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Comparison of NP and M Primers for Avian Influenza Virus Detection by Reverse Transcription Polymerase Chain Reaction

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ABSTRACT In detecting all subtypes of avian influenza viruses (AIVs), amplifications of nucleoprotein (NP) or matrix protein (M) genes are better than other genes because they are conserved more among the 8 genome segments in avian influenza virus. We compared the two published primer pairs targeting NP or M genes to detect 66 imported chicken meats and 13 feather samples using reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR (rRT-PCR). Two primer pairs, NP and M52, were used to compare the detection limits for a virus in allantoic fluid. The results showed that the method using the M52 primer pair was 100 times more sensitive than that using the NP primer pair for virus in allantoic fluid. By using these primers in RT-PCR and rRT-PCR, AIV detection results showed that all the samples tested were negative with the NP primer pair. The feather samples were negative with the M52 primer pair as well. However, 13 of 66 chicken meat samples showed positive with the M52 primer pair. The M52 primer pair products were sequenced and proved to be the chicken 28S rRNA gene, considered to be false positive. To avoid the interference of chicken gene, purified AIV without cell debris was used to evaluate these two primer pairs. The results showed that both primer pairs had the same detection limit. Thus, the NP primer pair but not the M52 primer pair is suitable for AIV detection. [Hong HY, Cheng IC, Chen HW, * Wang CH. Comparison of NP and M Primers for Avian Influenza Virus Detection by Reverse Transcription Polymerase Chain Reaction. Taiwan Vet J 38 (4): 213-220, 2012. * Corresponding author TEL: 886-2-33663859, FAX: 886-2-2363-1542, E-mail: chingho@ntu.edu.tw]

Key words: avian influenza virus, detection limit, false positive, M52 primer, NP primer

INTRODUCTION

Early detection of avian influenza virus is essential to control the spread of avian influenza viruses (AIV). A number of tests including virus isolation and detection of viral genetic materials by reverse transcription-polymerase chain reaction (RT-PCR) have been applied to confirm theses viruses [2,4]. Virus isolation is a specific method of diagnosis but it is not suitable for rapid detection as it takes several days to complete [7].

Various RT-PCR tests are available to identify AIVs [1,2,3,4]. These tests are rapid and highly sensitive but have not been extensively validated on clinical specimens. Some primers have been developed for AIV detection at the initial detection because virus subtypes are unknown in this stage. Primers for nucleoprotein (NP) and matrix (M) genes have been routinely used in Taiwan for the initial detection because NP gene and M gene are conserved in different sub-

types [7]. No comparison of the two primers has been reported although the M52 primer pair was thought to be more sensitive than the NP primer pair. However, Marti *et al*. reported that pair amplifies a fragment of chicken genome with the same size as that of the AIV genome [5]. That primer might result in a false positive. However, no tested clinical specimens have been reported. Here we report on the comparison of the two primer pairs for the imported chicken meat samples by RT-PCR as well as real-time RT-PCR (rRT-PCR). The M52 Primer might anneal chicken genome and not suitable for AIV detection.

MATERIALS AND METHODS

Viruses AIVs, strains A/duck/Yunlin/04 (H5N2), A/chicken/Taiwan/1209/03 (H5N2), A/chicken/ 2838v/00 (H6N1) and A/duck/Yilan/2904/99 (H6N1) were used for evaluating the primers in this study. The strain A/duck/Yunlin/04 (H5N2) was used for detection limit determination with NP and the M52 primers separately. This virus titer was 2^9 hemagglutination (HA) unit and 2.7×10^7 EID_{50}/mL in specific-pathogen-free (SPF) chicken eggs (Animal Health Research Institute, Chunan). In order to rule out the interference of cell genes, sucrose gradient purified AIV of this strain was used for this purpose.

Samples Sixty-six chicken meat samples and 13 feather samples were taken from a routine imported chicken meat AIV inspection from Keelung and Kaohsiung harbors in 2009 and 2010. AIV detection was conducted by RT-PCR and rRT-PCR with the two primer pairs. Virus isolation was performed in SPF chicken embryos for all the samples showing positive by RT-PCR with either primer. The sample was considered negative if no virus was isolated after two passages in embryo propagation.

Reverse transcription-polymerase chain reaction (RT-PCR) TriSolution (Genmedika Biotechnol Corp) was used to extract viral RNA from AIV-infected allantoic fluid. Two pairs of primers, NP1200/NP1529 (NP primer) and M52c/M253r (M52 primer) were used:
NP1200 (forward):5'-CAG(A/G)TACTGGGC (A/T/C)ATAAG(A/G)AC-3',
NP1529 (reverse): 5'-GCATTGTCTCCGAAGAAATAAG-3' [4] and
M52C (forward): 5'-CTTCTAACCGAGGTCGAAACG-3',
M253R (reverse): 5'-AGGGCATTTTGGACAAA (G/T)CGTCTA-3' [1].

The amplification program for the NP primers started with 1 cycle at 40℃ for 30 min and 1 cycle at 94℃ for 3 min, followed by 35 cycles at 94℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min and 10 seconds and 72℃ for 10 min. The amplification program for the M52 primer pair started with 1 cycle at 42℃ for 30 min and 1 cycle at 94℃ for 3 min, followed by 40 cycles at 94℃ for 1 min, 45℃ for 1 min, 72℃ for 3 min. The expected sizes were 244 bp and 330 bp with the M52 and the NP primer pairs, respectively.

Preparation of linear plasmid According to the cloning recommendations, the complete matrix gene of A/duck/Yunlin/04 (H5N2) was constructed using the TA cloning kit. In order to produce defined RNA transcripts, plasmid DNA was cut with *Xba*I, digesting the insert downstream, to become linear. An equal volume of phenol-chloroform was added to the solution. One tenth volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold absolute alcohol were added to the upper aqueous phase. The mixture was incubated at 4℃ for 30 min and then centrifuged for 15 min. The pellet was resuspended in diethyl pyrocarbonate (DEPC) water.

In vitro transcription The linear form plasmid DNA was transcripbed by TranscriptAid™ T7 High Yield Transcription Kit (Fermentas). To remove residual plasmid DNA, the products of RNA transcripts were incubated with DNase I at 37℃ for 40 min. After the reaction, the RNA transcripts were purified by phenol:chloroform:isoamylalcohol (25:24:1, pH 8.0) (Amresco). The RNA transcripts and phenol:chloroform:isoamylalcohol were blended, and the upper phase was collected and mixed with the same volume of 2.5 M LiCl. The pure transcribed RNA were precipitated at -20℃ overnight and harvested by centrifugation at 12,000 rpm for 20 min at 4℃.

Real-time reverse transcription-polymerase chain reaction (rRT-PCR) Viral RNA was extracted with TRIzol LS reagent (Genmedika Biotechnol Corp) according to the method described by the supplier. The NP and M52 primer pairs were used separately in this study. The rRT-PCR reaction was carried out in a 20 μL mixture of iScript one-step RT-PCR kit with SYBR Green (Bio-Rad) containing 0.25 μM of each primer, 0.5 μL of iScript reverse transcriptase and 2 μL of RNA template according to the published papers with modification [3,8]. The reverse transcription was performed on a MyiQ Thermal Cycler machine (Bio-Rad). cDNA was synthesized at 50℃ for 10 min in RT and then followed by 45 PCR cycles according to the manufacture procedure except 50℃ for 30 sec in the data collection step. Positive and negative results were determined by the iQ5 software and rechecked manually. Those with melting point at 84℃ in the melt-curve were considered to be positive with both NP and M52 primer pairs and those without to be negative. The specificity and detection limit of rRT-PCR with NP and the M52 primer pairs were performed with template RNA from AIVs tested.

Detection limits of RT-PCR and rRT-PCR for infected allantoic fluid AIV A/duck/Yulin/04 (H5N2) was prepared by inoculating into SPF chicken embryos. After 4 days of incubation at 37℃, the allantoic fluid was harvested and the virus titer was titrated with SPF chicken embryos by standard methods [7]. The 10 x serial dilutions of the allantoic fluid were detected by RT-PCR and rRT-PCR.

Detection limits of RT-PCR for purified AIV In order to avoid the contamination of chicken genome, the infected allantoic fluid was purified by 20-50% sucrose gradient centrifugation. The purified AIV solution was considered to be free from chicken cells. The 10x serial dilutions of the purified AIV were detected by RT-PCR and rRT-PCR. The RNA was extracted and the AIV genome was detected by RT-PCR with the same methods described above.

Sequencing of the positive band with the M52 primer The meat samples having positive band with the M52 primer pair was purified with gel extraction kit (Qiagen) and sequenced directly with a commercial service (Mission Co, Taipei). The sequence was compared with the reference sequences using BLAST in the GenBank.

RESULTS

Detection limits of RT-PCR and rRT-PCR for infected allantoic fluid The virus titer of A/duck/Yunlin/04 (H5N2) was calculated to be 2.7×10^7 EID_{50}/mL in SPF eggs, equal to 7.7×10^{10} copy numbers by rRT-PCR. The allantoic fluid contained AIV as well as chicken cells. The study found that RT-PCR with the M52 primer was 100 x more sensitive than that with primer NP, either in RT-PCR or rRT-PCR (Table 1, Fig. 1). The melting temperature of rRT-PCR was 84℃ (Fig. 2).

Detection limits of RT-PCR for purified AIV The concentration of purified A/duck/Yunlin/04 (H5N2) was 480 μg/mL. After serial dilutions of purified AIV, both NP and M52 primer pairs showed the same sensitivity in RT-PCR. They detected the same dilution of the pure AIV (10^{-4}) (Fig. 3).

Chicken meat and feather samples During routine avian influenza virus detection for imported chicken meat samples at our laboratory, all samples showed negative by RT-PCR and rRT-PCR with the NP primer pair. However, some chicken meat samples showed positive (Fig. 4). The positive results were obtained in the same lot but not other lots. The same results were obtained with triple repeats. The appearance of positive results depends on the contamination of chicken genomes in the purification of RNA from meat. Among the 66 chicken meat samples, 13 of them

Table 1. Detection limits of RT-PCR and rRT-PCR with different primers on AIV A/duck/Yunlin/04 (H5N2) in allantoic fluid.

Primer	RT-PCR	rRT-PCR
NP	10^{-3}	10^{-4}
M52	10^{-5}	10^{-6}

The virus titer is 2.7×10^7 EID_{50} /mL which equals to 7.7×10^{10} copy numbers by rRT-PCR. The M52 primer shows 100 x more sensitive than that with the NP primer by RT-PCR and rRT-PCR.

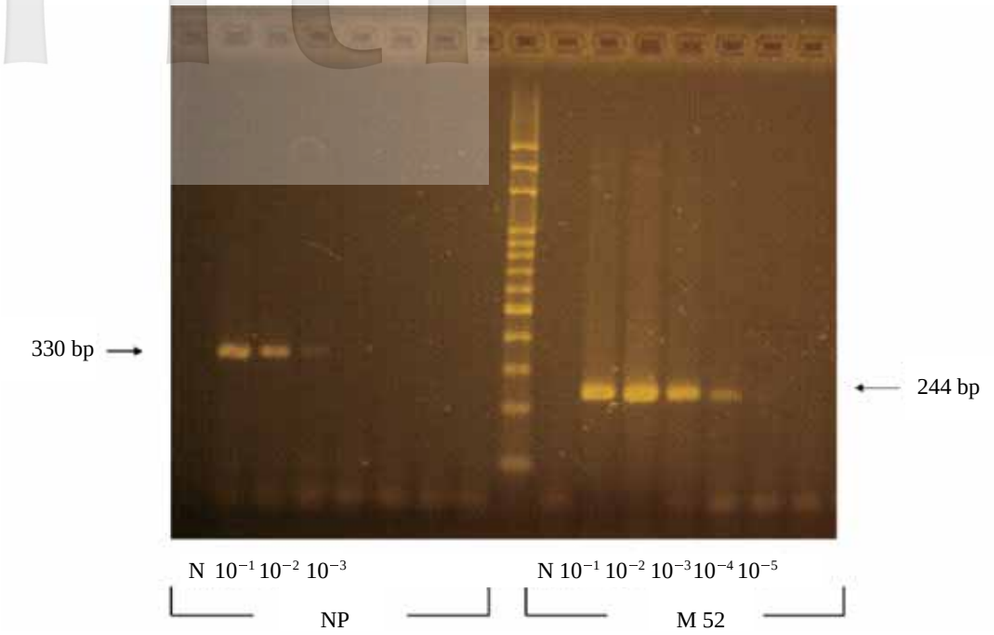

Fig. 1 Detection limits of RT-PCR with the NP and the M52 primers to AIV, A/duck/Yunlin/04 (H5N2). Note 100 times more sensitive with the M52 primer than with the NP primer (10^{-5} *v.s* 10^{-3} dilution).

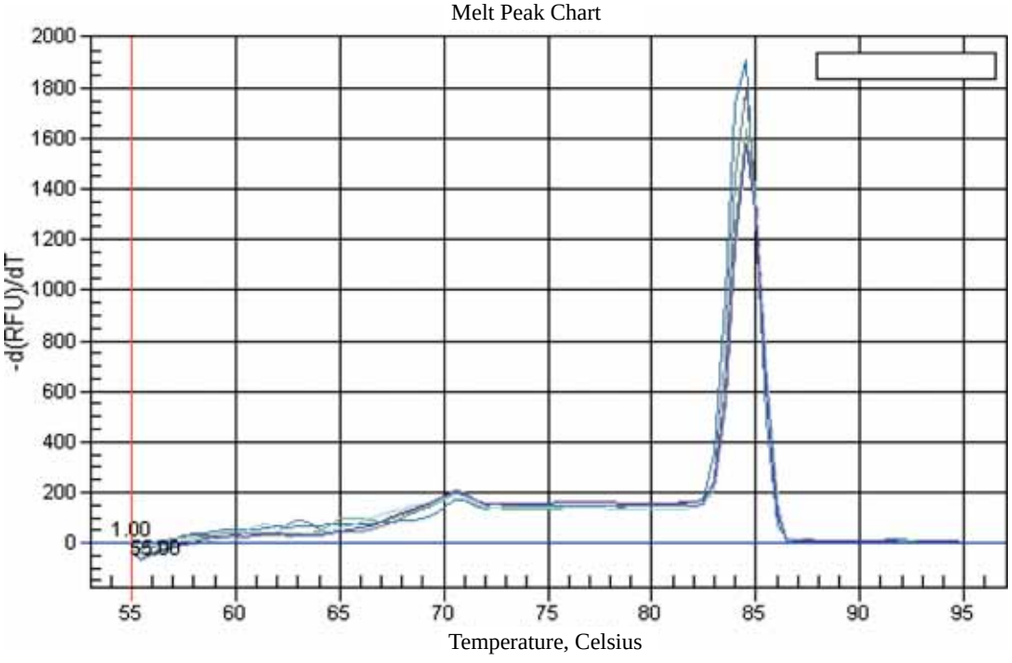

Fig. 2 Melting curve of the rRT-PCR product of A/duck/Yunlin/04 (H5N2) at different dilutions from 10^{-1} to 10^{-4}. The melting temperature is about 84℃ with the NP primer pair.

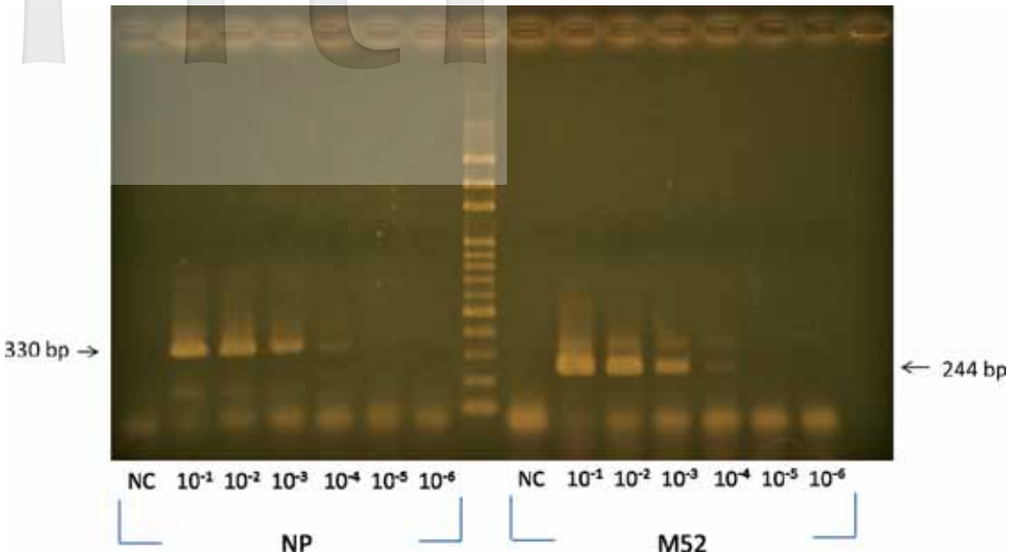

Fig. 3 Detection limits of RT-PCR with the NP and the M52 primer pairs to purified AIV A/duck/Yunlin/04 (H5N2). Note the detection limits with both primers are the same (10^{-4} dilution).

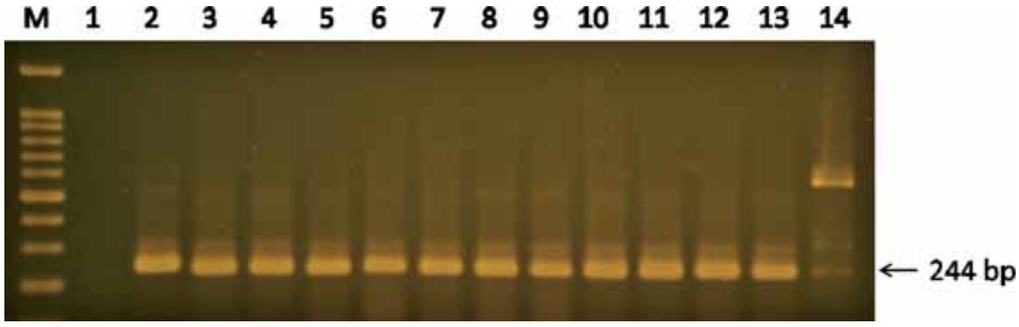

Fig. 4 RT-PCR results of 13 chicken meat samples in a lot with the M52 primer pair. Note all samples showed positive. Lane 1: negative control with water. Lane 2: positive control of A/duck/Yunlin/04 (H5N2) in allantoic fluid. Lane 3-13: sample numbers from 136-146. Lane 14 allantoic fluid from a SPF chicken embryo.

in the two lots showed positive (13/66 = 20%).

All the 13 feather samples were negative with both primers. All the samples were approved to be negative by further analysis by RT-PCR with the NP primer pairs and chicken embryo inoculation for two passages.

Sequencing of the positive band produced by RT-PCR with the M52 primer pair The RT-PCR products of 8 meat samples showing positive with the M52 primer pair were selected for sequencing directly. Due to the invalidity of the sequences near primers, only the central part of the fragment was sequenced. That sequence was about 195 bp and showed 98% identical to *Gallus gallus* 28S rRNA gene, clone GfLSU-1 (accession number FM165415.2) and *Gallus gallus* clone AY006 28S rRNA gene (accession number DQ018757). The bands from 2 positive meat samples were cloned and sequenced. Their sequence was 98% identity with the *Gallus gallus* too. Thus, all the samples tested were false positive.

DISCUSSION

Taiwan imports chicken meat from other countries, mostly the United State of America (USA). Since avian influenza viruses have been detected in the USA from time to time, imported chicken meat should be checked for the presence of AIVs at the border. The concern is that AIVs could intrude into Taiwan through imported meat. The first step in detect AIV is RT-PCR with primers targeting conserved genes, like NP and M. At the beginning we used NP and the M52 primer pairs to detect AIVs and found the sensitivity of the M52 primer pair was 100 x higher than that with the NP primer pair. However, the M52 primer pair was found to anneal chicken genome too at the same product size. Marti *et al*. revealed that RT-PCR with the M52 primer pair resulted in false positives in RT-PCR. The sequence of that RT-PCR products were highly homologous (98%) to *Gallus gallus* [5]. Thus, the RT-PCR with the M52 primer pair is not suitable for avian influenza detection.

We further confirmed that the M52 primer pair anneals to the chicken genome because the sequences of the positive bands are from chickens. This false positive didn't appear each time but when the sample was contaminated with chicken cell debris. To avoid the contamination, we used purified virus to compare the sensitivity of the two primers and found both sensitivities were the same (Table 1). The M52 primer pair could not be used for the routine surveillance due to its false positive reaction.

rRT-PCR provides several advantages over RT-PCR, i.e. speed and elimination of the possible contamination from new samples. However, rRT-PCR with SYB green can also detect the nonspecific chicken genome product that has a similar Ct value. If a probe is used in rRT-PCR such as Taqman system, the nonspecific results would not occur since the probe could only bind to the specific products.

For imported meat, the samples showing positive by RT-PCR should be performed by RT-PCR with H5 and H7-specific primers. Those showed positive by the M52 primer pair were detected by H5 and H7 RT-PCR and all samples were negative (data not shown). Virus isolation was used for those positive samples. However, the virus isolation only detects live virus. More likely, the virus has been destroyed during shipment from other countries.

The reason that all tested imported chicken meat showed negative to AIV by RT-PCR might be that the AIVs in the USA are low pathogenic. Low pathogenic AIVs are present only in respiratory and digestive tract and cannot invade into meat. The present rRT-PCR is not more sensitive than RT-PCR. These factors that affect the sensitivity of the rRT-PCR might be because of insufficient RNA amount and the rapid degradation before testing.

In order to perform rRT-PCR rapidly and on a large scale., a single-step RT-PCR was used. This method is less sensitive than the two-step RT-PCR procedure [6]. In addition, a single-step RT-PCR procedure also greatly reduces the risk of cross contamination because the tubes are not reopened once the template is added. There is also a risk for one-step instead of two-step since RNA is transcribed from template plasmid before testing because RNA-degrading enzymes are ubiquitous. The RNA might be degraded prior to testing. Finally, the negative results might be due to the low virus content in meat. Having more volume of the original sample might result in better sensitivity by RT-PCR. This study demonstrates that one should be careful to use the M52 as the primer for AIV detection.

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以 NP 及 M 引子逆轉錄聚合酶連鎖反應檢測禽流感病毒的比較

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摘要　禽流感病毒 8 段基因中，核蛋白質（NP）及基質蛋白質（M）基因較其他基因保守，因此利用此二基因可以檢測所有亞型的禽流感病毒。我們應用已發表的 NP 引子及 M52 引子，以逆轉錄聚合酶連鎖反應（reverse transcription polymerase chain reaction, RT-PCR）及即時逆轉錄聚合酶連鎖反應（real-time reverse transcription polymerase chain reaction, rRT-PCR）的方法進行禽流感的檢測，為了比較其靈敏度，選取尿囊液的病毒，進行序列稀釋，結果在 RT-PCR 及 rRT-PCR 的方法中，M52 引子比 NP 引子皆敏感 100 倍；以 NP 引子檢測 66 個進入雞肉及 13 個羽毛樣本皆為陰性，以 M52 引子檢測，有 13 個雞肉樣本為禽流感陽性，羽毛樣本為陰性；M52 引子產物經定序，證實為雞細胞的 28S rRNA 基因，有偽陽性的出現。為避免雞隻細胞核酸的干擾，以純化的病毒進行 RT-PCR，結果 M52 引子與 NP 引子的靈敏度相同，結論此 NP 引子適合禽流感病毒檢測，而此 M52 引子不適合。[洪惠雯、鄭益謙、陳慧文、*王金和。以 NP 及 M 引子逆轉錄聚合酶連鎖反應檢測禽流感病毒的比較。台灣獸醫誌 38 (4)：213-220，2012。* 通訊作者 TEL：886-2-33663859，FAX：886-2-2363-1542，E-mail：chingho@ntu.edu.tw]

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