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Research Article

Epidemiological Surveillance of Avian Influenza Virus by Taqman-Based Real-Time Reverse Transcription Polymerase Chain Reaction Assays in Hebei Province, China

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Abstract

Avian influenza virus (AIV) is one of the major zoonosis pathogens and is found chiefly in birds, but infections can occur in humans. In this study, a TaqMan-based real-time reverse transcription polymerase chain reaction (RT-PCR) assay targeting M1 gene of AIV was developed and their sensitivities and specificities were investigated. The results indicated that the standard curve had a wide dynamic range (10^1-10^7 copies/μL) with a linear correlation (R^2) of 0.992 between the cycle threshold (Ct) value and template concentration. The real-time RT-PCR assay is highly sensitive and able to detect 2.3×10^2 copies/μL of AIV RNA, as no cross-reaction was observed with other viruses from avian. Using the developed method, we conducted an epidemiological survey of AIV in Hebei province, northern China. Our investigation of clinical samples indicated that AIV was present in healthy chicken flocks at a frequency of 0.8% (2/240) in Hebei province from January 2013 to December 2013. Ten of 30 (33.3%) samples from diseased and deceased chickens were positive by the real-time RT-PCR. Our results demonstrates a prevalence of AIV in Hebei province and highlights the need for further epidemiological and genetics studies of AIV in this area.

INTRODUCTION

Avian influenza virus (AIV) usually refers to influenza A viruses, a member of the genus *Influenza A* viruse of the family *Orthomyxoviridae*, and has a worldwide distribution. There are many subtypes of AIV, but only some strains of four subtypes have caused great losses in the poultry industry. These are types H5N1, H5N2, H7N9, and H9N2 [1]

Among these, subtypes H7N9 and H5N2 are two novel avian influenza virus first reported to have infected humans or chickens in 2013 in China [2,3]

The current methods of detecting AIV include virus isolation, hemagglutination inhibition (HI) assay, and reverse transcription-polymerase chain reaction (RT-PCR) [4]. In contrast to conventional assays, real-time RT-PCR offers rapid results with potentially increased sensitivity and specificity of detection. It is also less prone to false positive results from amplicon contamination and is more amenable to the quantitative estimation of viral load. In this study, we developed a highly sensitive and specific TaqMan-based real-time RT-PCR method to target the matrix protein 1 (M1) genes for the rapid detection and quantitation of AIV in clinical specimens. Using the developed method, we performed an epidemiological survey of AIV in Hebei province from January 2013 to December 2013.

MATERIALS AND METHODS

Viruse: Inactivated AIV, including H5, H7, and H9 subtypes were purchased from State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Design of primers and probes: M1 genes are highly conserved in the genome of AIV. Primers and TaqMan probes were selected and designed from conserved M1 genes using the Primer Express Software (version 3.0; Applied Biosystems, USA) to generate a 149 bp amplicon. The probe was labeled with 5-carboxyfluorescein (FAM) at the 5'-end and N, N, N', N'-tetramethyyl-6-carboxyrhodamine (TAMRA) at the 3'end. Nucleotide information of each primer or probe is summarized in (Table 1). The nucleotide sequences and locations of the primers for A/Thailand/2(SP-33)/2004 (H5N1) (GenBank accession no. AY627893.1) are shown in (Table 1).

RNA extraction: Viral RNA was extracted from 150 μL of supernatant from tissue samples using the RNA extraction kit (Qiagen Inc., USA) following the manufacturer's instruction. The extracts were resuspended in 20 μL of distilled water, aliquoted and stored at -80°C before real-time RT-PCR amplification was carried out.

Construction of the plasmid DNA strandard: M1 genes were amplified using the forward (5'- CGT AGA CGC TTT GTC CAG AAT GC-3') and the reverse primers (5'- GTC CTC ATT GCC TGC ACC ATC -3') from H9 subtype AIV. The PCR products were subcloned into pEASY-Blunt cloning vectors (Transgen, Beijing, China) and subsequently subjected to automated sequencing reactions (Invitrogen, Beijing, China). The positive recombinant plasmid pEB- M1 was purified using a TIANprep mini plasmid kit (TIANGEN, beijing, China) and quantified using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Serial 10-fold dilutions of pEB-M1 were prepared in 10 mM Tris-EDTA buffer (pH8.0) and stored at -20°C prior to use in standard curve generation.

Real-time RT- PCR assay: AIV RNA was extracted as described above. Standard plasmid DNA was used as a template and 10-fold serially diluted in 10 mM Tris-EDTA buffer (pH8.0) to produce 1.8×10^{6} to 1.8×10^{1} copies/μL. The real-time RT-PCR assay was performed in a 25 μL reaction mixture containing 1 μL extracted RNA or standard plasmid, 12.5 μL FastTaqMan Mixture (with ROX) (Cwbiotech, Beijing, China), 0.5 μL AMV reverse transcriptase (Transgen, Beijing, China) 400 nM each of forward and reverse primer, and 200 nM of probe. Amplification and detection were performed with a Bio-Rad iQ5 real-time PCR detection system under the following conditions: RNA was reverse transcribed at 50°C for 20 min, followed by PCR activation at 95°C for 3 min and 40 cycles of amplification (5 sec at 95°C and 40 sec at 60°C). Analysis of each assay was conducted with iQ5 Standard Edition Optical System Software (version2.1; Bio-Rad).

Sensitivity and Specifity of the real-time RT- PCR. To examine the sensitivity of real-time RT-PCR for AIV amplification. The real-time RT-PCR reactions were conducted using various concentrations of AIV RNA as template. The RNA was quantified by NanoDrop 1000 (Thermo Scientific, USA) and was diluted serially 10-fold from 2.3×10^{6} to 2.3×10^{0} copies/μL as template. Real-time RT-PCR was performed using the optimized reaction parameters. RT-PCR was performed using AIV-specific primers (AIV-F: 5'-CGT AGA CGC TTT GTC CAG AAT GC-3'; AIV-R: 5'-GTCCTCATTGCCTGCACCATC-3'). Briefly, RT-PCR was performed by using 1μL of diluted RNA template and 10μmol of each primer in a 25 μL reaction volμme by following the manufacturer's protocol with the following cycling times and temperatures: 94°C for 3 min and 30 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 25 sec. Three microliters of RT-PCR products were analyzed by agarose gel electrophoresis and subjected subsequently to automated sequencing reactions (Invitrogen, Beijing, China). The size of AIV fragment amplified by RT-PCR was respectively 428 bp. Reactions with different viruses including AIV, infectious bursal disease virus (IBDV), newcastle disease virus (NDV), infectious bronchitis virus (IBV), and duck hepatitis virus (DHV) were performed to determine the specificity of the real-time RT-PCR assay.

Table 1: Primers and probe used in real-time RT-PCR assay for AIV.

Type	*Sequence (5'-3')*	Position[a]
Forward	TCGCACTCAGCTACTCAACC	344-363
Reverse	CTGTCTGTGAGACCGATGCT	473-492
Probe	FAM - ACCCATGCAACTGGCAAGTGCA-TAMRA	366-387

[a]Numbers represents the nucleotide position within the genes of Influenza A virus [A/Thailand/2(SP-33)/2004(H5N1)] matrix protein 1 (M1) (GenBank acession number: AY627893.1).

Table 2: Comparison of RT-PCR and real-time RT-PCR methods for detection of AIV from clinical samples.

Health status	*Location of samples*	*Type of tissue or samples*	No.positive/no.tested samples(%)	
			RT-PCR	Real-time RT-PCR
Healthy	Baoding	Swab	0/40	0/40
	Cangzhou		0/40	0/40
	Handan		0/40	0/40
	Xingtai		0/40	0/40
	Tangshan		0/40	0/40
	Shijiazhuang		0/40	2/40
Diseased and deceased	Baoding	Tonsils	8/30	10/30

Clinical specimens. Clinical specimens were collected from different chicken farms in the Hebei province in 2013. The samples mainly included throat/anal swab of healthy chickens, and tonsils of diseased chickens. Samples were stored in RNAlater RNA Stabilization Reagent (Qiagen Inc., USA). Tissue samples were homogenized and centrifuged at 3000 rpm for 15 min to obtain a cell-free supernatant. The sample RNAs were extracted as described above. The real-time RT-PCR were performed using the optimized reaction parameters. Conventional RT-PCR were preformed simultaneously.

RESULTS AND DISCUSSION

AIV are highly contagious, extremely variable viruses found in birds, humans, horses, pig and dogs. The viruses can be classified into two categories: low pathogenic (LPAI) that typically causes little or no clinical signs in birds and highly pathogenic (HPAI) that can cause severe clinical signs and/or high mortality in birds. Two surface antigens, the hemagglutinin (H) and neuraminidase (N) proteins are used to classify type A influenza viruses into subtypes. There are 16 hemagglutinin antigens (H1 to H16) and nine neuraminidase antigens (N1 to N9). In recent years, H5, H7, and H9 subtypes were reported to occur frequently in China. [2,3,5,6] The aim of this study was to establish a rapid method for surveillance and specific diagnosis of AIV-infection. Against H5, H7, and H9 subtypes in chicken flocks.

The optimization of the real-time RT-PCR reaction was performed by evaluating different concentrations of components and cycling conditions using DNA standards and AIV. Primers and probe were titrated to determine optimum concentrations and different annealing and data acquisition temperatures were also evaluated (data not shown). The optimum fluorescence and the lowest Ct values, in the absence of primer dimer or nonspecific amplification. The real-time RT-PCR assay was performed by the optimized reaction parameters in a 25 μL reaction mixture containing 1 μL of extracted DNA, 12.5 μL of 2×FastTaqMan Mixture, 1.0 μL of each primer (10 μm), 1.0 μL of probe (5 μm), 0.5μL of AMV reverse transcriptase, and 8.0 μL of Dnase/Rnase-free water.

Serial ten-fold dilutions of plasmids were used to construct a standard curve by plotting the logarithm of the plasmid copy number against the measured Ct values (Figure 1). The standard curve had a wide dynamic range of 10^1-10^7 copies/μL with a linear correlation (R^2) of 0.992, and a slope of -3.079 between the Ct value and the logarithm of the plasmid copy number.

The sensitivity of the real-time RT-PCR assay was evaluated by testing 10-fold serial dilution of RNA templates (2.3×10^6 to 2.3×10^0 copies/μL). The detection limit of real-time RT-PCR was 2.3×10^2 copies/μL, whereas that of RT-PCR was 2.3×10^3 copies/μL (Figure 2). Comparisons between the real-time RT-PCR and RT-PCR amplification indicated that real-time RT-PCR is 10-fold more sensitive than RT-PCR (Figure 2A and B). The specificity of the real-time RT-PCR assay was evaluated using other animal viruses, and a water negative control. Strong fluorescent signals were obtained only in the detection of AIV, including H5, H7, and H9 subtypes, whereas the signals from other four virus samples and the water control were equivalent to baseline levels under the optimized reaction conditions (Figure 3). Thus, AIV was clearly differentiated from other viruses by comparing the signal strengths at different levels.

Real-time RT-PCR was preformed simultaneously on 270 clinical samples. The results are shown in Table 2. Two of 240 (0.8%) throat/anal swabs from healthy chickens were positive by real-time RT-PCR. Ten of 30 (33.3%) samples from diseased and deceased chickens were positive by real-time RT-PCR. Eight samples (3.0%) were positive by two methods. Four sample (1.5%) were positive by real-time RT-PCR, but negative by RT-PCR analysis. No sample (0%) was positive by RT-PCR and negative by real-time RT-PCR. The results showed that real-time RT-PCR was more sensitive than the conventional RT-PCR assay. Our investigation of clinical samples indicated that AIV was present in healthy chicken flocks at a frequency of 0.8% (2/240).

The current methods of detecting AIV include virus isolation, hemagglutination inhibition (HI) assay, and reverse transcription-polymerase chain reaction (RT-PCR).[4]. Virus isolation is one of the conventional methods, but it is time-consuming and laborious. Serology represents the most popular technique; however, this method has some disadvantages because antibody titres can fall rapidly after infection [7]. Conventional RT-PCR is less time-consuming but prone to sample contamination occurring during PCR processing steps, which incresases the potential

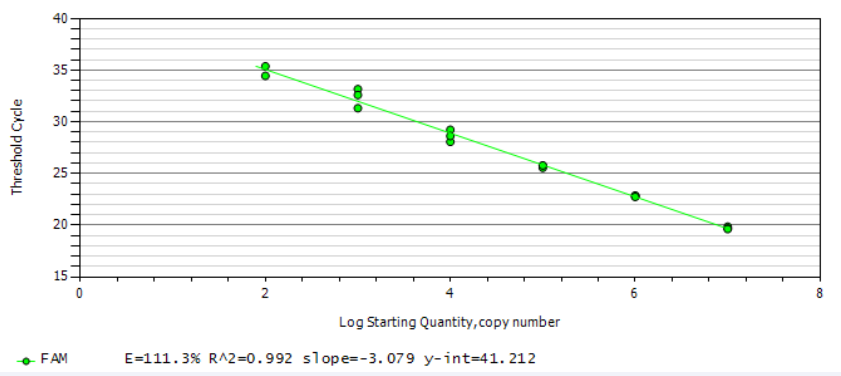

Figure 1a Data graph of the TaqMan-based real-time RT-PCR assay for AIV detection. (A) Standard graph. The assay was performed using the TaqMan method on serial 10-fold dilutions of the pEB-M1 standard plasmid (1.8×107 to 1.8×102 copies/μL). The standard curve produced using pEB-M1 was linear, with a correlation (R2) of 0.992 between the cycle threshold (Ct) value and template concentration, and a slope of -3.079.

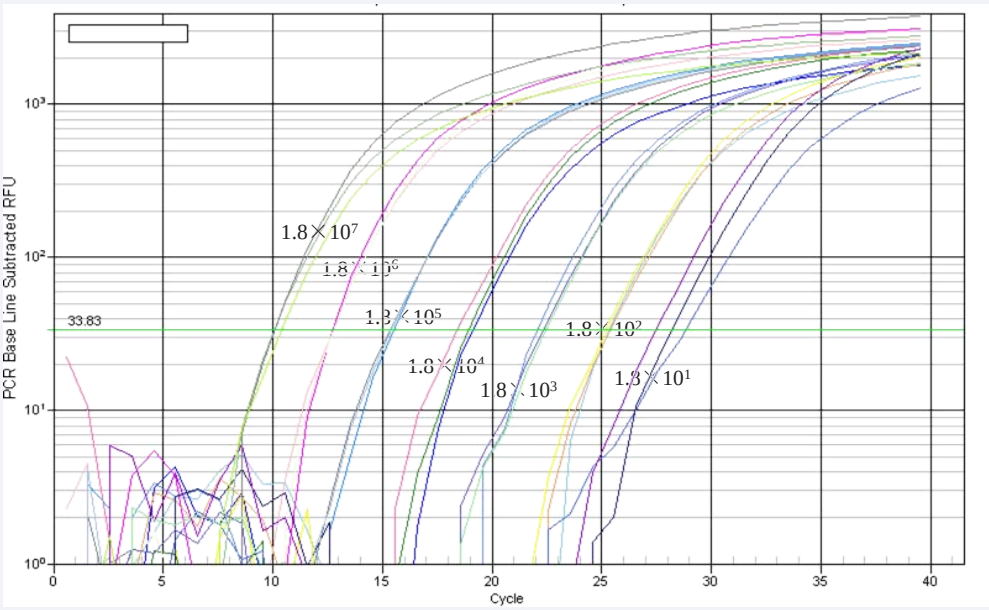

Figure 1b Data graph of the TaqMan-based real-time RT-PCR assay for AIV detection. (B) Amplification curve graph. Serial 10-fold dilutions of the standard plasmid (pEB-M1) containing the target nucleotide sequence were amplified using real-rime RT-PCR assay. Amplification plots of standard plasmid (1.8×107 to 1.8×101 copies/μL) were detected by the real-time RT-PCR.

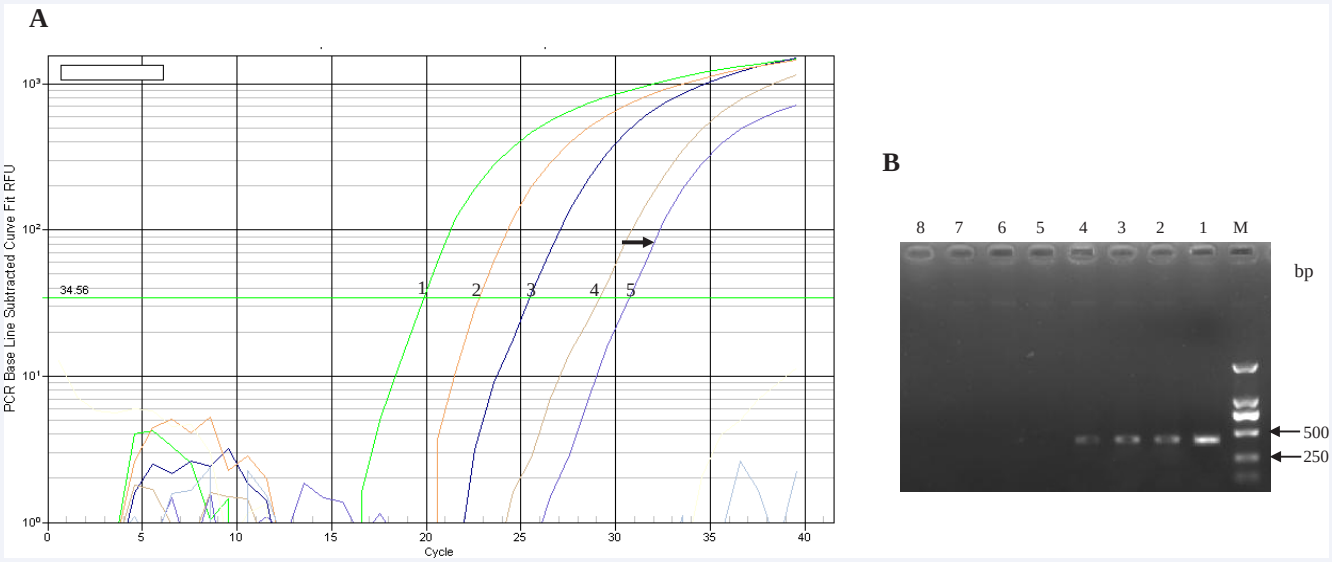

Figure 2 The sensitivities of real-time RT-PCR assays. A serial 10-fold dilution of AIV RNA derived from H9 subtype was used in the real-time RT-PCR assays. (A) Analysis of the real-time RT-PCR assay by amplification curve graph. The black arrow represents the crossing point. (B) Analysis of the RT-PCR assay by agarose gel electrophoresis. Lane 1: 2.3×10^6 copies/μL; Lane 2: 2.3×10^5 copies/μL; Lane 3: 2.3×10^4 copies/μL; Lane 4: 2.3×10^3 copies/μL; Lane 5:2.3×10^2 copies/μL; Lane 6: 2.3×10^1copies/μL; Lane 7: 2.3copies/μL; Lane 8: water control. All experiments were repeated three times and similar results were obtained.

for false-positive results. The development of real-time RT-PCR technology presents an opportunity for more rapid, sensitive and specific detection of RNA and is becoming widely used because the accumulated amplicons can be detected directly during the DNA amplification [8]. Real-time RT-PCR has been used in detecting viral RNA molecules from animals due to its simplicity and high sensitivity including influzenza A (H1N1) (Whiley et al., 2009), [9].transmissible gastroenteritis virus [10], foot-and-mouth disease virus [11,12], dengue virus 13], classical swine fever virus [6-14], and porcine reproductive and respiratory syndrome virus.[15,16]. In this study, a highly efficient and practical method for the detection of AIV was established. Since M1 genes of AIV is among the most conserved regions and has been chosen as a preferred target region for the detection of AIV RNA by RT-PCR, primers and probe was designed to amplify target sequences at the M1 genes region of the AIV genome for the real-time RT-PCR assay. The real-time RT-PCR assay is highly sensitive and able to detect 2.3×10^2 copies/μL of AIV RNA, as

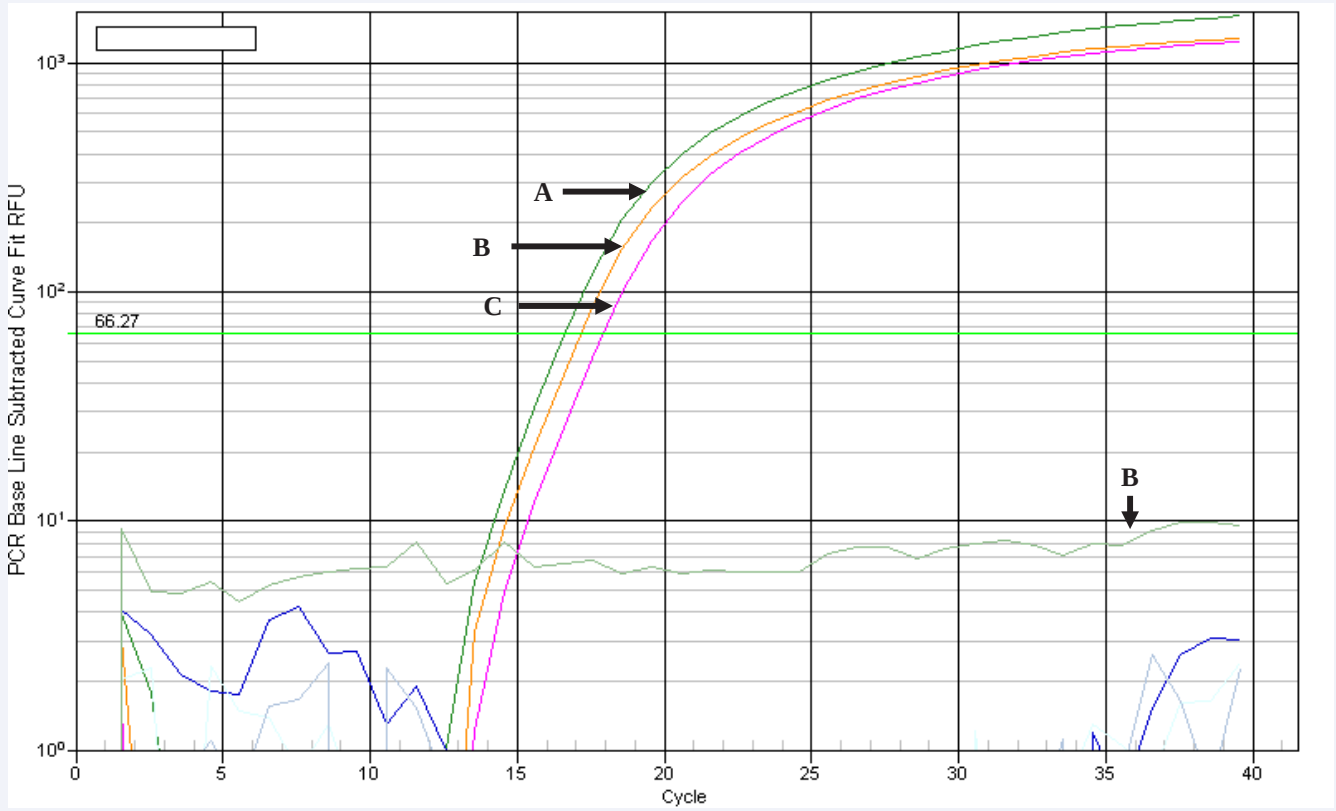

Figure 3 Specific evaluation of the real-time RT-PCR assay for the detection of AIV. The line of AIV amplification including H5, H7, and H9 subtype was indicated (A), (B), and (C), respectively. The other lines represented the amplifications using nucleic acids from other viruses (B), including NDV, IBDV, IBV, and DHV. The control reaction using DEPC-treated water as template was also included.

no cross-reaction was observed with other viruses. Most of the amplification reactions could be finished within 80 min. Thus, the real-time RT-PCR assay is faster than RT-PCR. This is the report to demonstrate the application of a TaqMan-based real-time RT-PCR amplification technique for the detection of AIV. This method can be used for preliminary diagnosis of AIV. Further study will be needed to determine the serotype of AIV.

In conclusion, a TaqMan-based real-time RT-PCR assay for AIV detection was developed. Using the developed method, we investigated the epidemiology of AIV in chicken flocks in the Hebei province of northern China. Our investigation demonstrates a prevalence of AIV in Hebei province and highlights the need for further epidemiological and genetics investigations of AIV in this area.

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