www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(11): 409-412

www.thepharmajournal.com
Received: 28-09-2018
Accepted: 30-10-2018

Athira S
M.Sc (Applied Microbiology) student, Dept of Veterinary Microbiology, College of Veterinary and Animal Sciences (CVAS), Mannuthy, Thrissur Dt, Kerala, India

Priya PM
Assistant Professor, Dept of Veterinary Microbiology, College of Veterinary and Animal Sciences (CVAS), Mannuthy, Thrissur Dt, Kerala, India

Sheikh Moin Ahmad
PhD Scholar, Dept of Veterinary Microbiology, College of Veterinary and Animal Sciences (CVAS), Mannuthy, Thrissur Dt, Kerala, India

Rinsha Balan
PhD Scholar, Dept of Veterinary Microbiology, College of Veterinary and Animal Sciences (CVAS), Mannuthy, Thrissur Dt, Kerala, India

Mini M
Professor and Head, Dept of Veterinary Microbiology, College of Veterinary and Animal Sciences (CVAS), Mannuthy, Thrissur Dt, Kerala, India

Correspondence
Priya PM
Assistant Professor, Dept of Veterinary Microbiology, College of Veterinary and Animal Sciences (CVAS), Mannuthy, Thrissur Dt, Kerala, India

Standardisation of multiplex PCR for simultaneous detection of *Riemerella anatipestifer, Escherichia coli* and *Salmonella enterica* in ducks

Athira S, Priya PM, Sheikh Moin Ahmad, Rinsha Balan and Mini M

Abstract
Riemerella anatipestifer, *E. coli* and *Salmonella* are the three bacterial agents which produce concurrent infection in ducks. These agents produce more or less similar lesions, and hence differential diagnosis makes it difficult. Conventional cultural methods are laborious and time consuming, and not recommended at the time of outbreak. Molecular techniques like polymerase chain reaction (PCR) are widely employed for rapid identification of the etiological agents. Hence, a study was framed with an objective to develop multiplex Polymerase Chain Reaction (m-PCR) for the rapid detection and differential diagnosis of these bacterial agents. The positive isolates of *R. anatipestifer, E. coli* and *Salmonella* were revived and their DNA was extracted. After checking the purity and concentration, they were subjected to individual PCR for optimization of annealing temperature and primer concentration. Primer based on *Dna*B gene, *Pho*A gene and *Inv*A gene were chosen to amplify the *R. anatipestifer, E. coli* and *Salmonella* genomic DNAs. Multiplex PCR was standardized in which the DNA of *R. anatipestifer* yielded an amplicon of 610 bp, whereas *E. coli* and *Salmonella* genomic DNA gave 720 bp and 256 bp product size. All the three primers were highly specific and no cross reactions were detected. Also they yield the products which are distinguishable. The developed m-PCR could be employed for the routine detection and differentiation *R. anatipestifer, E. coli* and *Salmonella* infection in ducks.

Keywords: Riemerellosis, *Riemerella anatipestifer*, *E. coli*, *Salmonella*, multiplex PCR, *Dna*B gene, *Pho*A gene, *Inv*A gene

Introduction
Riemerella anatipestifer infection causes new duck disease which was reported from 2008 onwards in Kerala (Priya *et al.*, 2008a) [12]. It is an acute / chronic infection in ducks, characterised by sudden death with ataxia and nervous disorders, fibrinous pericarditis, peri hepatitis and diarrhoea (Sandhu and Rimler, 1997) [20]. *Salmonella* infection in poultry is important as it causes diseases as well as act as source of food borne transmission to humans. Routine cleansing and disinfection often fail to eliminate *Salmonella* due to the emerging drug resistance and sometimes help the pathogen to survive much better by killing the competing commensal flora (Tsai and Hsiang, 2005) [26]. Ducklings suffering from infections with *Salmonella* might show signs and lesions identical to new duck disease/ colibacillosis like fibrinous pericarditis and peri hepatitis (Hu *et al.*, 2011) [9]. Collibacillosis, a common systemic infection, caused by *E. coli* is an economically important disease among poultry throughout the world (Saif *et al.*, 2003) [18]. Birds surviving the septicemic phase develop fibrinous pericarditis, perihepatitis and air sacculitis which often confuses with *R. anatipestifer* infection. As the three above mentioned infections produce more or less similar clinical signs and gross lesions, it is quite difficult to differentiate them based on symptoms and lesions. Identification by conventional methods like cultural and biochemical tests is time consuming and further confusing, as strains with similar biochemical characteristics are prevalent. Genomic characterisation techniques like Polymerase Chain Reaction (PCR) has supplemented or replaced the traditional cultural methods (Tenovar *et al.*, 1995) [24]. In case of concurrent infections, where the involvement of two or more microorganism, the use of multiplex PCR is best alternative for rapid, reliable and sensitive than individual PCR. Hence, a study was planned to develop a standard protocol for multiplex Polymerase Chain Reaction (m- PCR) assay for the differential diagnosis of *R. anatipestifer*, *E. coli* and *Salmonella* infection in ducks.

Materials and Methods

Revival of bacterial isolates

The lyophilized isolate of *R. anatipestifer* maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy was dissolved in distilled water and streaked on to the brain heart infusion agar (BHIA) supplemented with sterile defibrinated bovine blood (BA) at five to ten per cent level and incubated for 24 to 48 h at 37º C in a candle jar. It was identified as *R. anatipestifer* as per the method described by Surya *et al.* (2016) [23].

Avian isolates of *E. coli* obtained from Poultry Disease Diagnosis and Surveillance Laboratory (PDDSL), Veterinary College and Research Institute, Namakkal, Tamil Nadu, and one representative isolate of *Salmonella enterica* maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy on agar slants were streaked on to the Mac Conkey agar (MCA) plates and incubated for 24 h at 37º C. They were identified as per the method described by Merchant and Packer (2002) [11].

Isolation of genomic DNA

The genomic DNA of all isolates was extracted based on the method suggested by Chistensen *et al.* (1993) [3] and Sambrook and Russell (2001) [19]. By measuring the OD values at 260 nm and 280 nm by Nano drop, the concentration and purity of the DNAs were checked.

Polymerase Chain Reaction for *Riemerella anatipestifer*, *E. coli and Salmonella*

The published primers of Hu *et al.* (2001) [8] were used.

Organisms	Targeted gene	Sequence	Amplicon
Riemerella anatipestifer	*dna*B	AAACTCAGGCAAAGGTGGCAC	610 bp
		TGTATGGTAGTTTTGATGCTTTCAA	
E. coli	*pho*A	CGATTCTGGAAATGGCAAAAG	720 bp
		CGTGATCAGCGGTGACTATGAC	
Salmonella enterica	*Inv*A	AACCAGCAAAGGCGAGCAG	256 bp
		CAATACGATGCTGTTATCGTCCAG	

For standardisation of individual PCR, the respective positive control DNA of *Riemerella anatipestifer*, *E. coli* and *Salmonella enterica* were subjected to individual gradient PCR using different annealing temperatures (500 C to 56 0C) with varying concentrations of primers (10, 20 and 30 pmol/µL). Finally, an optimum annealing temperature and primer concentration were arrived by the following reaction mixture and protocols.

Components	Quantity (µL)
Emerald master mix	10
Primer: F (10 pmol / µL)	1
Primer: R (10 pmol / µL)	1
Nuclease free water	6
DNA	2
Total	20

The mixture was mixed properly and placed in an automated thermal cycle (MJ Mini, Bio Rad, Germany) for the amplification of the desired gene. Appropriate positive and negative controls were used for every PCR reaction.

Steps	Temperature	Time	Cycles
Initial denaturation	940C	4 min	1
Denaturation	940C	45 sec	30
Annealing	500C	45 sec	
Extension	720C	50 sec	
Final extension	720C	10 min	1

The amplified PCR products were subjected to agarose gel electrophoresis using one per cent agarose gel in TBE buffer, visualised in UV transilluminator and photographed in Gel-documentation system (Bio Rad laboratories, USA).

Development of multiplex PCR

Multiplex PCR was standardised using the same primers and protocol developed for individual PCR. The PCR mixture was reconstituted in 25 µL volume as shown below

Components	Quantity (µL)
PCR master mix	10
R. anatipestifer primer: F(10pmol/µL)	1
R. anatipestifer primer: R(10 pmol/µL)	1
E. coli primer: F (10 pmol/µL)	1
E. coli primer: R (10 pmol/µL)	1
Salmonella primer: F (10 pmol/µL)	1
Salmonella primer: R (10 pmol/µL)	1
Nuclease free water	7
DNA template	2
Total	25

Screening of clinical samples by multiplex PCR

Biomaterials like liver, heart blood and spleen were collected aseptically from the concurrent infection suspected ducks presented to the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy. The DNA was extracted from the pooled, processed samples using Nucleospin tissue DNA kit (Takara) as per the manufacturer's instructions and subjected to the developed m- PCR.

Results and Discussion

Revival of bacterial isolates

The standard isolates of *R. anatipestifer*, *E. coli* and *Salmonella* were revived successfully using specific selective media. Frommer *et al.* (1990) [5], Priya *et al.* (2008b) [13] and Surya (2011) [22] also employed bovine blood agar (BA) as enriched media for the isolation of *R. anatipestifer*. The *E.coli* isolates gave lactose fermenting rosy pink colored colonies on MCA. They had the biochemical identification tests and cultural characters consistent with the description of Merchant and Packer (2002) [11]; Giovanardi *et al.* (2005) [7]; Quinn *et al.* (1994 & 2002) [14, 15] and Priya *et al.* (2008b) [13]. The Salmonella isolate was confirmed as *S.* Gallinarum and the biochemical findings are in accordance with Ravishankar *et al.* (2008) [16] and Siddique *et al.* (2009) [21].

Isolation of genomic DNA

The DNA was extracted from the revived isolates of *R. anatipestifer*, *E. coli* and Salmonella as per the method elaborated by Christensen *et al* (1993) [3] and Sambrook and Russell (2001) [19]. Earlier workers (Surya, 2011; Sabnam, 2015) [22, 17] also followed the same method. The purity of the DNA samples was observed as 1.8 to 1.9 for all the isolates.

Polymerase Chain Reaction for *Riemerella anatipestifer*, *E. coli and Salmonella*

Selection of ideal primers plays a prime role in determining the sensitivity, specificity and reproducibility of PCR assay. In the present study, published primers (Hu *et al*., 2011) [9] based on *dna*B gene for *Riemerella anatipestifer*, *pho*A for *E. coli* and *Inv*A gene for *Salmonella* were employed.

While specificity checking of the *dna*B gene primers, it gave positive amplicons only from control DNA of revived *R. anatipestifer*. No amplicons were obtained from control *E. coli* and *Salmonella* DNA, which indicates it's specificity as mentioned by Hu *et al.* (2011) [9]. The sensitivity of these primers i.e., the minimum DNA concentration at which the amplification occurred was checked by using serial tenfold dilution of the positive control samples and arrived as 1018 dilution.

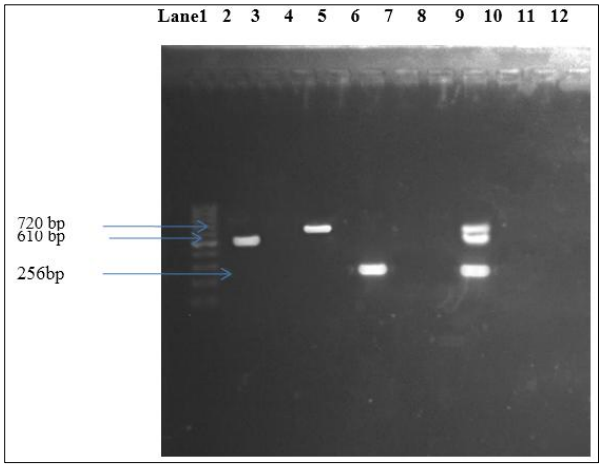
To identify the *E. coli* infection, a house-keeping gene of *E. coli*, *Pho* A gene targeted primers were used. The primers were found to be highly specific and did not show any cross reaction as reported by Kong *et al.* (1995) [10] and Thong *et al.* (2011) [25].

The primers targeting the *Inv*A gene were employed earlier by Siddque *et al.* (2009) [21] and Tuchili *et al.* (2005) [27] to identify Salmonella. They were found to be highly specific and did not show any cross reaction (Hu *et al.*, 2011) [9].

Standardisation of multiplex PCR

Multiplex PCR was a precise technique routinely employed by various workers to identify different microorganisms in a single reaction. Eyigor *et al.* (2003) [4] selected three sets of primers to identify different serovars of Salmonella in poultry. Germini *et al.* (2008) [6] described the development of multiplex PCR for simultaneous detection of Salmonella serovars, *Listeria monocytogenes* and *E. coli*. Hu *et al.* (2011) [9] developed an m-PCR that could discriminate *R. anatipestifer, E. coli and S. enterica* rapidly in field isolates. They opined the assay as a rapid and reliable test at the time of outbreak to differentiate the clinically similar infections. Kong *et al.* (1995) [10], Alvarez *et al.* (2004) [2] and Akiba *et al.* (2011) [1] also reported this technique for easier identification of different microorganisms.

In this study, the selected primers successfully amplified the genome of the three bacterial isolates. The multiplex PCR based on *Dna*B gene for *R. anatipestifer* yielded an amplicon with 610 bp; *Pho* A gene for *E. coli* showed a PCR product size at 720 bp and *Inv*A gene of *Salmonella* yielded a positive PCR band at 256 bp (Fig. 1). The assay also yielded three amplicons at a discernible size. Further, the specificity and reliability were confirmed by screening few clinical samples.

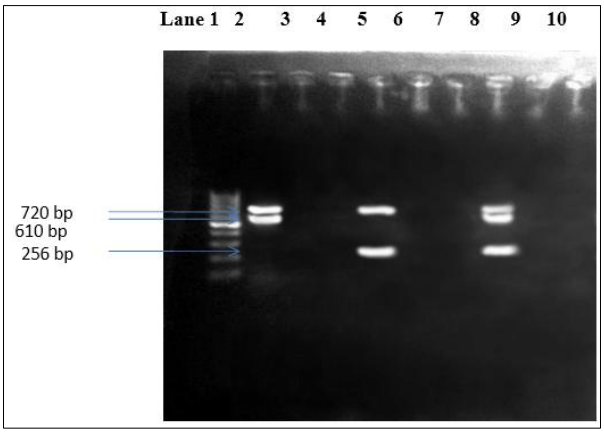


Lane 1: DNA molecular weight marker (100 bp)
Lane 2: Positive PCR amplicon specific for *R. anatipestifer*
Lane 4: Positive PCR amplicon specific for *E. coli*
Lane 6: Positive PCR amplicon specific for *Salmonella*
Lane 9: Positive m- PCR amplicons

Fig 1: Standardisation of Multiplex Polymerase chain reaction

Screening of clinical samples by multiplex PCR

The DNA extracted from the biomaterials collected randomly from suspected concurrent infections yielded corresponding amplicons of *R. anatipestifer*, *E. coli* and *Salmonella*. Few samples revealed the PCR bands specific for *R. anatipestifer* and *E. coli* while in some others, amplicons specific for *E. coli* and *Salmonella* were also detected (Fig. 2).



Lane 1: DNA molecular weight marker (100 bp)
Lane 2: Positive PCR amplicon specific for *R. anatipestifer* and *E. coli*
Lane 5: Positive PCR amplicon specific for *E. coli* and *Salmonella*
Lane 8: Positive m- PCR amplicons

Fig 2: Screening of clinical samples using m- PCR

To conclude, the multiplex PCR developed in this study could be used for rapid detection of concurrent infection caused by *R. anatipestifer, E. coli and S. enterica* in field outbreaks.

References

1. Akiba M, Masahiro K, Taketoshi I. Rapid identification of *Salmonella enterica* serovars, typhimurium, choleraesuis, infantis, hadar, enteritidis, Dublin and gallinarum, by multiplex PCR. J Microbiol. Method. 2011; 85:9-15.
2. Alvarez J, Mertxe S, Vivanco AB, Perale I, Cisterna R, Rementeria A, Garaizar J. Development of a multiplex PCR technique for detection and epidemiological typing of Salmonella in human clinical samples. J Clin. Microbiol. 2004; 24:1734-1738.
3. Christensen JP, Olsen JE, Bisgaard M. Ribotypes of *Salmonella enterica* serovar gallinarum biovargallinarum and pullorum. Avian Pathol. 1993; 22:725-738.
4. Eyigor A, Carli KT. Rapid detection of Salmonella from poultry by real-time polymerase chain reaction with fluorescent hybridization probes. Avian Dis. 2003; 47:380-386.
5. Frommer A, Bock R, Inbar A, Zemer S. Muscovy ducks as a source of *Pasteurella anatipestifer* infection in turkey flocks. Avian Pathol. 1990; 19:161-163.
6. Germini A, Masola A, Carnevali P, Marchelli R. Simultaneous detection of *Escherichia coli* O175: H7, *Salmonella* spp., and *Listeria monocyte genes* by multiplex PCR. Food control. 2009; 20:733-738.
7. Giovanardi D, Campagnari E, Ruffoni LS, Pesente P, Ortali G, Furlattini V. Avian pathogenic *Escherichia coli* transmission from broiler breeders to their progeny in an integrated poultry production chain. Avian. Path. 2005; 34:313-331.
8. Hu Q, Liu X, Miao J, Zhao D, Zhang L, Ding C. The epidemiology study of *Riemerella anatipestifer* infection in Jiangsa and Anhui provirus. Guide Chin. Poult. 2001; 18:18-19.
9. Hu Q, Tu J, Han X, Zhu Y, Ding C, Shengqing Y. Development of multiplex PCR assay for rapid detection of *Riemerella anatipestifer*, *Escherichia coli*, and *Salmonella* enteric simultaneously from ducks. J Microbiol. Methods. 2011; 87:64-69.
10. Kong RYC, Dung WF, Vrijmoed LLP, Wu RSS. Co-detection of three species of water-borne bacteria by multiplex PCR. Marine Pollut. Bull. 1995; 31:317-324.
11. Merchant IA, Packer RA. Veterinary Bacteriology and Virology. (7th Ed).Indian Ed., 2002.
12. Priya PM, Pillai DS, Balusamy C, Rameshkumar P, Senthamilselvan P. An Unusual Occurrence of Coli septicemia in Budgerigars (Melopsittacus Undulatus). Int. J Poult. Sci. 2008a; 7:191-192.
13. Priya PM, Pillai DS, Balusamy C, Rameshkumar P, Senthamilselvan P. Studies on outbreak of new duck disease in Kerala. India. Int. J Poult. Sci. 2008b. 7:189-199.
14. Quinn PJ, Cater ME, Markey BK, Carter GR. Clinical Vet. Microbiol. Wilf publications, 1994; 648p.
15. Quinn PJ, Markey BK, Carter ME, Donnelly WJ, Leonard FC. Veterinary Microbiology and Microbial Diseases. Blackwell Sciences Ltd. 2002; 648.
16. Ravishankar C, Priya PM, Pillai D, Mini M, Sharmadha MK, Rameshkumar P, Jayaprakasan V. Isolation Of Salmonella Gallinarum From Chicken And Quails. 2008; 12:25-75.
17. Sabnam VS. Serotyping and molecular charecterisation of *Riemerella anatipestifer*. MSc thesis, Kerala Veterinary and Animal Science University, Mannuthy, 2015; 75p.
18. Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE. Diseases of Poultry. (12th Ed.). Editorial Board for the American Association of Avian Pathologists, 2003.
19. Sambrook J, Russell DW. *Molecular Cloning. A Laboratory Manual.* (3rdEd.). Cold Spring Harbor Laboratory Press, New York. 2001; 2231p.
20. Sandhu TS, Rimler RB. *Riemerella anatipestifer* infection. In: Calnek, B. W. (ed.), Diseases of Poultry. (10th Ed.). Iowa State University Press, USA, 1997; pp. 161-166.
21. Siddique RA, Saxena M, Lakhchaura BD. PCR based rapid detection of *Salmonella* from poultry samples. Indian J Public Hlth. 2009; 53:226-228.
22. Surya PS. Isolation and Characterization of *Riemerella anatipestifer* from ducks from ducks. M.V. Sc. thesis, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, 2011; 82 p.
23. Surya PS, Priya PM, Mini M. Biotyping and antibiogram of *Riemerella anatipestifer* from ducks in Kerala. Bioscience Biotechnology Research Communications. 2016; 9:462.
24. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. Interpreting chromosomal DNA restriction patterns produced by pulse-field gel electrophoresis: criteria for bacterial strain typing. J Clin. Microbiol. 1995. 33:2233-2239.
25. Thong KL, Lai MY, Chua KH. Simultaneous detection of methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii*, Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa by multiplex PCR. Tropibiomedi, 2011; 28:21-31.
26. Tsai HJ, Hsiang PH. The prevelance and antimicrobial susceptibilities of *Salmonella* and *Campylobacter* in ducks in Taiwan. J Vet. Med. Sci. 2005; 67:7-12.
27. Tuchili LM, Kodama H, Izumoto Y, Mukamoto M, Fukata T, Baba T. Detection of *Salmonella* Gallinarum and *S.* Typhimurium DNA in experimentally infected chicks by polymerase chain reaction. J Vet. Med. Sci. 1995; 57:59-63.