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Multiple RT-PCR Detection of H5, H7, and H9 Subtype Avian Influenza Viruses and Newcastle Disease Virus

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ARTICLE INFO	ABSTRACT
	Objective: This paper focuses on the multiple detection RT-PCR technology of H5, H7, AND H9 subtype avian influenza viruses and Newcastle disease virus, and points out the specific detection methods and detection procedures of avian influenza and Newcastle disease virus. **Methods:** The genes of Newcastle disease virus carrying out the HA gene sequence of H5, H7 and H9 subtype AIV in GenBank were used to establish a strategy for simultaneous detection of three subtypes of avian influenza virus and Newcastle disease virus. **Results:** The results showed that the program can detect and distinguish H5, H7 and H9 subtype avian influenza viruses and Newcastle disease virus at one time. **Conclusion:** Multiple RT-PCR detection method has high detection sensitivity and can detect and determine different subtypes of avian influenza virus and Newcastle disease virus quickly and accurately, therefore, it has a crucial role in the detection and control of avian influenza H5, H7 and H9 subtypes and Newcastle disease.

1. Introduction

AIV is a poultry strong infectious disease caused by the Orthomyxoviridae influenza Type A virus. The susceptible animals mainly include poultry and wild birds such as seabirds, waterfowl and wild birds. It also poses a serious threat to the health of humans and lower mammals, causing a variety of serious diseases. The spread of AIV can cause respiratory infections in poultry and in large cases cause large-scale deaths of poultry, causing serious economic losses to aquaculture enterprises. Avian influenza virus strains have large variations and many subtypes, which are characterized by rapid spread and difficult to control. As an internationally recognized Class A infectious disease, the avian flu virus will cause serious damage to social development and people's health,

therefore, it is necessary to strengthen the research on avian influenza virus, and to identify various subtypes of avian influenza virus through scientific and reasonable detection methods, and provide data support for the prevention and treatment of avian influenza diseases.

Both Newcastle disease virus and avian influenza virus are infectious diseases with poultry as the main carrier. They are all respiratory diseases. The avian influenza virus and Newcastle disease virus have a high mortality rate and strong contagiousness, once poultry infects these two viruses; it is prone to widespread spread and brings very serious economic losses to the aquaculture enterprises. Diseased poultry infected with Newcastle disease virus or avian influenza virus manifests as neurological symptoms and respiratory symptoms. It is difficult to determine the relevant symptoms and the cause of the disease in time

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for anatomical examination, which brings certain difficulties to the prevention and treatment work. Conventional epidemic detection methods mainly include serological laboratory tests and pathogen isolation and identification. These methods are relatively complicated in operation and cumbersome in steps, and it is difficult to simultaneously diagnose and diagnose multiple pathogens.

2. Overview of Multiple RT-PCR

Multiple RT-PCR generally refers to an experimental method of amplifying multiple templates by using multiple pairs of primers in one PCR reaction system or adding multiple pairs of primers to one PCR reaction system to amplify several regions of a single template. Multiple RT-PCR technology can realize the effect of one PCR reaction monitoring multiple viral RNAs, which is indispensable in clinical virus monitoring and infection source determination process. Especially in the field of clinical infection differential diagnosis and import and export animal quarantine and immunization, there are very wide applications. Many scholars at home and abroad have carried out a lot of research on multiple RT-PCR technology. For example, Chinese scholars have established double PCR technology to detect IBV, NDV, double PCR detection of MG and MS, and multiple PCR technology to detect IBV, NDV, MG, ILTV, etc[1]. Zou et al. used RT-PCR technology in the 1990s in addition to the A-type avian influenza virus, the B-type avian influenza virus, and the C-type avian influenza virus[2]. In the 21st century, Ming et al. identified 15 avian influenza subtypes using RT-PCR technology[3].

The application of multiple RT-PCR detection method for the clinical diagnosis of diseases can significantly speed up the detection speed and detection accuracy, reduce the detection cost, and provide sufficient data support for the prevention and treatment of related infectious diseases.

This experiment uses multiple RT-PCR detection technology to establish a rapid detection method for H5, H7 and H9 subtype avian influenza viruses and Newcastle disease virus, which can timely obtain the types of related diseases and identify suspected cases of AIV and NDV infection, which provides theoretical guidance for the clinical diagnosis of avian influenza H5, H7 and H9 subtypes and Newcastle disease.

3. Materials and Methods

3.1 Materials and Reagents

AIV-H5 strain, AIV-H7 strain, AIV-H9 strain, and chicken Newcastle disease virus were purchased by Harbin Vet-

erinary Research Institute. RT-PCR kit, RNA extraction kit, gel recovery kit, reverse transcriptase AMV, reaction buffer, and reverse transcription random primers were all provided by Nanjing Kingsray Biotechnology Co., Ltd.

3.2 Primer

Using DNAstar software, multiple pairs of primer designs were performed according to the H5, H7, AND H9 subtype avian influenza virus genes and Newcastle disease virus gene sequences registered in GenBank. After repeated experiments, a number of pairs of specific primers, H5 subtype avian influenza primers P1 and P2, were selected and the fragment length was 427 bp; H7 subtype avian influenza primer P3, P4, fragment length 501 bp; H9 subtype avian influenza primer P5, P6, fragment length 303 bp. Newcastle disease virus primers NDVP1, NDVP2, product length 221 bp. The primers were synthesized by Nanjing Kingsray Biotechnology Co., Ltd. and stored at -20 °C.

3.3 Viral RNA Extraction

50 mg of the sample to be inspected was weighed and placed in a tissue grinder by a sterile operation, and the mixture was ground and ground, and after grinding for a while on ice, 1.5 mL of physiological saline was added, and the ground mixture was centrifuged. Take tissue microfilarum 300 μL in 1.5 mL EP tube, add 750 mL Trizol, mix, place at room temperature, and add 0.1 mL of chloroform after 5 min. Then, the liquid was mixed, placed at room temperature for 10 min, centrifuged at 12000 rpm for 15 min at 4 °C, and centrifuged, and about 2 mL of the supernatant was placed in a centrifuge tube. Add 500 μL of isopropanol, shake well and mix well. Leave at room temperature for 10 min, continue centrifugation for 10 min, remove the supernatant, wash the precipitate with ethanol solution and separate the precipitate. Thereafter, 10 μL of RNase-free trihydrogenated water treated with DEPC was used to dissolve the precipitate, and then 0.5 μL of HRP RNA inhibitory enzyme was added to the solution for RT-PCR detection[4].

3.4 Single RT-PCR Amplification

RT-PCR amplification of four primers for a single primer-RNA was extracted with four mixed antigens, and RT-PCR amplification was performed using primers FP1/FP2, P3/P4, P5/P6, and NDVP1/NDVP2, respectively.

3.5 Multiple RT-PCR Reactions

The multiple PCR reaction was carried out using the H5, H7, AND H9 subtype avian influenza viruses and New-

castle disease virus as templates and after the reaction was completed, the amplified product was detected by agarose gel electrophoresis. The primer ratio, primer concentration, reaction temperature and time were repeatedly optimized to determine the reaction system of H5, H7, AND H9 subtype avian influenza viruses and Newcastle disease virus.

3.6 Specificity Test

The multiple PCR reaction of the RNA of H5, H7, and H9 subtype avian influenza viruses, Newcastle disease virus, Duck tembusu virus (DTMUV), avian infectious laryngotracheitis virus (ILTV), avian infectious bronchitis virus (IBV), and Egg drop syndrome virus (EDS) were carried out using the designed reaction system to verify the specificity of the constructed system.

3.7 Sensitivity Test

The multiple RT-PCR reaction was carried out by 10-fold dilution of H5, H7, AND H9 subtype avian influenza virus and Newcastle disease virus RNA, and the sensitivity of the one-step multiple RT-PCR to the minimum detection amount of RNA was determined.

4. Results

4.1 Single RT-PCR Results

RNA was extracted with four mixed antigens, and then amplified by RT-PCR with primers FP1/FP2, P3/P4, P5/P6 and NDVP1/NDVP2, respectively, and positive bands were amplified, as shown in Figure 1.
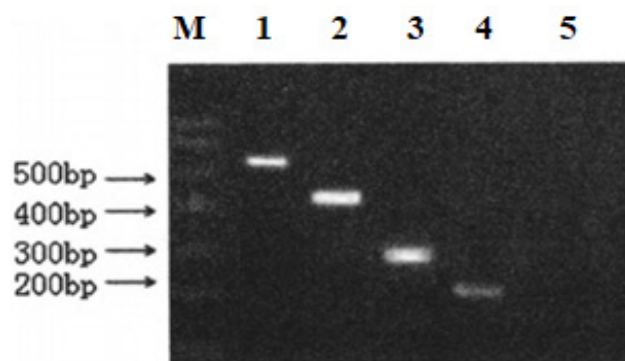

Figure 1. Single RT-PCR results

4.2 Establishment of Multiple RT-PCR Reaction System

The multiple RT-PCR reaction was carried out using H5, H7, AND H9 subtype avian influenza virus and Newcastle disease virus as templates, and after the reaction was completed, the amplified product was detected by agarose gel electrophoresis. After repeated optimization of primer ratio, primer concentration, reaction temperature and time, the reaction system of H5, H7, and H9 subtype avian influenza virus and Newcastle disease virus can be determined: The total reaction system is 25 μL. The system contains 15 μL of double distilled distilled water, 2 μL of magnesium chloride solution, 2.5 μL of PCR Buffer, 2 μL of template cDNA and 0.25 μL of EX TAQ, P1 (or P2, P3, P4, P5, P6, NDVP1, NDVP2) 4 μL, dNTP. The reaction system was placed in a 90 °C environment for 5 min, and then denatured at 94 °C for 1 min, annealed at 55 °C for 50 s, extended at 72°C for 1 min, 30 cycles, and extended at 72 °C for 10 min.

4.3 Specificity Test

The multiple PCR reaction was carried out with the designed reaction system using H5, H7, H9 subtype avian influenza virus, Newcastle disease virus, Duck tembusu virus (DTMUV), avian infectious laryngotracheitis virus (ILTV), avian infectious bronchitis virus (IBV), and Egg drop syndrome virus (EDS) RNA to verify the specificity of the constructed system. The results showed that only the H5, H7, H9 subtype avian influenza virus, Newcastle disease virus template as a template can amplify the target band and the other cannot amplify the corresponding band, as shown in Figure 2.
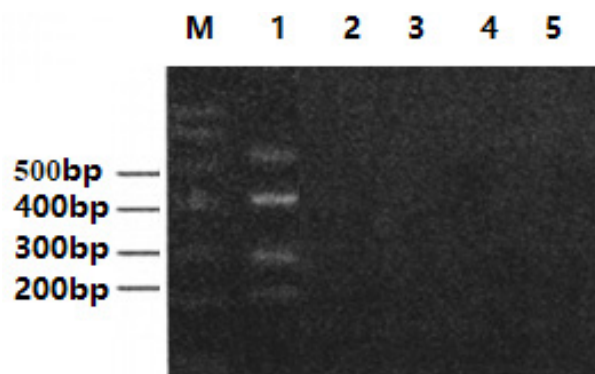

Figure 2. Specificity test results

Notes: M: Marker, 1: H5, H7, and H9 subtype avian influenza virus, Newcastle disease virus, 2: DTMUV, 3: ILTV, 4: IBV, 5: EDS

4.4 Sensitivity Test

The multiple RT-PCR reaction was carried out by 10-fold dilution of H5, H7, and H9 subtype avian influenza virus and Newcastle disease virus RNA, and the sensitivity of the one-step multiple RT-PCR to the minimum detection amount of RNA was determined. The test results show that H5, H7, H9 subtype avian influenza virus and Newcastle disease virus can reach 10^{-4}, and the sensitivity of

H9 subtype avian influenza virus can reach 10^{-6}.

5. Discussion

5.1 Necessity of Multiple RT-PCR Detection of Avian Influenza and Newcastle Disease Virus

Both avian flu and Newcastle disease are avian infectious diseases caused by viruses, which pose a great threat to aquaculture and human health and have been designated as Class A infectious diseases by the International Office of Epizootics, and included in the list of animal infectious diseases of the International Biological Weapons Convention. The incident of bird flu infection in Hong Kong in the 1990s caused panic and highlighted the need for avian flu prevention.

Although there have been many reports on multiple RT-PCR detection methods for H5, H7 and H9 subtype avian influenza, however, since H3 subtype AIVs derived from ducks are prevalent in chickens in China and have been isolated from chickens in China and other countries, the detection data and methods related to other countries are highly biased. The mature method is not fully applicable to China's national conditions, and there are also fewer methods for distinguishing H5, H7, H9 subtype AIVs and NDVs, therefore, it is necessary to establish a scientific, flexible, rapid, convenient and accurate detection method to detect and distinguish these diseases[5].

5.2 Inadequacies and Disadvantages of Current Detection Methods

HA and HI tests, virus isolation and identification, virus neutralization test (VNT), enzyme-linked immunosorbent assay (ELISA) avian influenza, NIT test, immunofluorescence technique (IFT), agar gel diffusion test (AGP) are Newcastle disease Commonly used differential diagnostic techniques. The application of these techniques often requires a long time to obtain accurate test results, making it difficult to perform rapid differential diagnosis. Moreover, the virus content in animal products is low, and it is difficult to detect whether or not a virus is present by the above technique. If a simple PCR reaction is used, multiple PCR reactions are required to detect multiple viruses, which is laborious and costly[6].

5.3 Advantages of This Method

The multiple RT-PCR rapid detection method takes only a few hours, but the conventional detection method often takes several days, and the multiple RT-PCR detection method costs less, the operation is simpler, and the equipment requirements are not high, therefore, multiple RT-

PCR technology as a rapid detection technology has a wide range of applications in clinical detection[7]. This experiment combined with relevant literature and case to screen four pairs of specific primers, which can be quickly distinguished in the same reaction system and can accurately diagnose suspected infection cases. The RNA mixed with the virus antigen was extracted, and four pairs of primers were used for multiple RT-PCR, and the results showed three clear target bands of different sizes. It is indicated that simultaneous detection of four viral antigens in the same system is feasible. Consistent with the virus isolation and identification results, the time is only 4 hours. This multiple RT-PCR method has shown great advantages in the differential diagnosis of suspected cases. It is of great practical significance to rapidly differentiate and diagnose H5, H9 subtype AIV and NDV infections in clinical diagnosis[8].

6. Conclusion

The avian influenza virus has the characteristics of high mortality, strong contagiousness and serious harm, which will bring a very heavy blow to the aquaculture industry, therefore, it is very necessary to strengthen the research and prevention of avian influenza virus. Because the avian influenza virus has many subtypes and is easy to mutate, it brings great difficulty to the identification and identification of viruses. The traditional detection method has a long duration, high cost, insufficient detection precision, and it is difficult to meet the needs of current disease prevention and control. Therefore, this study is mainly aimed at the identification and identification methods of subtype avian influenza virus and Newcastle disease virus. By using a variety of RT-PCR detection methods, it can quickly and accurately identify the virus type and create a good environment for avian influenza prevention and treatment.

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