Original Research

Detection of Riemerellosis in Ducks by *gyr*B Gene Based Polymerase Chain Reaction

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Abstract

The new duck disease or riemerellosis is predominantly a disease of ducks caused by a gram negative organism Riemerella anatipestifer. The present study was focused on the standardisation of gyrB gene based PCR for its investigation in Kerala. Evolutionary rate of gyrB gene created by mutation is faster than any other protein-coding genes. Hence, the gyrB gene was targeted for primer designing and by gradient PCR optimum conditions were arrived, which yielded an amplicon with 162 bp size. Throat swabs were collected from 60 apparently healthy ducks and 56 throat swabs revealed positive reaction by gyrB PCR whereas the bacteria were isolated from only four cases. The throat swabs of experimentally infected ducklings were found to be negative by gyrB PCR, but the liver and heart blood yielded positive amplicons and also the bacteria was isolated in pure culture. The results revealed that gyrB PCR was more accurate, sensitive and specific than the conventional culturing methods.

Key words: Ducks, *gyr*B PCR, Kerala, New Duck Disease, Riemerellosis

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Introduction

Infection with *Riemerella anatipestifer* in ducklings produces a contagious disease, known as new duck disease or riemerellosis which was reported in Kerala from 2008 onwards (Priya *et al.,* 2008; Soman *et al.,* 2014; Somu *et al.,* 2016 and Surya *et al.,* 2016). During earlier periods, *R. anatipestifer* was mis-identified as *Pasteurella multocida* due to their morphological and cultural similarities. Identification of these agents based on conventional methods such as cultural and biochemical tests were time consuming and further confusing. Hence, molecular techniques are much more needed for earlier diagnosis. Genomic

characterisation techniques like PCR which replaced these traditional cultural methods for rapid identification of pathogenic bacteria (Tenovar *et al.,* 1995). It is also possible that clinically healthy ducks carries *R. anatipestifer* in their respiratory tract. Yamamoto and Harayama (1995) found that gyrase B-encoding gene (*gyr*B), a type II DNA topoisomerase, is universally present in all bacterial strains and hence could form a suitable gene for bacterial identification. Hence, a study was framed with the objectives of standardisation of *gyr*B gene based PCR and its utility for preliminary identification of isolates and epidemiological surveys.

Materials and Methods

Revival and Identification of Bacteria

R. anatipestifer isolate (RA1) maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, as lyophilized form was revived by experimental inoculation of 0.5 ml each of suspension with 3×10^8 cfu/ml concentration into two ducklings of one-week of age. Liver, heart blood and throat swabs collected from dead birds were utilized for both isolation and molecular studies. The identity of the revived bacteria was confirmed by conventional methods (Surya *et al.,* 2016) and *R. anatipestifer* species-specific PCR assay (Kardos and Kiss *et al*., 2006).

Extraction of DNA

The DNA was extracted from revived *R. anatipestifer* culture and also from pooled tissue samples of liver and heart blood from inoculated ducklings (Sambrook and Russell, 2001). The DNA from throat swabs was extracted using Multi sample DNA Purification Kit of Himedia (Mumbai, India) as per the manufacture's instruction. The concentration and purity were measured using Nanodrop (Thermo Scientific).

Standardisation of gyrB Gene PCR

The DNA from RA1 was used for the standardization of *gyr*B gene based PCR. The primers were designed using primer 3 software were forward (F) 5' GGCTAAGGCAAGACAAGCTG 3' and reverse (R) 5' GCAGTTCCTCCTGCAGAGTC 3'. The DNA from RA1 was used to perform gradient PCR using different annealing temperatures (60ºC to 70ºC) with varying concentrations of primers (10, 20 and 30 pmol/µL). An optimum annealing temperature and primer concentration could be arrived by the following reaction mixture and protocols. Composition of single reaction mix for amplification was 10 µL of EmeraldAmp® GT PCR Master Mix, 1 µL each of forward and reverse primer (10 pM/µL), 2 µL of template DNA and 6 µL of nuclease free water. The cycling protocol followed with initial denaturation at 95ºC for 5 min, followed by 30 cycles of denaturation at 95ºC for 45 sec, annealing at 65.1ºC for 30 sec, extension at 72ºC for 30 sec with a final extension at 72ºC for 10 min. The PCR amplification was carried

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out in a thermo cycler (Biorad, USA-Model: MJ Mini). The amplified products were detected by submarine gel electrophoresis in three per cent agarose, visualized under UV transilluminator and the results were documented in a gel documentation system (Bio- Rad laboratories, USA). Specificity and sensitivity of the designed *gyr*B primers were estimated.

Screening of Throat Swabs for gyrB Gene by PCR

Throat swabs were collected from 60 apparently healthy ducks of University Poultry and Duck Farm, Mannuthy were used for both isolation and screening of *gyr*B by PCR. Genomic DNA extracted from the liver, heart blood and throat swabs of dead ducklings were also checked.

Results and Discussion

Revival and Identification of *R. anatipestifer*

The RA1 was revived successfully from both the inoculated ducklings, which developed clinical signs suggestive of riemerellosis like loss of appetite, staggering gait, listlessness, nervous disorder, greenish diarrhoea and death. The BHIA supplemented with sterile defibrinated bovine blood (BA) at five to ten per cent level was used for isolation. The culture plates of heart blood and liver of inoculated ducklings revealed smooth, convex, moist, greyish-white, translucent and butyrous colonies measuring (1-3 mm) after 24 h incubation at 37ºC. No growth could be observed from throat swabs even after 3 days of incubation. Frommer *et al*. (1990), Priya *et al*. (2008), Surya (2011), Sabnam (2015) and Shancy (2015) also used bovine BA as an enriched medium for primary isolation of the organism from clinical samples. Segers *et al.* (1993) observed smooth, non-pigmented colonies on chocolate agar while Songer and Post (2005) observed the colonies as convex, transparent and butyrous on BA upon micro aerophillic conditions with incubation at 37ºC. The organism was Gram-negative, non-motile and morphology varied from cocco-bacilli, short rods to filamentous forms, non-haemolytic on BA, grew micro aerobically and did not grow on Mac Conkey agar. Similar colony characters were also observed by Rimler and Nordholm (1998) and Shome *et al.* (2004).

Riemerella anatipestifer was differentiated from *P. multocida* based on their biochemical characteristics such as indole production, gelatin liquefaction and ornithine decarboxylase utilization. *P. multocida* was positive for indole and ornithine decarboxylase utilization and did not liquefy gelatin (OIE, 2010). The isolate was found to be positive for gelatin liquefaction, catalase and oxidase tests. It was negative for IMViC and ornithine decarboxylase test. The results were in accordance with Vancanneyt *et al.* (1999), Shome *et al.* (2004), Surya (2011) and Shancy (2015). Based on the morphological, cultural and biochemical characteristics, the RA1 isolate was identified as *R. anatipestifer.*

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Riemerella anatipestifer Species-Specific PCR

Genomic DNA was extracted from *R. anatipestifer* culture, pooled tissue samples of liver and heart blood and throat swabs from inoculated ducklings as per Sambrook and Russell (2001) and kit method, respectively. As they had desired purity and concentration, it was utilized for *R. anatipestifer* species specific PCR and the amplicons were noticed at 546 bp from RA1, liver and heart blood. Similar results were reported by Soman *et al*. (2014) and Shancy (2015). The throat swab showed negative result.

Standardisation of *gyr*B Gene PCR

James (2010) and Kuhn *et al*. (2011) utilized 16S rRNA as molecular marker to detect pathogenic bacteria. As it has low mutation rate, it was often difficult to differentiate closely related bacteria. Later, *gyr* B gene was identified as an equally good marker sequence by Yamamoto and Harayama (1995), Kumar *et al*. (2006) and Takeda *et al.* (2010) for the classification of bacteria at the species and subspecies level. Wang *et al.* (2012) designed a pair of PCR primers to amplify *gyr*B gene sequence of *R. anatipestifer* and compared it with 16S rRNA sequence-based PCR and concluded that *gyr*B-based PCR was more accurate. Evolutionary rate of *gyr*B gene created by mutation is faster than any other protein-coding genes. This was considered as main criteria behind its selection and application. Hence, the *gyr*B gene was targeted for primer designing and by gradient PCR optimum conditions were arrived, which yielded an amplicon with 162 bp size. Wang *et al.* (2012) obtained 194 bp product size by *gyr*B gene PCR. Also Wang *et al.* (2012) employed 1.5 per cent agarose gel to view the product. As the current study yielded amplicon of lesser molecular weight, 3 per cent agarose was found to be best to view the product by gel documentation system. While evaluating the specificity of *gyr*B primers, it was noticed that positive amplicons were obtained only from the DNA of the *R. anatipestifer* culture (Fig. 1).

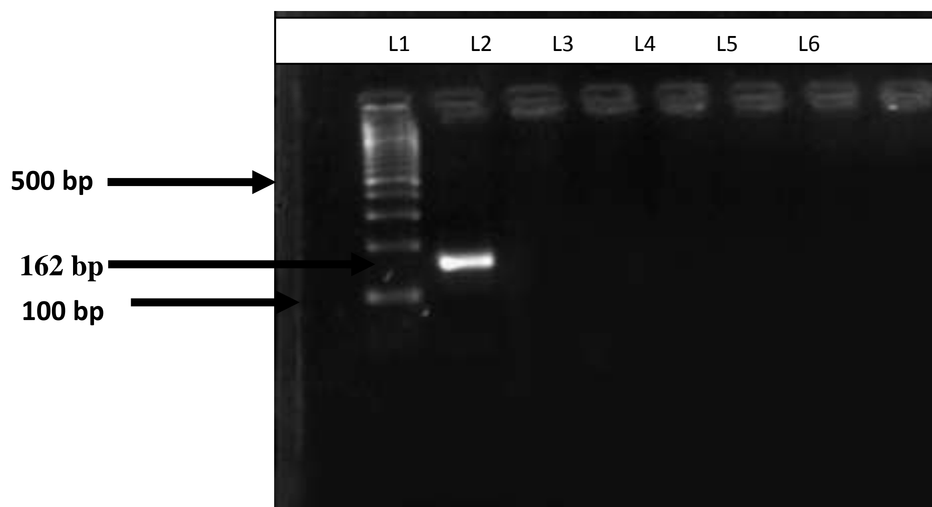

Fig. 1: Specificity of *gyr*B primers (Lane1-100 bp ladder, Lane 2- RA1, Lane 3- *E. coli*, Lane 4- *Salmonella,* Lane 5- *P. multocida*, Lane 6- DNA from Duck plague)

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The DNA from *E. coli, Salmonella, P. multocida* and duck plague virus did not yield any amplicons. This clearly showed the specificity of the designed *gyr*B primers. The selection of primers plays a major role in determining the specificity of the primers as mentioned by Kardos *et al.* (2006). The sensitivity was found to be at 10^8dilution (Fig. 2). The *gyr*B gene based PCR was successfully standardised using the DNA from the RA1 isolate and later utilized for screening the biomaterials. The DNA from liver and heart blood yield positive amplicons for *gyr*B gene PCR at 162 bp whereas the throat swab from inoculated ducklings showed negative result.

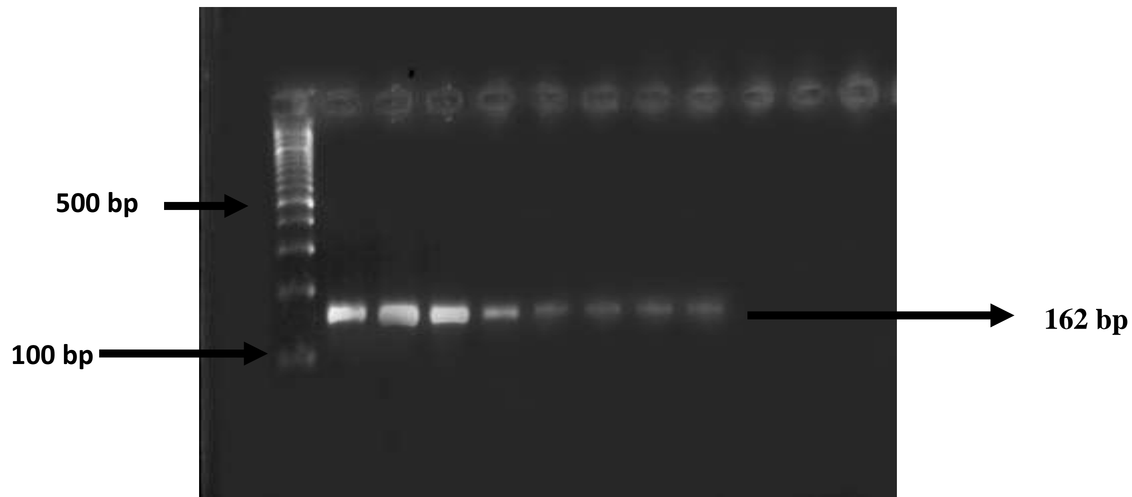

Fig. 2: Sensitivity of *gyr*B primers

Isolation of *R. anatipestifer* from Throat Swabs

Out of 60 throat swabs streaked directly on to blood agar, only 4 samples (6.7 per cent) yielded *R. anatipestifer.* It was confirmed based on the assessment of morphology, cultural characteristics, biochemical reactions and RA specific PCR.

Screening of Throat Swabs by *gyr*B PCR

In the present study, 56 throat swabs out of 60 samples revealed positive reaction (93.3 %) by *gyr*B gene based PCR though the bacteria were isolated only from 4 cases (Fig. 3). Wang *et al*. (2012) also extracted DNA from throat swab of 85 healthy ducks and found 67 samples (78.8 per cent) as positive by *gyr*B gene based PCR. They also noted 46 percent (26/56) positivity on testing 56 duck livers. The throat swabs of experimentally infected ducklings were found to be negative by *gyr*B PCR and by isolation studies. But the liver and heart blood yielded positive amplicons and the bacteria was isolated in pure culture. Hence it has been assumed that the *R. anatipestifer* is present in the throat region as commensal organism like *P. multocida*. Under stress, the organism may undergo rapid multiplication, followed by dissemination to internal organs through the blood stream. The development of clinical signs occurs when the organisms enters into septicemic phase and thereby the throat could be free of the organism.

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Fig. 3: Screening of throat swabs by *gyr* B gene based PCR

The presence of *R. anatipestifer* in most of the healthy birds revealed its commensal nature, necessary preventive measures should be taken to avoid stress conditions. These findings revealed that *gyr*B PCR could be employed for epidemiological studies. The results suggested that the *gyr*B gene based PCR was a specific and rapid tool for the detection of *R. anatipestifer* isolates.

Conclusion

In the present study, out of sixty throat swabs tested 56 throat swabs revealed positive reaction by *gyr*B PCR whereas only 4 cases were positive for bacterial isolation. The throat swabs of experimentally infected ducklings were found to be negative by *gyr*B PCR and by isolation studies, but their liver and heart blood yielded positive amplicons and the bacteria was isolated in pure culture. Hence, it was concluded that the *gyr*B gene based PCR could be used as a specific and rapid tool for the detection of *R. anatipestifer* isolates.

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