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Molecular characterization of the hypervariable regions of S1 gene of infectious bronchitis virus isolates

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ABSTRACT

A preliminary study was conducted for the detection of the infectious bronchitis virus circulating among the poultry flocks of Kerala employing a reverse transcriptase polymerase chain reaction, where, a high prevalence of the virus was reported. The present study is the first comprehensive study pertaining to the molecular characterization of hypervariable regions of the S1 gene of IBV in Kerala. The positive samples were sequenced with primers targeting the three hypervariable regions of the S1 subunit of spike gene. It was observed that the isolates of Kerala shared more than 95% similarity with isolates from different parts of India. On phylogenetic analysis of hypervariable regions 1 and 2, all the Kerala isolates clustered with isolates from other parts of the country. Phylogenetic analysis of the hypervariable region 3 revealed that the Kerala isolates branched separately from the isolates belonging to different parts of India. The study advocated the need of an area specific vaccine in Kerala, employing the potential candidate vaccine strain isolated in the study.

Keywords: Hypervariable region, Infectious bronchitis virus, Phylogenetic analysis, Sequencing

Avian infectious bronchitis virus (IBV) belongs to the genus *Gammacoronavirus* of subfamily Orthocoronavirinae and family Coronaviridae (Siddell *et al*. 2019). The genome of IBV encodes four structural proteins namely nucleocapsid (N), membrane (M) glycoprotein, spike (S) glycoprotein and envelope (E) protein (Cavanagh 2005). Among them, S protein shows remarkable diversity in its nucleotide sequence (Haqshenas *et al*. 2005). Chicken of all ages are susceptible to IBV infection, however the severity was found to be greater in chicks compared to adults (Bande *et al*. 2016).

Despite implementing rigorous vaccination programmes, control of infectious bronchitis (IB) still remains a problem (Jackwood *et al*. 2005, Icochea *et al*. 2023). This is attributed to the fact that there is continuous emergence of genetic and antigenic variants of IBV, thereby deterring protection against the newly emerged strains (Martin *et al*. 2007, Al-Mubarak and Al-Kubati 2020). Though inactivated vaccines are safe, they are expensive and less effective as compared to live attenuated vaccines (Zhou *et al*. 2003), however there is chance of reversion of virulence in case of live attenuated vaccines (Jia *et al*. 1995). Thus, IB continues to be a huge threat to the poultry industry as new variants of the virus are still being isolated and that to from the vaccinated flocks (Vermeulen *et al*. 2023). According to Gallardo *et al*. (2021), IBV variants may not be entirely genetically distinct from the usual strains affecting a particular geographic region, but are phenotypically distinct and hence immune evading to the immunity produced by typical serotype specific vaccines. These variations can be more than or equal to 5% at the genomic level in the S1 gene's hypervariable region. Majority of the times, these mutant strains are typically limited to specific geographic areas. Hence, frequent monitoring is mandatory for tracking the emergence of new variants of IBV, to establish more efficient and effective vaccination strategies. Previous study has confirmed a high prevalence of IB (Rajalakshmi *et al*. 2023) and vaccination against the disease is usually not practiced in the state.

According to Raja *et al*. 2020, sequencing of HVRs of S1 gene has a strong correlation with viral neutralisation test and can be used to genotype IBV isolates. Shyma *et al*. (2018) sequenced the HVR 3 of S1 subunit of spike gene of the field isolates from Thrissur district of Kerala and found that the sequences shared 98% homology with H120 vaccine strain, the current IBV vaccine strain in India.

Thus, the present study aims to characterise the field isolates of Kerala by sequencing all the three HVRs of S1 subunit of spike gene and comparing the sequences with current vaccine strain and also with the strains isolated from different parts of the country. This would be a pilot study to pave the way for identifying the suitable candidate vaccine strain pertaining to Kerala, which in turn could aid in formulating the effective strategies for controlling the

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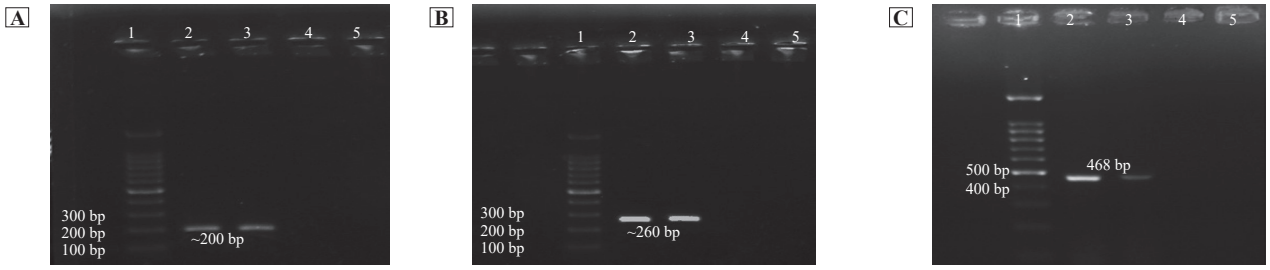

Fig. 1. Agarose gel electrophoresis of PCR amplified products: A. Hypervariable region 1 of S1 subunit of spike gene of *Infectious bronchitis virus* (200 bp); B. Hypervariable region 2 of S1 subunit of spike gene of Infectious bronchitis virus (260 bp); C. hypervariable region 3 of S1 subunit of spike gene of Infectious bronchitis virus (468 bp) [Lane 1: 100 bp ladder, Lane 2: positive control, Lane 3: positive sample, Lane 4: negative control, Lane 5: negative sample].

disease in the state.

MATERIALS AND METHODS

Tissue samples (trachea, lung, liver, spleen, oviduct and kidney) taken from 95 suspected cases of IB, confirmed by RT-PCR (TRIzol method) targeting the 5' UTR (Rajalakshmi *et al*. 2023), were subjected to isolation in 9-11 day old embryonated chicken eggs by allantoic route of inoculation. The harvested allantoic fluid was used for genotypic characterization.

The allantoic fluid was subjected to RNA extraction (TRIzol method) and cDNA synthesis (Bio-rad iScript™

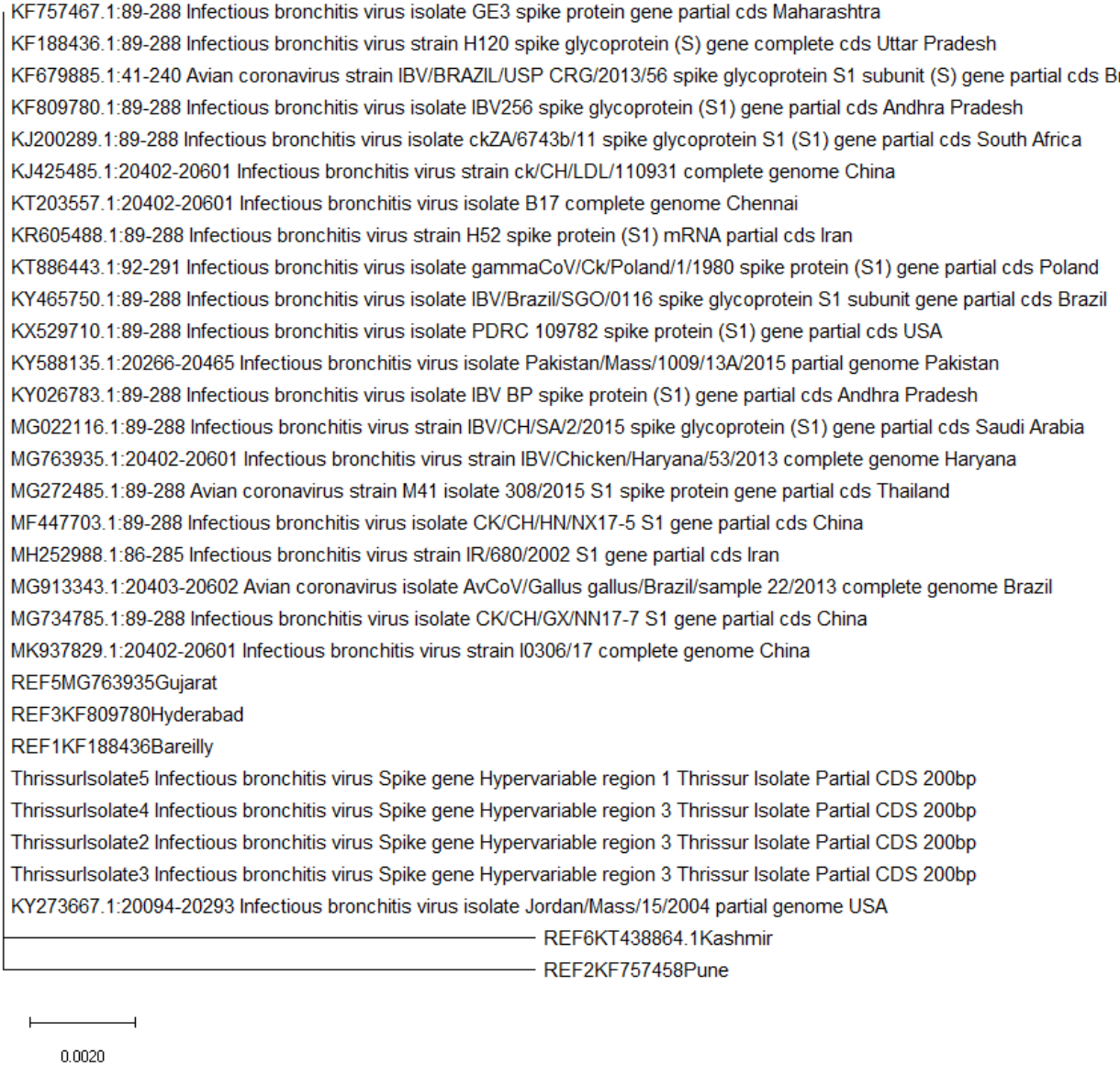

Fig. 2. Phylogenetic analysis of hypervariable region 1 of S1 subunit of spike gene of Infectious bronchitis virus.

cDNA kit (1708891) followed by RT-PCR targeting the 5'UTR and positive cDNA were subjected to RT-PCR targeting the hypervariable regions (HVR) of the S1 subunit of spike gene. The primers targeting the HVR of S1 subunit were designed in-house (Primer 3 software). Complementary DNA from Nobilis intervet vaccine (H120) was used as the positive control. Negative control was made up with nuclease free water.

For HVR 1 and 2, initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58.9°C for 45 s and extension at 72°C for 45 s and final extension for 72°C for 5 min. For HVR3, initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 1 min and extension at 72°C for 45 s and final extension for 72°C for 5 min.

The PCR products were purified by elution in 1% agarose gel using QIAquick Gel Extraction Kit (28704) and subjected to sequencing. The sequences of hypervariable regions 1, 2 and 3 of S1 subunit were amplified using in-house designed primers, IBS1F and S1R, IBS2F and S2R, IBS3F and S3R, which corresponds to amplicon size of 200 bp, 260 bp and 468 bp, respectively. The amplicons were further sequenced using forward and reverse primers by automated sequencer using Sanger's dideoxy chain termination method, which was outsourced. The obtained sequences were aligned with other sequences of IBV available in GenBank using BLASTn. The nucleotide sequences and predicted amino acid sequences were analysed using appropriate bioinformatics tools. Phylogenetic analysis of nucleotide sequences was carried out using "Mega7" programme of Lasergene software using neighbour joining method.

RESULTS AND DISCUSSION

The existence of multiple serotypes of IBV makes it an arduous challenge for the prevention and control of the disease. For preliminary screening of samples, 5'UTR was chosen to be amplified, considering its highly conserved nature (Andreasen Jr *et al*. 1991).

Between the various IBV serotypes, variations exist in the form of very few amino acid differences, which is predominantly limited to the S1 subunit of the spike gene of IBV (Cavanagh 2005). Furthermore, altered tissue tropism and antigenicity in various IBV strains have been attributed to variations in the S1 sequence (Cavanagh and

Fig. 3. Phylogenetic analysis of hypervariable region 2 of S1 subunit of spike gene of Infectious bronchitis virus.

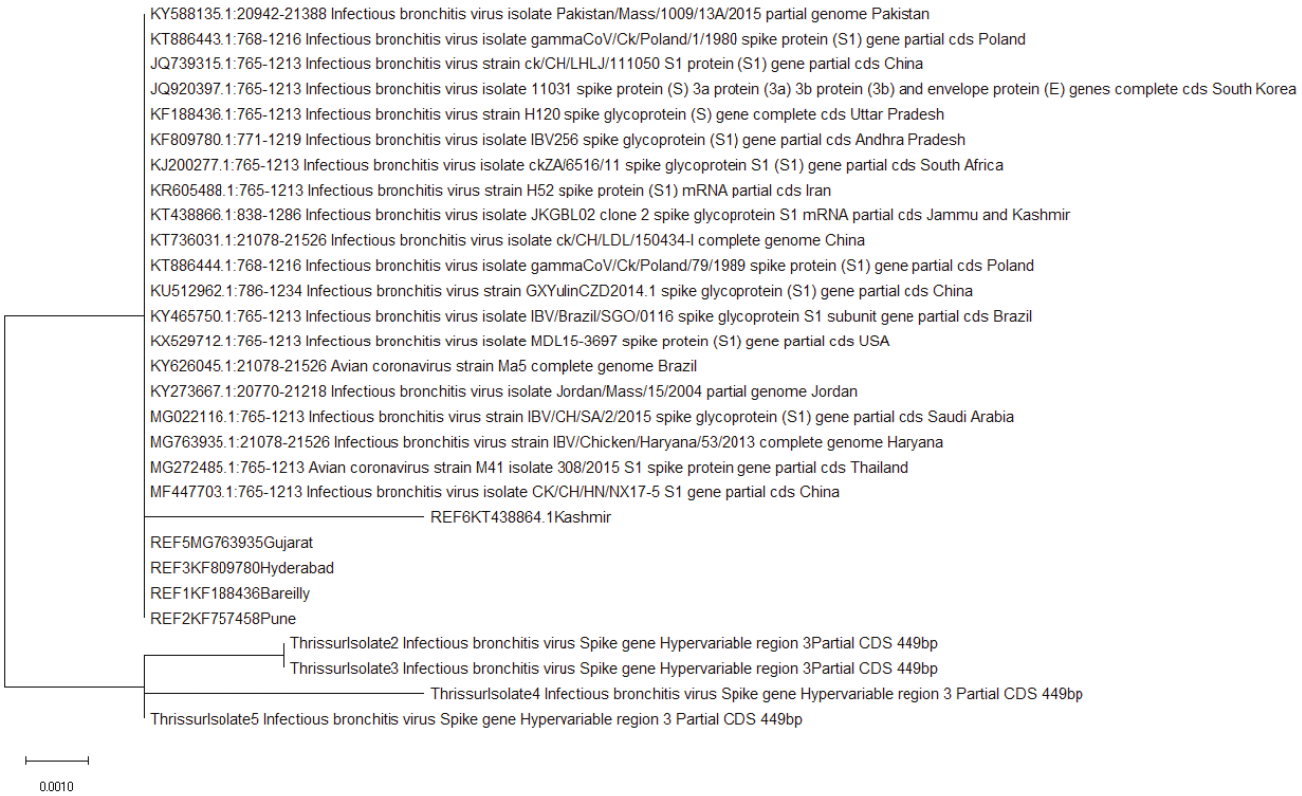

Fig. 4. Phylogenetic analysis of hypervariable region 3 of S1 subunit of spike gene of *Infectious bronchitis virus.*

Naqi 1997). More specifically, serotypic determinants have been identified in the first 395 amino acid region of the S1 subunit, which includes the three major HVRs. The HVR 1 is located between the amino acid residues 56 to 69, HVR 2 between 117 to 131 and HVR 3 between 274 to 387 (Montassier, 2010). Taking these into account, three in-house primers were designed targeting the three HVRs of S1 subunit (Fig. 1. A-C).

Strain identification by sequencing the HVRs of the S1 subunit of spike gene of IBV had been reported to be successful in studies (Lee *et al*. 2003). Previous studies indicated that the genotyping of IBV based on HVR 1 of the S1 subunit could represent the grouping method of the whole S1 subunit (Wang *et al*. 2000). Several studies had been conducted by amplifying both the HVR1 and HVR2 of S1 subunit for identifying the IBV strains prevalent in a given geographical region (Pohuang *et al*. 2009). Also, it is important to note that the HVR 3 occupies a considerably large region of the S1 subunit of spike gene of IBV, compared to the HVR 1 and HVR 2 and thus, in some studies the HVR3 was also targeted (Fellahi *et al*. 2016). Hence, all the three HVRs are equally significant in determining the strain variations of IBV and in the present study, all the three HVRs of the S1 subunit were targeted and sequenced. EMBOSS merger was used to merge the sequence reads to obtain coding sequence (cds) of the hypervariable regions 1, 2 and 3 of S1 subunit of spike gene and then analyzed. The sequences were queried in the nucleotide database and blasted in order to confirm that the BLASTn hits were of HVRs of S1 subunit.

The isolates obtained in the present study did not reveal any variation in the nucleotide sequences with respect to each other. Since H120 strain of IBV is the current vaccine strain in India, it was used as a reference strain in the study. The partial cds of S1 subunit of spike gene obtained from positive samples showed more than 95% homology on comparison with the complete cds of S1 subunit of H120 vaccine strain, as well as the sequences of Indian isolates deposited in GenBank. On phylogenetic analyses of the hypervariable regions 1 and 2 of S1 gene, the Thrissur isolates were clustered with each other and also with other Indian IBV isolates from Pune, Andhra Pradesh, Uttar Pradesh, Bareilly, Gujarat and Kashmir. Whereas, on phylogenetic analysis of hypervariable region 3, all the four representative isolates of the present study were branched separately among each other and also with the isolates from Andhra Pradesh, Pune, Bareilly, Gujarat, Jabalpur and Haryana. The size of the hypervariable region 3 was 342 bp, whereas the sizes of the hypervariable regions 1 and 2 were 42 bp and 45 bp, respectively. More variations were noted in the hypervariable region 3 compared with the other two hypervariable regions. Similar results were obtained by Jakhesara *et al*. (2018) when they made a phylogenetic analysis of the IBV isolate from Haryana. Their results showed that the Haryana isolate clustered with the other Indian IBV isolates. In contrast, a study in Tamil Nadu in small-holder chicken flocks revealed the presence of QX-like strain and on phylogenetic analysis, this isolate clustered with previously reported QX strains (Ganapathy *et al*. 2020). Phylogenetic trees are presented in Figs. 2-4.

It can be inferred from this study that the isolates of Kerala shared more than 95% similarity with isolates from different parts of India and the phylogenetic analysis of the hypervariable region 3 revealed that the isolates branched separately from others belonging to different parts of India. With respect to Kerala, the study is the pilot one of its kind and stressed the importance of an area specific vaccine employing the local isolates. Further studies are going on targeting the full S1 subunit of spike gene employing a greater number of samples, which will be helpful in designing an effective candidate vaccine against IBV specific to the region.

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