Short Communication

SCANNING ELECTRON MICROSCOPY OF A NOVEL INDIAN NEPHROPATHOGENIC INFECTIOUS BRONCHITIS STRAIN, IND/AHL/16/01 IN EXPERIMENTALLY INFECTED EMBRYONATED CHICKEN EGGS

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ABSTRACT

Avian infectious bronchitis (IB) is a common, highly contagious, acute, and economically important viral disease of chickens caused by avian infectious bronchitis virus (IBV) of Coronaviridae. Nephropathogenic strains have emerged from mutation of widely used classical, live attenuated IB vaccines which mainly protects from respiratory form of the disease. The present work is directed to study the ultrastructure of the new strain of IBV, the IND/AHL/16/01, that was first isolated from an outbreak of nephritis and gout related mortality cases of coloured layer pureline birds at Indian Council of Agricultural Research - Directorate of Poultry Research (ICAR-DPR). Molecular characterisation and phylogenetic analysis was carried out and it was confirmed that the isolate is a novel nephropathogenic strain. Nine-day-old Embryonated Chicken Eggs (ECE) obtained from in-house hatchery of ICAR-DPR were inoculated by allantoic route and observed for embryonic lesions and mortality. The allantoic fluid from these ECE were collected into 2.5% glutaraldehyde and stored at 4 degree Celsius until processing. Scanning Electron Microscope (SEM) imaging by negative staining technique, revealed electron dense, pleomorphic Virus Like Particles (VLP's) of 120 nm diameter.

Key words: ECE, IBV, Nephropathogenic, SEM

IBV is a common viral infection in poultry farms of India. IBV infection in addition to causing serotypic changes and genetic variations may also alter the tissue tropism and pathogenicity of viruses and lead to the generation of new IBV pathotypes.

Different pathotypes of IBV have been reported by earlier workers which include the respiratory form in the

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year 1930 (Cavanagh and Naqi, 1997), nephropathogenic strains (Winterfield and Hitchner, 1962), enteropathogenic strains (Ambali and Jones, 1990), strains resulting in deep muscle myopathy (Gough *et al*., 1992) and proventriculitis (Yu *et al*., 2001).

Electron microscopy provides a direct means of detecting and identifying IBV in biological samples based on morphological characteristics of corona virus. Positive cultures are confirmed based on the presence of coronavirus-like pleomorphic structures with spike projections, following negative staining with phosphotungstic acid. Importantly, the shape and diameter of the virus are taken into consideration when making diagnostic judgements (Bande *et al.,* 2016).

The objective of the present study is to observe the morphology of novel Indian IBV nephropathogenic isolate IND/AHL/16/01 in the allantoic fluid of ECE by SEM.

The virus

Infectious bronchitis virus (IBV) isolate (IND/AHL/16/01) isolated from the outbreak of nephritis and gout related mortality cases from coloured layer pure lines maintained at avian health lab, ICAR-Directorate of Poultry Research (ICAR-DPR), Hyderabad was used in this study. The isolate was initially passaged in ECE and showed IBV specific embryo curling, dwarfing and haemorrhage. Allantoic fluid was confirmed for IBV with S1 gene specific primers by RT- PCR (Gelb *et al*., 2005). The full length S1 gene region was amplified (1.6 kbp), sequenced and analysed with other IBV types for its phylogenetic relationship. The present IBV variant isolate clustered with nephropathogenic variants like 4/91 and 793B. The allantoic fluid was also tested for other viruses and found to be negative.

Virus cultivation in ECE and processing for SEM

Embryo inoculations through the allantoic cavity route were carried out using the method described by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012; de Wit *et al.,* 2011). Nine day old embryonated chicken eggs procured from DPR, Hyderabad and were candled. The air cell margin and eyespot were marked with pencil and the point of inoculation was marked opposite to the eye spot, 2-3 mm below the air cell margin. The shell was disinfected by swabbing with 70% ethanol. At the inoculation site a hole was punched with a sterile egg puncher and 0.1 mL of homogenised and clarified virus was inoculated and incubated at 37 ^{0}C for 5 days. The embryos that died within 24 h were discarded and on the 5th day the all the eggs were chilled at 4 ^{0}C for overnight and the allantoic fluid harvested in a biosafety cabinet.

Chilled eggs were swabbed with 70% alcohol and the egg shell was opened carefully above the air cell with a pair of sterile forceps and picked away till the air cell margin, then the inner shell membrane was peeled carefully back to expose the chorio-allantoic membrane (CAM). A Pasteur pipette was used to pierce the CAM and the allantoic fluid collected.

Allantoic fluid samples were collected and preserved in 2.5% glutaraldehyde (PBS based EM grade) at 4 degree Celsius and processed for SEM by negative staining technique as per the standard protocol (Bozzala and Russels, 1998) for virus detection.

The ECE inoculated with the virus isolate, IND/AHL/16/01 showed curling, dwarfing, congestion of embryos (Fig. 1). The allantoic fluid showed round electron dense virion like particles (VLP's) of approximately 120 nm in diameter under SEM (Fig. 2). The VLP's showed pleomorphism although most of them are circular in outline with diameter of 80- 120 nm (Fig. 3). Few particles have invaginations at their centres indicating infolding of the membrane (Fig. 4). These findings were similar to the findings of Berry *et al*. (1964), Bingham and Almeida (1977) and Bande *et al*. (2016) and they have reported an electron dense, pleomorphic, approximately 80-160 nm in diameter with distinctive projections for IBV.

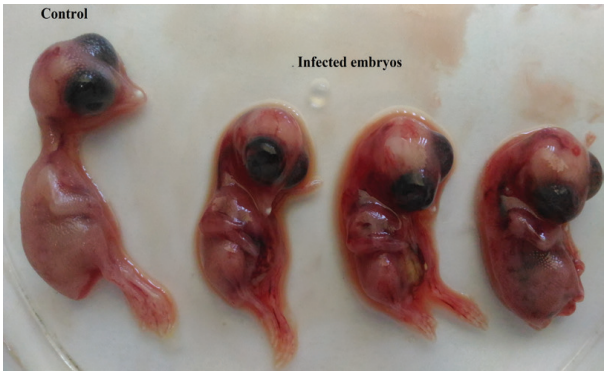

Fig. 1. IND/AHL/16/01inoculated chicken embryos showing curling, dwarfing, congestion of embryos

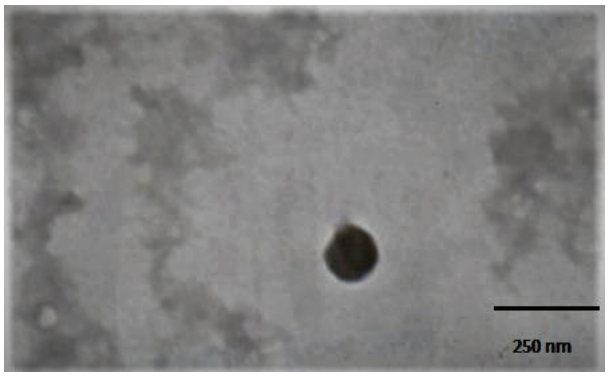

Fig. 2. SEM of IBV infected allantoic fluid showing virus like particle (VLP). UA & LC 250 nm

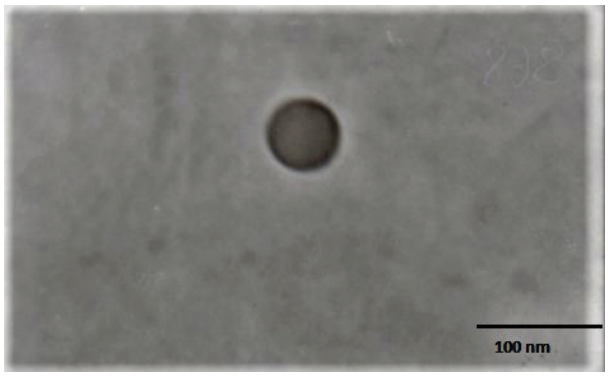

Fig. 3. SEM of IBV infected allantoic fluid showing virion with circular in shape. UA & LC 100 nm

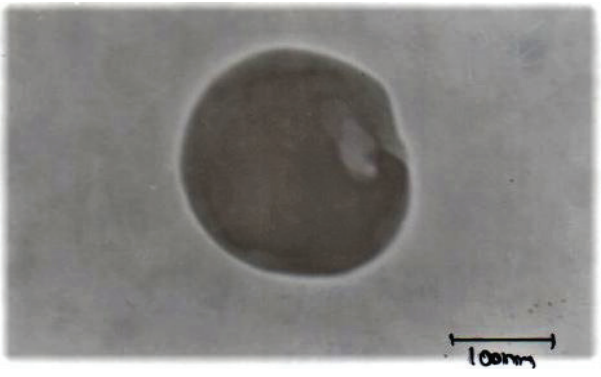

Fig. 4. SEM of IBV infected allantoic fluid showing virion with circular in shape and showing invaginations at their centres indicating in-folding of the membrane.UA & LC 100 nm (Note: Photomicrograph scale marked manually; therefore, may not indicative of accurate size)

With the emergence of novel strains of IBV, it became difficult to detect them in samples with conventional detection techniques like neutralisation assay, serological tests. Hence the modern techniques like electron microscopy, RT-PCR, RFLP aids in identification of new strains.

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