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Combination of multiplex reverse transcription recombinase polymerase amplification assay and capillary electrophoresis provides high sensitive and high-throughput simultaneous detection of avian influenza virus subtypes

Shou-Kuan Tsai [1,†], **Chen-Chih Chen** [2,3,†], **Han-Jia Lin** [1], **Han-You Lin** [4], **Ting-Tzu Chen** [4], **Lih-Chiann Wang** [4,*]

[1]Department of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung 20224, Taiwan
[2]Institute of Wildlife Conservation, College of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan
[3]Animal Biologics Pilot Production Center, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan
[4]School of Veterinary Medicine, National Taiwan University, Taipei 10617, Taiwan

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***Corresponding author:**
Lih-Chiann Wang
School of Veterinary Medicine, National Taiwan University, No. 1, Section 4, Roosevelt Rd, Da'an District, Taipei 10617, Taiwan.
E-mail: lcwang@ntu.edu.tw

[†]Shou-Kuan Tsai and Chen-Chih Chen contribute equally to this work.

ORCID iDs
Shou-Kuan Tsai
https://orcid.org/0000-0002-3618-7503
Chen-Chih Chen
https://orcid.org/0000-0002-9001-5323

ABSTRACT

The pandemic of avian influenza viruses (AIVs) in Asia has caused enormous economic loss in poultry industry and human health threat, especially clade 2.3.4.4 H5 and H7 subtypes in recent years. The endemic chicken H6 virus in Taiwan has also brought about human and dog infections. Since wild waterfowls is the major AIV reservoir, it is important to monitor the diversified subtypes in wildfowl flocks in early stage to prevent viral reassortment and transmission. To develop a more efficient and sensitive approach is a key issue in epidemic control. In this study, we integrate multiplex reverse transcription recombinase polymerase amplification (RT-RPA) and capillary electrophoresis (CE) for high-throughput detection and differentiation of AIVs in wild waterfowls in Taiwan. Four viral genes were detected simultaneously, including nucleoprotein (NP) gene of all AIVs, hemagglutinin (HA) gene of clade 2.3.4.4 H5, H6 and H7 subtypes. The detection limit of the developed detection system could achieve as low as one copy number for each of the four viral gene targets. Sixty wild waterfowl field samples were tested and all of the four gene signals were unambiguously identified within 6 h, including the initial sample processing and the final CE data analysis. The results indicated that multiplex RT-RPA combined with CE was an excellent alternative for instant simultaneous AIV detection and subtype differentiation. The high efficiency and sensitivity of the proposed method could greatly assist in wild bird monitoring and epidemic control of poultry.

Keywords: Capillary electrophoresis; influenza virus; reverse transcription

Han-Jia Lin
https://orcid.org/0000-0002-4929-6573
Han-You Lin
https://orcid.org/0000-0002-0858-6160
Ting-Tzu Chen
https://orcid.org/0000-0002-2297-3618
Lih-Chiann Wang
https://orcid.org/0000-0001-7302-4714

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Conflict of Interest

BiOptic Inc. supplied the CE analyzer in this study. BiOptic Inc. played no role in the study design or in the collection, detection and interpretation of data, nor in the decision to submit this manuscript for publication. None of the authors has any relationships that could inappropriately influence or bias the content of this paper.

Author Contributions

Conceptualization: Lin HJ, Lin HY, Wang LC; Data curation: Tsai SK, Chen CC, Chen TT; Formal analysis: Tsai SK, Chen CC, Chen TT; Investigation: Tsai SK, Chen CC, Chen TT; Methodology: Tsai SK, Chen CC, Chen TT; Project administration: Tsai SK, Chen CC, Chen TT, Wang LC; Software: Tsai SK, Chen TT; Supervision: Lin HJ, Lin HY, Wang LC; Validation: Chen TT, Wang LC; Visualization: Chen TT, Wang LC; Writing-original draft: Tsai SK, Chen CC, Wang LC; Writing-review & editing: Tsai SK, Chen CC, Lin HJ, Lin HY, Wang LC.

INTRODUCTION

Avian influenza is a highly contagious disease caused by the type A influenza virus, which has many subtypes determined by the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [1]. Until now, 18 known HA subtypes and 11 known NA subtypes have been found in nature. Except the bat-derived influenza H17 and H18 viruses, all AIV subtypes are found in waterfowls which serve as the primordial reservoir of the influenza A viruses [2]. Clade 2.3.4.4 H5 highly pathogenic avian influenza (HPAI) viruses (novel H5N2, H5N3 and H5N8) have caused outbreaks in poultry and wild birds throughout Taiwan since 2015 [3,4], leading to tremendous economic loss. According to the official website data of the Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ), the HA gene of these novel H5 viruses was closely related to that of the H5N8 virus isolated from ducks in Korea in 2014, and the other genes were close to avian influenza viruses (AIVs) isolated in China, Japan and Korea [5]. These novel H5 viruses were therefore verified as from migratory birds and not from the evolution of local viruses in Taiwan.

H6N1 influenza virus has been frequently isolated from poultry in Taiwan since the 1970's [6]. The virus has been enzootic and made latent infections in chickens. In 2013, the first H6N1 human case in the world was reported in Taiwan [7]. The dog H6N1 natural infection case was also reported in Taiwan in 2015 subsequently [8]. These incidences unveil the extensiveness and impact of the H6N1 virus in Taiwan.

H7N9 virus causing human infection was first reported in China in 2013, which was generated by multiple interspecies transmissions and reassortment among the viruses resident in domestic ducks and the H9N2 virus enzootic in chickens [9]. This raises serious concerns in neighboring areas with poultry farming industry, including Taiwan, as it poses the high possibility of spreading the virus through the migration of wild birds.

Since AIVs continuously undergo genetic reassortment among different subtypes and clade 2.3.4.4 H5 HPAI viruses have spread rapidly and globally through wild birds since 2014 [10], it is imperative to make intensified surveillance in migratory birds and wild endemic waterfowls. High throughput and high sensitivity measures for multitasking detection of AIVs is extremely worth developing for outbreak prevention and control.

The recombinase polymerase amplification (RPA) assay is an approach for rapid and specific DNA/RNA amplification. Unlike polymerase chain reaction (PCR), it works under isothermal conditions and completes amplicon production within 30 min [11,12]. The RPA product can be visualized using traditional plate agarose gel electrophoresis [13]. With the assistance of an extra detection probe, it can be detected using a real-time device [14] or lateral flow dipstick [12]. Single influenza A virus HA gene detection using RPA for H7N9 [15] or H5N1 [16] has been reported. Nevertheless compared to PCR, RPA is much prone to produce non-specific signals [11,17] which render it difficult for multiplex molecule investigation. Multiplex RPA for more than three targets has never been attempted, not to mention the quadruplex reverse transcription recombinase polymerase amplification (RT-RPA) for the extremely diverse AIVs. Therefore, a powerful device with high specificity and accuracy used for post multiplex RT-RPA detection is needed.

Capillary electrophoresis (CE) is a DNA sizing system, which is widely applied in detection due to its automation, superior accuracy, high speed and ease of use [18]. More than 10

products can be simultaneously detected within 15 min [19,20]. In recent years, CE has been progressively applied to high-throughput nucleic acid analysis applications, including pathogen detection [21], genotyping [22], discrimination of the alleles [23], and species identification [24]. However, CE is limited mostly to analyze PCR products but has never been applied to RPA product detection. Lightweight and portable CE machine has been developed in recent years [25], making it an excellent potential post-RPA apparatus for future field sample tests on site.

Since H6 and clade 2.3.4.4 H5 AIVs are pervasive in Taiwan and the island is substantially under the threat of H7 from China, immediate virus detection from either wild birds or domestic poultry is a critical issue. A system combining multiplex RT-RPA plus CE was developed in this study for the detection of all AIV subtypes via the conserved nucleoprotein (NP) gene and simultaneous differentiation of clade 2.3.4.4 H5, H6 and H7 subtypes via each of the HA gene. Multiplex RT-RPA provided rapid multitasking for RNA amplification, and CE supplied high-precise and high-throughput capacity for post-RPA analysis. Quadruple gene detection employing multiplex RT-RPA and CE is a novel, highly efficient and sensitive approach, enabling fast diagnosis of diversified AIVs in field samples on a large scale and prompting epidemic control of the country.

MATERIALS AND METHODS

Viral RNA and field samples

Viral RNA of H5N2 (A/chicken/Taiwan/a2888/2015) (clade 2.3.4.4) and H7N9 (A/WB/TW/WB2498/2011) viruses were obtained from Division of Epidemiology, the Animal Health Research Institute, Council of Agriculture, Tamsui, Taiwan. Viral RNA of H6N1 virus (A/chicken/Taiwan/2838v/00), avian infectious bronchitis virus (IBV, GenBank Accession No. AY606316), Newcastle disease virus (NDV, GenBank Accession No. AF164966) and clade 2.2.1 low pathogenic avian influenza virus H5N2 (A/chicken/Taiwan/1209/03) were obtained from the Poultry Disease Laboratory, School of Veterinary Medicine, National Taiwan University. Human H1N1 and H3N2 viruses were from Taiwan Centers for Disease Control. Migratory and endemic waterfowls were trapped using funnel trap [26] in Aogu Wetland Forest Park (Coordinate: 23.496118, 120.127495), Chiya county, Taiwan, a major wintering site for migratory waterfowls. Birds trapped in this study were species migrated through East Asian-Australasian Flyway from Alaska or Siberia in the winter spanning 2017-2018 (**Supplementary Table 1**). Oral and cloacal swabs were collected from 60 trapped waterfowls using FLOQSwabs (Copan Diagnostics, USA) and were preserved in the UTM transport media (Copan Diagnostics) [27] in the cool box. The procedures for wild waterfowls trapping and sample collection were approved by Institutional Animal Care and Use Committee of National Pingtung University of Science and Technology (Approval no.: NPUST-106-029). Viral RNA of the field samples was extracted using QIAamp viral RNA kit (Qiagen, USA).The RNA was immediately stored at −80°C until used for the RT-RPA assay.

RPA primer design

The RPA primer design followed the recommended rules [12] .The NP gene sequence of all AIVs and HA gene sequences of clade 2.3.4.4 H5, H6 and H7 AIVs in domestic poultry and wild birds in Taiwan, China and northeastern Asia from Genbank or GISAID (The Global Initiative on Sharing All Influenza Data) were aligned using the MegAlign program (DNASTAR Lasergene 7.2.1,USA). Primer candidates were selected under the assistance of Multiple Primer Analyzer website [28]. The RPA primer employed in this study is listed in **Table 1**.

Table 1. The designed recombinase polymerase amplification primers for multiple detection of AIV genes

Primer name	Sequence (5′→3′)	Direction	Targeted gene	Product size (bp)
NP 217F	ARCACYCTTGARCTRAGAAGYAGATAYT	Forward	NP gene of all AIVs	217
NP 217R	CCATCATYCTTATGATTTCWGTCCTCAT	Reverse		
H5 173F	ATGCCATTCCACAATATACAYCCYCTCAC	Forward	HA gene of clade 2.3.4.4 H5 AIVs	173
H5 173R	ATTCCYTGCCATCCTCCCTCTATRAAMCCTGC	Reverse		
H6 199F	GACTGGAATGATAGATGGGTGGTATGGC	Forward	HA gene of H6 AIVs	199
H6 199R	GGAATGATAGATGGGTGGTATGGCTATC	Reverse		
H7 137F	GTGCATGTAGGAGATCAGGATCTTCATT	Forward	HA gene of H7 AIVs	137
H7 137R	TCCCCATACTATCAGAGCTGGGTCTCTC	Reverse		

AIV, avian influenza virus; NP, nucleoprotein; HA, hemagglutinin.

Plasmid cloning and *in vitro* transcription

The NP gene of AIVs and the HA genes of clade 2.3.4.4 H5, H6 and H7 viruses were amplified using one-step RT-PCR (Qiagen, Germany) with each of the designed RPA primer pairs. The RT-PCR products were purified using the PCR cleanup kit (GeneMark, Taiwan) and cloned into pGEM-T Easy Vector (Promega, USA). The recombinant plasmid was linearized and the 3' overhang was conversed with the DNA polymerase Klenow (Promega). In vitro transcription was performed using Riboprobe in vitro Transcription Systems (Promega) with T7 RNA Polymerase according to the manufacturer's recommendations. DNase (Promega) was added to remove the remaining template DNA. The produced RNA was purified using RNeasy MiniElute Cleanup Kit (Qiagen) and verified by electrophoresis gel. The RNA was quantified using a spectrophotometer (Thermo Fisher Scientific, USA) and the copy number was calculated.

RT-RPA reaction

RPA reactions were carried out using the TwistAmp basic kit (TwistDx Limited, UK). The singleplex RT-RPA was carried out and had good performance (data not shown). The multiplex RT-RPA reactions were modified based on the manufacturer's manual. For each reaction, 29.5 μL of Rehydration Buffer, 1 μL of RNase inhibitor (Promega), 1 μL of Moloney murine leukemia virus reverse transcriptase (Protech), and 10 μL of 4 M Betaine (Sigma-Aldrich, USA) were added to dissolve the freeze-dried pellet. Afterwards, 0.5 μL of each 10 μM RPA forward and reverse primers and 2 μL of RNA template were added and mixed. Two point five μL of 280 mM magnesium acetate was then added to form a total 50 μL solution and start the reaction. After incubation at 39°C for 10 min, the solution was sent to a vortex for 2 sec and spun down, and then incubated for another 20 min. The final multiplex RT-RPA product was purified using the QIAquick PCR Purification kit (Qiagen) for the following CE and plate agarose gel electrophoresis.

CE and plate agarose gel electrophoresis

The purified multiplex RT-RPA products were subjected to CE. Traditional plate agarose gel electrophoresis was also implemented to compare. The CE was performed using Qsep400 Bio-Fragment Analyzer S1 cartridge (BiOptic, Taiwan) based on the manual instruction. Samples were injected in 4 KV voltage for 20 seconds and ingredients were separated in 6 KV voltage for 300 seconds. The alignment markers were 20 bp and 1,000 bp. The migration time versus relative fluorescent unit (RFU) of each ingredient peak was read and analyzed using Q-Analyzer software (BiOptic Inc.). The plate electrophoresis was performed using 4% agarose gel with 100 bp ladder marker in 1X TE buffer for 50 min, and visualized under UV light.

Detection limit comparison between the two electrophoresis systems

The standard RNA obtained form in vitro transcription was 10-fold serially diluted (10^8 to 10^0 copies) in RNase-free water and used as templates for the multiplex RT-RPA reaction. The multiplex RT-RPA detection limits visualized on plate electrophoresis gel bands and CE peaks were compared.

Field sample tests

The field wild waterfowl samples were tested using the multiplex RT-RPA and electrophoresis. Traditional plate agarose gel electrophoresis and CE were both manipulated and the detection sensitivity was compared.

RESULTS

Detection limit tests of multiplex RT-RPA

The detection limit of multiplex RT-RPA between traditional plate agarose gel electrophoresis and CE was compared. In vitro transcribed RNA from the NP gene, clade 2.3.4.4 H5 HA gene, H6 HA gene and H7 HA gene was quantified and used as the template for multiplex RT-RPA reactions. RNA was 10-fold serial diluted from 10^8 to 10^0 copy numbers. The results showed that the detection limit on plate agarose gel after multiplex RT-RPA for NP, H6, H5 and H7 was 10^4, 10^4, 10^5, 10^5 copy numbers per reaction, respectively. The detection sensitivity was lower than singlex RT-RPA whose detection limit was 10^3 copy numbers for each gene on agarose gel (data not shown). However, if CE was employed after multiplex RT-RPA, the detection limit could be restored and attained 10^0 copy numbers per reaction for all of the four targeted genes (**Fig. 1**). Other common avian respiratory viruses, such as IBV and NDV, were used as the negative control. Other common influenza subtypes in Taiwan, for example clade 2.2.1 low pathogenic avian influenza virus H5N2 (A/chicken/Taiwan/1209/03) and human H1N1 and H3N2 viruses, were used as control which only have corresponding NP bands or peaks (**Fig. 1**). This indicated that the developed multiplex RT-RPA had good specificity. Moreover, CE was a much more susceptive platform than agarose gel for post-multiplex RT-RPA detection, providing instant signals for simultaneous quadruple target gene identification.

Field sample tests

Oral and cloacal swabs were collected from 60 wild waterfowls in the wetlands of southern Taiwan. Simultaneous AIV detection and virus subtyping were performed using the developed multiplex RT-RPA approach. Multiplex RT-RPA products were submitted to the traditional plate agarose gel electrophoresis and CE, and the results were compared (**Supplementary Table 1** and **Supplementary Fig. 1**). The CE detection rate was much higher than that for plate agarose gel electrophoresis. The agarose gel bands corresponding to the CE peaks were referred to sequencing, and the correctness was verified through GenBank BLAST processing. High similarity but not identity between the field sample sequences and the positive control sequences also confirmed that the positive signals were authentic and not from the contamination with the positive control (**Supplementary Table 2**). **Fig. 2** showed the representative CE results from field samples. All of the positive field sample peaks (blue line) were precisely overlapped with the positive standards (red line) at the correct corresponding size point, proving that the these peaks were not from noises due to the non-complete RPA reaction (**Fig. 2** and **Supplementary Fig. 1**).

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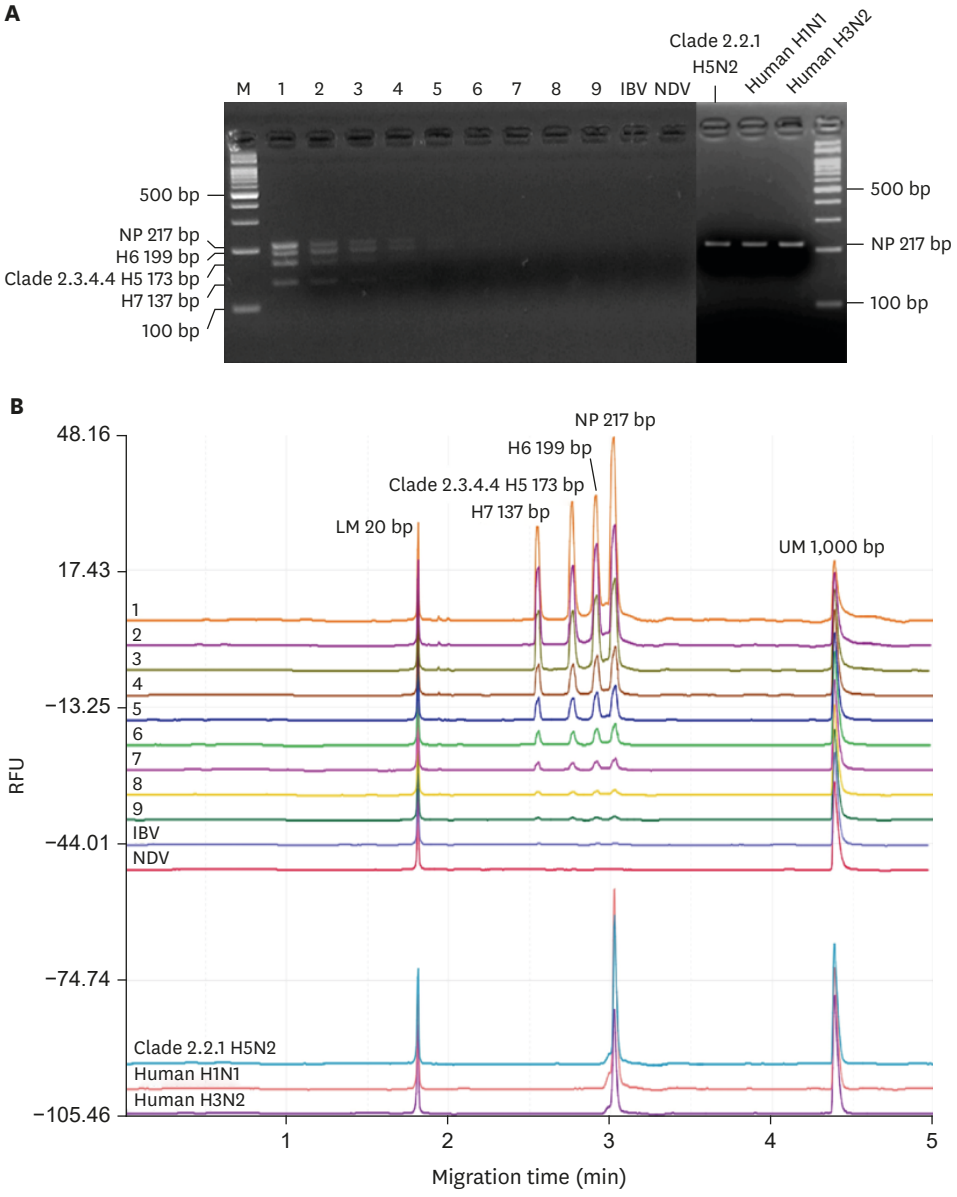

Fig. 1. Detection limit comparison between traditional plate agarose gel electrophoresis (A) and capillary electrophoresis (B). *In vitro* transcribed RNA from each targeted viral gene was quantified and used for multiplex reverse transcription recombinase polymerase amplification reaction templates.
M, 100 bp ladder marker; LM, lower alignment marker (size = 20 bp); UM, upper alignment marker (size = 1,000 bp); 1, 10^8 copy numbers; 2, 10^7 copy numbers; 3, 10^6 copy numbers; 4, 10^5 copy numbers; 5, 10^4 copy numbers; 6, 10^3 copy numbers; 7, 10^2 copy numbers; 8, 10^1 copy numbers; 9, 10^0 copy numbers; IBV, infectious bronchitis virus; NDV, Newcastle disease virus. Other common avian respiratory viruses (IBV and NDV) and type A influenza viruses (clade 2.2.1 low pathogenic H5N2, human H1N1 and human H3N2) were used as control.

All of the results indicated that simultaneous AIV detection and differentiation of H5, H6 and H7 subtypes could be achieved in field samples employing the multiplex RT-RPA plus CE system, providing high-throughput simultaneous quadruple gene determination with superior sensitivity, accuracy and efficiency.

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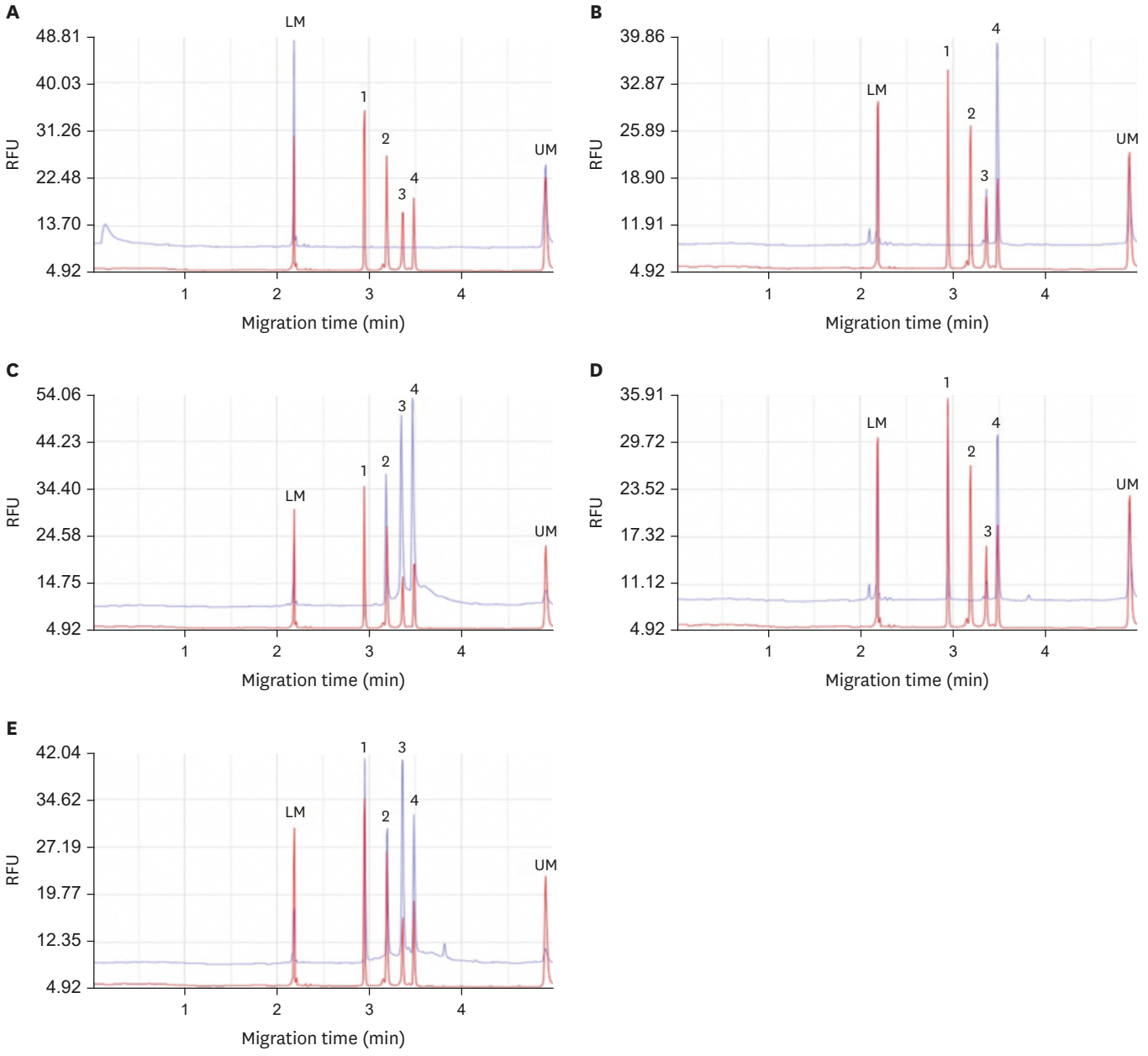

Fig. 2. The representative capillary electrophoresis results of the field samples (Blue line, tested field sample; Red line, positive control which was from the in vitro transcribed RNA standards). (A) All negative. (B) NP and H6 positive. (C) NP, H6 and H5 positive. (D) NP, H6 and H7 positive. (E) All of the four genes (NP, H6, H5, and H7) were positive.
LM, lower alignment marker (20 bp); UM, upper alignment marker (1,000 bp); Peak 1, H7 HA gene (137 bp); Peak 2, H5 HA gene (173 bp); Peak 3, H6 HA gene (199 bp); Peak 4, NP gene (217 bp); NP, nucleoprotein.

DISCUSSION

The detection platforms mostly used for post-RPA detection are lateral flow dipstick, real-time fluorescence machine and plate argarose gel electrophoresis [11,12,29]. The former two need sophisticatedly designed probe with fluorophore tag and residue modifications. With regard to the real-time approach, an intelligent scanning device with fluorescence excitation and emission is a necessity.

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Multiplex RPA reactions have been performed for three intestinal protozoa species using lateral flow dipstick [30] or for three bacterial pathogens using biochip [31]. Compared to singlex RPA, the detection limit of multiplex RPA was significantly worse and only hundreds of copy numbers could be obtained [30].

Nevertheless, CE, a relatively simpler, more universal and wider applied instrument in laboratories, has never been employed as a platform for post-RPA detection so far. Multiplex RT-RPA assays for simultaneous detection of quadruple RNA targets have never been attempted as well. The detection limit of the proposed system could achieve as low as one copy number for each of the four viral gene targets in this study, greatly promoting the detection efficacy and efficiency.

The working time of multiplex RT-RPA and CE for simultaneous detection of 4 viral genes of 60 samples was 3 h. If the process included RNA extraction, RT-RPA product purification and the final CE dada analysis, the whole working time would be completed within 6 h for total 60 samples. Lightweight and portable CE machine has currently been available, which makes on-site detection possible in the near future.

The specificity of RPA is not satisfactory compared to PCR. Nonspecific amplification is prone to occur in RPA if the primers have not been rigorously designed. The addition of 0.8 M betaine, the PCR performance improver, could significantly increase specificity of RPA applied to hepatitis B virus DNA detection [32]. In this study, final concentration of 0.8 M betaine was added to the multiplex RT-RPA reaction and showed improved signal outputs (data not shown), benefiting the accuracy and high-throughput application of CE.

In this research, we identified the coinfection of majority of our field sample collected from migratory wild duck or endemic waterfowls in the same habitat. Simultaneously infection with multiple AIV strains in wild waterfowls is considering as one of the primary mechanisms for genetic reassortment which plays an important role for AIV diversity [33]. Studies had revealed the coinfection of AIVs in wild birds [34,35]. Nevertheless, the limitation of sensitivity, resolution and complicated processes for method used for viral antigen or antibody screening restricted the study of AIV coinfection. The integration of multiple RT-RPA and CE as we demonstrated in the study can largely increases the sensitivity and efficiency in the screening of AIV infection. The high-throughput simultaneous detection of AIV subtypes in wild waterfowls would greatly facilitate the disease monitoring and the epidemic control of poultry.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

The multiplex reverse transcription recombinase polymerase amplification results of 60 field samples displayed by plate agarose gel and CE

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Supplementary Table 2

The comparison of the targeted gene sequences between field samples and the positive control sequences

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Supplementary Fig. 1

The RT-RPA results from 60 field samples displayed on plate agarose gel electrophoresis (A) and CE (B). M: 100 bp ladder marker; P: positive control which was from the in vitro transcribed RNA standards; N: ddH_2O as negative control; 1-60: sample no. 1-60. Blue line: tested field sample. Red line: positive control of the in vitro transcribed RNA standards. The 6 major peaks on the CE from left to right were lower alignment marker (20 bp), H7 HA gene (137 bp), H5 HA gene (173 bp), H6 HA gene (199 bp), NP gene (217 bp) and upper alignment marker (1000 bp), respectively.

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