Detection of Newcastle disease virus and H5-subtype Influenza virus in swiftlet houses by Multiplex reverse transcription PCR assay

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ABSTRACT

The purpose of this study was to investigate the presence of Avian paramyxovirus 1 (APMV-1), also known as Newcastle disease virus (NDV), and Avian influenza type A, especially H5 subtype (AIV-H5) in swiftlet houses and swiftlet nest by multiplex RT-PCR (mRT-PCR). The assay used two specific primer pairs designed to detect the conserved sequence of the F gene of NDV and the HA gene of the AIV-H5, with product sizes of 282 bp and 420 bp, respectively. The mRT-PCR was established with the detection limit of 25 copies/reaction for each target virus. The thermal cycle was optimized as follows: cDNA synthesis at 45°C for 20 min, an initial denaturation at 95°C for 5 min, followed by 35 cycles of amplification encompassing denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 45 sec, ending by a final extension step at 72°C for 7 min. Eighty-eight field samples including feces and swabs of the nest surface were examined and all samples were confirmed to be negative for these two viruses. The results of this study indicated that the swiftlet nests and the environment of swiftlet houses were not contaminated with NDV or AIV-H5 viruses. Moreover, the established mRT-PCR protocol had good specificity and detection limit, and can be used for routine veterinary diagnosis.

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1. Introduction

In recent years, the swiftlet farming and swiftlet products have become a fast-growing industry in Vietnam. The products related to swiftlets on the market have high economic values due to its traditionally considered values in nutrition and health supports. Thus, understanding infectious pathogens is important to protect this new and prosperous profession. Among the diseases that can cause huge losses in poultry and/or wild birds, Newcastle disease and AIV-H5 influenza A are in the list of big concerns.

Newcastle disease virus (NDV) a member of the genus *Avulavirus* within the *Paramyxoviridae* family, is a negative sense, single-stranded, non-segmented, enveloped RNA virus (Alexander & Senne, 2008). Chambers et al. (1986) stated that the NDV genome is composed of six genes that code for six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the RNA polymerase (L). F and HN proteins are considered as central immunogenic proteins of the virion (Meulemans et al., 1968).

Avian influenza virus is enveloped, single-stranded RNA virus with eight segmented genes, belong to the genera influenza virus A in the family Orthomyxoviridae (Lamb, 2001). Avian influenza A virus contains two surface antigens, the hemagglutinin (HA) and neuraminidase (NA) proteins, classified into 18 hemagglutinin (H1-H18) and 11 neuraminidase (N1-N11) subtypes. Classification according to pathogenicity,

influenza A virus has two types of low pathogenic avian influenza (LPAI) causing no signs of disease or only exhibit mild illness in birds and high pathogenic avian influenza (HPAI) that can cause infection and death in wild birds, domestic poultry, and other mammals (Alexander, 2007). In particular, highly pathogenic AIV-H5 virus strains cause high mortality rates in both poultry and humans (Claas et al., 1998). In 1996, the H5N1 virus was first detected in geese and was the major cause of avian viral respiratory diseases outbreaks in China. In 2003, the first outbreak of avian influenza H5N1 in Vietnam, millions of poultry were culled only in a short time.

Several investigations found the presence of NDV and AIV-H5 AIV in wild birds (Schelling et al., 1999; Hoye et al., 2010; Hoque et al., 2012). Birds infected with NDV or highly pathogenic AIV-H5 virus may manifest similar symptoms, including acute respiratory disorders, decrease in egg production and high mortality. Conventional diagnostic methods such as clinical examination or gross lesions may not be confirmative enough to differentiate these two viruses.

Currently, diseases in swiftlets have not been carefully examined in the world as well as in Vietnam. Many swiftlet houses were built within or near poultry farms which poses a potential risk of the transmission of pathogenic viruses. Thus, understanding the presence of some viral agents in swiftlet houses is important for a good disease control strategy in the future. Some studies have shown possibility to detect AIV and NDV by multiplex reverse transcription-polymerase chain reaction using throat wash, oral swab, choanal swab samples (Xie et al., 2006; Chen et al., 2008; Tao et al., 2009). In this study, we developed a multiplex RT-PCR method and used for investigation and differentiation of NDV and AIV-H5 subtype in samples collected in swiftlet houses.

2. Materials and Methods

2.1. Controls and field samples

Positive controls for AIV and NDV: H5 gene sequence of 1745 bp of AIV was synthesized by IDT (Integrated DNA Technologies - USA); for NDV, a vaccine containing NDV and Infectious-Bronchitis Virus (containing B1 Type, B1 Strain, Connecticut Type 1,000 doses, Zoetic, USA) was used.

Negative controls: for specificity evaluation, DNA/RNA of bacteria or viruses that are popularly circulating in poultry farm and potentially contaminate the samples such as IBDV, IBV, *Staphylococcus* spp., *Clostridium perfringens*, *Streptococcus* spp., Salmonella spp., *Escherichia coli* were used. These controls were provided by Biomin Veterinary Diagnostic Laboratory in Vietnam (DSM group).

Field samples: Eighty-eight samples were collected from swiftlet houses in Vietnam including feces and swab samples of the nest-surface. Feces were collected at five positions for each floor (one sample for each corner and 1 sample at the center of the floor) and pooled into one sample per floor. For swab, sterile cotton swab was moisturized with physiological saline and used to wipe on the surface of the nest. The nests chosen for sampling were distributed at five spots as described for fecal samples. Samples were stored at 4°C and transported to the laboratory within 24 h. At the laboratory, the samples were stored at -20°C and nucleic acid extraction was performed within 3 days since their arrival.

2.2. DNA/RNA extraction

DNA/RNA from the samples (feces, swab, and vaccine) was extracted using WizPrep^{TM} Viral DNA/RNA Mini Kit, according to instructions of the manufacturer. RNA/ DNA was stored at -20°C until being used.

2.3. Primers

Primer information for NDV and AIV-H5 were obtained from Tang et al. (2012) and were listed in Table 1. For NDV, primers were specific for the fusion (F) gene with a product size of 282 bp. For AIV-H5, specific primers were designed on hemagglutinin (HA) gene with a product size of 420 bp.

2.4. Multiplex RT-PCR (mRT-PCR)

MyTaq^{TM} One-Step RT-PCR Kit was used to optimize primer concentrations and annealing temperature for the mRT-PCR. After a dozen rounds of optimization, the best ratio of primers concentration was determined to be 0.4 µM : 0.2 µM for NDV : AIV-H5 primers, which equivalent to the ratio of 2/1 respectively. mRT-PCR was performed in 20 µL reaction volume con-

Table 1. Primer sequences and estimated product sizes

Virus	Target gene	Primer sequences (5'-3')	Product size (bp)	Reference
NDV	F	F: TCACTCCTCTTGGCGACTC R: CAAACTGCTGCATCTTCC	282	Tang et al., 2012
AIV-H5	HA	F: ACCCAGCCAATGACCTCT R: CACTTTGCCCGTTTACTT	420	

taining 10 µL of MyTaq One-step mix (2X), 2 µL of DEPC-treated water, 2.4 µL of the primer mix, 5 µL of RNA templates, 0.4 µL of RiboSafe RNase Inhibitor, 0.2 µL of Reverse transcriptase enzyme. The target genes were amplified using the following conditions: a reverse transcription at 45°C for 20 min, an initial denaturation at 95°C for 5 min, followed by 35 cycles of amplification encompassing denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 45 sec, ending with a final extension step at 72°C for 7 min. The amplified products were analyzed by 1.2% (w/v) agarose gel in 0.5X Tris-Acetic-EDTA (TAE) and stained with Midori Green Advance DNA (Cat#MG04, Nippon Genetics), electrophoresis at 70 volts for 45 min, and visualized under UV light.

2.5. Examination of primer specificity

In reality, environmental samples can contain all types of microorganism existing in an area. Thus, beside the target viruses, contamination with other pathogens from the environment of swiftlet houses into the collected samples can be inevitable. To assure that the mRT-PCR specifically detect NDV and AIV-H5, but not interact with unrelated DNA/RNA, some viruses and bacteria such as IBDV, IBV, *Staphylococcus* spp., *Clostridium perfringens*, *Streptococcus* spp., *Salmonella* spp., *Escherichia coli* were used as negative control samples in the reactions.

2.6. Determination of detection limit in mRT-PCR

To further determine the detection limit of the mRT-PCR assay, the positive control was diluted 10 times continuously from 10^6 to 10^0 copies/µL and used in the reactions. The minimum number of template copies that could give a positive result on gel electrophoresis was considered as the limit of detection of the mRT-PCR.

3. Results

3.1. Optimal annealing temperature for the mRT-PCR

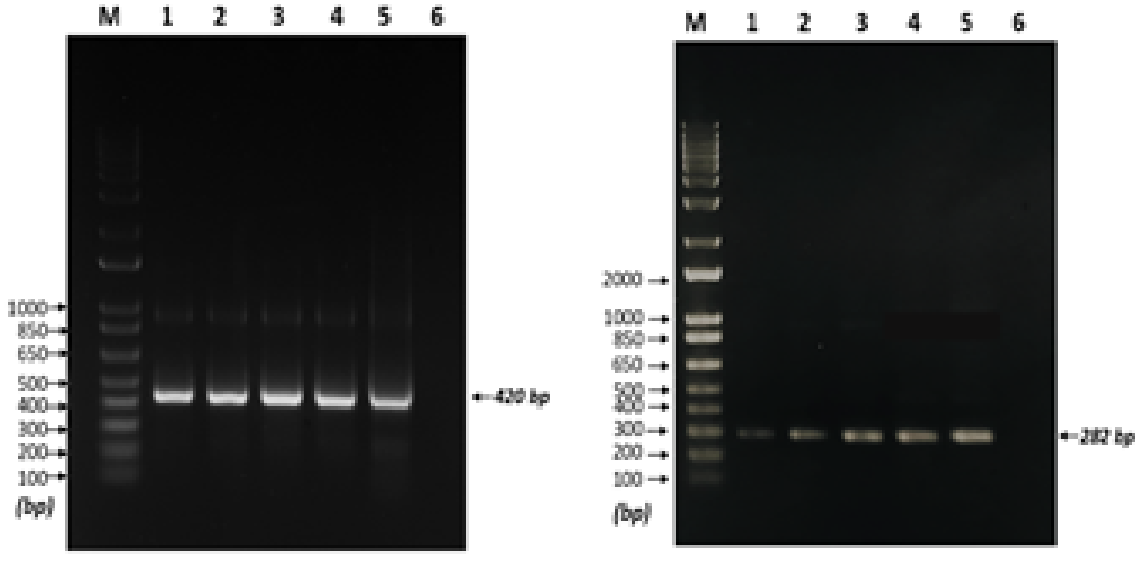
In this study, we established a PCR protocol for laboratory diagnosis of NDV and AIV-H5 in field samples of swiftlet houses and optimized the annealing temperature for the best amplification efficacy. Each primer pair was tested in single RT-PCR (sRT-PCR) at different annealing temperatures: 52°C, 54°C, 56°C, 58°C, 60°C. Results of gel electrophoresis in Figure 1 showed that sRT-PCR reactions successfully amplified the F gene of NDV with product size 282 bp and 420 bp for the HA gene of the AIV-H5. Both primer pairs worked well at the temperature range of 52 - 60°C. The temperature of 58°C was selected for subsequent experiments. In mRT-PCR reaction, the primer pairs generated precisely two amplicons of the expected sizes observed in gel electrophoresis (Figure 2).

3.2. Optimization of the primer concentration for the mRT-PCR

Based on the amplification efficacy when the two primer pairs used equally, the optimization of primer concentration used in mRT-PCR was performed with adjusted ratios in which the primer concentrations of NDV - AIV-H5 were: 1:1 (0.2 µM: 0.2 µM); 2:1 (0.4 µM: 0.2 µM); 2.5:1 (0.5 µM: 0.2 µM); 3:2 (0.6 µM: 0.4 µM). Figure 3 showed the corresponding products, indicating that the ratio 2:1 for NDV - AIV-H5 to be the best and was selected for further examinations.

3.3. Specificity and detection limit of the mRT-PCR

Swiftlet houses are often built in the same areas with other domestic animals, posing a risk of pathogen transmission from poultry to swiftlet and vice versa. Therefore, the specificity of the



(A) (B)

Figure 1. Optimization of annealing temperature in s-PCR. (A) AIV-H5 subtype, (B) NDV. (Lane M): DNA ladder 1 Kb plus, (Lane 1): 52°C, (Lane 2): 54°C, (Lane 3): 56°C, (Lane 4): 58°C, (Lane 5): 60°C, (Lane 6): negative control with pure water. The thermal cycling conditions were cDNA synthesis with 45°C/20 min and 95°C/5 min; 35 cycles of 95°C/30 sec, 52-60°C/30 sec and 72°C/45 sec, a final extension at 72°C/7 min.

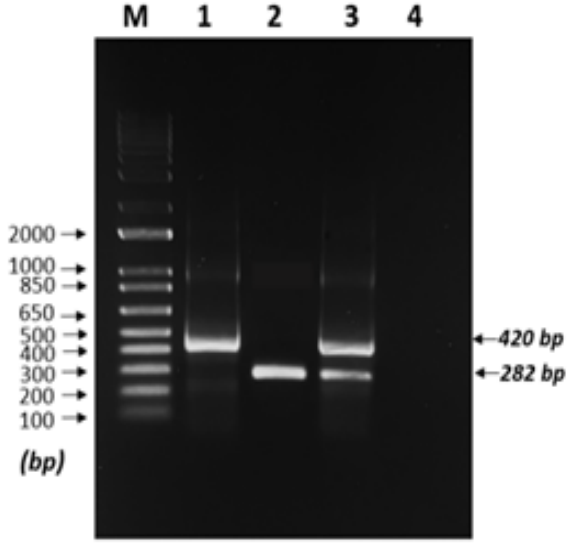


Figure 2. Products of sRT-PCRs and mRT-PCR. (Lane M): DNA ladder, (Lane 1): AIV-H5 420 bp, (Lane 2): NDV 282 bp, (Lane 3): mRT-PCR of all two targets, (Lane 4): negative control with pure water. The thermal cycling conditions were cDNA synthesis with 45°C/20 min and 95°C/5 min; 35 cycles of 95°C/30 sec, 58°C/30 sec and 72°C/45 sec, a final extension at 72°C/7 min.

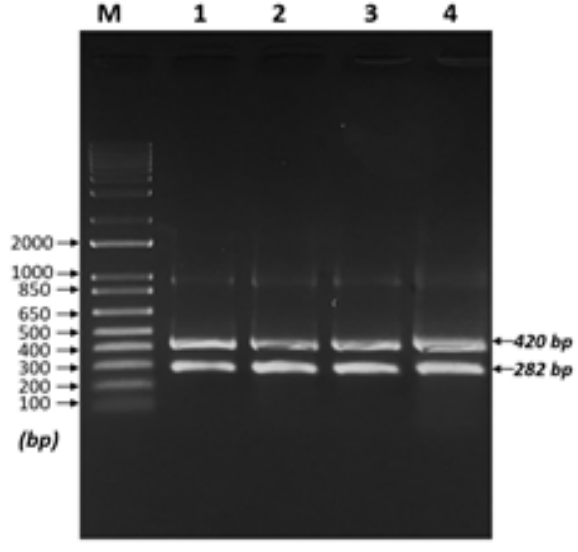
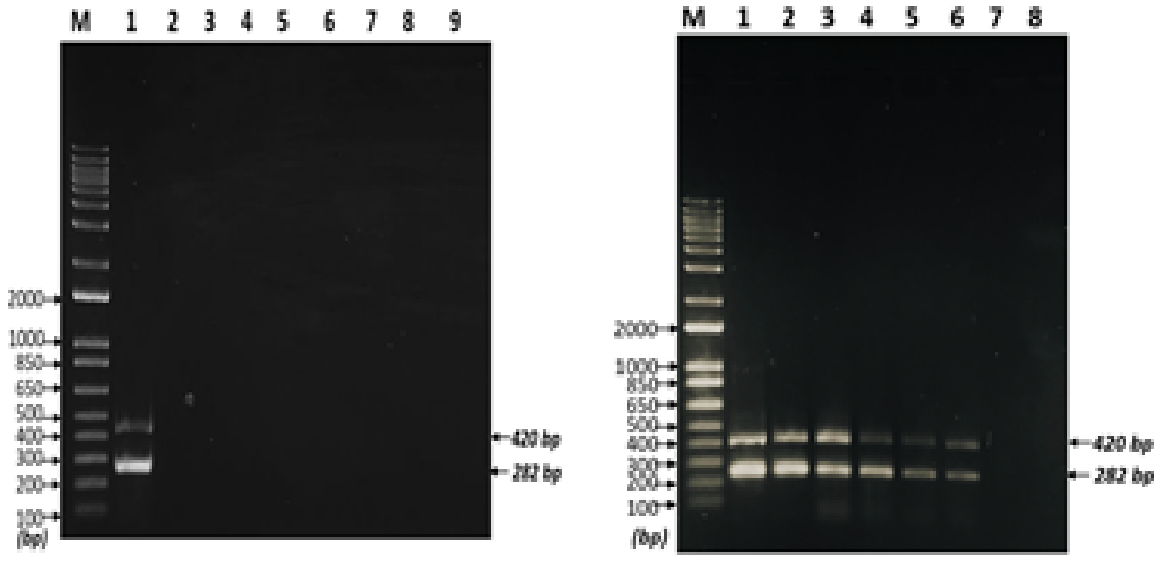


Figure 3. Electrophoresis results of the mRT-PCR with different NDV - AIV-H5 primers ratio. (Lane M): DNA ladder, (Lane 1): 1:1 (0.2 µM: 0.2 µM), (Lane 2): 2:1 (0.4 µM: 0.2 µM), (Lane 3): 2.5:1 (0.5 µM: 0.2 µM), (Lane 4): 3:2 (0.6 µM: 0.4 µM).

mRT-PCR is important to avoid false positive results due to misbinding of the primers to unrelated pathogens. To evaluate the specificity of this mRT-PCR, DNA/RNA extracted from some other pathogenic bacteria and viruses that potentially contaminate in samples such as IBDV, IBV, *Staphylococcus* spp., *Clostridium perfrin-*



(A) (B)

Figure 4. Specificity and detection limit of the mRT-PCR. (A) Result of specificity primers test. (Lane M): DNA ladder, (Lane 1): positive control, (Lane 2): E. coli, (Lane 3): Streptococcus spp., (Lane 4): Staphylococcus spp., (Lane 5): C. perfringens, (Lane 6): IBDV, (Lane 7): IBV, (Lane 8): Salmonella spp., (Lane 9): negative control. (B) The detection limit of the mRT-PCR. (Lane M): DNA ladder, (Lane 1-7): DNA template of AIV-H5 and NDV at $2.5*10^6$ - $2.5*10^0$ copies/reaction of each pathogen, (Lane 8): negative control with pure water.

gens, *Streptococcus* spp., *Salmonella* spp., *Escherichia coli* were used. Results showed that none of these templates were amplified by the primers specific for NDV and AIV-H5 (Fig 4A) confirming the specificity of primers for NDV and AIV-H5.

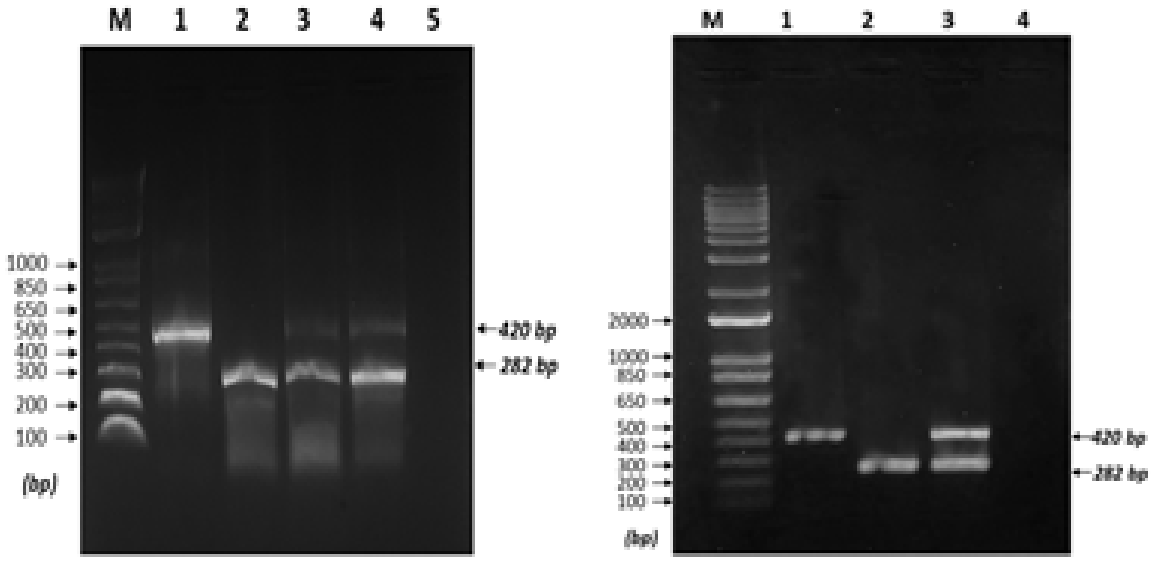
To check the detection limit of the mRT-PCR, templates of the positive controls were diluted 10-fold serially within the range of 10^6 to 10^0 copies/µL. Afterwards, mRT-PCR reactions were performed with the number of templates of each virus decreasing from $2.5*10^6$ to $2.5*10^1$ copies/reaction. As shown in Figure 4B, the two expected bands of NDV and AIV-H5 appeared clearly in lane 1-6. Nevertheless, lane 7 with the amount of $2.5*10^0$ copies/virus/reaction did not present any products. The experiments were repeated three times with similar results. Therefore, the detection limit of mRT-PCR was $2.5*10^1$ copies per reaction for each pathogen.

3.4. Examination of NDV and AIV-H5 virus in clinical samples

The purpose of this study was to use mRT-PCR to investigate the presence NDV and AIV-H5 subtype in the environment of swiftlet houses.

Figure 5. Electrophoresis results of PCR products of some clinical samples. (Lane M): DNA ladder, (Lane 1): positive control, (Lane 2-10): clinical samples, (Lane 11): negative control with pure water.

After the mRT-PCR has been established successfully. It was applied for 88 samples consisting of 44 samples of feces and 44 swab samples of the nest collected from 11 swiftlet houses located in Dong Nai and Lam Dong provinces. Surprisingly, neither NDV nor AIV-H5 was detected in the tested samples as shown partially in Figure 5. The assay was done twice, and negative results were very consistent. To further confirm the negativity as well as to remove all doubts that the viral nucleic acid may be existing, but the mRT-PCR protocol was unable to detect pre-



(A) (B)

Figure 6. Electrophoresis results of PCR products of field samples. (A) Field samples supplemented with positive control samples. (Lane M): DNA ladder, (Lane 1): AIV-H5 (420 bp), (Lane 2): NDV (282 bp), (Lane 3-4): control positive with field samples, (Lane 5): negative control with pure water; (B) Field samples of poultry infected with AIV-H5 and NDV. (Lane M): DNA ladder, (Lane 1): AIV-H5 (420 bp), (Lane 2): NDV (282 bp), (Lane 3): positive control (Lane 4): negative control with pure water.

cisely, some field samples were selected randomly, and the positive control were mixed into the samples before extraction step at the level of 10^{10} copies/extraction and $5*10^{10}$ copies/extraction for NDV and AIV-H5, respectively. Then the extract was used for mRT-PCR following the same protocol performed in Figure 5. Figure 6A demonstrated some of the assays and the results were positive when either one or both viral templates were incorporated into the samples. Additionally, when the mRT-PCR was applied for fecal samples of poultry infected with NDV or AIV-H5, it could function well in detection of the viral target (Figure 6B). The experiments in Figure 6 further confirmed that the negative results of the swiftlet samples were true and there was not any inhibitory effect caused by the sample platform.

4. Discussion

mRT-PCR methods for the identification of AIV-H5 subtype and NDV have been established worldwide (Malik et al., 2004; Xie et al., 2006; Chen et al., 2008). Hoye et al., (2010) showed that AIV-H5 could be transmitted between domestic poultry and wild birds, especially for H5 subtype. Newcastle disease virus is one of the most important viral diseases in domestic birds as well as wild birds (Snoeck et al., 2013; Hirschinger et al., 2021). Even though hundreds of reports about AIV-H5 and NDV surveillance in wild birds are available but there is limited or no information of AIV-H5 in swiflet bird.

El Zowalaty et al. (2021) found 4.1% of cloacal and oropharyngeal swab samples positive for NDV but negative for AIV-H5 in wild birds using RT-PCR. Investigating 380 cloacal swab samples of chickens and ducks for AIV and NDV, Tang et al. (2012) discovered 0.51% (2/380) positive with NDV and 1.02% (4/380) positive with AIV-H5. Some other research could detect AIV-H5 at 4.61% (25/542 samples) (Verhagen et al., 2017); 1.3% (63/4279 samples) (Bevins et al., 2016). While NDV could be detected by RT-PCR at 3.06 - 3.5% for live bird samples (N = 1461) and 0.4% for feces samples (N = 1157) respectively in Africa and in North Queensland of Australia, (Hoque et al., 2012; Cappelle et al., 2015). These studies indicated that there are opportunities to detect NDV or AIV in fecal samples, but the investigation may require a large amount of sample.

The detection limit of our mRT-PCR was 25 copies per reaction for each RNA of pathogen, and specificity primer for reaction. Thus, our

mRT-PCR procedure established can be useful for detecting pathogens presence and investigation for pathogen occurrence before further viral analysis of field samples.

5. Conclusions

In summary, we successfully developed an mRT-PCR procedure for detecting the presence of NDV and AIV-H5 virus in field samples. The mRT-PCR was specific and functioned well at the detection limit of 25 copies/target virus per reaction. Application of this mRT-PCR in diagnosis of NDV and AIV-H5 viruses in the environment of swiftlet houses and the surface of bird nests temporarily confirmed the absence of these viruses in the samples tested. The result of this study demonstrated that the environment of swiftlet houses and the bird nests were quite safe from NDV and subtype H5 of AIV.

Conflict of interest

All authors have read the journal's policy on disclosures of potential conflicts of interest, and we declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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