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RAPID DETECTION OF AVIAN INFECTIOUS BRONCHITIS VIRUS BY USING COMMERCIAL INSULATED ISOTHERMAL POLYMERASE CHAIN REACTION (iiPCR) AND ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) IN SRI LANKA

Wanasinghe W. M. L. A.[1] and Fouzi M. N. M.[2]*

[1]Department of Animal Science, Faculty of Animal Science and Export Agriculture, Uva Wellassa University, Sri Lanka.
[2]Department of Farm Animal Production and Health, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka.

**Corresponding Author: mnmf@vet.pdn.ac.lk (https://orcid.org/0000-0002-6993-7163)*
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ABSTRACT

Infectious bronchitis (IB) is considered one of the most important poultry diseases worldwide. Many cases have been presented in Sri Lanka with similar clinical symptoms to those of IB in poultry belt areas. Therefore, the present study aimed to develop the IBV rapid detection methods using commercial insulated isothermal Polymerase Chain Reaction (iiPCR) and enzyme-linked immunosorbent assay (ELISA) in broilers, commercial layers, and backyard poultry flocks in Sri Lanka. Tracheal swabs and blood samples (n=413) were collected from vaccinated (n=107) and unvaccinated (n=306) birds exhibiting clinical signs attributable to IBV. Viral RNA was extracted from tracheal swabs and IBV was detected by iiPCR. Serum was separated from blood samples, and ELISA detected antibodies against IBV. It was reported that 46% (190/413) of birds were serologically positive for IBV by ELISA and 41.88% (173/413) of birds were positive for IBV by iiPCR. The total prevalence of IBV was 20.56% (22/107) of vaccinated and 49.34% (151/306) of unvaccinated poultry flocks by iiPCR. Furthermore, ELISA detected 30.84% (33/107) of vaccinated and 51.30% (157/306) of unvaccinated poultry flocks. Thus, IBV was more prevalent in unvaccinated birds comprised of backyard chickens and broilers in sampled areas. Moreover, the Batticaloa district had the highest prevalence of IBV (>75%). Thus, iiPCR assay could be used for rapid detection of IBV infection in clinical requirements

Keywords: Antibody, ELISA, Infectious bronchitis, iiPCR, Sri Lanka

INTRODUCTION

Infectious bronchitis virus (IBV) is a coronavirus that causes significant economic loss in the poultry industry across the world (Ennaji *et al*., 2020). IBV affects poultry of all ages, resulting in acute respiratory signs such as sneezing, coughing, gasping, nasal discharge, tracheal rales increased morbidity as well as a decrease in egg yield and quality (Cavanagh, 2007; De Wit, 2000). However, routine vaccination against IBV is being done on commercial poultry farms in many countries including Sri Lanka.

The poultry industry is recognized as a rapidly expanding livestock sub sector in Sri Lanka (DAPH, 2022). In many districts of Sri Lanka, poultries especially chickens are reared under the intensive system for egg production (commercial layer), meat production (commercial broiler) and backyard chicken (village chicken) have been reared in Sri Lanka since ancient times (Silva *et al*., 2016; Mokanapriya *et al.*, 2021). Most of the intensive poultry farms are located in Kurunegala and Gampaha areas, while other districts have fewer intensive farms. Districts in the Northern and Eastern provinces of Sri Lanka are known for backyard chicken farming and new intensive farms have also grown up. Jaffna (Northern Province), Trincomalee and Batticaloa (Eastern province) are important districts where poultry farming is now becoming popular with small-scale intensive farming with traditional backyard operations using backyard chickens (JICA, 2012).

Intensive commercial poultry farms are mainly located in two districts of Sri Lanka, namely Kurunegala and Gampaha. However, Jaffna district poultry operations are mainly in small-scale farms, including backyard chicken flocks. Vaccination against IBV is mainly done in intensive farming in Kurunegala and Gampaha. However, despite the continued IBV vaccination on commercial farms, IBV outbreaks are observed in many districts mainly Kurunegala and Gampaha based on the gross lesions as there is no established laboratory close by to confirm IBV by molecular methods. However, IBV has not been extensively studied in Sri Lanka previously. There are limited studies on IB cases in Sri Lanka and Ball *et al*. (2016) reported that over 50% of the IBV positive samples were related to divergent from vaccinal strains in Sri Lanka with his

limited sampling. Furthermore, currently there is no established laboratory facility to confirm IB cases in Sri Lanka. Thus, it is important to know the IBV status in Sri Lanka to take adequate control measures if the disease is present in Sri Lanka.

Virus isolation in embryonated eggs is routinely used to diagnose IB, followed by immunological identification of the isolates (De Wit, 2000) and by molecular identification by PCR (Bijlenga *et al*., 2004). However, the serological tests and the RT-PCR, which are commonly used to detect IBV (Ball *et al*., 2016; De Wit, 2000), are complex and time-consuming laboratory procedures. Moreover, reverse transcription (RT)-PCR, which is a commonly used and prevailing tool to detect IBV (Ball *et al*., 2016; De Wit, 2000), requires specialized procedures such as RNA stabilization and cDNA synthesis, which are time-consuming laboratory procedures. However, PCR techniques have been shown to be very efficient in the detection of IBV (Bijlenga *et al*., 2004). The newly developed insulated isothermal PCR assay (iiPCR) is rapid and sensitive technique to detect many animal diseases (Chua *et al*., 2016; Go *et al*., 2016). This recently developed and widely accessible iiPCR method is based on the fluorescent probe hydrolysis and the detection can be completed in an hour with analytical specificity and sensitivity of up to 10 copies per reaction (Du et al., 2020; GeneReach Biotechnology Corporation (Taichung City, Taiwan). Currently, the iiPCR assays are being developed and used to diagnose several poultry diseases, such as *Mycoplasma* and *Salmonella* (Chua *et al*., 2016; Kubota *et al*., 2013). However, IBV has not been extensively studied using iiPCR in Sri Lanka, previously. Therefore, the present study was aimed at finding the prevalence of IBV in broilers, commercial layers and village/backyard poultry, both in non-vaccinated and vaccinated flocks, using iiPCR technology and ELISA.

METHODOLOGY

Study area and sample collection

Ethical clearance was obtained from the Animal Ethical Review Committee of the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya (Ethical clearance certificate No. VERC-21-03). Suspected cases of IB were selected based on clinical signs such as loss of appetite, coughing, sneezing, nasal discharges, diarrhea, pasted vent, low egg quality and production, postmortem lesions such as haemorrhages in the nephritis, kidney, hemorrhagic tracheitis, caseous or catarrhal exudates in the nasal passages. Tracheal swabs and blood samples were collected from unvaccinated (n=306) and vaccinated (n=107) IB suspected chickens from randomly selected poultry flocks in Jaffna (9.6615° N, 80.0255° E) (Unvaccinated; broilers (n=57) and layers (n=53)), Trincomalee (8.5874° N, 81.2152° E) (Unvaccinated; broilers (n=05) and layers (n=98)), Kurunegala (7.4818° N, 80.3609° E) (Vaccinated; broilers (n=50) and layers (n=57)), and Batticaloa (7.7310° N, 81.6747° E) (Unvaccinated; broilers (n=31), layers (n=12) and backyard chicken (n=50)) districts of Sri Lanka during the period of June 2019 to May 2021. Two milliliters of blood were collected from each bird from the wing venipuncture. Serum was separated from each blood sample and stored at-20^{0}C until further use. Samples were transported to the laboratory on ice and stored at -80^{o}C at the Molecular Laboratory, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya until further processing.

Enzyme-linked immunosorbent assay (ELISA)

The commercial infectious bronchitis virus antibody test kit (AffiniTech Ltd., Bentonville, United States) was used to identify antibodies against IBV in serum samples. The ELIZA antibody test kit (infectious bronchitis virus antibody test kit, Affini Tech ltd) is highly specific for detecting antibodies against all known serotypes of Infectious bronchitis virus in chicken serum. One part sample diluent (4X) was diluted with 3 parts deionized water. Samples were diluted with sample diluent at a 1:400 ratio. A hundred microliters of negative and positive samples were dispensed into duplicate wells. A hundred microliters of diluted test samples were placed in each well of a 96-well micro-ELISA plate and incubated at room temperature for 30 minutes. One part of the wash solution (20X) was diluted with 19 parts of deionized water. After incubation, the ELISA plate was washed three times with 300 µl of wash solution per well, and a hundred microliters of conjugate were dispensed per well. Then, the ELISA plate was incubated at room temperature for 30 minutes. Again, the ELISA plate was washed three more times with wash solution (300 µl) in each well. A hundred microliters of substrate were dispensed into each well and incubated at room temperature for 30 minutes. After incubation, 100 µl of stop solution was added to each well. A microplate reader (BIORAD, Tokyo, Japan) was used to read an ELISA plate at 405 nm and 630 nm wavelengths.

RNA Extraction

Viral RNA was extracted from the tracheal swabs samples by using the Taco^{TM} DNA/RNA Extraction Kit (GeneReach Biotechnology Corporation, Taichung City, Taiwan) according to the manufacturer's protocol. Samples and reagents were loaded into a 96-well extraction plate. The loaded 96-well plate and mixing sleeve/mixing comb were installed by using mini–Automatic Nucleic Acid extraction system (Taco^{TM}, Taiwan) for one hour. The RNA sample was stored at -80^{0}C and used for downstream applications.

Detection of IBV by iiPCR

The POCKIT IBV detection kit manufactured by GeneReach Biotechnology Corporation (Taichung City, Taiwan) uses insulated isothermal polymerase chain reaction (ii PCR) technology to detect the RNA of infectious bronchitis virus (Change et al., 2012; Tsai et al., 2012). This detection kit is specifically designed to be used on an iiPCR compatible instrument, POCKIT™ Nucleic Acid Analyzer (Taiwan). The sensitivity and specificity of iiPCR (POCKIT™ Nucleic Acid Analyzer) are equivalent to real-time PCR, with an analytical sensitivity of up to 10 copies per reaction. The samples were placed into the Nucleic Acid Analyzer for one hour at 520 nm and 550 nm wavelengths.

Statistical analysis

Kappa statistic was used to determine the agreement between the ELISA and the iiPCR in detecting the IBV infection in tested chickens.

RESULTS AND DISCUSSION

Suspected cases of IB exhibited the typical postmortem lesions such as hemorrhages in the nephritis, swollen kidney, salpingitis, undeveloped oviduct with accumulation of clear fluid or purulent, hemorrhagic tracheitis, caseous or catarrhal exudates in the nasal passages (Fig 1). A total of 173 out of 413 sampled birds (41.88%) were positive for IBV by iiPCR and a total of 190 out of 413 sampled birds (46%) were serologically positive for IBV as determined by ELISA test (Fig. 2).

The present study was carried out with sampling in different districts where poultry farming is abundant in Kurunegala, Jaffna, Trincomalee, and Batticaloa districts (Fig. 2). Batticaloa district had the highest IBV prevalence (>75%) in broilers, layers and backyard chickens than other districts. The prevalence of IBV in backyard chickens is high in the Batticaloa district, although backyard chickens possess valuable traits such as disease resistance, adaptation to harsh environments and the ability to utilize poor quality feed (Abeykoon *et al*., 2013). Furthermore, all the broiler chickens and layers were collected from Jaffna, Trincomalee and Batticaloa districts have not been vaccinated, whereas the majority of intensive poultry farms in the Kurunegala district practice strict vaccination programs.

Based on the bird type, IBV screened by iiPCR were highly positive in unvaccinated flocks of commercial broilers, layers and backyard chickens. Thus, the prevalence of IBV in unvaccinated chicken was higher than that of vaccinated chicken in the districts of Trincomalee and Batticaloa except for layers. In contrast to a report in a previous study by Ball *et al*. (2016), the present study shows the prevalence of IBV in broilers was higher in unvaccinated flocks than vaccinated flocks (Fig. 3). This could be due to very closely situated farms with high density in unvaccinated farms than in vaccinated farms and prevalence of virus variants from vaccine strains (De Wit *et al.,* 2011). From a global perspective, it is important to have a better diagnosis to prevent and control the emergence of the avian infectious bronchitis virus and its variants (Dhama *et al*., 2014). In the same context, farmers in the Kurunegala district face serious problems (clinical disease of IB based on the clinical signs and the postmortem lesions) even after the vaccination of different types of vaccines. This could be coupled with a re-detection of vaccinal strains of IBV as per the finding of Ball *et al*. (2016), and De Wit *et al*. (2011). Meanwhile, Ball *et al.* (2016) found an overall 19 out of 34 (55.88%) IBV positive cases as determined by RT-PCR and sequencing in Sri Lanka. The same authors reported higher IBV prevalence in unvaccinated backyard chickens. This could be true as the clinical disease is easily maintained in the unvaccinated population rather than in hyper-immune commercial birds. However, Ball *et al*. (2016) report that extracted RNA from chicken tissues was subjected to RT-PCR to detect the IBV S1 gene, and the product was sequenced. However, iiPCR targets the specific gene and only detects the antigen level of the respective all-vaccine and field virus strains of IBV (up to 10 copies per reaction for iiPCR) in each sample under the wavelengths of 520 nm and 550 nm. POCKIT IBV detection kit uses insulated isothermal polymerase chain reaction (iiPCR) technology to detect the RNA of infectious bronchitis virus (Change *et al*., 2012; Tsai *et al*., 2012). ii PCR is based on qualitative IBV detection. The primers and probes only target specific sequences of IBV.

The ELISA test is a relatively sensitive test to detect early IgG (Chen *et al.,* 2011). The ELISA test was a popular method for detecting antibody responses to IBV infection in chicken flocks (Chen *et al*., 2003; Wing *et al*., 2002). In Taiwan, type-specific antibody detection of local IBV strains was developed to serve as a rapid and reliable diagnostic method for the characterization of IBV clinical infections in the field (Chen *et al.*, 2011).

Our previous study (Fouzi *et al*., 2018) revealed the seroprevalence of avian infectious bronchitis virus in Sri Lanka. In the current investigation, 46% (190/413) of the suspected flocks had high levels of IBV antibody titers measured by ELISA. The present study showed that seropositive of IBV were found by ELISA in unvaccinated flocks of backyard chickens 72% (36/50), layers 46.63% (76/163) and commercial broilers 48.38% (45/93), and in vaccinated flocks of layers 52.63% (30/57) and commercial broilers 6% (03/50). Similarly, Sabrarinath *et al* (2011) also reported that the unvaccinated flocks of backyard

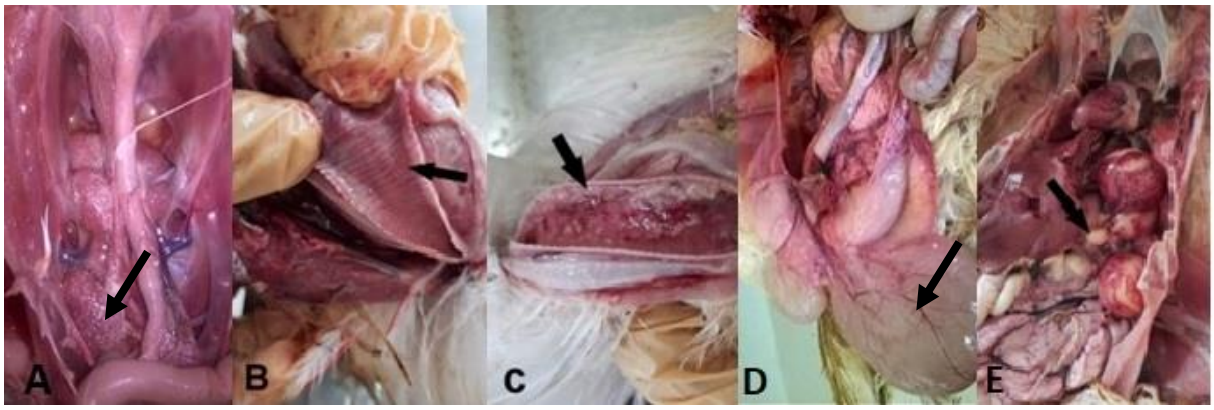

Figure 1: Characteristic lesions in the different organs of chickens suspected of infectious bronchitis based on postmortem lesions such as nephritis and enlarged kidney (A), hemorrhages in the trachea (B), and catarrhal exudates in the larynx (C), salpingitis and fluid filled oviduct (D), and low egg quality and production (E).

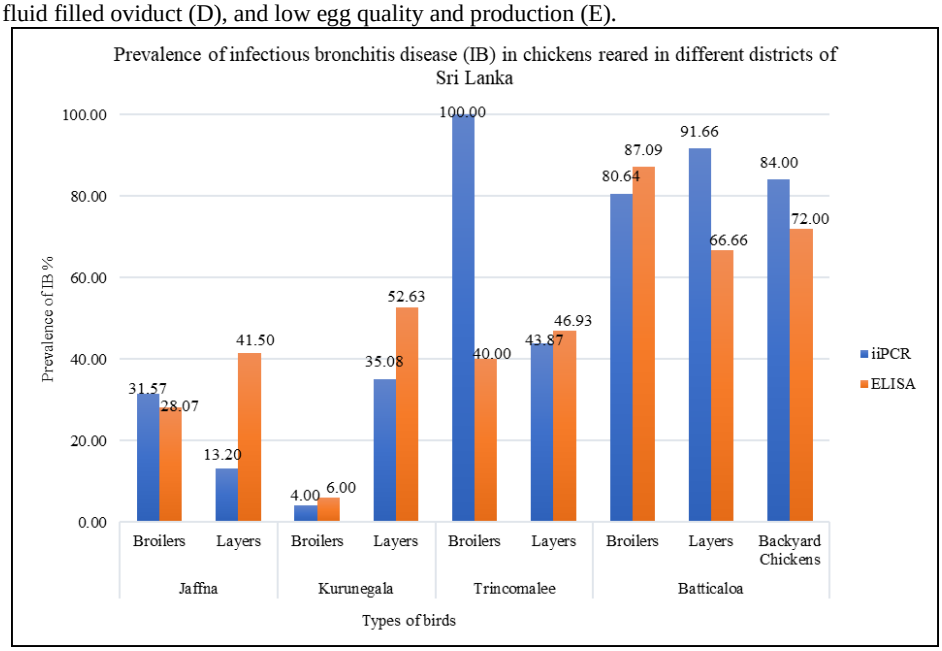

Figure 2: Prevalence of IB in Jaffna, Kurunegala, Trincomalee and Batticaloa districts confirmed by iiPCR and ELISA tests.

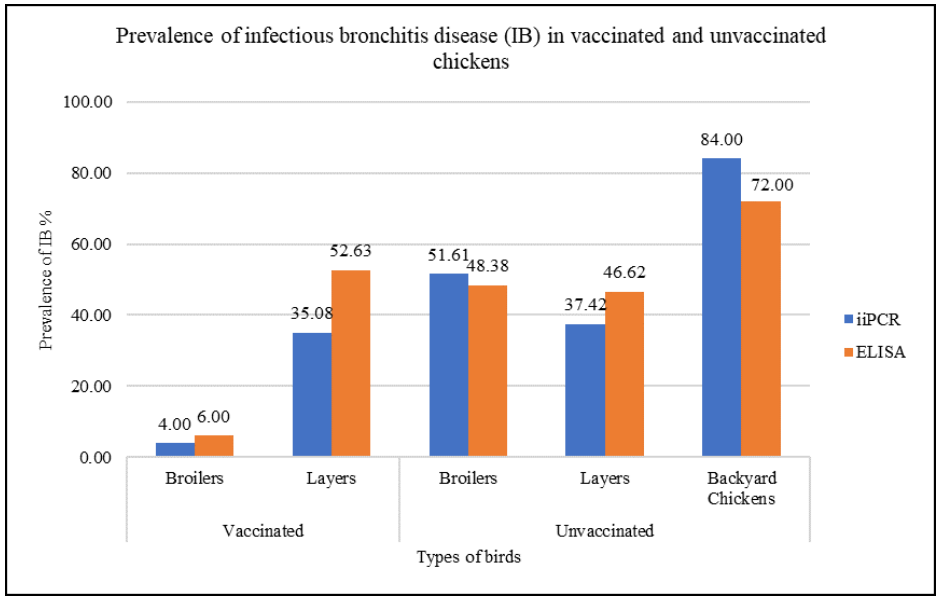

Figure 3: Prevalence of IBV in vaccinated and unvaccinated groups of broilers, layers and backyard chickens confirmed by iiPCR and ELISA tests.

chickens 54.28% (114/210) and 18.02% (31/172) broilers were positive for IBV-antibodies in Grenada. Moreover, unvaccinated flocks of layers 91.67% (176/192) and indigenous chickens 78.32% (177/226), and vaccinated flocks of breeders 90.91% (140/154) were seropositive for IBV in Southwestern Nigeria (Emikpe *et al*., 2010). In Iran, unvaccinated flocks of 82.43% of broilers and 85.3% of backyard hens showed significant anti-IBV antibody titers despite no clinical indications of IBV (Hadipour *et al*., 2011; Mahzounieh *et al*., 2006). Similarly, 88% of commercial poultry flocks were seropositive in Pakistan (Ahmed *et al*., 2007), 92.9% of flocks were seropositive for antibodies in Jordan (Roussan *et al*., 2009), and 82.7% of chickens were seropositive in South Western Nigeria (Emikpe *et al*., 2010). In the present study, a high percentage of seroprevalence was observed in unvaccinated flocks of backyard chickens and vaccinated flocks of layers. This might be an expected finding given the disease's highly contagious nature among Sri Lankan backyard flocks. Moreover, the IBV infection was more prevalent in the backyard chickens and less in the broilers by detection of both iiPCR and ELISA techniques.

CONCLUSIONS

The current investigation found that infectious bronchitis virus (IBV) was found in Sri Lankan poultry flocks, mostly among unvaccinated backyard hens and broilers. It was also observed that vaccinated flocks were also affected with IBV, but lesser extent than unvaccinated flocks. The iiPCR technology could be used for rapid detection of IBV infection in Sri Lanka settings.

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