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Establishment and Application of Digital RT-PCR Assay for Detection of Avian Influenza Virus H9 Subtype

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Abstract

A digital RT-PCR method for rapid detection of H9 subtype influenza was established by comparing the two methods of digital RT-PCR and real-time quantitative RT-PCR. The sensitivity, specificity and reproducibility of the two methods for H9 were determined by gradient dilution using the same pair of primers and probes. Both methods were able to detect 10^4 times diluted H9 pathogens, while digital RT-PCR could detect H9 in single droplets, and its sensitivity was higher than real-time quantitative RT-PCR. At the same time, the specificities of both methods were very strong, with no amplification reactions for H3N2, H4N2, H6N2. The reproducibility of the two methods were also good. Digital RT-PCR has a higher sensitivity than real-time quantitative RT-PCR and could play an important role in the rapid detection of H9 subtype influenza virus.

Keywords

Avian Influenza Virus H9 Subtype (H9), Digital RT-PCR, Real-Time Quantitative RT-PCR, Sensitivity, Specificity

1. Introduction

Avian influenza (AI) is a highly contagious avian influenza disease caused by the orthomyxovirus class A influenza virus, with 16 HA subtypes and 10 NA subtypes [1]. According to the avian influenza virus (Avian influenza virus, AIV), pathogenicity can be divided into highly pathogenic avian influenza and low pathogenic avian influenza virus [2]. H9 subtype low pathogenic AIV (hereinafter referred to as H9) is widely found in China's Liaoning, Anhui, Shandong,

Guangdong, Fujian, Jiangsu, Henan, Hunan, Hubei, Shanghai, Guangxi, Yunnan, Sichuan, Xinjiang and other regions [3], so it is very important to detect and diagnose it.

As a novel nucleic acid detection method, digital PCR (dPCR) technology independently performs PCR reactions by dividing the reaction system into a large number of reaction units and calculating the number of nucleic acids according to the Poisson distribution and the positive ratio [4].

Currently, there are two kinds of digital PCR tests, droplets and chip. Unlike traditional quantitative PCR techniques, dPCR is independent of the circulatory threshold (CT) of the amplification curve and is not affected by amplification efficiency, with good accuracy and reproducibility, and can achieve absolute quantitative analysis [5]. In this paper, dRT-PCR was established by comparing the sensitivity, specificity and reproducibility of dRT-PCR and real-time quantitative RT-PCR (qRT-PCR), and it was applied to the detection of actual samples, so as to achieve rapid detection of H9 subtype low pathogenic avian influenza.

2. Materials and Methods

2.1. Materials

2.1.1. Virus and Samples

The viruses used in the experiments (H9 subtype, influenza virus, H3N2 subtype influenza virus, H4N2 subtype influenza virus, H6N2 subtype influenza virus), and clinical samples were all preserved in our laboratory.

2.1.2. Instruments and Reagents

Real time fluorescence quantitative RT-PCR: Roche's production of LC4800, Real-time, RT-PCR, System;

Digital RT-PCR: QX200Droplet, Generator, PX1, RT-PCR, Sealer, S1000, Thermal, Cycler, QX200, Droplet, Reader, Plate produced by Bole company;

One step prime script RT-PCR Kit (perfect real time) Kit: from treasure biological company;

One-step RT-ddRT-PCR kit for probes Kit: bought from Bole company.

2.2. Method

2.2.1. Primers and Probes (See Table 1)

2.2.2. Extraction of RNA

TaKaRa RNA extraction kit was used to extract H9 virus RNA. After extraction, RNA was used as the original template and diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} imes, respectively, and stored in −20˚C refrigerator.

2.2.3. dRT-PCR—Determination of Annealing Temperature

The reaction liquid is configured according to **Table 2**, and the annealing temperature of the amplification reaction is determined according to the reaction conditions set out in **Table 3**, respectively, between −50˚Cand −60˚C.

Table 1. The sequence of H9 PRIMERS AND Taqman probe.

Name	Sequence
Forward primer	5'-CCATTGRACATRGCCCAG-3'
Reverse primer	5'-CYATTTATTCGACTGTCGCCTC-3'
TaqMan probe	5'–(FAM)-FRGAAGGCAGCRAACCCCATTGCAAP-(TAMRA)-3'

Table 2. The reaction system of digital RT-PCR assay.

Reagents	Volume
2X One-Step RT-ddRT-PCR supermix	10 μL
25 mM manganese acetate solution	0.8 μL
RT-PCR Forward Primer (10 uM)	0.4 μL
RT-PCR Reverse Primer (10 uM)	0.4 μL
TaqMan Probe	0.4 μL
DEPC H_2O	6 μL
RNA	2 μL
Total	20 μL

Table 3. The reaction conditions of digital RT-PCR assay.

Temperature	Time	Cycle
60˚C	30 min	
95˚C	5 min	
94˚C	20 s	6
60˚C	35 s	
94˚C	20 s	36
50˚C - 60˚C	35 s	
98˚C	10 min	1
12˚C	∞	1

2.2.4. qRT-PCR Test

A qRT-PCR method for the determination of H9 × 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, H3N2, H4N2, H6N2, NTC (H_2O) amplification curve and Ct value, repeated three times.

2.2.5. dRT-PCR Test

The sensitivity and specificity of the dRT-PCR method for H9 were determined, wherein the specificity test was controlled by H3N2, H4N2, H6N2, according to **Table 2** reaction system (20 μL) and reaction conditions (annealing temperature 2.2.3, temperature determined by **Table 3**).

2.2.6. Detection of Samples

Select the appropriate samples and validate them using the above two methods (2.2.4, 2.2.5).

2.2.7. Data Analysis

After the amplification of dRT-PCR reaction, Droplet Reader was used to read the results, and to judge the presence of amplified droplets as the basis, and to analyze the. qRT-PCR response data, and to determine the result by looking at the amplification curve and Ct value.

3. Results and Analysis

3.1. Determination of Annealing Temperature at dRT-PCR

The amplification of temperature at 50˚C - 60˚C (0.5˚C) was measured. The results showed that when the annealing temperature was 59˚C, the amplification reaction could occur. Therefore, the annealing temperature of the primer was 59˚C.

3.2. Sensitivity Analysis

3.2.1. Sensitivity of qRT-PCR

H9 RNA will be diluted 10 times, were detected by qRT-PCR. The results showed that (see **Figure 1**): the concentration of template 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} in the range of 7 orders of magnitude, the growth curve of qRT-PCR were "S" type, and the exponential growth period of qRT-PCR curve are almost parallel, reflect the amplification efficiency of similar template $\times 10^{-5}$, 10^{-6} Ct value is greater than 28, similar to the blank control group, Ct value, so the detection sensitivity for the original template $\times 10^{-4}$.

3.2.2. Sensitivity of dRT-PCR

The H9 RNA template 10 times dilution series, dRT-PCR detection. Read each dilution was amplified from **Figure 2** can droplet number, with increasing dilution the amplification gradually decreased from 10^{0} to10^{-4}. The number of amplified were 3980, 406.3, 60.4, 8.9, 2.2 copies/μL, from the10^{-5} dilution started close to the amount of amplification, and in 0 - 1 copies/μL, although the content of the H9 template is very few, but can still detect pathogens.

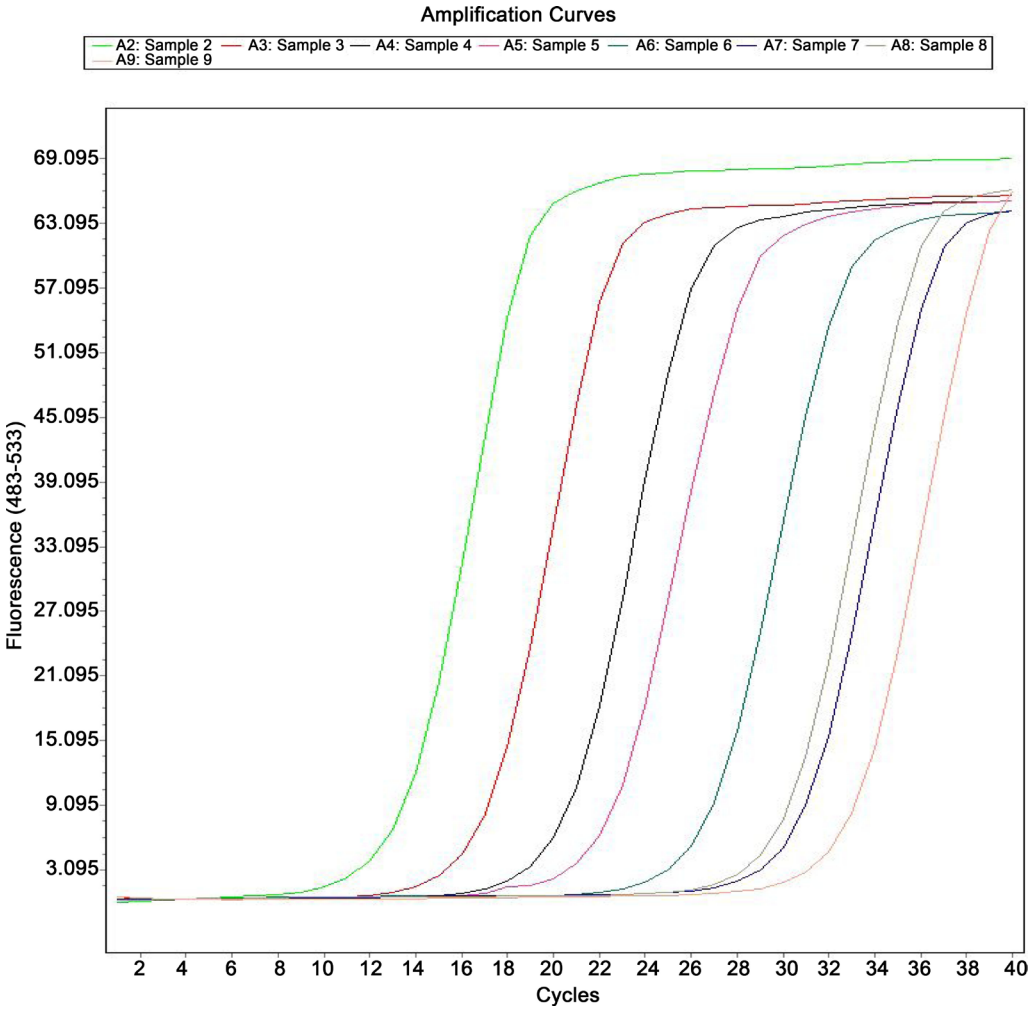
3.3. Specificity Analysis

3.3.1. The Specificity of qRT-PCR

H9 were extracted from H3N2, H4N2, H6N2, RNA, qRT-PCR were detected. The results showed that the amplification curve of H9 is "S" (see **Figure 3**), while H3N2, H4N2, H6N2 is not a typical "S" shape amplification curve showed that H3N2, H4N2, H6N2 were therefore selected primers amplification. The probe and specificity of H9 is good.

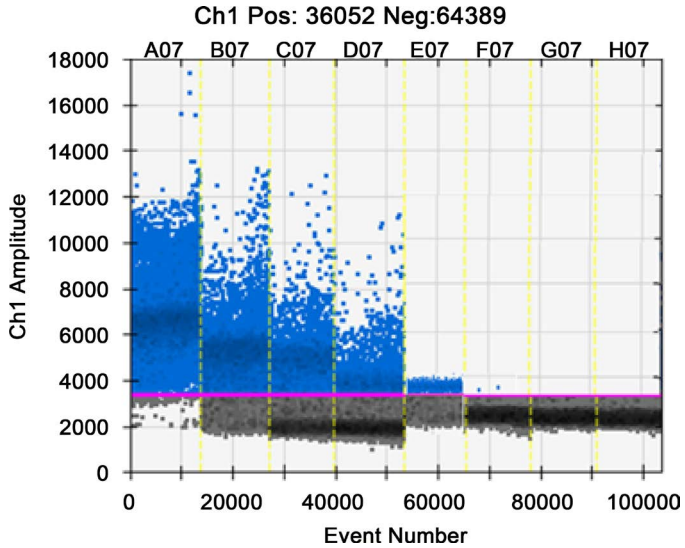
3.3.2. The Specificity of dRT-PCR

H9, H3N2, H4N2, RNA, H6N2 of the original template was shown in **Figure 4**,



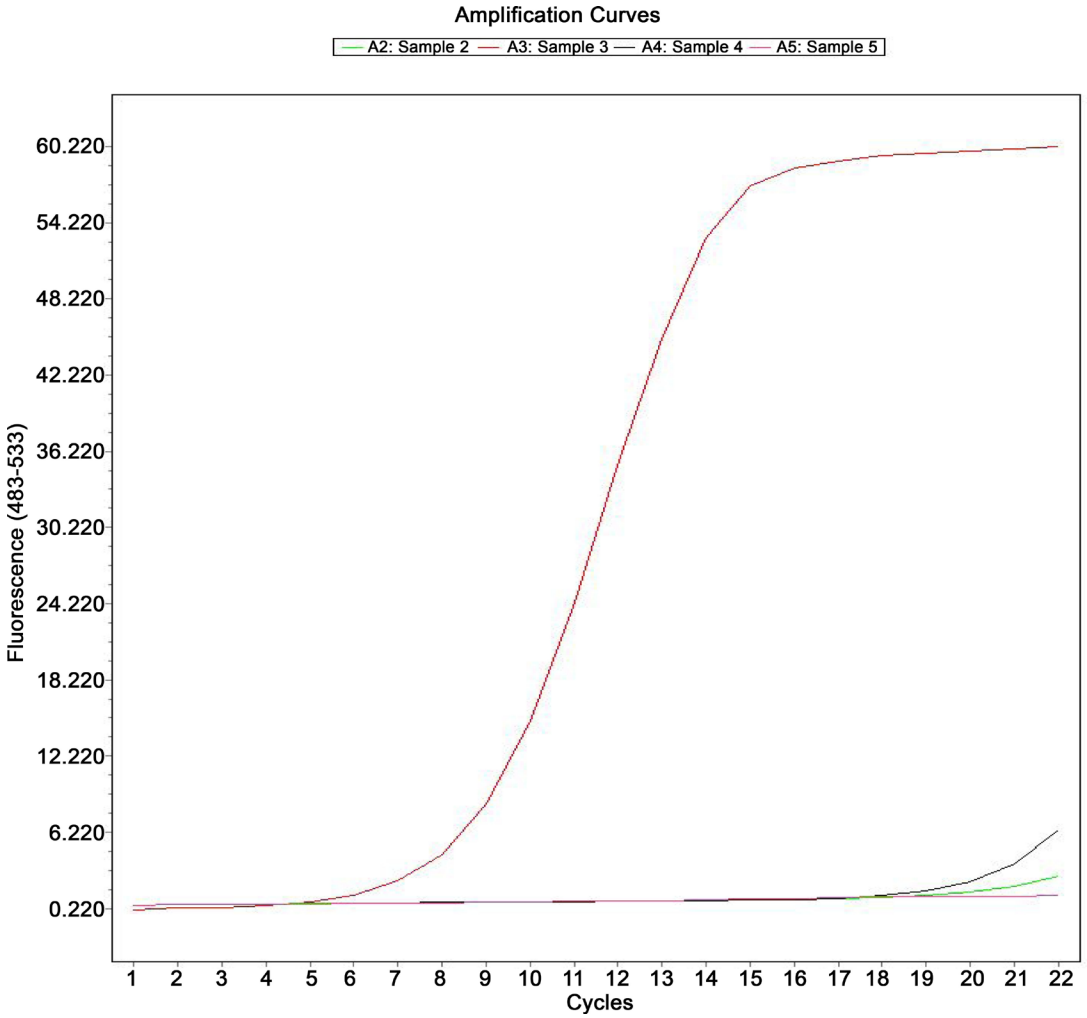
Note: Sample A2-A7 represent the original template 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, Sample A2 is blank control.

Figure 1. Amplification plot of real-time RT-PCR assay for the detection of H9.



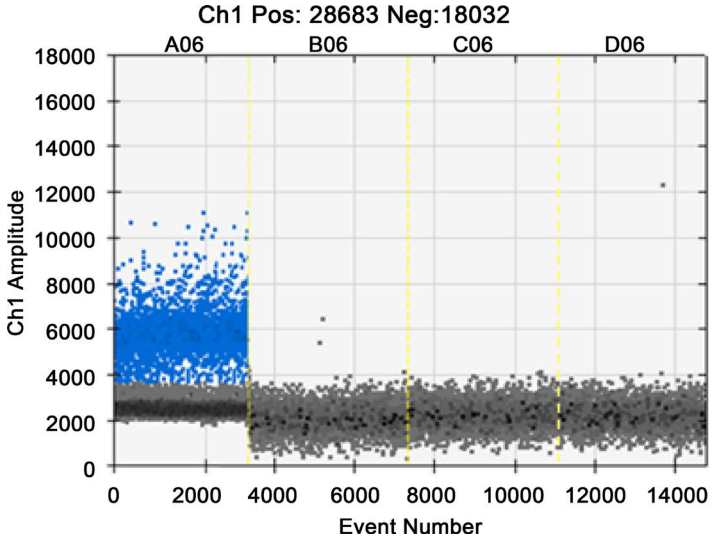
Note: A07-G07 represent the original template 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, H07: blank control.

Figure 2. The sensitivity of digital RT-PCR assay for the detection of H9.



Note: Sample A2: H9 Sample A3: H3N2 Sample A4: H4N2 Sample A5: H6N2.

Figure 3. Specificity test of real-time RT-RT-PCR assay for H9.



Note: A06: H9 B06: H3N2 C06: H4N2 D06: H6N2.

Figure 4. The specificity of digital RT-PCR assay for the detection of H9.

Table 4. The repeatability of Real-time RT-PCR and Digital RT-PCR (mean ± SE).

Concentration	Ct Value (Real-Time RT-RT-PCR)	Number of Droplets (Digital RT-PCR)
H9 × 100	12.41 ± 0.36a	4026.15 ± 172.46a
H9 × 101	15.70 ± 0.64a	465.32 ± 94.76b
H9 × 102	18.24 ± 0.27a	59.42 ± 1.21c
H9 × 103	21.74 ± 0.97b	8.64 ± 0.67d
H9 × 104	25.68 ± 0.89bc	3.19 ± 1.01d
H9 × 105	28.4 ± 1.08c	2.74 ± 2.28d
H9 × 106	30.23 ± 1.17c	3.04 ± 3.10d
NTC (H20)	35.02 ± 0.42d	0d

the specificity of H9 is better, a large number of droplets occurred in the amplification reaction, while the other three kinds of virus H3N2, H4N2, H6N2 were only 0.23, 0.15, 0.27 copies/μL, can be neglected.

3.4. Reproducibility Analysis

Through the 8 test dRT-PCR and qRT-PCR reproduce the reproducibility of the results obtained, as shown in **Table 4**. **Table 4** shows, 8 small reproduction error test, the test of good reproducibility. The qRT-PCR Ct values of 8 replicates standard error smaller between 0.36 - 1.17, standard error dRT-PCR; droplet number 8 replications is less than the total droplet number (total droplet number Droplet Reader read 2000/μL) 2.4%, good reproducibility.

3.5. Clinical Samples Were Detected

3.5.1. Detection of Samples by dRT-PCR

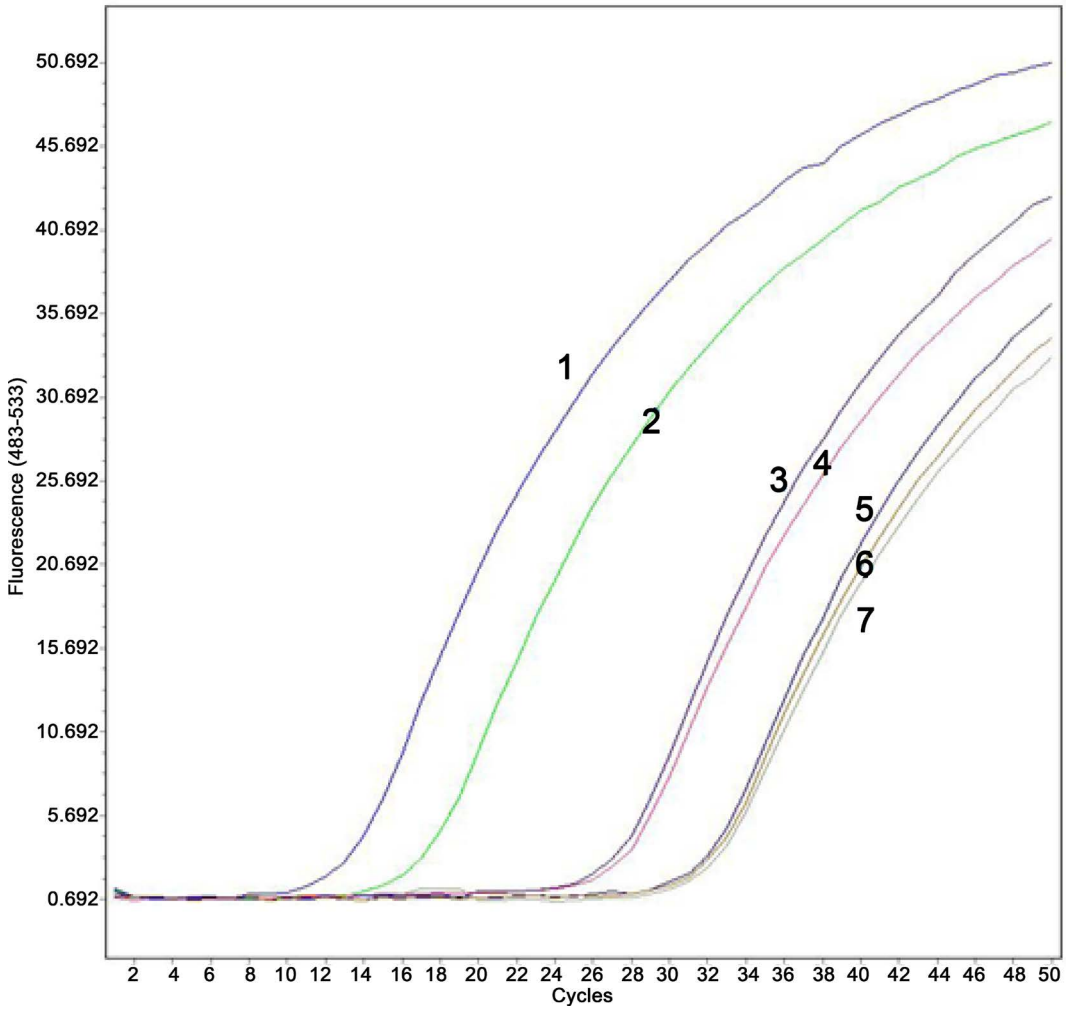
The dRT-PCR method was used to detect the sample to be detected by H9, RNA, RNA Health Organization, water as a positive control, negative control and blank control. The results showed that samples were amplified, samples containing H9 pathogens, see **Figure 5**.

3.5.2. Detection of Samples by qRT-PCR

The qRT-PCR method was used to detect samples with H9, RNA, RNA Health Organization, water were used as positive control, negative control and blank control. 1 H9 and RNA 2, 3, 4 were observed in the sample of "S" type amplification curve, showed detectable H9 H9.5 in sample, see **Figure 6**.

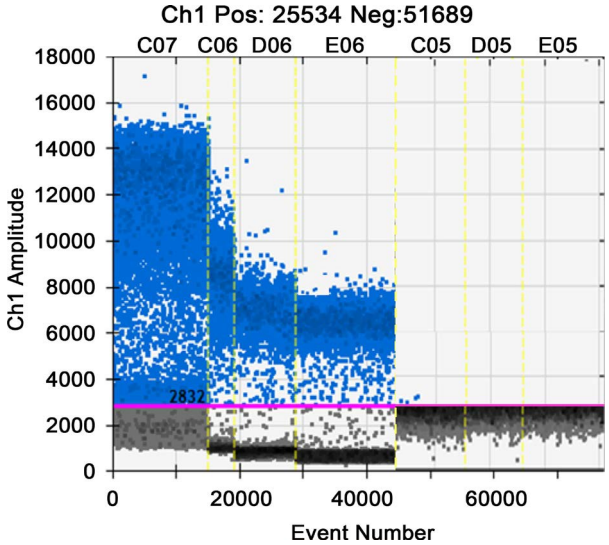
4. Discussion

H9 subtype avian influenza is widespread and spreads rapidly, which can cause recessive infection of chicks and chickens and decrease in hen egg production.H9 subtype avian influenza virus itself on the chick mortality rate is low, but once mixed with bacteria and other infected chickens, the situation will be unable to control [6]. While the traditional PCR method is not sensitive, prone to missed detection.



Note: 1: H9 RNA, 2 - 5: sample, 6: negative control, 7: blank control.

Figure 5. Amplification plot of real-time RT-RT-PCR samples with H9.



Note: C07: H9 RNA, C06-C05: sample, D05: negative control, E05: blank control.

Figure 6. Digital RT-PCR reaction for infection detection test samples with H9.

5. Conclusion

In this paper, by comparison of the two methods of qRT-PCR and dRT-PCR, the following conclusions can be drawn: dRT-PCR is more sensitive than qRT-PCR and can detect the target gene in single droplet, and can detect trace pathogen, which is more widely used than qRT-PCT method. The designed primers and probes are specific and have only amplification reaction to H9 and can be used for daily H9 detection. Compared with qRT-PCR, dRT-PCR reproducibility is higher, and can be used as a confirmation method in routine testing.

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