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Original Research Article

Simultaneous Detection of *Mycoplasma gallisepticum, Mycoplasma synoviae, Infectious bronchitis virus* and *Infectious laryngotracheitis virus* in Poultry by Multiplex SYBR Green Real Time PCR

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Abstract: Multifactorial respiratory problems are very common in poultry due to intensive rearing methods and caused by coinfection with bacteria (MG, MS, *E. coli* and *Avibacterium spp*.) and viruses, such as IBV and ILTV. The conventional procedures are tedious and time taking, to overcome the difficulties in diagnosis of mixed respiratory pathogens, this study was undertaken with the following objective simultaneous detection of MG, MS, IBV and ILTV by Multiplex SYBR Green Real Time PCR. The SYBR Green Real time PCR assay was standardized for simultaneous detection of respiratory pathogens in poultry by targeting *IGSR* gene for MG, *vlhA* gene for MS, *N* gene for IBV and *ICP4* gene for ILTV in single tube. The optimized conditions for all genes was initial denaturation at 94^{o}C for 3.59 mins, followed by 40 cycles of denaturation at 94^{o}C for 20 seconds, annealing at 55^{o}C for 45 seconds and extension at 72^{o}C for 1 min and final extension step was at 72^{o}C for 10 mins. The amplifications of all four genes was observed and recorded the ct values of 30.28 (*IGSR* gene), 24.16 (*vlhA* gene), 19.39 (*ICP4* gene) and 11.81 (*N* gene) and also the recorded Tms were 74.0 (*IGSR* gene), 82.5 (*vlhA* gene), 85.5 (*ICP4* gene) and 80.5 (*N* gene). The samples with below 35 recorded threshold cycles value were considered as positive. Out of 19 farms screened, 2 were positive for ILT, 10 for MG,7 for MS and 5 for MG and MS and all were negative for IB. In sreened samples, the recorded ct values for *IGSR* genes ranged between 11.81 to 30.28, product Tms ranged between 74 to 87, for *vlhA* gene ct values ranged between 17.39 to25.81, product Tms ranged between 81 to 87, for *ICP4* gene ct value was 19.08 and 19.23,product Tms was 81 and 80.5.

Keywords: Ct value, MG, MS, ILTV, IBV, SYBR Green real time PCR, Tms.

INTRODUCTION

Growth in poultry sector is being challenged due to increased incidence and re-emergence of diseases caused due to evolution of several pathogens and use of live vaccines. The avian respiratory system hosts a wide range of commensal and potential pathogenic bacteria and viruses that interact with each other. Such interactions could be either synergistic or antagonistic, which subsequently determines the severity of the disease complex. The intensive rearing methods of poultry are responsible for the marked increase in avian respiratory diseases worldwide. Because of similarities in exhibition of clinical signs and lesions in mixed infections, it is often difficult to diagnose and get mislead the specific disease condition. To reduce the economic losses in poultry industry, rapid and confirmatory differential diagnosis is essential and to implement control strategies. So this study was undertaken with the following objective simultaneous molecular detection of MG, MS, IBV and ILTV by Multiplex SYBR Green Real Time PCR.

MATERIAL AND METHODS

Samples

A total of 19 suspected farms with history of vaccination against IB and RD in different poultry pockets of Andhra Pradesh and samples were collected, includes 12 cloacal swabs, 12 tracheal swabs, 8 tracheal tissues, 8 lung tissues and 4 oviducts are collected from each farm and labelled farm wise and organ wise.

Nucleic acid extraction

The DNA and RNA extraction was done by Trizol Method[1] and the Complementary DNA was synthesized using QIAGEN cDNA synthesis kit (GERMANY).

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Multiplex SYBR Green Real Time PCR

The facilities in Department of Virology, SVIMS were utilized, Aria Mx Real –Time PCR machine and Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo scientific) was used for this study. Multiplex SYBR Green Real Time PCR test was standardized using Nobilis H120 vaccine for IBV, the modified live infectious laryngotracheitis vaccine (Merial) for ILTV, the *Mycoplasma gallisepticum*, live, freeze dried vaccine (Nobilis MG 6/85) (Intervet) for MG and the *Mycoplasma synoviae* live vaccine (Bioproperties pvt ltd, (MS-H strain)) was used as positive control for MS, by targeting the following genes (Table1) and reaction mixture (Table 2).

Table-1: Primers used for simultaneous detection of IBV, ILTV, MG and MS

Target gene	Primer	Primer sequence	Amplicon size	Reference
vlhA	Forward	5'-TACTATTAGCAGCTAGTGC-3'	350-400 bp	**Jeffery *et al. [2]***
	Reverse	5'-AGTAACCGATCCGCTTAAT-3'		
IGSR	Forward	5'-ACTGATAGCTTTTCGTACAGCACG-3'	812bp	**Raviv *et al. [3]***
	Reverse	5'-CATCGGGACATTCTCCAGGTAGCA-3'		
ICP4	Forward	5^1-CTTCAGACTCCAGCTCATCTG-3^1	635bp	**Chacon and Ferreira [4]**
	Reverse	5^1-GTCATGCGTCTATGGCGTTGAC-3^1		
N	Forward	5'-TCATGGCAAGCGGTAAGG-3'	316 bp	**Andresen *et al. [5]***
	Reverse	5'-TTCAGGTTAGCGGCTGGTC-3'		

Table-2: PCR mixture optimised for the amplification of MG, *VlhA*, IB *N*, and *ICP4* genes:

S. No.	Constituents	Volume (µL)
1.	Template DNA each	2
2.	Forward primer (10 p*M*/µL) MG gene	0.5
3.	Reverse primer (10 p*M*/µL) MG gene	0.5
4.	Forward primer (10 p*M*/µL) *ICP4* gene	0.5
5.	Reverse primer (10 p*M*/µL) *ICP4* gene	0.5
6.	Forward primer (10 p*M*/µL) *vlhA* gene	0.5
7.	Reverse primer (10 p*M*/µL) *vlhA* gene	0.5
8.	Forward primer (10 p*M*/µL) *N* gene	0.5
9.	Reverse primer (10 p*M*/µL) *N* gene	0.5
10.	Maxima SYBR Green /ROX q PCR Master Mix	25
11.	Nuclease free water	8.5
	Total	50

The Optimised Multiplex SYBR Green Real Time PCR conditions for the amplification *IGSR*, *vlhA*, *N* and *ICP4* genes was initial denaturation at 94^{o}C for 3.59 mins, followed by 40 cycles of denaturation at 94^{o}C for 20 seconds, annealing at 55^{o}C for 45 seconds and extension at 72^{o}C for 1 min and final extension step was at 72^{o}C for 10 mins.

RESULTS AND DISCUSSION

In the present study the affected birds showed respiratory rales, dyspnoea, sneezing, mucoid nasal discharges, swelling of lower eyelids, oedema of facial sub-cutis, growth retardation and lameness. Similar clinical manifestations were reported by Ley [6] and Ramdass *et al.* [7], based on these clinical signs, the flocks were suspected for respiratory infections like *Mycoplasma*, ILT and IB or mixed infections. The isolation methods is gold standard method for diagnosis of diseases but time consuming, Hence, the molecular detection methods could be better alternatives for amplifying even low levels of nucleic acids from the samples [8]. In the present study genomic DNA and RNA was extracted from the samples and vaccines by TRIsol method. The extracted genomic DNA and RNA (cDNA) was used as template for each Multiplex SYBR Green PCR reaction. The Multiplex SYBR Green Real time PCR assay was standardized for simultaneous detection of MG, MS, IB and ILTV in single test by targeting specific genes for each pathogen *i.e IGSR* gene for MG , *vlhA* gene for MS, *N* gene for IBV and *ICP4* gene for ILTV in single tube(Table 1).Similarly Jarquin *et al [9]* developed a Real Time SYBR Green assay for the simultaneous detection of MG and MS and Raviv *et al. [3]* targeted species specific and intraspecific conserved genes,16S to 23S IGSR genes of MS and MM, 16S ribosomal DNA for MI and highly conserved *mgc2* gene of MG for detection mycoplasma mixed infections.

The optimized conditions, initial denaturation at 94^{o}C for 3.59 mins, followed by 40 cycles of denaturation at 94^{o}C for 20 seconds, annealing at 55^{o}C for 45 seconds and extension at 72^{o}C for 1 min and final extension step was at 72^{o}C for 10 mins. At standardized conditions all the amplifications of all four genes was observed and recorded the ct values of 30.28 (*IGSR* gene), 24.16 (*vlhA* gene), 19.39 (*ICP4* gene) and 11.81 (*N* gene) and also the recorded Tms were 74.0

(*IGSR* gene), 82.5 (*vlhA* gene), 85.5 (*ICP4* gene) and 80.5 (*N* gene) (Fig 2). The ct values inversely correlates with the concentration of DNA in the samples. Higher the ct value lower is the concentration of DNA in the sample and vice versa. A ct value less than or equal to 35 is considered as the cut off in real time PCR similarly Phillips *et al. [10]* also taken same cut off value for detection of Rota viral infections by using real time PCR.

Out of 19 farms screened, 2 were found to be positive for ILT, 10 were for MG,7 were for MS and 5 farms were for MG and MS and all were negative for IB. The recorded ct values for *IGSR* genes ranged between 11.81 to 30.28, product Tms ranged between 74 to 87, for *vlhA* gene ct values ranged between 17.39 to25.81, product Tms ranged between 81 to 87, for *ICP4* gene ct value was 19.08 and 19.23,product Tms was 81 and 80.5 (Fig 2). Similarly Fraga *et al. [11]* detected 67 positives for MG and 64 for MS out of 78 farms from different poultry flocks in Brazil by targeting species specific primers and probes specific for each pathogen by multiplex Real Time PCR and showed 100% specificity and sensitivity in the MG analysis and 94.7% sensitivity and 100% specificity in the MS analysis. Similarly Haque *et al. [12]* detected individual infections and both infections of MG and MS infections by using real-time PCR by targeting *mgc2* and *vlhA* genes.

A SYBRGreen real time PCR test was developed for detection of IBV, ILTV, MG and MS mixed infections similarly Laamiri *et al. [13]* also used multiplex PCR for detection of AIV, IBV, NDV and ILTV. So this study is useful for rapid and specific detection of co-infections in field samples based on that, can implement the control methods. Respiratory disease caused by infection with multiple pathogens was the most common cause of death or illness, the presence of both bacterial and viral pathogens makes identification of the inciting cause (primary agent) often impossible. Infectious agents such as MG, MS, IBV and ILTV can each cause respiratory disease on their own and also can predispose birds to other respiratory infections. Rapid, accurate and simultaneous detection of the respiratory pathogens and adoption of appropriate control strategies in the poultry farms could greatly help in combating the economic losses in the poultry industry.

In this study the SYBR green Real Time PCR assay useful in terms of specificity and capacity of detection of more than one target in a sample and no cross reactions was observed similarly Sprygin *et al. [14]* used real time PCR test for detection of more than one pathogen in samples especially mycoplasma mixed infections. In this study, 100% specificity was observed by using other than target DNA (Pox DNA) and no amplifications in NTCs.

CONCLUSION

In the present study an attempt was made to standardise the multiplex real time PCR using SYBR green chemistry for the simultaneous detection of Mycoplasma(MG,MS), IB and ILT. However future studies should aim at development of Taqman probe based multiplex Real time PCR technique for accurate diagnosis of these respiratory pathogens in the clinical samples.

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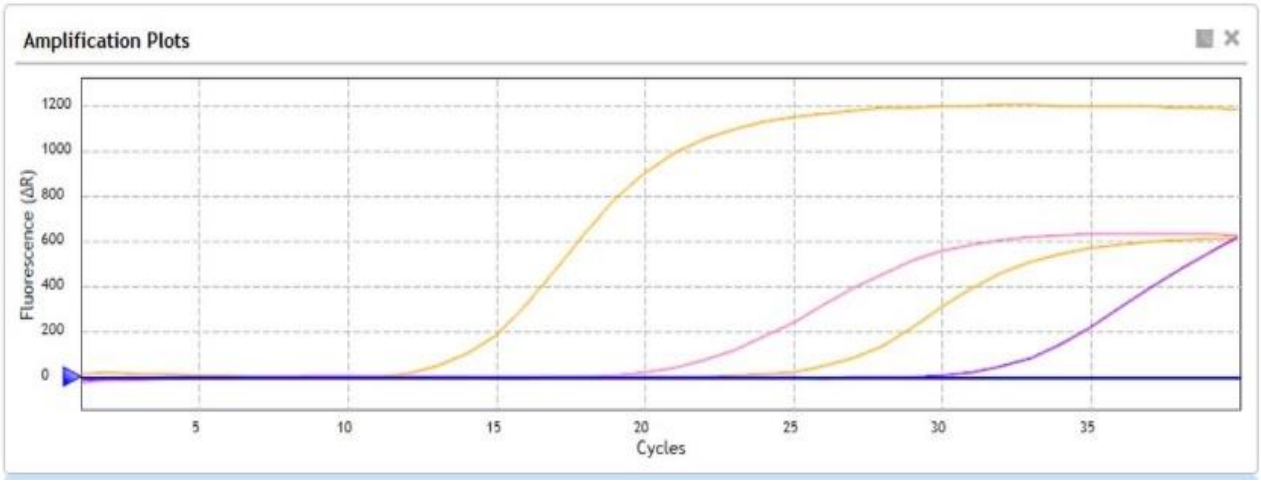

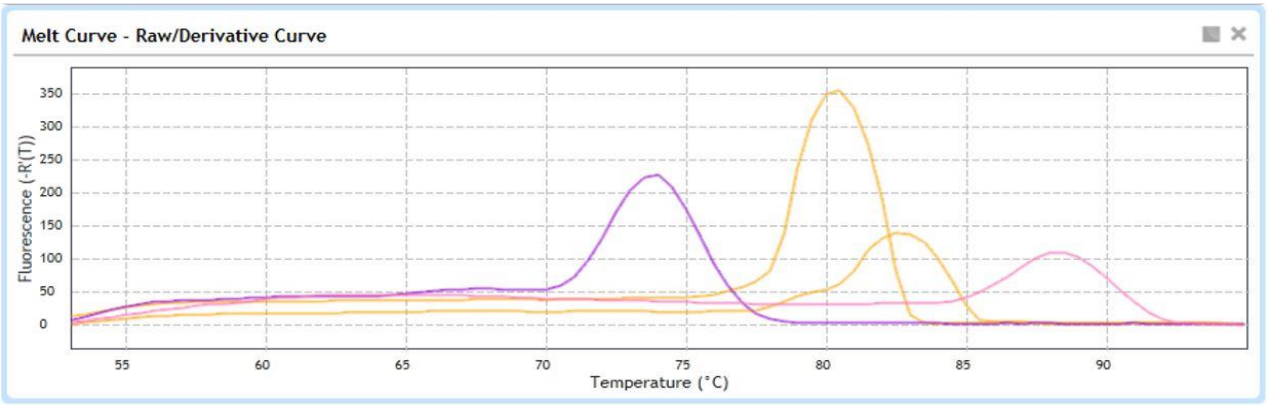

Fig-2: Standardization of SYBR Green Real Time Multiplex PCR for simultaneous detection of MG, MS, ILTV and IBV (Amplifation plots and meltcurves for four pathogens)

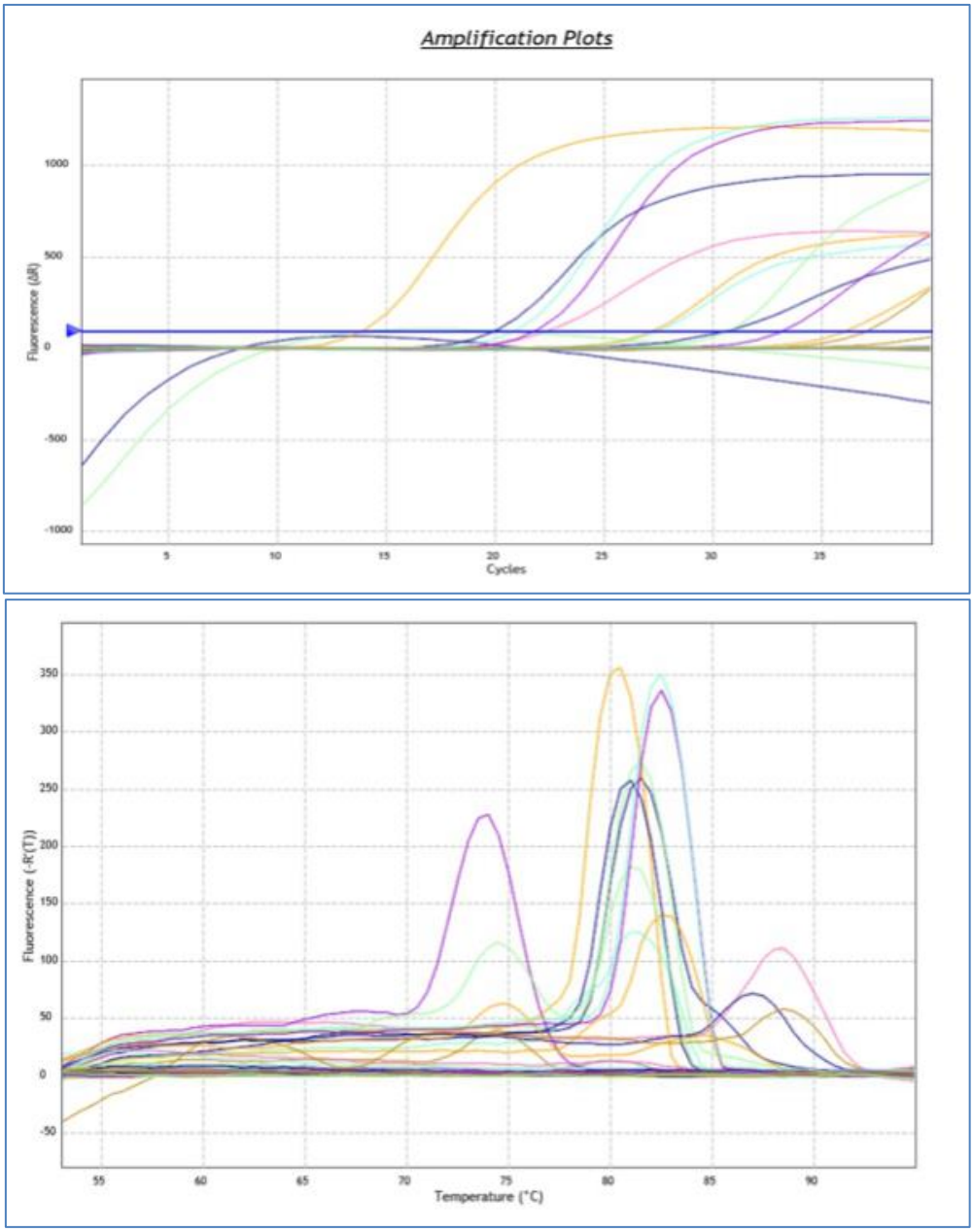

Fig-3: Amplification plots and melts curves of *IGSR, ICP4* and *vlhA* genes in SYBR Green Real Time Multiplex PCR

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