severe respiratory disease with a high mortality rate (Quan et al., 2018; World Health Organization, 2018). Based on the two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which are expressed on the surface of virions, AIV can be classified into different subtypes, such as H5N1, H5N6, H7N9 and H9N2 (Wright et al., 2013). AIV can evolve through reassortment when two or more different virus strains co-infect the same host and swap gene segments resulting in progeny with different genomic combinations than the parental strains (Gerloff et al., 2014; Wu et al., 2013). AIV has also evolved through an inherently rapid rate of genomic mutation (i.e., $> 10^{-3}$ substitutions per site per year) because of the viral error-prone polymerase complex and short replication cycle (Chen and Holmes, 2006). Accelerated viral evolution may also facilitate mammalian adaptation of AIV and may lead to new strains of virus with more efficient replication, enhanced transmissibility, and increased virulence in mammalian hosts. AIV has caused H5N1 virus infections in humans since 1997 and annual H7N9 epidemics since 2013 (Lai et al., 2016; Su et al., 2017). Therefore, AIV posts a long-term pandemic threat to public health (Richard et al., 2014; Tanner et al., 2015). Monitoring AIV detected in mammalian hosts for molecular signatures of mammalian adaptation, antigenic variation and antiviral drug resistance is a critical component of influenza surveillance, pandemic preparedness and vaccine development. Ferrets are a commonly used animal model for mammalian infection,

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transmissibility and virulence with AIV and for generation of anti-influenza virus immune sera, testing vaccine efficacy and therapeutic drugs (Belser et al., 2011, 2009). Increasingly, AIV research, such as intra- and inter-host evolutionary studies, requires next-generation sequencing (NGS) analysis of viral genomes from samples collected on sequential days from the same host. Ferret nasal wash samples are non-invasive and easy to collect on a daily basis for AIV genome extraction and sequencing to identify genetic variations post-inoculation.

The influenza A viral genome consists of eight gene segments that are known to be translated into as many as 12 proteins. The two largest gene segments are polymerase basic gene 1 and 2 (PB1 and PB2, ~2.3 kb), followed by polymerase acidic gene (PA, ~2.2 kb), HA gene (~1.8 kb), nucleoprotein gene (NP, ~1.6 kb) and NA gene (~1.4 kb). The two smallest gene segments are matrix gene (M, ~1 kb) and non-structural protein gene (NS, ~0.9 kb) (Wright et al., 2013). Traditional Sanger sequencing on amplified gene segments usually provides less than 5-fold (5X) coverage of the influenza genome at each nucleotide position (Deng et al., 2015). This low coverage is not sufficient for accurate variant calling, especially for variants at low abundance. NGS provides hundreds- to thousands-fold coverage of the influenza genome per nucleotide position and can enhance the identification of known and unknown genetic markers that emerge or remain fixed in a virus population following mammalian infection (Croville et al., 2012; Poon et al., 2016; QuiÑOnes-Mateu et al., 2014; Van den Hoecke et al., 2015). Amplification of influenza gene segments prior to NGS is an important step used to enrich influenza genomes especially when high coverage depth is required for variant analysis (Lin et al., 2014). Among the current methods of influenza genome amplification, multi-segment reverse transcription-PCR (M-RTPCR) is widely used for NGS of influenza virus genomes because it can simultaneously amplify all 8 RNA segments from any influenza A virus in a single reaction with one universal primer set. This primer set was designed to target the conserved regions in the 5′ and 3′ termini of each segment and was robust in amplification of influenza genomes directly from clinical swab specimens (Zhou et al., 2009; Zhou and Wentworth, 2012; Zou et al., 2016).

As part of a study to assess influenza virus replication in a ferret model, nasal wash samples were collected from ferrets inoculated with different subtypes of AIV on various days post-inoculation. The nucleic acids extracted from these nasal wash samples were subjected to M-RTPCR and NGS using Illumina Mi-Seq. Despite being influenza A virus-positive by real-time RT-PCR (rRT-PCR), nasal wash samples, especially those collected on later times post-inoculation, had poor NGS outcome, such as low read coverage and incomplete assembly. A previous NGS study on nasal wash samples collected from ferrets inoculated with seasonal influenza virus also demonstrated poor coverage depth in some of the nasal wash samples collected on later days post-inoculation (Frise et al., 2016). In another study, influenza genomes were only obtained from a small portion of respiratory samples collected from AIV (H7N9) infected patients following nucleic acid extraction, M-RTPCR amplification and NGS. Among the genomes obtained, 20% of the samples had at least one segment incomplete due to very poor coverage (less than 10X) (Zou et al., 2018).

In this study, nasal wash samples from ferrets inoculated with either highly pathogenic avian influenza (HPAI) H5N1 or HPAI H7N9 viruses were collected on various days post-inoculation. Five aliquots were made from each nasal wash sample and each aliquot was subjected to one of five commercially available nucleic acid extraction methods. All the extracted nucleic acids were amplified by M-RTPCR and sequenced by Illumina Mi-Seq. For each nasal wash sample, the number of influenza gene segments assembled completely at the required coverage depth for variant calling were compared among aliquots subjected to the five different extraction methods. Nucleic acids extracted by MagNA Pure compact RNA isolation demonstrated the optimal NGS outcome with complete assembly of all 8 gene segments at high coverage depth from nasal wash samples collected at all the time points. DNase treatment included in the automated procedure for the MagNA pure compact RNA isolation was determined to be critical to the success of influenza virus NGS from ferret nasal wash samples.

2. Materials and methods

2.1. Viruses

Highly pathogenic avian influenza (HPAI) H5N1 virus (A/chicken/Cambodia/Z9P1E1W7M1/2015) was received from Cambodia as an egg-isolated virus (passage E2) and was propagated in 10 day old embryonated chicken eggs. The allantoic fluid was harvested at 24 h post-infection, centrifuged and the clarified supernatant was stored at −80 °C as virus stock (passage E2/E1). The virus infectivity titer, determined by the Reed-Muench method using 50% Egg Infectious Dose per milliliter (EID_{50}/ml), was $10^{7.63}$ EID_{50}/ml. HPAI H7N9 virus (A/Taiwan/1/2017) was received from Taiwan as an egg-isolated virus (passage E1) and was propagated, harvested and stored as described above. The infectivity titer of the virus stock (passage E1/E1) was $10^{9.8}$ EID_{50}/ml.

2.2. Ferret inoculation and nasal wash sample collection

For each virus used in this study, two male ferrets (Triple F Farms) that were 6–12 months old and tested negative for currently circulating influenza viruses were inoculated intranasally with 1 ml of virus diluted to 10^6 EID_{50}/ml. Post-inoculation, ferret nasal wash samples were collected in sterile collection cups from anesthetized animals by intranasal instillation of 1 ml of Phosphate Buffered Saline (PBS) supplemented with Bovine Serum Albumen (BSA) and penicillin/streptomycin on days 3, 5 and 7. All animal work in this study was conducted in an Enhanced Biosafety Level 3 facility and approved by the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee (IACUC).

2.3. RNA extraction and DNase treatment

Five 100 µl aliquots were made from each nasal wash sample. One aliquot was lysed in 300 µl MagNA Pure LC TNA Isolation-Lysis/Binding Buffer (Roche) and extracted by MagNA Pure compact nucleic acid isolation kit I, following the manufacturer's Total-NA-plasma-external protocol (Roche). One aliquot was lysed in 250 µl MagNA Pure compact RNA isolation-Lysis/Binding buffer (Roche) and extracted by MagNA Pure compact RNA isolation kit following the manufacturer's RNA-Tissue-V3-1 protocol (Roche). One aliquot was lysed in 500 µl buffer RLT (QIAgen) containing 1% β-mercaptoethanol and extracted by RNeasy mini kit according to the manufacturer's instructions (QIAgen). One aliquot was lysed in 560 µl buffer AVL (QIAgen) and extracted by QIAamp viral RNA mini kit according to the manufacturer's instructions (QIAgen). The last aliquot was lysed in 1000 µl TRIzol reagent (Invitrogen) and extracted using the TRIzol/chloroform protocol provided by Invitrogen. RNase-free glycogen (12 µg, Thermo Fisher) was added to the aqueous phase to facilitate the precipitation of RNA. The elution volume was 50 µl for all extraction methods and the extracted nucleic acids were aliquoted and stored at −80 °C.

Turbo DNA-free kit (Ambion) was used to treat extracted nucleic acids with DNase. Briefly, 35 µl of extracted nucleic acids were mixed with 4 µl of 10X buffer and 1 µl of Turbo DNase. After incubation at 37 °C for 30 min, 4 µl of resuspended DNase inactivation reagent was added and the reaction was incubated at room temperature for 5 min with occasional mix. After centrifugation at 10,000 g for 1.5 min, the supernatant was stored as DNase-treated RNA.

2.4. Real-time qRT-PCR

The presence of influenza A virus RNA (vRNA) in the extracted

Table 1
Comparison of five extraction methods for NGS of ferret nasal wash samples containing H5N1 vRNA.

Extraction Methods	Nasal Wash Sample	Ct Value[a]	Total NGS Reads	Influenza Reads (%)[b]	Assembled Influenza Genome Segments							
MagNA pure-RNA	Ferret-1-D3	25.15	543,004	99.41%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-2-D3	23.63	504,960	99.34%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-1-D5	26.48	235,808	99.27%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-2-D5	26.48	496,402	98.72%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-1-D7	29.46	653,106	92.82%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-2-D7	27.94	585,844	95.63%	PB2	PB1	PA	HA	NP	NA	MP	NS
QIAgen RNeasy	Ferret-1-D3	26.88	522,656	52.27%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-2-D3	22.06	526,142	57.33%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-1-D5	28.35	668,610	13.19%					NP		MP	NS
	Ferret-2-D5	28.04	566,034	17.70%					NP		MP	NS
	Ferret-1-D7	30.2	600,538	0.81%								
	Ferret-2-D7	28.44	541,388	11.36%					NP		MP	NS
QIAamp viral RNA	Ferret-1-D3	24.05	404,000	62.35%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-2-D3	23.48	443,676	44.36%				HA	NP	NA	MP	NS
	Ferret-1-D5	26.59	329,836	20.55%					NP		MP	NS
	Ferret-2-D5	27.31	309,766	20.61%					NP		MP	NS
	Ferret-1-D7	28.06	315,802	1.30%								
	Ferret-2-D7	28.4	342,444	7.08%							MP	NS
TRIzol/Chloroform	Ferret-1-D3	24.27	474,736	54.06%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-2-D3	23.07	549,920	56.00%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-1-D5	27.31	348,576	15.03%					NP		MP	NS
	Ferret-2-D5	26.44	310,652	35.30%	PB2			HA	NP		MP	NS
	Ferret-1-D7	29.61	510,714	0.37%								
	Ferret-2-D7	27.44	516,580	12.64%					NP		MP	NS
MagNA pure-NA	Ferret-1-D3	24.29	573,780	24.14%				HA	NP	NA	MP	NS
	Ferret-2-D3	24.81	531,764	10.94%					NP		MP	
	Ferret-1-D5	24.3	462,758	13.48%					NP		MP	NS
	Ferret-2-D5	26.38	588,166	9.22%					NP		MP	NS
	Ferret-1-D7	26.27	557,656	1.16%								
	Ferret-2-D7	27.45	493,106	2.84%								

[a]Real-time qRT-PCR with primers and probes targeting the influenza A matrix gene conserved region.
[b]Percentage of total NGS reads that contributed to the influenza genome assembly.

nucleic acids was confirmed and quantified with the SuperScript III Platinum one-step qRT-PCR kit (Invitrogen). Briefly, a 25 µl reaction was set-up containing the extracted nucleic acids (5 µl), enzyme mix (0.5 µl), 2X PCR master mix (12.5 µl), nuclease-free water (5.5 µl), oligonucleotide forward and reverse primers (0.5 µl each) and a FAM-labeled TaqMan hydrolysis probe (0.5 µl) designed to detect the universal matrix (M) gene of influenza A viruses (Shu et al., 2011). The reactions were performed on a Stratagene Mx3005 P instrument using the following conditions: reverse transcription at 50 °C for 30 min, inactivation at 95 °C for 2 min, 40 cycles of PCR amplification at 95 °C for 15 s and 55 °C for 30 s.

2.5. Multi-segment-RT-PCR (M-RTPCR) of the influenza A genome

SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase HiFi (Thermo Fisher) was used to simultaneously amplify the eight influenza A vRNA genomic segments from the extracted nucleic acids with universal influenza A primers designed to target the conserved regions in the 5′ and 3′ termini of each segment (Zhou et al., 2009; Zhou and Wentworth, 2012). Briefly, a 25 µl reaction was set up containing nuclease-free water (8 µl), 2X reaction mix (12.5 µl), enzyme mix (0.5 µl), universal influenza A primer mix (1 µl) and extracted nucleic acids (3 µl). The thermocycler (BioRad) was programed at 42 °C for 50 min, 50 °C for 10 min, 94 °C for 2 min, followed by 5 cycles of 94 °C for 30 s, 43 °C for 30 s, 68 °C for 3 min 50 s, and then another 31 cycles of 94 °C for 30 s, 57 °C for 30 s, 68 °C for 3 min 30 s with an extension of 10 s per cycle, plus a final extension at 68 °C for 10 min. To analyze the amplicons, 10 µl of the amplification products were loaded on the QIAxcel advanced instrument with QIAxcel DNA Screening Kit (QIAgen) and visualized using the QIAxcel ScreenGel software. Nucleic acids extracted from an H1N1-pdm virus stock (A/California/07/2009) were used as a positive control (pos) and a mock extraction for each method was used as a non-template control (ntc) for M-RTPCR.

2.6. NGS library construction and data analysis

Primers and single-stranded DNA were removed from the M-RTPCR amplicon's using Exonuclease I reagent following the manufacturer's protocol (Applied Biosystems). Subsequently, the amplicons were quantified using Quant-iT High-Sensitivity dsDNA Assay Kit (Invitrogen) according to the manufacturer's recommendation and diluted to 0.2 ng/µl. A total of 0.5 ng amplicons for each sample were

subjected to library preparation using half-volumes of the Nextera XT DNA library preparation kit (Illumina) following the manufacturer's protocol. The resulting indexed paired-end libraries were purified, quantified, size-estimated, normalized and pooled as previously described (Shepard et al., 2016). A total of 6 pmol pooled libraries spiked with 5% PhiX were sequenced on the Illumina MiSeq platform using the MiSeq v2 300 cycle kit (Illumina). The output reads were filtered, sorted and assembled into influenza genome segments using the iterative refinement meta-assembler (IRMA) pipeline as previously described (Shepard et al., 2016). Only the assembled segments with 400X minimum average coverage and 100X minimum coverage at each nucleotide (except for the 5% region at the 5′ and 3′ ends) were qualified as successfully assembled. Because the NexTera tagmentation cannot add an adapter at the termini of an influenza gene segment, the 5% terminal region of each assembled segment was allowed to have 80X minimum coverage at each nucleotide position. IRMA pipeline was also used to identify variants in the assembled segments using the following minimum requirements: 1% frequency, average quality of 24, 2 allele count, 100X coverage and 80% confidence. The identified variants (if any) and coverage depth of each assembled segment were visualized in the IRMA output (Shepard et al., 2016).

3. Results

3.1. Comparison of nucleic acid extraction methods from ferret nasal wash samples infected by H5N1 virus

To determine the optimal nucleic acid extraction method for NGS of infected ferret nasal wash samples, five commercially available extraction methods were selected for comparison. For each sample, the Ct values of vRNA extracted by different methods did not vary dramatically. The Ct values of all samples were < 30 except for one (Ct = 30.2) (Table 1). This suggested that all five nucleic acid extraction methods were efficient in extracting vRNA from ferret nasal wash samples.

As the first step in the influenza NGS library preparation pipeline, M-RTPCR was used to amplify the eight influenza genome segments from the extracted nucleic acids. For each extraction method, the M-RTPCR products for each sample were separated and visualized using the QIAxcel advanced electrophoresis system. Five M-RTPCR bands were detected in the positive control representing influenza vRNA segments. Due to the resolution limitation of the QIAxcel advanced electrophoresis system, segments PB2 (~2.3 kb), PB1 (~2.3 kb) and PA (~2.2 kb) were indistinguishable and appeared as a single band (Fig. 1A). Segments NP (~1.6 kb) and NA (~1.4 kb) also often migrate closely together and appear as one wide band (Fig. 1A). Among the five extraction methods, nucleic acids extracted by MagNA Pure-RNA kit outperformed all the others in amplifying influenza vRNA segments in each nasal wash sample (Fig. 1A). Nucleic acids extracted by TRIzol/Chloroform or column-based methods amplified nearly all the influenza segments in the day 3 (D3) nasal wash samples, but failed to amplify many segments in the day 5 (D5) and especially the day 7 (D7) nasal wash samples (Fig. 1B–1D), which have lower levels of nucleic acid present. Nucleic acids extracted by MagNA pure-NA kit failed to amplify many of the influenza vRNA segments in all nasal wash samples. Instead, many non-specific bands were detected (Fig. 1E). All the M-RTPCR products were then used for library construction using Illumina NexTera technology and sequenced using Illumina Mi-Seq. The total NGS reads for all nasal wash samples regardless of extraction method were above 300,000 with one exception at 235,808 (Table 1). However, for each nasal wash sample, the percentage of reads that contributed to the final influenza vRNA genome assembly (influenza reads) varied dramatically among different extraction methods. All the nasal wash samples subjected to MagNA pure-RNA extraction consistently yielded more than 90% influenza reads. The percentage of influenza reads in samples subjected to TRIzol/Chloroform or column based extraction started at a range of 44–63% in D3 samples, decreased to 13–36% in D5 samples, and further decreased to 0.37–13% in D7 samples. Samples subjected to MagNA pure-NA extraction performed poorly, yielding 9–25% influenza reads in D3 and D5 samples and decreased to 1–3% in D7 samples (Table 1). The influenza reads for each sample were used to assemble the gene segments and the read coverage for each segment was analyzed. Nasal wash samples extracted with MagNA pure-RNA displayed a higher depth of coverage for each gene segment compared to samples extracted by other methods (Supplementary Fig. 1). To increase the accuracy of variant calling in assembled gene segments, only the assembled segments with at least 400X average coverage over each segment and 100X coverage at each nucleotide position (80X for the termini) were considered as qualified segments. Among different extraction methods tested, all the nasal wash samples subjected to MagNA pure-RNA extraction assembled all eight influenza vRNA segments, and met the coverage requirements. Samples subjected to TRIzol/Chloroform or column based extraction assembled almost all influenza segments in D3 samples, but only the smaller vRNA segments (NP, MP, NS) in D5 samples, and no segments at all in one of the D7 samples. Samples subjected to MagNA pure-NA extraction assembled mainly the smaller segments (NP, MP, NS) in D3 and D5 samples and no segments at all in both D7 samples (Table 1). These data correlated well with the M-RTPCR results and suggested that among the five selected extraction methods, samples subjected to MagNA pure-RNA extraction had the best NGS outcome with all the influenza vRNA segments assembled at the desired coverage. In contrast, samples subjected to MagNA pure-NA extraction resulted in the worst NGS outcome with less than 25% influenza reads and few influenza vRNA segments assembled at the desired coverage.

3.2. Comparison of nucleic acid extraction methods from ferret nasal wash samples infected by H7N9 virus

To further confirm the observed differences in NGS outcome from nasal wash samples extracted by different methods, two additional ferrets were inoculated with HPAI H7N9 virus (A/Taiwan/1/2017). The Ct values for nucleic acids extracted from the D3 and D5 nasal wash samples were all < 25 and those extracted from the D7 samples were all < 28 (Table 2). This confirmed that all five extraction methods were efficient in extracting vRNA from ferret nasal wash samples. For each extraction method, the M-RTPCR products for each sample were separated and visualized, as described above. Similar to that observed for HPAI H5N1 nasal wash samples, the nucleic acids extracted by MagNA pure-RNA kit outperformed the other methods with efficient amplification of all influenza segments for all nasal wash samples (Fig. 2A). Nucleic acids extracted by TRIzol/Chloroform showed strong influenza vRNA segment amplification from all samples except for one of the D7 samples, which still had faint bands (Fig. 2D). Nucleic acids extracted by column based methods showed only faint influenza vRNA segment bands in one of the D5 samples and failed to amplify most of the influenza vRNA segments in one of the D7 samples (Fig. 2B–C). Nucleic acids extracted by MagNA pure-NA kit failed to amplify most of the influenza segments in one of the D5 and both D7 samples (Fig. 2E). After library construction and Mi-Seq sequencing, nasal wash samples subjected to MagNA pure-RNA extraction had the highest percentage of influenza reads in the range of 96–99% and all the influenza segments were assembled at the desired coverage in all samples. Samples subjected to TRIzol/Chloroform extraction had the second highest percentage of influenza reads in the range of 66–97%, and all the influenza segments assembled at desired coverage in all samples (Table 2 and Supplementary Fig. 2). In contrast, samples subjected to MagNA pure-NA or column based extraction had much lower percentages of influenza reads in one of the D5 and both D7 samples (< 25%) with samples subjected to MagNA pure-NA extraction being the lowest (< 10%). Correspondingly, many influenza vRNA segments, especially the longer polymerase genes, failed to assemble at the desired coverage in these samples (Table 2 and Supplementary Fig. 2). These data agreed with the

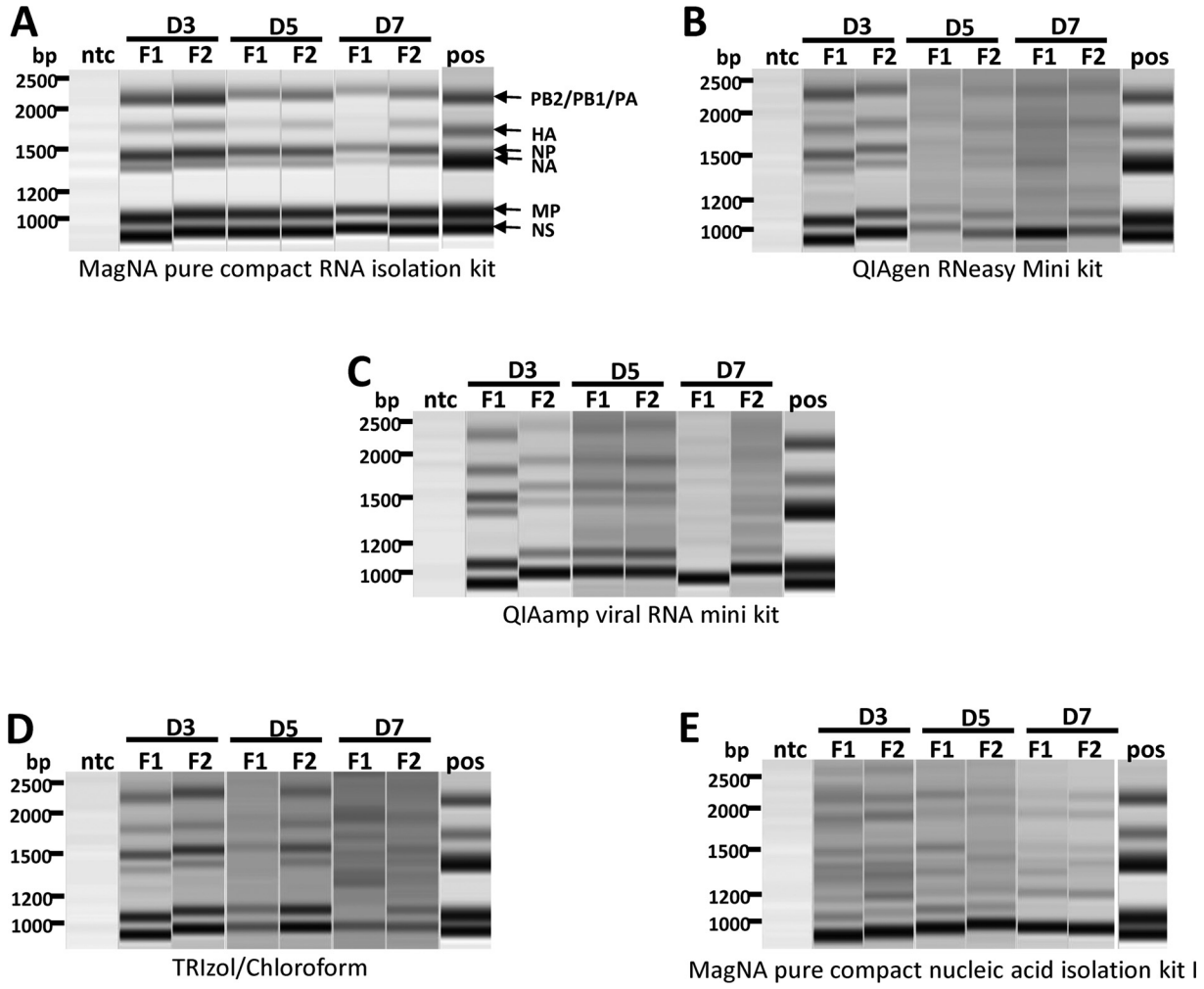


Fig. 1. Amplification of influenza A(H5N1) genome segments from nucleic acids extracted by five different methods. Two male ferrets (F1 and F2) were inoculated intranasally with HPAI A(H5N1) virus. Five 100 µl aliquots were made from each nasal wash sample and extracted by MagNA pure-RNA (A), QIAgen RNeasy mini (B), QIAamp viral RNA mini (C), TRIzol/Chloroform (D) and MagNA pure-NA (E), respectively. The segment(s) represented by each band were labeled for the positive control. The internal size markers were labeled on the side in bp.

data obtained from the nasal wash samples of HPAI H5N1 virus inoculated ferrets and further confirmed that among the five selected extraction methods, the MagNA pure-RNA extraction consistently yielded the best NGS outcome.

3.3. DNase treatment is critical to influenza A vRNA NGS from ferret nasal wash samples

MagNA pure-RNA extraction contains an additional DNase treatment step in the automated protocol that is not included in the other extraction methods. To test whether DNase treatment was critical to the NGS outcome, aliquots of the nasal wash samples containing HPAI H5N1 vRNA were also extracted by the MagNA pure-RNA protocol without the DNase treatment step (MagNA pure-RNA w/o DNase). Interestingly, by removing the DNase treatment step, M-RTPCR failed to amplify most of the influenza vRNA segments from nearly all nasal wash samples; instead, non-specific bands were observed (Fig. 3B). Correspondingly, the percentage of influenza reads after NGS decreased dramatically, especially in the D5 and D7 samples (Table 3). Few to no influenza vRNA segments were assembled at the desired coverage in all samples except for one (Table 3). When treating these failed RNA extractions manually with Turbo DNase, almost all the influenza vRNA segment bands were detected in all but one of the D7 samples after M-RTPCR at similar levels as nucleic acids extracted by MagNA pure-RNA (Fig. 3A and C). Turbo DNase treatment also restored the percentage of influenza reads to the same levels (> 90%) as nucleic acids extracted by MagNA pure-RNA (Table 3). As a result, all influenza vRNA segments were assembled at the desired coverage in all but the D7 samples (Table 3). The coverage of the segments is critical to variant calling. For example, when a D5 sample was subjected to MagNA pure-RNA extraction including the DNase treatment, a minimum of 100X coverage was achieved at each nucleotide in the NA CDS region and the average coverage was above 1200 × . Seven variants were identified based on the parameters described in the Material & Methods (Fig. 4A). In contrast, when the same sample was subjected to MagNA pure-RNA extraction, but the DNase treatment step was removed, the average coverage was 15 times lower (< 80X) and no variant was identified due to low coverage (Fig. 4B). Taking together, these data suggested that DNase treatment is critical to the success of influenza A vRNA NGS from nasal wash samples, which in turn, greatly facilitated analysis of major and minor nucleotide variants.

3.4. Background DNA inhibited amplification of influenza vRNA segments and negatively affected NGS outcome

Amplification products that did not correspond to the size of any known influenza gene segments were observed after M-RTPCR in many samples that failed NGS. For example, those samples extracted by the

Table 2
Comparison of five extraction methods for NGS of ferret nasal wash samples containing H7N9 vRNA.

Extraction Method	Nasal Wash Sample	Ct Value[a]	Total NGS Reads	Influenza Reads (%)[b]	Assembled Influenza Genome Segments							
MagNA pure-RNA	Ferret-3-D3	19.46	195,038	96.34%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-4-D3	17.94	310,762	98.56%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-3-D5	24.22	287,198	99.06%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-4-D5	19.55	344,614	98.97%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-3-D7	27.78	350,544	98.39%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-4-D7	27.41	379,344	98.91%	PB2	PB1	PA	HA	NP	NA	MP	NS
QIAgen RNeasy	Ferret-3-D3	17.98	644,176	84.45%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-4-D3	16.91	622,544	80.50%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-3-D5	23.52	470,106	15.44%		PB1				NA	MP	NS
	Ferret-4-D5	20.75	593,818	52.08%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-3-D7	26.95	441,278	10.30%							MP	NS
	Ferret-4-D7	25.57	267,864	12.33%							MP	NS
QIAamp viral RNA	Ferret-3-D3	17.21	579,082	73.38%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-4-D3	16.19	549,284	62.40%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-3-D5	22.58	360,162	20.68%		PB1		HA	NP		MP	NS
	Ferret-4-D5	18.69	447,628	55.47%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-3-D7	25.48	438,784	21.00%	PB2	PB1		HA			MP	NS
	Ferret-4-D7	23.86	419,324	24.03%		PB1		HA	NP	NA	MP	NS
TRIzol/Chloroform	Ferret-3-D3	17.49	487,252	96.53%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-4-D3	15.24	427,796	94.05%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-3-D5	22.57	355,396	81.17%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-4-D5	19.42	484,366	97.41%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-3-D7	25.17	428,748	66.07%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-4-D7	24.73	471,802	88.51%	PB2	PB1	PA	HA	NP	NA	MP	NS
MagNA pure-NA	Ferret-3-D3	18.67	501,156	80.15%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-4-D3	17.19	577,666	74.55%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-3-D5	23.26	543,562	7.87%							MP	NS
	Ferret-4-D5	20.36	363,840	44.94%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-3-D7	26.37	653,186	7.65%							MP	NS
	Ferret-4-D7	25.08	537,816	9.94%		PB1					MP	NS

[a]Real-time qRT-PCR with primers and probes targeting the influenza A matrix gene conserved region.
[b]Percentage of total NGS reads that contributed to the influenza genome assembly.

MagNA pure-NA (Fig. 1E) and MagNA pure-RNA kits w/o DNase had amplicons that did not correspond to any of the eight vRNA segment sizes (Fig. 3B). These irrelevant amplification products were absent from samples subjected to DNase treatment during extraction (Figs. 1A, 2 A and 3 A) or after extraction (Fig. 3C). This suggested that the background DNA in the extracted nucleic acids may bind the influenza universal primers and potentially compete with the amplification of influenza gene segments during M-RTPCR. To test this hypothesis, TRIzol/Chloroform extracted nucleic acids, which amplified all influenza segments and showed good NGS outcome from a D7 nasal wash containing HPAI H7N9 vRNA, were gradually replaced in the M-RTPCR reaction with MagNA pure-NA extracted nucleic acids from the same sample, which contained 1.47 ng/μl DNA and had poor NGS outcome. By replacing 1 μl of nucleic acid extracted by TRIzol/Chloroform with 1 μl of nucleic acid extracted by MagNA pure-NA, M-RTPCR failed to amplify most of the influenza vRNA segments (Fig. 5A). This correlated with the NGS outcome showing a dramatically decreased percentage of influenza reads (from 71% to 14%) and less assembled influenza segments at the desired coverage (Table 4). The percentage of influenza reads continued decreasing as more nucleic acid extracted by TRIzol/Chloroform were replaced in the M-RTPCR reaction (Table 4). However, when gradually replacing the nucleic acids extracted by TRIzol/Chloroform with nuclease-free water in the M-RTPCR reaction, only a dilution effect, but not an inhibitory effect, was observed. All the influenza vRNA segment bands were detected after M-RTPCR until two-thirds of the nucleic acids extracted by TRIzol/Chloroform were replaced with water (Fig. 5B). This also correlated well with the NGS outcome (Table 4). To further confirm the inhibitory effect, instead of replacement, 1 μl of nucleic acid extracted by MagNA pure-NA was added in addition to 3 μl nucleic acids extracted by TRIzol/Chloroform in the M-RTPCR reaction. Again, M-RTPCR failed to amplify the influenza segments efficiently and the bands detected were faint (Fig. 5C), which correlated with the NGS outcome (Table 4). When using another D5 nasal wash sample containing H7N9 vRNA, the same inhibitory effect was also observed by adding 1 μl of nucleic acid extracted by MagNA pure-NA (contain 1.63 ng DNA) in addition to 3 μl of nucleic acid extracted by TRIzol/Chloroform in the M-RTPCR reaction (Fig. 5D and Table 4). These data suggested that background DNA in the nucleic acids extracted from ferret nasal wash samples could inhibit M-RTPCR amplification of influenza vRNA segments.

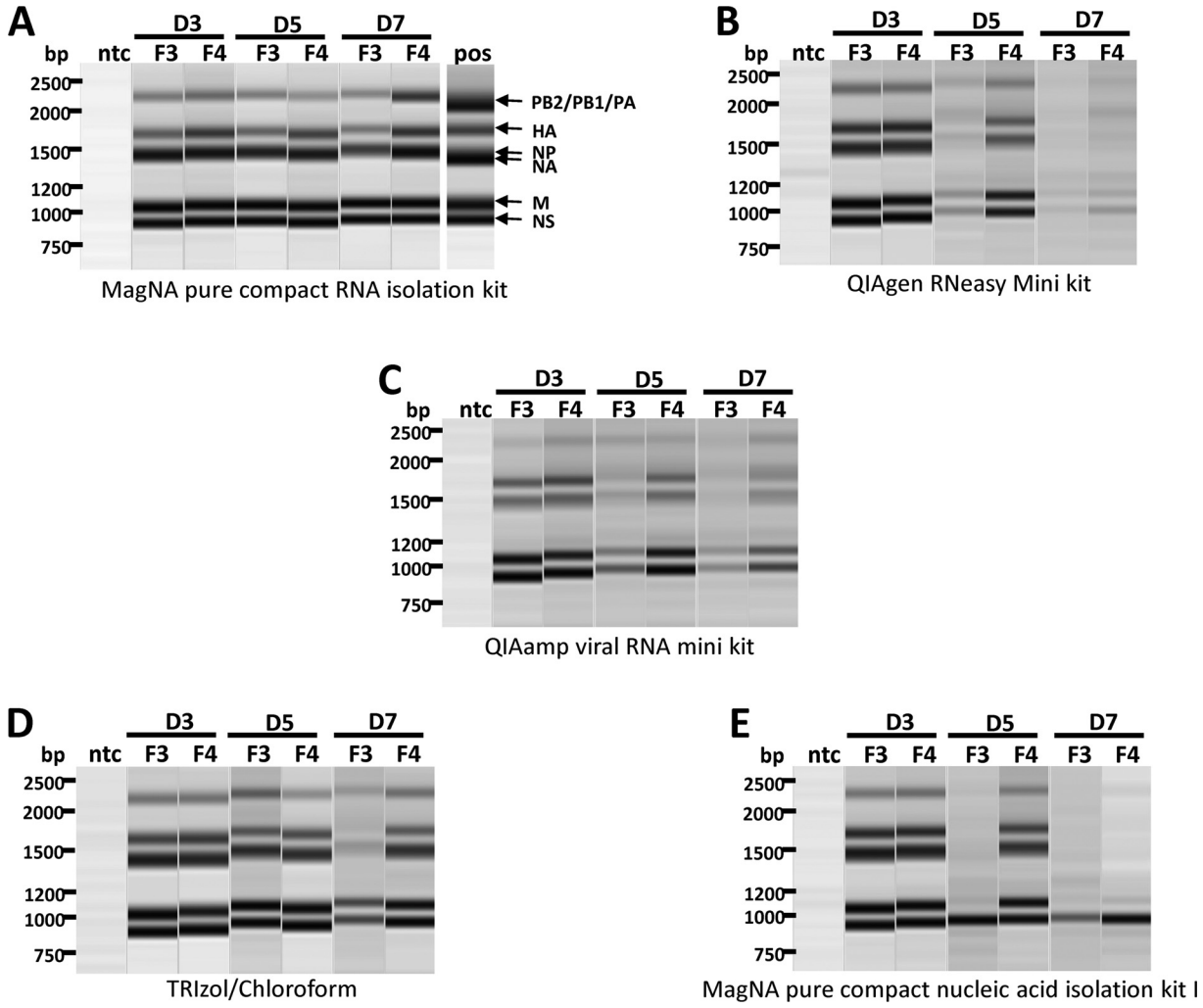


Fig. 2. Amplification of influenza A(H7N9) genome segments from nucleic acids extracted by five different methods. Two male ferrets (F3 and F4) were inoculated intranasally with HPAI A(H7N9) virus. Five 100 µl aliquots were made from each nasal wash sample and extracted by MagNA pure-RNA (A), QIAgen RNeasy mini (B), QIAamp viral RNA mini (C), TRIzol/Chloroform (D) and MagNA pure-NA (E), respectively. The segment(s) represented by each band were labeled for the positive control. The internal size markers were labeled on the side in bp.

4. Discussion

The wide use of NGS has enhanced studies on influenza virus infections and led to increased numbers of intra-host and inter-host evolution studies (Frise et al., 2016; Quinones-Mateu et al., 2014; Sobel Leonard et al., 2016; Zou et al., 2018). Nasal wash/swab sample collection from patients or animals is widely used for longitudinal studies of influenza virus replication and infectivity. This allows for sample collection at multiple, sequential data points from the same host without the need for invasive techniques. Several studies have employed NGS analysis of nucleic acids extracted from host nasal wash/swab samples and reported difficulty obtaining all eight influenza A vRNA segments assembled with adequate length and nucleotide coverage. A previous study extracted nucleic acids from seasonal influenza-positive patients' nasopharyngeal swab samples using QIAamp viral RNA mini kit and sequenced the viral genomes using M-RTPCR combined with Illumina Mi-Seq. Approximately 20% of the samples failed to assemble at least one influenza vRNA segment under the high assembly threshold (minimum contig length ≥800 bp and minimum reads ≥1000). On the other hand, contigs of human genome were assembled in these samples (Zhao et al., 2016). Another study extracted nucleic acids from nasal wash samples collected from seasonal influenza virus challenged human subjects. After M-RTPCR and NGS using Hi-Seq, only 46% of virus-containing nasal wash samples were successfully sequenced (Sobel Leonard et al., 2016).

Given the routine use of NGS in more research laboratories, variant nucleotide calling has become more standard with NGS data obtained from nasal wash/swab samples allowing for more insightful viral evolutionary studies of pathogens with high mutation rates. For confident variant calling, 100X coverage at each nucleotide was a minimum requirement for most variant calling software, as well as in previous publications (Moncla et al., 2016; Wilker et al., 2013). Assembled viral genomes with regions having less than 100X coverage were unable to detect variants in these regions (Fig. 4). Therefore, for this study we set our coverage threshold for successful assembly accordingly. After comparing the NGS outcome of five commercially available extraction methods from two sets of ferret nasal wash samples containing different subtypes of AIV, we determined that the MagNA pure-RNA extraction outperformed other methods. Optimal NGS outcome was consistently obtained from high quality, purified RNA extracted by this method from samples collected at various days post-inoculation, which contain a wide range of viral loads. The coverage depth obtained for each gene segment was sufficient for variant calling. This resulted in the ability to obtain sufficient influenza genome coverage for variant analysis from nasal wash samples collected on later days post-inoculation when viral load and vRNA concentrations were low, which was important in order to assess the virus population change after several days of replication in the host. Thus, the method described herein will benefit future intra-

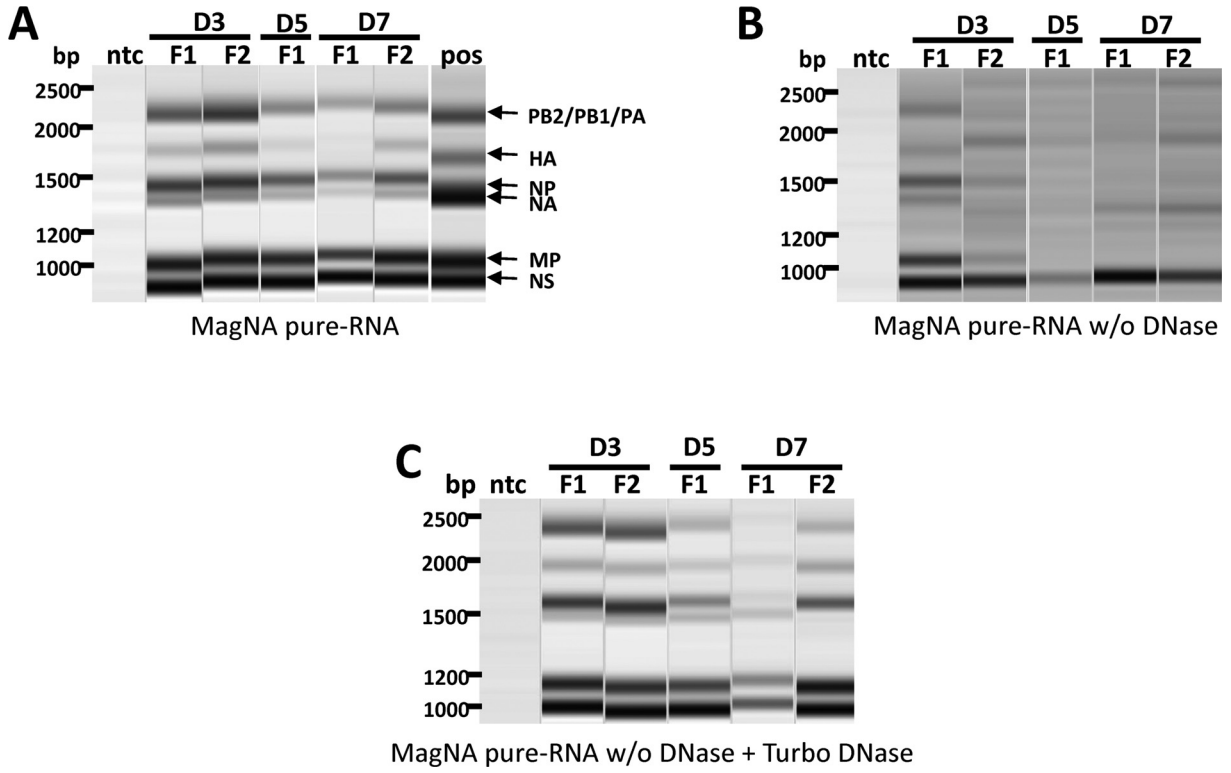


Fig. 3. DNase treatment improved M-RTPCR amplification of influenza vRNA segments. Two male ferrets (F1 and F2) were inoculated intranasally with HPAI A(H5N1) virus. Two 100 µl aliquots were made from each nasal wash sample except for F2-D5 sample due to low volume left. One aliquot was extracted by MagNA pure-RNA (A) and the other aliquot was extracted by the same method but skipping the DNase treatment (B). A portion (35 µl) of the nucleic acids extracted by skipping the DNase treatment were later treated with Turbo DNase after extraction (C). The segment(s) represented by each band were labeled for the positive control. The internal size markers were labeled on the side in bp.

host and inter-host evolution studies that assess host adaptation, selection of antigenic variants and, potentially, emergence of antiviral resistance.

MagNA pure-RNA extraction is a magnetic bead-based robotic method that includes a DNase treatment step in the automated procedure. DNase treatment was not part of the other extraction methods and was later determined to be critical to the success of influenza vRNA segment amplification and downstream NGS outcome. DNase treatment can reduce background DNA in the nucleic acids extracted from ferret nasal wash samples. Although influenza universal primers were used in the M-RTPCR amplification of the viral genome for enrichment, our study showed that the background DNA could compete with and inhibit

Table 3
DNase treatment improved influenza A(H5N1) NGS outcome from ferret nasal wash samples.

Extraction Method	Nasal Wash Sample	Total NGS Reads	Influenza Reads (%)[a]	Assembled Influenza Genome Segments							
MagNA pure-RNA	Ferret-1-D3	543,004	99.41%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-2-D3	504,960	99.34%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-1-D5	235,808	99.27%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-1-D7	653,106	92.82%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-2-D7	585,844	95.63%	PB2	PB1	PA	HA	NP	NA	MP	NS
MagNA pure-RNA w/o DNase	Ferret-1-D3	430,250	40.37%	PB2		PA	HA	NP	NA	MP	NS
	Ferret-2-D3	480,076	13.14%					NP		MP	NS
	Ferret-1-D5	589,700	2.98%								NS
	Ferret-1-D7	269,516	0.15%								
	Ferret-2-D7	529,600	0.84%								
MagNA pure-RNA w/o DNase + Turbo DNase	Ferret-1-D3	515,034	99.58%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-2-D3	574,400	99.39%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-1-D5	665,056	99.38%	PB2	PB1	PA	HA	NP	NA	MP	NS
	Ferret-1-D7	335,006	90.97%		PB1					MP	NS
	Ferret-2-D7	233,124	99.44%			PA	HA	NP		MP	NS

[a]Percentage of total NGS reads that contributed to the influenza genome assembly.

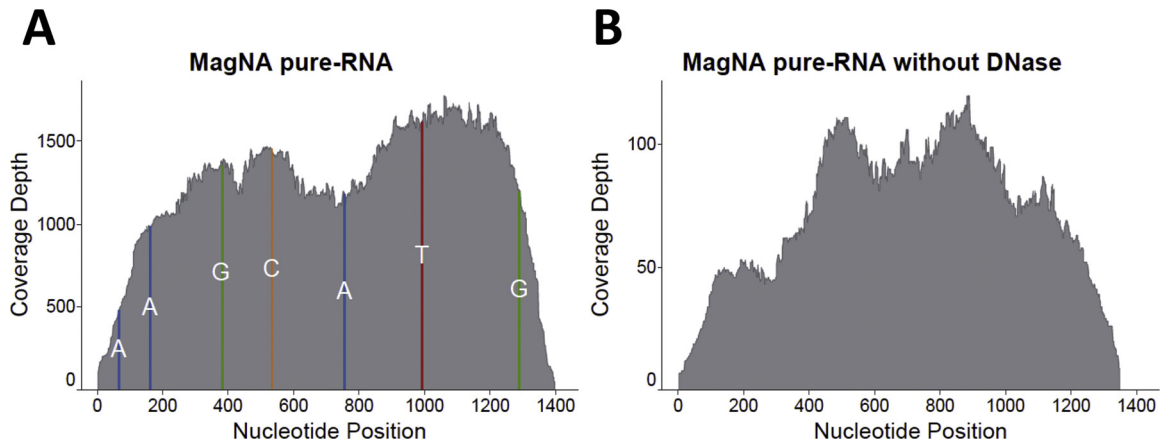
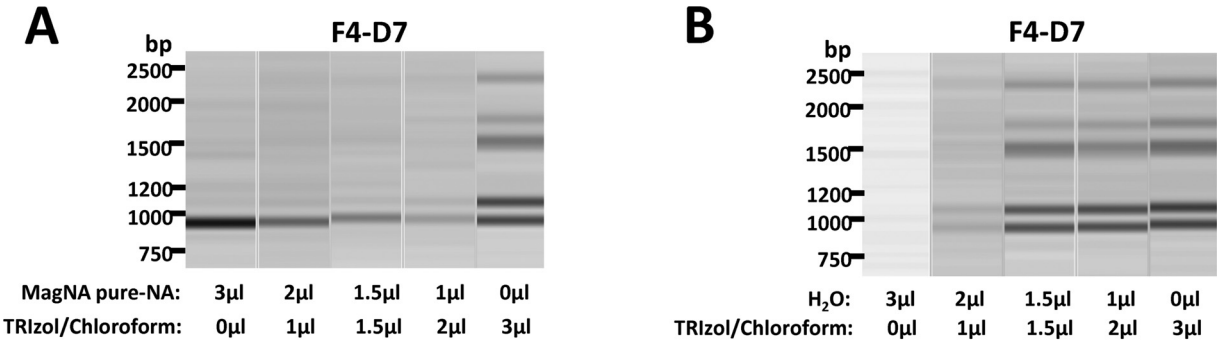


Fig. 4. DNase treatment improved coverage depth of assembled influenza gene segments after NGS. Ferret F1 was inoculated intranasally with HPAI A(H5N1) virus. Aliquots (100 µl) from the D5 nasal wash sample were extracted by MagNA pure-RNA with (A) or without DNase treatment (B). The coverage diagram of the assembled NA segment was shown here as a representative. The grey area represented the read coverage across the segment with the nucleotide position on the X-axis and the coverage depth on the Y-axis. Minor variants (> 1% frequency) were labeled on vertical lines according to their nucleotide position.



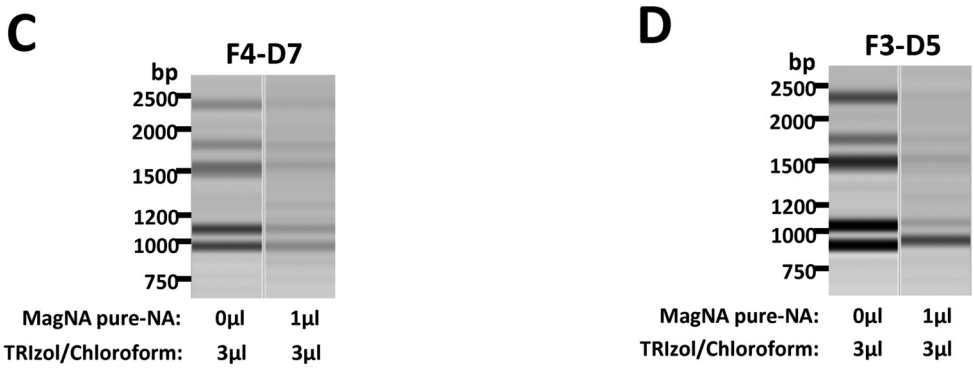


Fig. 5. Background DNA extracted from ferret nasal wash samples inhibited the M-RTPCR amplification of influenza genome segments. Two male ferrets (F3 and F4) were inoculated intranasally with HPAI A(H7N9) virus. Aliquots (100 µl) from each sample were extracted by MagNA pure-NA or TRIzol/Chloroform. For F4-D7 sample, the nucleic acids extracted by TRIzol/Chloroform was gradually replaced by either nucleic acids extracted by MagNA pure-NA (A) or by nuclease-free water (B) in the M-RTPCR reaction. Nucleic acids (1 µl) extracted by MagNA pure-NA was also added in addition to nucleic acids (3 µl) extracted by TRIzol/Chloroform in the M-RTPCR reaction for F4-D7 (C) and F3-D5 (D) samples. The internal size markers were labeled on the side in bp.

the amplification of influenza genome segments, especially in samples collected on later days post-inoculation when vRNA concentrations were low. Previous studies on influenza pathogenesis in ferrets have shown that the viral titer in the nasal wash samples collected on day 7 decreased more than 1000-fold compared to those collected on day 3 (Belser et al., 2016; Kaul et al., 2018). Similar decreasing levels of viral RNA extracted from nasal wash samples was also detected by real-time qRT-PCR in our study (Table 2). Although the influenza universal primers used in the M-RTPCR have high specificity against influenza vRNA, off-target amplification of background DNA may interfere with the amplification of viral gene segments when the vRNA to background DNA ratio is very low. Nasal wash samples collected on later days post-inoculation usually contained very low vRNA to background DNA ratio, but the viral population at these time points may begin to show clear signs of immune selection, host adaptation or other late stage infection mutations. Therefore, DNase treatment is necessary for nasal wash samples collected at later days post-inoculation to eliminate background DNA and ensure sufficient amplification of influenza virus

Table 4
Background DNA extracted from ferret nasal wash samples negatively affected influenza A(H7N9) NGS.

Nasal Wash Sample	H_2O	MagNA pure-NA[a]	TRIzol/ Chloroform[b]	Total NGS Reads	Influenza Reads (%)[c]	Assembled Influenza Genome Segments							
Ferret-4-D7		3 µl		714,814	4.88%							MP	NS
		2µl	1 µl	614,420	9.07%		PB1					MP	NS
		1.5 µl	1.5 µl	613,390	11.10%		PB1					MP	NS
		1 µl	2µl	361,544	14.09%		PB1					MP	NS
			3 µl	429,892	71.00%	PB2	PB1	PA	HA	NP	NA	MP	NS
	3 µl			8,900	0.53%								
	2µl		1 µl	505,540	19.95%	PB2	PB1	PA		NP		MP	NS
	1.5 µl		1.5 µl	399,062	50.13%	PB2	PB1	PA	HA	NP	NA	MP	NS
	1 µl		2µl	521,298	54.49%	PB2	PB1	PA	HA	NP	NA	MP	NS
			3 µl	558,092	69.65%	PB2	PB1	PA	HA	NP	NA	MP	NS
		1 µl	3 µl	443,888	17.42%		PB1		HA	NP		MP	NS
Ferret-3-D5			3 µl	363,710	71.40%	PB2	PB1	PA	HA	NP	NA	MP	NS
		1 µl	3 µl	390,758	13.39%							MP	NS

[a] The volume of nucleic acids extracted by MagNA pure NA kit was added into the M-RTPCR reaction.
[b] The volume of nucleic acids extracted by TRIzol/Chloroform was added into the M-RTPCR reaction.
[c] Percentage of total NGS reads that contributed to the influenza genome assembly.

genome for a successful downstream NGS analysis.

Host genomic DNA background affecting the pathogen genome amplification and downstream NGS quality is a serious concern. A previous study found possible human DNA sequences in 72% (145/202) of published microbial and viral metagenome data. The background human DNA sequences could exhaust amplification reactions and consume NGS reads of pathogen genomes to as high as 64% of the total read sequences (Schmieder and Edwards, 2011). In this study, more NGS reads mapped to the ferret genome than to the influenza genome in almost all the nasal wash samples extracted by MagNA pure RNA without DNase treatment. In contrast, very few reads were mapped to the ferret genome in the samples treated with DNase. This suggested that DNase treatment was effective in preventing preferential sequencing of excessive host genomic DNA in ferret nasal wash samples. The iterative refinement meta-assembler (IRMA), a robust read classifier and assembler designed for viral NGS by iterative optimization of read gathering and assembly, was used to sort and assemble NGS reads for samples extracted with or without DNase. Interestingly, compared to samples extracted with DNase, there were more unclassified reads that did not map to either ferret or influenza virus genome in samples extracted without DNase treatment. A recent study examined taxonomy on ferret and human nasal wash/swab samples collected before and after influenza A infection using NGS. They detected 16 s rRNA sequences from a wide variety of bacterial taxa that represented the host upper respiratory tract microbiome. Results indicated a temporary shift in the microbiome composition in ferrets and patients before and after influenza A infection (Kaul et al., 2018). Genomes of bacteria in this microbiome may consist partly of the large amount of unclassified reads we obtained from the nasal wash samples extracted without DNase treatment, which further addresses the importance of DNase treatment in eliminating background bacterial and host DNA contamination in nucleic acids extracted from nasal wash samples.

TRIzol/Chloroform manual extraction effectively separates DNA, RNA and protein into three fluid-phases (Chomczynski and Sacchi, 1987). RNA extracted by TRIzol/Chloroform can amplify influenza vRNA efficiently and yield optimal NGS outcome, as seen in the NGS of nasal wash samples containing H7N9 vRNA (Fig. 2D and Table 2). However, the aqueous RNA phase can become contaminated with DNA during the manual phase transfer or if the phase separation is not complete. Therefore, TRIzol/Chloroform extraction is neither as consistent nor as reliable as MagNA pure-RNA extraction in eliminating background DNA for the optimal NGS outcome. Also, TRIzol/Chloroform extraction requires the usage of toxic phenol and chloroform, and the procedure is labor intensive and not amenable to automation and high-throughput (Knepp et al., 2003). The silica-membrane based QIAgen column can preferentially bind RNA under defined buffer conditions. However, an additional on-column or post-extraction DNase treatment is still required to further remove residual DNA (Karp et al., 1998). By comparison, MagNA pure RNA extraction automatically performs the DNase treatment and clean-up on magnetic beads as part of the robotic extraction procedure. This is not only more efficient, but also prevents potential RNA degradation or loss associated with post-extraction DNase treatment. In addition, compared to the silica-membrane based extraction, magnetic bead-based extraction usually provides more thorough binding, mixing, washing and elution of nucleic acids due to its large surface area and the ability to be fully re-suspended in solution (Berensmeier, 2006; Fang et al., 2007; Yoon et al., 2016). Although the MagNA pure compact RNA isolation kit used in this study is designed to extract 8 samples at once, MagNA pure 96 cellular RNA kit that also includes an automatic DNase treatment can be used to extract 96 samples at once for high-throughput testing.

In conclusion, our study showed that the background DNA in nucleic acids extracted from nasal wash samples could negatively affect AIV genomic amplification and, hence, the NGS outcome of AIV genomes of interest. This negative effect was more severe in nasal wash samples collected on later days post-inoculation when ferrets began to clear viral infections. Therefore, selecting an RNA extraction method that can effectively eliminate DNA background, such as the MagNA pure-RNA extraction, is critical to achieving high quality NGS data with sufficient coverage for variant calling. DNase treatment is likely to facilitate NGS studies of any respiratory tract RNA pathogen found in ferret nasal wash samples or in human nasal swabs, nasopharyngeal, and/or bronchoalveolar lavage samples.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.04.014.

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