



Evaluation of a quantitative real-time PCR for rapid detection of *Riemerella anatipestifer* infection in birds

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ABSTRACT. To establish an accurate, rapid, and a quantifiable method for the detection of *Riemerella anatipestifer* infection, a widespread infectious disease in birds, we developed a TaqMan-based real-time PCR assay by using *DtxR* gene-specific primers and a TaqMan probe. The standard curve established with a linear correlation (R^2) of 0.998 and efficiency of 99% between the Ct value and the logarithm of the plasmid copy number. The reproducibility and specificity of the real-time PCR assay were confirmed by using plasmids containing *DtxR* genes or DNAs extracted from well-known bacteria or viruses causing duck diseases. The real-time PCR assay was 100 times more sensitive than the conventional PCR. The results reveal that the established real-time PCR assay might be a useful method for diagnosis and quantitative detection of *Riemerella anatipestifer* in birds.

KEY WORDS: conventional PCR, TaqMan-based real-time PCR, *Riemerella anatipestifer*

Riemerella anatipestifer (RA) is a gram-negative bacterium in the family of Flavobacteriaceae [13, 14]. RA can infect various poultry species of birds and cause serious economic losses especially to the duck industry [4, 13]. RA is a worldwide duck disease with high mortality, weight loss, and contamination of carcass. At least 21 serotypes have been described in different countries [8, 10, 11, 13, 15, 18], of which serotypes 1, 2, 3, 5, 10 and 15 are responsible for most major outbreaks in China [5, 20, 22, 23].

Many methods have been developed for detection RA infection in birds, including traditional method to differentiate RA isolates [11], PCR based on 16S rRNA or *rpoB* genes [3, 18], repetitive-sequence polymerase chain reaction (Rep-PCR) [6], multiplex PCR [5], and PCR-restriction fragment length polymorphism (PCR-RFLP) [22]. However, these assays are labor-intensive, less sensitive, and require agarose gel analysis for the detection of amplification products and had a risk of contamination. Recently, an excellent diagnostic tool with high sensitivity, specificity, and time saving had been used extensively for detection of amplicons during the PCR cycling in real time.

The transcriptional regulatory protein (DtxR) evolved in mediating metal ion homeostasis and pathogenesis of bacteria [2, 9, 16, 17, 21], which is highly conserved and might be another suitable gene for bacteria identification and classification. In this study, we developed a quantitative real-time PCR for the detection of RA infection based on *DtxR* gene. To evaluate the developed a TaqMan-based real-time PCR for diagnosing and monitoring duck RA infection, we compared the results of conventional PCR and real-time PCR by using 158 clinical samples from different areas of China. This Real-time PCR might be used as a good method to perform quantitative and qualitative analyses of RA infection.

MATERIAL AND METHODS

Bacterial Strains, virus strains, and DNA Preparation

RA strains [RA-1 (sera type 1), RA-2 (sera type 2), RA-3 (sera type 3), RA-5 (sera type 5), RA-10 (sera type 10), RA-15 (sera type 15), RA-17 (sera type 17)], *Escherichia coli* (*E. coli*), *Salmonella anatum* spp. (*S. anatum*) and *Pasteurella avium* (*P. avium*) isolates, and virus strains [duck circovirus and waterfowl parvoviruses (including the Muscovy duck parvovirus and goose parvovirus)] were collected in our laboratories. RA strains were cultured in tryptic soy broth (Difco Laboratories, Detroit, MI, U.S.A.). *E. coli*, *S. anatum*, *P. avium* and *S. aureus* were cultivated in Luria-Bertani broth culture. Bacterial genomic or viral DNA

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was extracted using a Genomic DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Primers and probe design

For RA real-time PCR, specific primers and a TaqMan probe to detect RA were designed using Primer Express 2.0. The target sequence (143-bp long) was selected from conserved region on nucleotide sequences of the *DtxR* gene of RA ATCC 11845 (GenBank no. CP003388.1) and GD05 (GenBank no. EU541215.1) strains. Primers *TaqMan1F*:5'-CTGCTGTAAGTGTAGATAC-3', *TaqMan1R*:5'-GAAGGTGTGAGAAATACC-3', and RA probe primer 5'-(FAM) CGGTTACCA TCATAGAAGCGTCAA (Eclipse)-3' were used for real-time PCR. Primers *R1F*:5'-TATTTATTTTTGTGTCTATGAACT-3' and *R1R*:5'-TCTTGGCTGAGTTTAAATCT-3' were used to amplify 700 bp PCR products, which were cloned to generate RA standard plasmids. Primers *R1F* and *R1R* were also designed or selected from conserved region on target *DtxR* gene.

Preparation of standard plasmids for real-time PCR

To generate a RA standard curve for the real-time reaction, a 700 bp PCR products by using primers *R1F* and *DR1R* were cloned into the vector pMD18-T vector (Takara, Dalian, China) according to the manufacturer's instructions. The transformed plasmids were purified by using a QIAGEN plasmid purification kit. The plasmids concentration were determined by measuring the OD at 260 nm by using a spectrophotometer (Eppendorf, Hamburg, Germany) and the number of copies of the plasmids were calculated as described previously [16]. Serial 10-fold dilutions of plasmid DNA were used in amplification reactions.

Standard curve for the real-time PCR

The real-time PCR for the *DtxR* gene was conducted by using Premix Ex *Taq*TM kit (Probe qPCR, TaKaRa, Dalian, China). The optimized reaction was carried out in a 25 μ l reaction system containing 12.5 μ l of supplied master mix, 0.5 μ l of each primers (10 μ M of *TaqMan1F* and *TaqMan1R*), 1 μ l of probe (5 μ M of probe primer), 2 μ l template DNA, and 8.5 μ l of sterile water. The thermal profile for the real-time PCR was 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec, 56°C for 10 sec, 72°C for 20 sec. Real-time PCR was performed on Mastercycler ep realplex (Eppendorf). The assay was repeated at least 3 times with each template and the negative control.

Determining the specificity of the real-time PCR

To determine the specificity of the real-time PCR, the RAs (RA-1, RA-2, RA-3, RA-5, RA-10, RA-15, RA-17), 6 well-known bacterial and viruses causing infectious diseases in ducks, including *Escherichia coli* (*E. coli*), duck cholera (*Pasteurella multocida*), *Salmonella* spp., duck circovirus and waterfowl parvoviruses (Muscovy duck parvovirus and goose parvovirus) were tested under the conditions described above.

Sensitivity of the real-time PCR

To determine the detection limit, the standard plasmid stock was diluted with sterile water. Four replicates of each dilution and two negative controls (blanks) were then tested by the real-time PCR and conventional PCR.

Reproducibility of the real-time PCR

To test the reproducibility of the real-time PCR, standard plasmids in 3 different concentrations (4.3×10^7 copies/ μ l, 4.3×10^5 copies/ μ l, and 4.3×10^3 copies/ μ l) were used as templates for evaluate the coefficients of variation (CVs) of the real-time PCR. Intra-assay (three times) and inter-assay (three times) CVs for Ct values were both included.

Conventional PCR reaction

The RA-*TaqMan1F* and RA-*TaqMan1R* primer-pair were used to test sensitivity of conventional PCR. Amplifications was carried out in a 25 μ l reaction system containing 2.5 μ l of $10 \times$ rTaq Buffer, 2 μ l of dNTP (2.5 mM), 1 μ l of each primers (10 mM of *TaqMan1F* and *TaqMan1R*), 2 μ l DNA solutions, 0.5 μ l of rTaq polymerase, and 16 μ l of RNase free ddH₂O. The thermal profile for the PCR was 95°C for 4 min, followed by 30 cycles of 95°C for 20 sec, 56°C for 15 sec, 72°C for 30 sec. Amplicons of 143 bp were separated through 2.0% agarose gel.

Detection of clinical samples

158 clinical RA samples were tested using real-time PCR under optimal conditions and compared with the conventional PCR methods using as described previously [1], and subsequent sequencing was performed to avoid false-positive results in related bacterial species. The forward primer 190f (5'-GTATTGAAAGCTCTGGCGG-3') and reverse primer 843r (5'-TCGCTTAGTCTCTGAACCC-3') were used to amplify a 654-base pair segment of the 6Srna gene of RA. All 158 clinical RA samples were streaked on 10% blood agar plate and then incubation at 37°C for 36 hr with 5% CO₂. The cultures that contained mixed bacteria were collected in 2-ml microcentrifuge tubes with phosphate-buffered saline (PBS). A 200- μ l aliquot was used for DNA isolation and then identified by the established real-time PCR method.

RESULTS

Establishing a standard curve for real-time PCR

Ten-fold serial plasmid dilutions were used to construct the standard curve by plotting the logarithm of the plasmid copy number against the measured Ct values (Fig. 1). The standard curve generated had a wide dynamic range of 4.3×10^1 – 4.3×10^6 DNA copies/ μ l with a linear correlation (R^2) of 0.998 and efficiency of 0.99 between the Ct value and the common logarithm of the plasmid copy number.

Specificity of the assay

The specificity of the assay was examined with regard to the nucleic acids extracted from RAs (RA-1, RA-2, RA-3, RA-5, RA-10, RA-15 and RA-17), *E. coli*, duck *P. multocida*, *Salmonella* spp., duck circovirus, Muscovy duck parvovirus and goose parvovirus. RAs (RA-1, RA-2, RA-3, RA-5, RA-10, RA-15 and RA-17) were tested positive, while other samples tested negative (Fig. 2).

Real-time PCR sensitivity

The detection limit of the real-time PCR was 4.3×10^1 copies (Fig. 3A), while the conventional PCR assay showed positive results only when more than 4.3×10^3 copies of the template were used (Fig. 3B). Therefore, the sensitivity of the real-time PCR is about 100 times greater than that of conventional PCR.

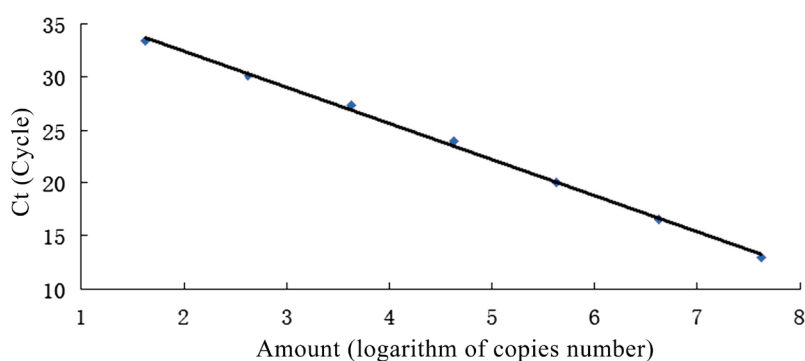


Fig. 1. The standard curve of Real-time PCR. The X-axis represents the common logarithm copies of the plasmids, and the Y-axis represents the cycle threshold (Ct). The assays were linear from 4.3×10^7 to 4.3×10^1 template copies; R^2 was 0.998, and the reaction efficiency was 99% for the RA *DtxR* gene.

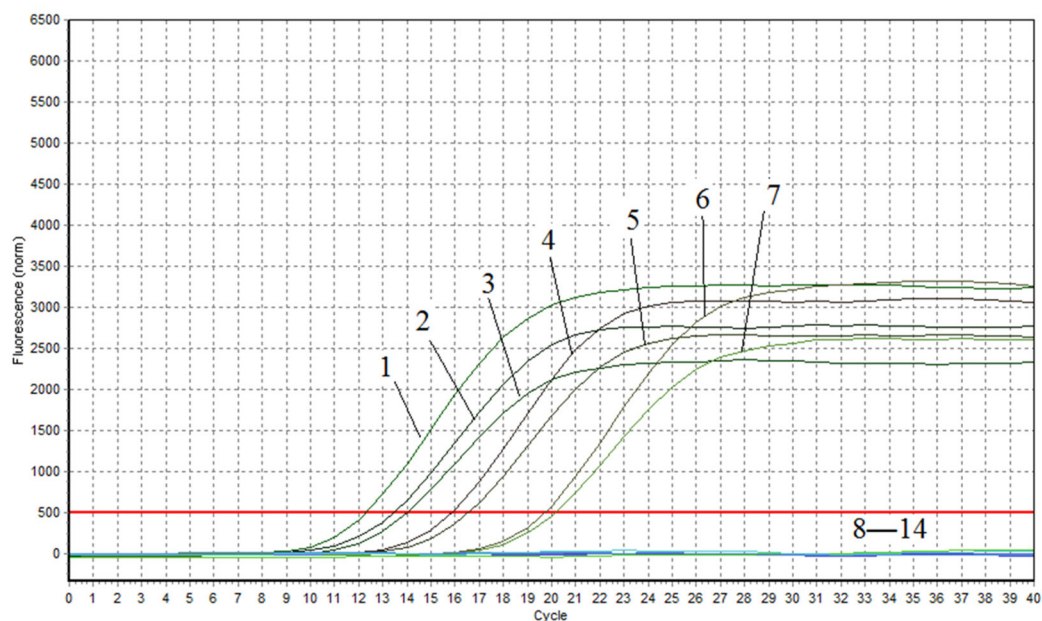


Fig. 2. The specificity real-time PCR. The X-axis represents cycles, and the Y-axis represents the fluorescence data. 1, RA-1; 2, RA-2; 3, RA-3; 4, RA-5; 5, RA-10; 6, RA-15; 7, RA-17; 8, *E. coli*; 9, *P. Multocida*; 10, *Salmonella* spp.; 11, duck circovirus; 12, Muscovy duck parvovirus; 13, goose parvovirus; 14, negative control.

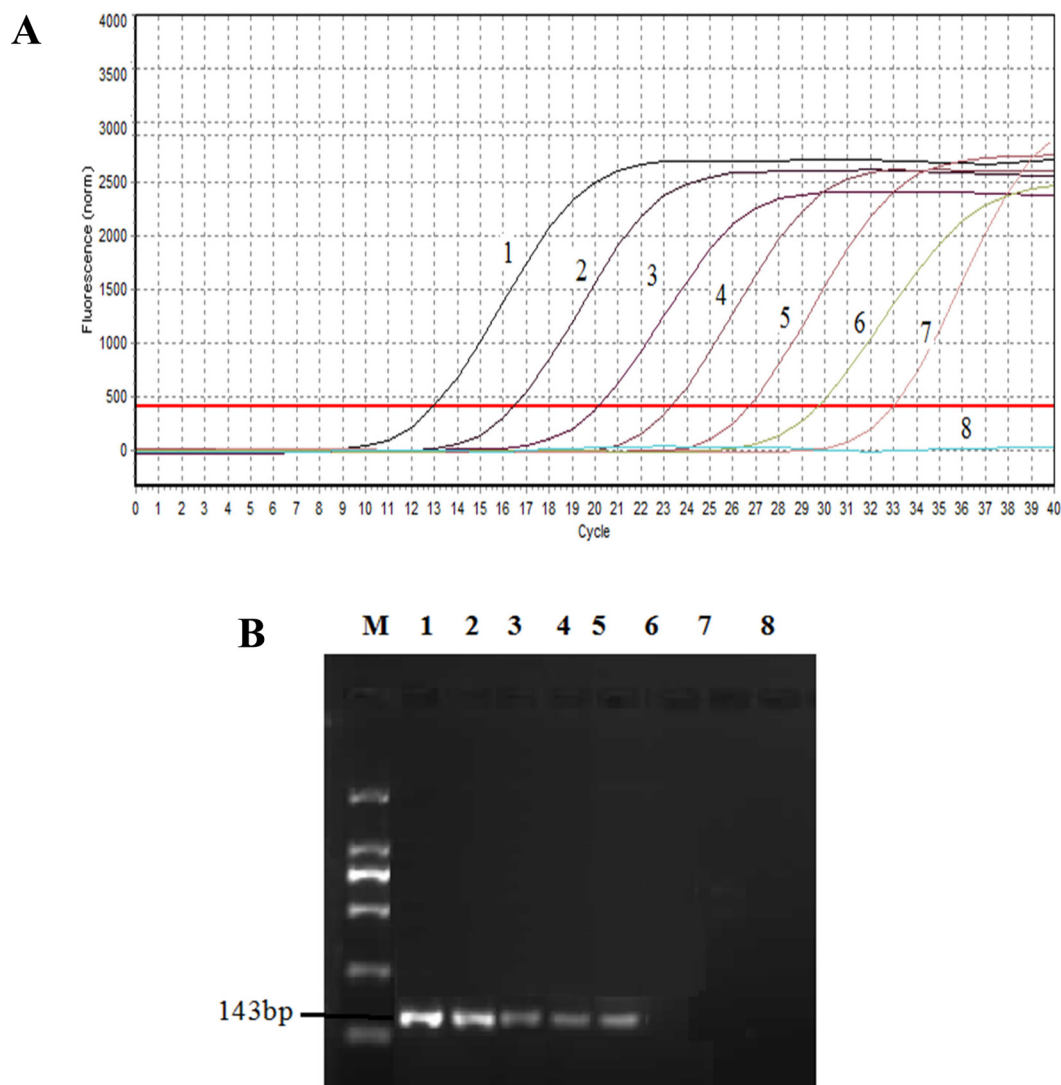


Fig. 3. Comparison of The sensitivity of real-time RT-PCR and conventional PCR. (A) Sensitivity of real-time PCR. Plot of the amplification of a 10-fold standard plasmid DNA serial dilution ($4.3 \times 10^7 - 4.3 \times 10^1$) copies. The X-axis represents cycles, and the Y-axis represents the fluorescence data. The detection limit was 4.3×10^1 copies. (B) Sensitivity of conventional PCR with RA-TaqMan1F and RA-TaqMan1R primers. Lanes 1-7: Amplification results of 4.3×10^7 to 4.3×10^1 template copies. lane 8: H₂O (control). Lane M: DNA molecular marker.

Table 1. Variance analysis of CT values of real-time PCR

Copies of standard plasmids	CT values of Intra-assay			CT values of Inter-assay		
	Mean	SD	CV (%)	Mean	SD	CV (%)
4.3×10^3	27.23	0.21	0.77	27.74	0.37	1.32
4.3×10^5	20.03	0.05	0.23	20.25	0.19	0.95
4.3×10^7	12.94	0.06	0.47	12.98	0.12	0.93

Table 2. Clinical samples detection for detection RA

Test result	RA isolation	Conventional PCR	Real-time PCR
Positive	75	123	127
Negative	83	35	31
Total		158	

Reproducibility of the real-time PCR for RA

When the standard RA plasmid DNA was used for evaluation of the coefficients of variation (CVs) of the real-time PCR, the intra- and inter-assay CVs for CT values ranged between 0.23 and 0.77%, and 0.93 and 1.32%, respectively (Table 1).

Clinical samples detection and RA isolation

Results of the real-time PCR and conventional PCR assays are shown in Table 2. One hundred fifty eight total samples, 123 samples (positive ratio 77.85%) were detected positive for RA DNA in conventional PCR, but 127 samples (positive ratio 80.38%) were positive in real-time PCR assay, with all conventional PCR positive samples were tested positive by the real-time PCR assay.

RA isolation and identification showed 75 RA strains were isolated, whereas the 4 samples (which tested positive for the PCR assay and negative for the real-time PCR assay) had no RA strains isolated, shared the same results with the PCR negative samples after RA isolation.

DISCUSSION

Riemerella anatipestifer (RA), previously known as *Pasteurella anatipestifer* or *Moraxella anatipestifer*, is a bacterial zoonosis of world-wide concern, which can infected birds are characterized by respiratory symptoms, septicemia, pericarditis, perihepatitis, meningitis and salpingitis [13]. Currently, RA had been reported to cause disease in waterfowl (including domestic ducklings and goslings), turkeys, pigeons and chickens [1, 7, 12]. It has also been isolated from wild birds, including wild ducks (Anatidae), geese (Anatidae), black swans (*Cygnus atratus*), pheasants (Phasianidae), guinea fowl (Numididae), quail (Galliformes), guillemot (*Uria aalge*) and gulls (Laridae) [7, 13]. These observations indicate that RA could cause large losses of wild migrating birds or resident waterfowl.

Traditional methods such as serological testing and RA isolation are available for the diagnosis of RA infections. RA isolation is time consuming and serological testing requires specific antibodies to avoid serological cross reactivity between different RAs. To diagnose RA infection quickly, molecular diagnostic method such as polymerase chain reaction (PCR) has been successfully used to diagnose RA, but these requires a cleaner laboratory environment to prevent pollution. TaqMan-based real-time PCR is considered a good method to perform quantitative and qualitative analyses of bacterial or virus infection [19].

In this study, the primers and probe were selected from the *DtxR* gene of the RA genome because this region is relatively conserved among RA isolates. When the RAs and 6 other duck pathogens were tested using real-time PCR, only the RAs tested positive and no cross-reaction signals were detected when using 6 other duck diseases pathogens, indicating that the real-time PCR assay is specific to RA. Compared with published sequences, the PCR products showed 100% identity with the target sequences of RA. The established real-time PCR assay had a detection limit of 43 copies for RA, which is 100 times more sensitive than conventional PCR. Tests on the reproducibility and specificity of the method suggest that the established real-time PCR system appears to be reliable and stable. The intra-assay CVs were equal or less than 0.77% and the inter-assay CVs were equal or less than 1.32%. We may conclude that the real-time PCR described here is sufficiently sensitive and specific for the diagnosis of RA in infected ducks.

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