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Detection of infectious bronchitis virus in chicken in Kerala by real time Taqman RT-PCR assay

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ABSTRACT

Chickens with signs of respiratory infection were screened for the presence of infectious bronchitis virus (IBV). Tissue samples were collected from dead and ailing chickens and were propagated intra-allantoically in embryonated chicken eggs and the allantoic fluid was harvested. The tissue samples and harvested allantoic fluid were probed with reverse transcriptase polymerase chain reaction (RT-PCR) with primers targeting 5'UTR of IBV. A total of 95 samples were tested, by RT-PCR. A Taqman probe labelled real time PCR assay targeting the 5' UTR and Nucleocapsid (N) gene of IBV was standardised for the detection of IBV in all the 95 tissue and allantoic fluid samples to assess its efficiency. The real time PCR could detect IBV in all the 95 allantoic fluid and tissue samples including the samples, which were negative in preliminary detection. Hence, in the present study, the real time PCR assay was found to have equal efficacy on clinical samples and allantoic fluid and with a sensitivity of 100% and specificity of 90.90% in comparison with the conventional RT-PCR assay.

Keywords: Embryonated chicken egg, Infectious bronchitis virus, N gene, Real time PCR, 5'UTR

The poultry industry in India faces constant challenges from infectious diseases (Ahmed *et al.* 2022). The rapid and steady emergence of strains of viral agents with increased virulence possesses a serious threat to the poultry industry (Dhama *et al*. 2014). The losses encountered include decreased feed conversion, reduced production, mortality and loss of market value.

Infectious bronchitis virus (IBV) is a positive-sense, single stranded, RNA virus which belongs to the family Coronaviridae (Cavanagh 2005) and has an envelope and a helical nucleocapsid. Although mortality is not the primary concern in poultry affected with infectious bronchitis (IB), the disease is listed by WOAH, as it leads to severe decline in egg production, meat and quality of eggs. As new strains of IBV emerge, a rapid and sensitive detection method is crucial and need of the hour to combat the losses incurred to the farmers (Liu *et al*. 2019).

Despite the implementation of rigorous vaccination programmes, the control of IB still remains a concern (Jackwood *et al*. 2005). Inactivated vaccines are safe, but are expensive and less effective when compared to live attenuated vaccines (Zhou *et al*. 2003). On the contrary, live attenuated vaccines possess the threat of reversion to virulence (Jia *et al*. 1995) and also drive IBV evolution (Legnardi *et al*. 2019). Thus, IB continues to be a huge challenge to the poultry industry as new variants of the virus are being isolated from the vaccinated flocks as well (El Houadfi *et al*. 1986).

Reports of high prevalence of IB exist in Kerala. The high prevalence along with the fact that vaccination is not usually practiced in the state makes the disease of prime importance. These warrant a rapid and sensitive detection of IB to develop efficient vaccination strategies in order to combat the disease. Although virus isolation using embryonated chicken eggs (ECE) is the gold standard test diagnostic test for IB, the process is time consuming and laborious. In the current study, we have standardised a real time Taqman RT-PCR assay targeting the nucleocapsid and untranslated region (UTR) of IBV with a view to aid in more sensitive detection of the virus from clinical samples. A total of 95 tissue samples and allantoic fluid were screened for IBV, of which, 45 were found positive.

MATERIALS AND METHODS

Collection of tissue samples: Tissue samples for the isolation of IBV were collected from both dead and ailing chickens that were brought with a history of respiratory ailments to the Department of Veterinary Pathology and Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy. The ailing chickens were sacrificed in a humane manner. Tissue samples (lung, trachea, oviduct, kidney, liver and spleen) were collected in Sigma-Aldrich RNA later (R0901) and PBS. The pooled tissue samples were thoroughly triturated

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with a mortar and pestle under sterile conditions and filtered with 0.22 µm sterile disposable syringe filter. Antibiotic-antimycotic solution was subsequently added to the tissue suspension and stored at -20ºC.

Isolation in embryonated chicken eggs: Nine to eleven day-old ECE were used for the study, to which tissue suspension was inoculated allantoically. The eggs were candled twice daily to ensure the viability of the embryos. Three blind passages were done before discarding the samples as negative. After 48 h post inoculation, the eggs were chilled at 4ºC overnight and the allantoic fluid was harvested and stored in sterile RNase free vials.

Ribonucleic acid extraction and complementary DNA synthesis: Ribonucleic acid (RNA) was extracted from the pooled tissues and allantoic fluid with Sigma–Aldrich TRI Reagent LS (T3934) reagent. Complementary DNA (cDNA) was synthesized from the extracted RNA with Bio-Rad iScript cDNA synthesis kit (1708891). The synthesised cDNA was stored at -70ºC.

Polymerase chain reaction: The primers specific for the highly conserved 5'UTR gene was employed for the detection of IBV. Complementary DNA synthesised from Nobilis intervet vaccine (H120 strain) was used as the positive control. The sequences and other parameters of the primers are depicted in Table 1. Primers were custom synthesised commercially (Sigma Aldrich) and obtained in lyophilised form.

Table 1. Sequences of primers used for the detection of 5' UTR

Sequences of primers	Product size in bp
F 5' GCTTTTGAGCCTAGCGTT 3' R 5' GCCATGTTGTCACTGTCTATT 3'	143

The working solution of the primer was used at a concentration of 10 pM/µL. The PCR conditions for the amplification of 5'UTR of IBV are mentioned in Table 2. The amplicons were finally visualised in 1% agarose gel. The representative amplicons were sequenced for further confirmation.

Table 2. Polymerase chain reaction conditions optimised for the amplification of 5'UTR (143 bp)

Steps	Temperature (°C)	Time	No. of cycles
Initial Denaturation	95	10 min.	
Denaturation	95	45 sec.	30 cycles
Annealing	53	1 min.	
Extension	72	1 min.	
Final extension	72	10 min.	

Real time Polymerase Chain Reaction: Primers targeting the highly conserved 5'UTR and N gene were used for real time PCR, which were selected as per Callison *et al*. (2006) and Meir *et al*. (2010) respectively and is shown in Table 3. The sensitivity and specificity of real time PCR in comparison with conventional RT-PCR for the detection of infectious bronchitis virus were calculated.

Table 3. Sequences of primers and probes used for real time PCR assay targeting 5' UTR and N gene

Primers	Sequence (5'-3')
IBUTRF	GCTTTTGAGCCTAGCGTT
5'UTR gene IBUTRR	GCCATGTTGTCACTGTCTATTG
IBNF IBNR N gene	GCCGATCACTTAAGGGCC CAATGTTTTCTCGCATCGCA
IB UTR probe	FAM-CACCACCAGAACCTGTCACCTC-BHQ1
IBN probe	FAM-TTGGAAGTAGAGTGACGCCCAAACTTCA-BHQ1

RESULTS AND DISCUSSION

Infectious bronchitis continues to be a never-ending threat in several parts of the world including India. Given the poor cross protection between the different serotypes of IBV, it is arduous and challenging to control the disease (Ramakrishnan and Kappala 2019, Houta *et al*. 2021). As far as Kerala is concerned, vaccination against IB is generally not practiced and numerous unpublished reports are available regarding the prevalence of the disease. A quick as well as accurate detection of the disease is absolutely necessary to implement appropriate control measures. In our study, a Taqman probe labelled real time PCR assay has been developed to ensure the rapid as well as sensitive detection of the disease.

Allantoic route is preferred for the inoculation of IBV as it is an epitheliotropic virus, which extensively replicates in the chorio-allantoic membrane epithelium, resulting in high titres of the virus (Jordan and Nassar 1973, Guy 2020). Moreover, allantoic route of inoculation is easily done when compared with other routes. Even though inoculation in ECE has these advantages; the ensuing diagnosis takes time until the occurrence of the characteristic lesion of curling and dwarfing in inoculated ECE. A combination of ECE

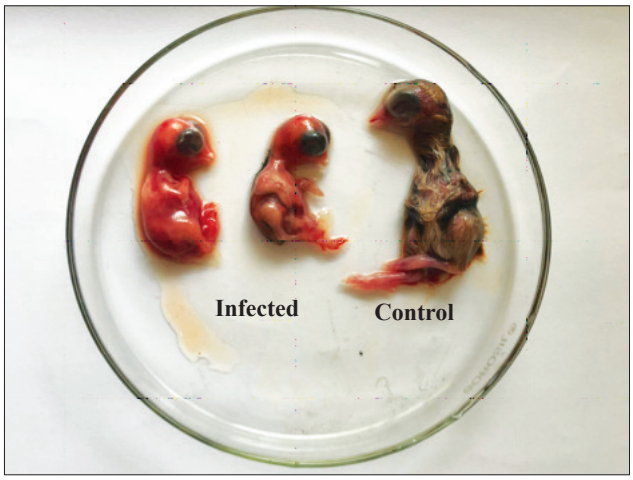

Fig. 1. Infectious bronchitis virus inoculated ECE with characteristic lesions on sixth passage. Control: 15 day-old healthy embryo; Infected: Embryo infected with IBV showing curling and dwarfing.

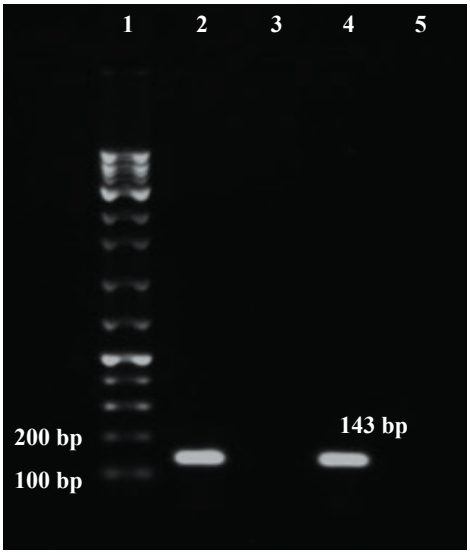

Fig. 2. Agarose gel electrophoresis of PCR amplified products of 5'UTR of IBV (143 bp). Lane 1: 100 bp ladder, Lane 2: positive control, Lane 3: negative control, Lane 4: positive sample, Lane 5: negative sample.

inoculation and antigen detection method such as RT-PCR can facilitate a rapid detection (Raja *et al.* 2020). Hence, in the present study, the allantoic fluid after first passage was subjected to RT-PCR for detection of the highly conserved 5'UTR gene of IBV with detection primers. These samples were discarded as negative only after the third passage.

On ECE, the infected embryos exhibited the characteristic curling and dwarfing from fifth passage onwards, whereas, the control ones appeared normal (Fig. 1). Additionally, an increased allantoic fluid volume and subcutaneous hemorrhages in embryos could be appreciated in the infected embryos. ECE inoculation was preferred over cell and organ culture because the former has been reported to have more sensitivity than the latter (Lukert 1965).

Complementary DNA from both tissue samples and the harvested allantoic fluid were at first amplified with PCR targeting the 5'UTR of IBV. On agarose gel electrophoresis, a 143 bp product was obtained (Fig. 2). Since, conserved region in the 5' UTR is present in all the strains of IBV; the same was used for preliminary detection purpose (Andreasen *et al*. 1991). Darbyshire *et al*. (1975) stated that maximum virus concentration in allantoic fluid could be achieved one to two days post inoculation (PI), similar results were observed in the present study with allantoic fluid harvested two days PI. Out of 95 samples tested, 40 tissue samples and 45 allantoic fluid samples were positive in preliminary detection with 5'UTR. The representative amplicons were confirmed by sequencing. Five tissue samples were negative in preliminary detection. However, their corresponding allantoic fluids were positive.

Following preliminary detection, the tissue and allantoic fluid samples were subjected to Taqman labelled real time PCR's (qRT-PCR) targeting 5'UTR and N gene of the IBV genome. Real time PCR assay is better when compared to the conventional RT-PCR assay as large number of samples can be processed in a relatively short run time (Ramakrishnan and Kappala 2019). Furthermore, with Taqman probe, the assay produces results with relatively high sensitivity and eliminates the need for melt curve analysis. However, when compared to virus isolation, the real time PCR assay may be less sensitive when cDNA from clinical samples are directly used (Kwon *et al*. 1993).

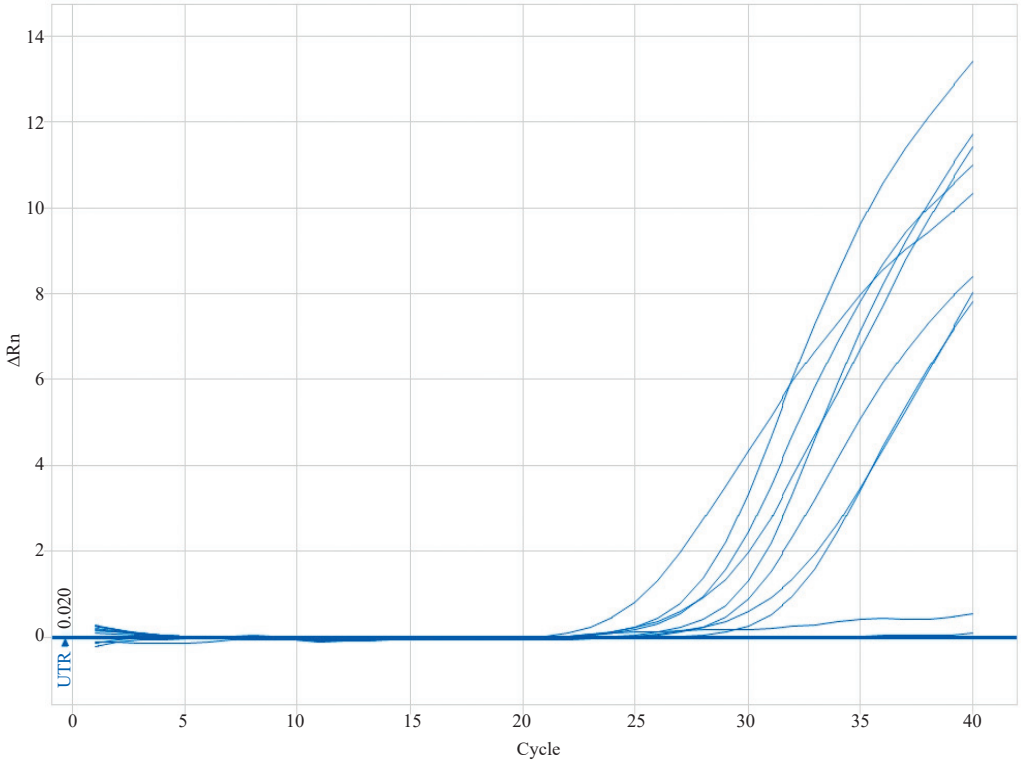

Fig. 3. Amplification plot of real time PCR assay targeting 5'UTR of IBV.

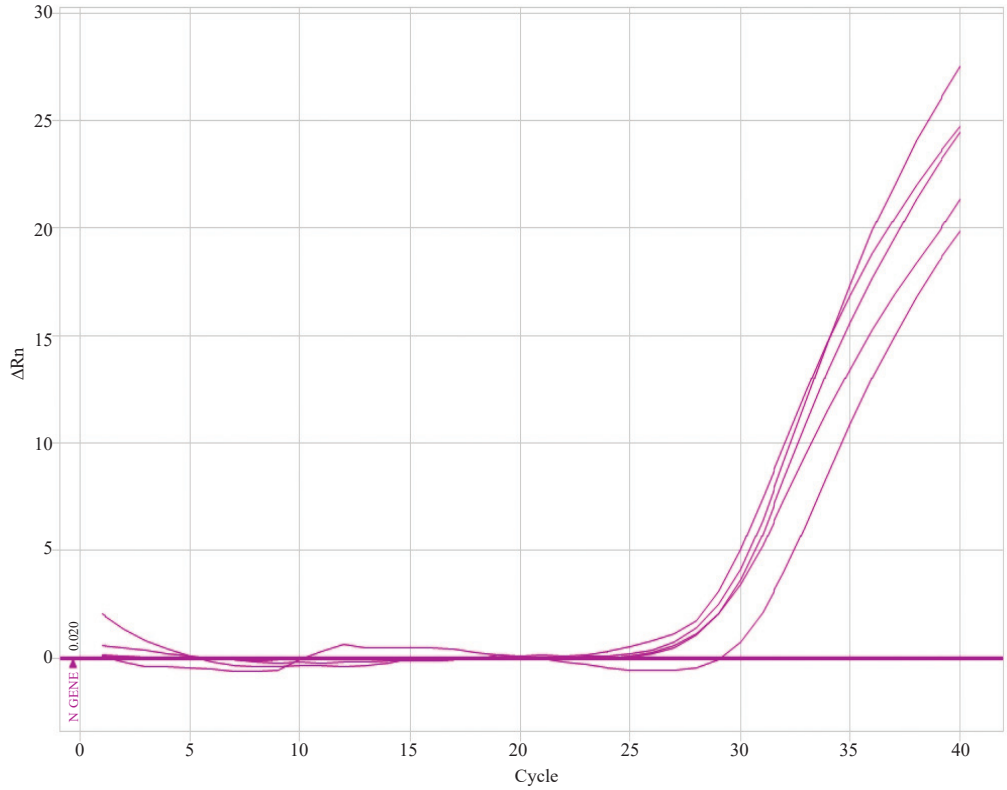

Fig. 4. Amplification plot of real time PCR assay targeting N gene of IBV.

In the present study, part of the tissue suspensions from clinical samples were propagated in ECE and the RNA extracted from the harvested allantoic fluid were also subjected to qRT-PCR assay along with the cDNA synthesized from the clinical samples, to investigate if there is any variation based on the samples used. However, no such dissimilarity could be observed. Out of 95 samples, 45 samples each of tissue and allantoic fluid were found positive.

The primers targeting 5'UTR and N gene of IBV were selected as per Callison *et al*. 2006, Meir *et al*. 2010, respectively. In order to amplify a conserved region of the IBV, Callison *et al*. (2006) designed primers and Taqman labelled probe targeting a fragment of the highly conserved 5'UTR, which made amplification of nearly all the strains of IBV possible. The assay developed by Callison *et al*. (2006) was highly sensitive, but it was not specific since it detected Turkey coronaviruses (TCoV) too. To overcome this limitation, Meir *et al*. (2010) amplified the conserved N gene sequence of IBV, considering the amplification of N gene to produce more specific results. The abundance of N gene in infected cells may be a pivotal factor for a relatively high sensitivity for real time PCR assays targeting the N gene (Spencer and Hiscox 2006).

In our study, both 5'UTR and N genes as per Callison *et al*. 2006, Meir *et al*. 2010 were targeted to find if there were any differences/variations in the result. However, the samples that were positive for 5'UTR were also positive for the N gene, i.e. all the 45 samples including the five tissue samples that were negative in the preliminary RT-PCR were positive for both 5'UTR and N gene real time PCR. The sensitivity and specificity of real time PCR in comparison with conventional RT-PCR assay were 100% and 90.09% respectively. The amplification plots generated is depicted in Fig. 3 and fig. 4. The present result is in agreement with Meir *et al*. (2010) who reported that real time RT-PCR is more sensitive than the conventional RT-PCR.

Real time PCR, which allows simultaneous quantification and detection of DNA, paves way for quick detection of the disease in a particular region. Although virus isolation is the gold standard test for the detection of IB, it is both time-consuming and laborious. It could be inferred from the present study that the real time RT-PCR has equal efficacy on clinical samples and allantoic fluid and was more efficient than conventional RT-PCR assays, which facilitates both sensitive and rapid detection of the disease, thereby permitting suitable control measures to be executed in a timely manner.

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