

Development and application of specific polymerase chain reaction assay targeting the *gyrB* gene for rapid detection of *Riemerella anatipestifer*

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ABSTRACT A pair of PCR primers was designed and synthesized to amplify a *gyrB* gene sequence from *Riemerella anatipestifer* (RA). A fragment of 194 bp was detected in RA-positive isolates, whereas other isolates were negative, which confirmed the high specificity of the primers and PCR conditions. As little as 1.6×10^4 cfu/mL of cultural liquid was required by this method. We compared a 16S rRNA sequence-based PCR method and a Biolog bacterial identification system used in the detection and identification of suspicious isolates

of RA in clinical tests. The results showed that the *gyrB*-based PCR was consistent with the results of the Biolog identification system and was more specific. By applying the *gyrB*-PCR to detect RA strains in 56 duck livers, a positive rate of 46% (26/56) was observed, whereas the positive rate of 85 throat swabs from clinically healthy ducks was 11%. Thus, this method could be used for the epidemiological investigation and preliminary isolate identification of RA.

Key words: *Riemerella anatipestifer*, *gyrB* gene, polymerase chain reaction

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INTRODUCTION

Riemerella anatipestifer (RA) is a gram-negative pathogen that was first isolated by Riemeer in 1932 (Hendrickson and Hilbert, 1932). *Riemerella anatipestifer* can cause acute and chronic infections, which are a serious global problem of wild waterfowl, domestic ducks, turkeys, and other birds (Sandhu, 2008). There are at least 21 known serotypes of RA, and no significant cross-protection has been reported, also no vaccine cross-protection (Loh et al., 1992; Pathanasophon et al., 1995; Higgins et al., 2000). This has caused high morbidity, mortality, and great economic losses in the poultry industry (Glunder and Hinz, 1989). In addition, RA clinical symptoms and autopsy changes are similar to those caused by *Pasteurella*, *Escherichia coli*, and *Salmonella*, which may cause diagnostic problems.

Traditional identification methods of RA aim to analyze phenotypic, physiological, and biochemical characteristics and are laborious and require several days to

complete. Various existing test methods, such as the fluorescent antibody technique (Guo et al., 1982) and immunohistochemistry methods (Liu et al., 2004), have failed to be widely used due to restrictions in serotype differences.

The PCR technique has allowed the development of novel and powerful tools that can be applied to the detection of microbiological agents in clinical conditions. The sequence of the 16S rRNA gene has been widely used as a molecular marker to detect pathogenic bacteria (James, 2010; Kühn et al., 2011). However, 16S rRNA has a low mutation rate, which occasionally makes discrimination between closely related species difficult (Wang et al., 2007). Recent studies found that gyrase B-encoding gene (*gyrB*) is another suitable gene for bacteria identification and classification. The *gyrB*, a type II DNA topoisomerase, is universally distributed in all strains and cannot spread horizontally among different bacterial species (Yamamoto and Harayama, 1995). One of the principal advantages of *gyrB* is that its evolutionary rate caused by mutation is not only faster than ribosomal genes but also appears to be faster than other protein-coding genes. The *gyrB* gene has an average base change rate of 0.7 to 0.8% per 100 million years, faster than 16S rRNA for each 1% change in 5,000 million years (Ochman and Wilson,

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1987). Therefore, the *gyrB* gene sequence was considered more suitable to determine genetic relationships of bacteria and identification than 16S rRNA (Kumar et al., 2006; Parkinson et al., 2009; Takeda et al., 2010). In this study, we compared the findings of *gyrB*-PCR with 16S rRNA-PCR (Tsai et al., 2005) and the Biolog bacterial identification system used in detection and identification of RA-suspected isolates in clinical tests. The results showed that the *gyrB*-PCR was more specific and could be useful for both preliminary isolate identification and epidemiological surveys.

MATERIALS AND METHODS

Bacterial Strains and DNA Preparation

Riemerella anatipestifer, *Escherichia coli*, *Salmonella anatum*, and *Pasteurella avium* isolates were provided by the Key Laboratory of Animal Disease and Human Health of Sichuan Province, China. *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 7953), and *Bifidobacterium breve* (ATCC 15700) were provided by the American Type Culture Collection.

All RA strains were cultured in tryptic soy broth (Difco Laboratories, Detroit, MI) and were incubated at 37°C in a 5% CO₂ incubator for 24 to 48 h. *Escherichia coli*, *S. anatum*, *P. avium*, *S. aureus*, and *B. subtilis* were cultivated in Luria-Bertani broth culture in an incubator at 37°C for 24 h. *Bifidobacterium breve* was cultivated in tryptone peptone yeast broth culture at 37°C in anaerobic culture for 72 h.

Bacterial genomic DNA was extracted using a Genomic DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Genomic DNA templates were stored at -70°C until further use.

RA Isolates and Samples

Sixty-seven strains of gram-negative bacteria were classified by shape, size, and biochemical reaction characteristics. These strains were collected from sites of major duck industries in China. These samples were provided by the College of Veterinary Medicine of Sichuan Agricultural University, China.

PCR Amplification

Riemerella anatipestifer-specific PCR DNA primers were designed using the *gyrB* gene (GenBank accession no. CP003388) of RA (Wang et al., 2012). The length of the amplified product was 194 bp. The gene sequence was analyzed by the Primer 5.0 software program to design the following PCR primers: *gyrBP1* (forward primers) 5'-AGAGCGAGAAGAAAAACCT-3', *gyrBP2* (reverse primers) 5'-CTCCCATAAGCATAGAGAA-GA-3'.

The PCR amplifications were carried out in a Biosystems 2720 thermal cycler (Foster City, CA). A 25-μL reaction volume contained 2.5 μL of 10× PCR buffer,

1.5 μL *Taq* DNA (1 U/μL), 1.5 μL dNTP (10 mmol/L), 0.5 μmol/L of each primer, and 1.5 μL of template DNA. An initial denaturation step was followed by 30 cycles at 95°C for 5 min, 60°C for 30 s, and 72°C for 30 s, and a final extension cycle at 72°C for 10 min. The amplified products were electrophoresed on a 1.5% agarose gel and visualized under Gel Doc XR+ UV light by staining with Goldview.

Specificity of the PCR Assay

The specificity of the PCR assays was examined using other avian pathogens, including *E. coli*, *S. anatum*, *P. avium*, *S. aureus*, *B. subtilis*, and *B. breve*. Furthermore, RA standard strains and clinical isolates were also examined by PCR. The results of the PCR amplifications were observed under UV light by staining with Goldview.

Sensitivity of the PCR Assay

The sensitivity of the PCR assay was determined using a series of DNA templates from 1.6×10^8 , 1.6×10^7 , 1.6×10^6 , 1.6×10^5 , 1.6×10^4 , 1.6×10^3 , 1.6×10^2 cfu of RA strains, respectively, following the optimized conditions previously described. The amplified products were subjected to electrophoresis on 1.5% agarose gel and visualized under Gel Doc XR+ UV light by staining with Goldview.

DNA Sequencing

After electrophoresis, a single band of 194 bp was excised and purified with a DNA purification kit (Qiagen, Hilden, Germany). The products were cloned into a pMD18-T vector using an applied DNA sequence (Takara, Dalian, China). A sequence similarity search was carried out using the BLAST server at the National Center for Biotechnology Information.

Comparison of *gyrB*-PCR, 16Sr RNA-PCR, and Biolog

According to the morphological characteristics and physiological and biochemical analyses, 67 strains were identified as RA isolates by conventional microbiology method (33 strains from duck livers and 34 strains from duck brains). Detection levels by *gyrB*-PCR, 16S rRNA-PCR, and the Biolog bacterial identification system were compared.

Detection of Infections from Suspicious Duck Livers by *gyrB*-PCR

The *gyrB*-PCR was applied to test 56 duck livers for RA strains. The birds had shown clinical symptoms such as fibrinous pericarditis, perihepatitis, and airsacculitis.

Testing of Clinically Healthy Ducks by *gyrB*-PCR

The *gyrB*-PCR was applied to detect and identify the RA in the testing of clinically healthy ducks. The 85 throat swabs collected from healthy commodity ducks (7 to 54 d old) and egg-laying ducks (200 to 300 d old) were tested by the *gyrB*-PCR assay.

RESULTS AND DISCUSSION

Commonly used isolation and identification methods for RA detection are time-consuming and the results leave room for improvements. In 1982, Bisgaard established a slide and tube agglutination test through serology testing methods to detect RA (Bisgaard, 1982). However, this method is limited because of serospecificity. At present, various methods for the detection of RA have been developed, including a fluorescent antibody technique (Guo et al., 1982). Although this technique is intuitive and sensitive, it is time-consuming and less convenient because it requires fluorescence antibodies to be prepared. Real-time PCR is considered a good method to perform quantitative and qualitative analyses of RA. However, this method requires high-quality training of technical staff and more serious condition on primer design; furthermore, the synthesis of the probe is costly. In addition, the loop-mediated isothermal method is a sensitive, specific, and rapid method for bacterial detection, but it can be more easily contaminated, and it requires a cleaner laboratory environment to prevent pollution. In this study, we established a specific PCR method that is fast, accurate, reliable, and cost-effective. It has the required specificity and sensitivity for accurate diagnosis and provides a better choice of different methods.

Currently, about 2,500 species have been discovered through 16S rRNA-PCR sequencing. However, due to the highly conservative nature of 16S rRNA, some studies have struggled to distinguish closely related bacteria (Fox et al., 1992; Stackebrandt and Goebel, 1994). The gene *gyrB* is estimated to evolve faster than 16S rRNA and could thus provide an alternative effective target gene for the identification of bacteria at the species or subspecies level (Yamamoto and Harayama, 1995). After optimizing the PCR conditions, we could amplify a differential fragment about 194 bp from RA. This product was cloned, sequenced, and compared with published sequences. The results showed that the PCR product showed 100% identity with the target sequence of RA, whereas it could not be amplified in samples of *E. coli*, *S. anatum*, *P. avium*, *S. aureus*, *B. subtilis*, and *B. breve* (Figure 1). Sensitivity was detected by culture liquid. As shown in Figure 2, it had very good sensitivity. The limit of culture liquid detection was 1.6×10^4 cfu/mL, which was more sensitive than the 16S rRNA-PCR assay (Yang et al., 2007).

The PCR detection of RA using 16S rRNA sequence could lead to false-positive results (Christensen and

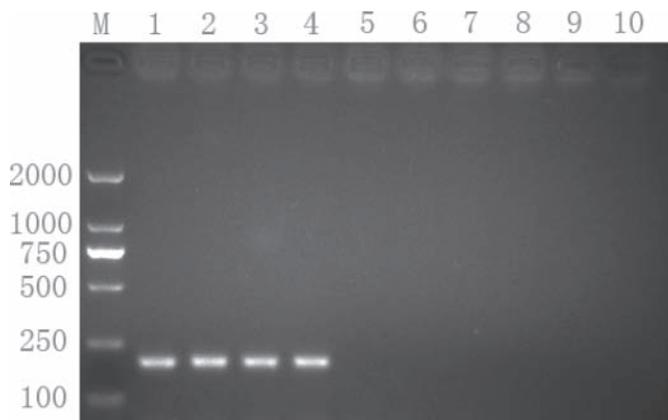


Figure 1. Specificity test results of PCR for the detection of *Riemerella anatipestifer*. M: DNA marker DL2000; 1, 2, 3, 4: products from *Riemerella anatipestifer* strains; 5: *Escherichia coli*; 6: *Salmonella anatum*; 7: *Pasteurella avium*; 8: *Staphylococcus aureus*; 9: *Bacillus subtilis*; 10: *Bifidobacterium breve*.

Bisgaard, 2010), which we also found in this study. We compared the results of *gyrB*-PCR with 16S rRNA-PCR and the Biolog bacterial identification system used in detection and identification of 67 isolates suspected of containing RA. Both the sensitivity and specificity rate of the *gyrB*-PCR was 100%, which is consistent with the Biolog bacterial identification system, whereas the sensitivity and specificity rates of the 16rRNA-PCR method were 80.7 and 63.6%, respectively, which indicates that the *gyrB*-PCR had a better specificity and could overcome interference caused by similar bacteria strains. The reduced conservative sequences of the designed primers were more accurate for identifying strains of RA (Table 1).

The *gyrB*-PCR was used to test 56 duck livers suspected of infection with RA strains. Among these, 26 were positive, resulting in a positive rate of 46% (26/56). This shows that clinical symptoms such as fibrinous pericarditis, perihhepatitis, airsacculitis, and clinical pathological changes of the ducks were not due to RA infections. Investigators should be aware that clinical symptoms might be caused by *Pasteurella*, *Escherichia coli*, and *Salmonella*, organisms that are not eas-



Figure 2. The PCR susceptibility test results of *Riemerella anatipestifer* bacilluculture. M: DL2000 DNA marker; 1: 1.6×10^8 cfu/mL; 2: 1.6×10^7 cfu/mL; 3: 1.6×10^6 cfu/mL; 4: 1.6×10^5 cfu/mL; 5: 1.6×10^4 cfu/mL; 6: 1.6×10^3 cfu/mL; 7: 1.6×10^2 cfu/mL.

Table 1. Comparison of the results of the Biolog identification system and of 2 PCR methods

Sample	Total	<i>gyrB</i> -PCR ¹	16S rRNA-PCR	Biolog system
		+, -	+, -	+, -
RA ²	67	35, 32	46, 21	35, 32
Sensitivity (%)		100	80.7	—
Specificity (%)		100	63.6	—

¹*gyrB* = gyrase B.

²*Riemerella anatipestifer*.

ily detected in RA infection. Thus, we should pay more attention to that.

Due to the possibility that clinically healthy ducks could still carry RA, we tested 85 throat swabs from clinically healthy ducklings, species ducks, and merchandise ducks. Of these, 10 were positive, resulting in a positive rate of 11% (10/85). This indicated a certain carrying rate of RA in healthy ducks, and ducks with pathological changes are not necessary for the positive detection of RA. Therefore, to strengthen control measures, we can detect healthy ducks by *gyrB*-PCR to avoid the occurrence of RA infection, thereby effectively reducing the risks of the duck industry.

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