Chapter 1

Literature Review

1.1 Introduction

The commercial poultry industry is confronted by a number of diseases. Despite the development of vaccines and vaccination protocols against viral diseases, emerging variant strains continue to be a threat to the poultry industry. Infectious bronchitis virus is an excellent example of such viral infections.

Infectious bronchitis virus (IBV) is a highly infectious and contagious pathogen of chickens worldwide. IBV is the member of the coronaviridae family and contains a single stranded positive sense RNA of 27.6 kb (Boursnell *et al.*, 1989). The disease is prevalent in all countries with an intensive poultry industry. Rapid mutation and evolution of new strains makes the study of this virus more difficult. It is an economically important disease associated with mortalities in young chickens and decreased egg production accompanied by inferior egg quality in laying flocks (Broadfoot *et al.*, 1956). The commercial egg laying chicken has a level of productivity second to none. However an outbreak of IBV in developing pullets may have a devastating effect on future productivity. In adult laying hens, IBV has a varying effect on production. In 1931, Schalk & Hawn observed a mortality rate of up to 40-80% in chicks with severe respiratory signs and post-mortem lesions such as congestion, with mucus exudate, in the bronchi and trachea. The disease proved to be rapidly transmissible. The cause of the disease was confused with infectious laryngotracheitis (ILT) until the study of Beach and Schalm (1936). They differentiated between IBV and ILT by cross-immunity studies. Later, Beaudette and Hudson (1936) first cultivated the virus in chicken embryos inoculated by the chorioallantoic route. In Australia, the virus was first isolated from cases of uraemia in Armidale, NSW, Australia by the late Associate Professor Robin Cumming in 1961. After facing considerable scepticism, he finally published his findings at the end of the year 1962 (Cumming, 1962). It is generally considered that, in breeders and commercial layers, the respiratory and nephritic forms of IB are controlled satisfactorily, although their uterotropic protection is has not been studied.

Also, the role of IBV in cases of watery whites and poor shell quality is unknown (Ignjatovic, 1998)

Unfortunately, the study of the pathogenesis of IBV has been regarded as a low priority area of research although there is an urgent need to know the exact pathogenesis of the disease, particularly under field conditions. The mechanism of pathogenicity was recently placed at the top of research objectives proposed for priority treatment by an adhoc group on vaccinology (Bourne *et al.*, 1996). Significant drops in egg production owing to disease in layers have caused great economical losses. IBV continues to be an economically-significant problem despite the wide spread use of live and inactivated vaccines (Hofstad, 1981). Production losses could be a greater concern than mortality. Occurrence of multiple serotypes has complicated the prevention of disease by vaccination (Cavanagh and Naqui, 1997)

In layers IBV can cause severe declines in egg production and also deterioration in egg shell quality and egg internal quality (MacDougall, 1968; Seovian and Levine, 1957). Such effects may be accompanied by mild (Munner *et al.*, 1987) or no respiratory signs (Cook, 1984). The severity of the production decline varies with the period of lay, the virulence of the virus involved and other non-specific factors. The response of individual hens also varies greatly (McMartin, 1968b). Production may start to increase 2 to 3 weeks after infection but, when laying is resumed, egg production remains subnormal (Ignjatovic and Sapats, 2000)

Studies regarding the detailed pathogenesis and isolation of IBV in the respiratory tract and kidney of chickens have received much attention. The effect of IBV on egg production has been reported by several workers but associated changes in the different parts of the reproductive tract of adult layer birds have not been elucidated properly.

In the reproductively active hen, the oviduct is the biological machinery which daily undergoes a series of biochemical, cellular and hormonal changes. The fully-functional oviduct occupies a large part of the abdominal cavity. Being such a complex organ, it is very important to understand the normal structure and function of the normal mature oviduct before studying its response to infectious bronchitis virus.

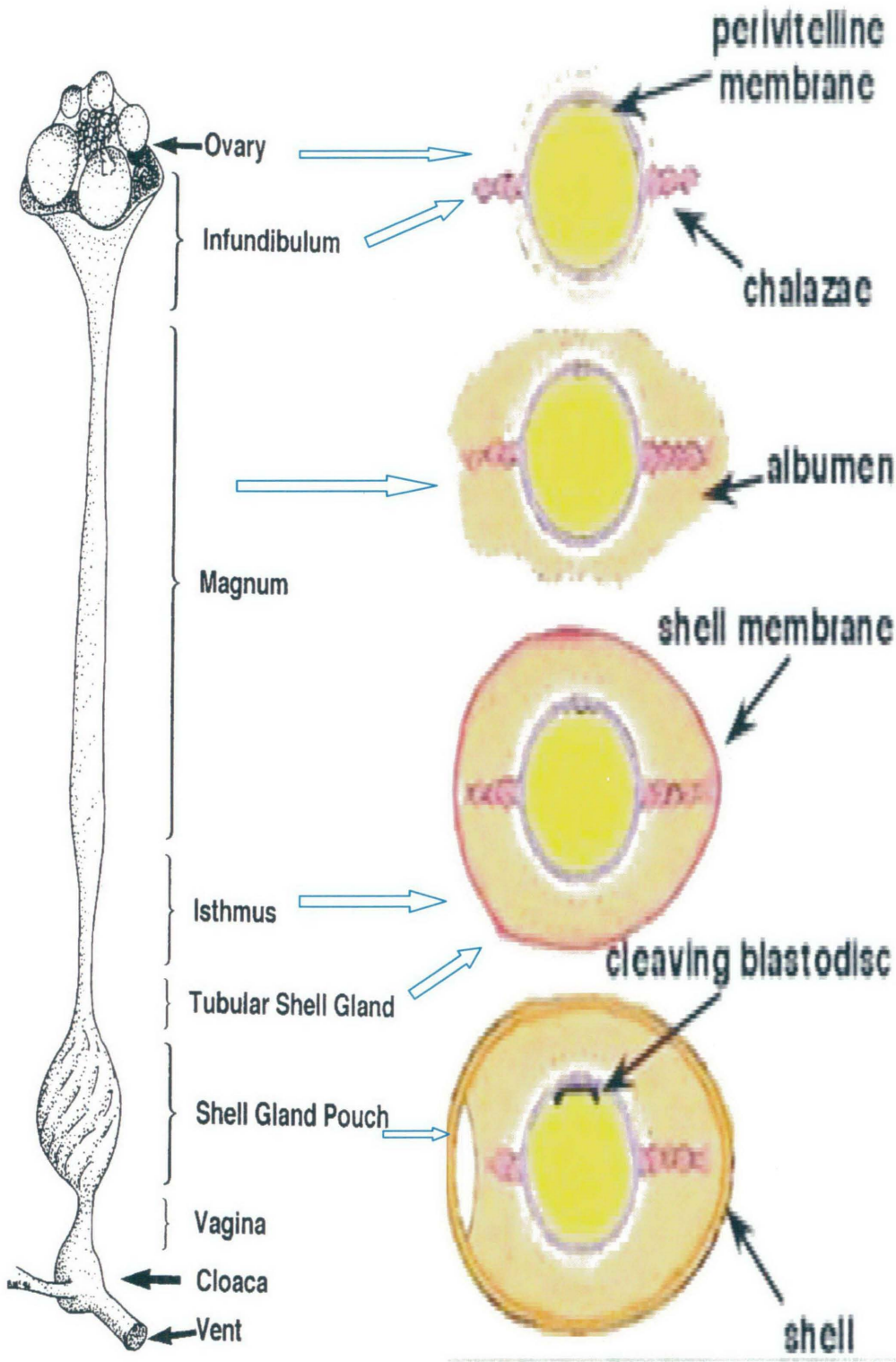

Plate 1.1: Structure of the oviduct and developmental stages of the egg

Source: Roberts and Brackpool (1995),

www.wisc.edu/ansci_repro/lec/lec1/female_hist.html

1.2 Oviduct

The oviduct of layer birds is derived from the left Mullerian duct. In chickens, the left duct develops more rapidly after 16 weeks of age and becomes fully functional just prior to the onset of egg production. Cilia line the entire oviduct and are thought to offer local immunity as well as being responsible for sperm transport in the oviduct. Both α and β adrenergic receptors are present throughout the length of the oviduct, inducing the oviduct motility (Crossley *et al.*, 1980). The role of oviduct motility during the formation of the egg is not fully understood. Shimada and Asai (1978) recorded electrical activity and motility in the magnum, isthmus and shell gland and found that the highest electrical stimulus was recorded in the shell gland during oviposition. The ovulation-oviposition cycle usually ranges from 24 to 28 hrs and the eggs laid on successive days is called a sequence (Johnson, 2000). Each sequence is generally separated by a pause, which may be of one or more days. This has also been described as the resting phase of the oviduct (Aitken, 1971). The time interval between oviposition and subsequent ovulation is 15 to 75 min (Johnson, 2000). Reproductive hormones play a vital role during egg formation. Follicle stimulating hormone is responsible for follicular development and luteinising hormone (LH) stimulates ovulation. According to Wilson and Sharp (1976), a combination of oestrogen and progesterone primes the LH release. Oestrogen not only enhances the growth of the oviduct but also induces the production of important albumen proteins such as ovalbumen, conalbumen and lysozyme by promoting the formation of tubular secretory glands (Palmiter, 1972). According to Johnson (2000), the presence of progesterone receptors in the surface and glandular epithelial cells of the myometrium may be evidence of a role for progesterone in shell formation as, along with the ciliated epithelium, the wall of the oviduct also comprises the lamina propria, inner connective tissue layer, inner circular muscle layer, outer connective tissue layer, outer longitudinal muscle layer and serosal layer (Hodges, 1974). The muscle layer increases caudally and is usually abundant in the outer anterior of the vagina. Scattered plasma cells and lymphocytes are also present in all parts of the oviduct (Biswal, 1954).

The oviduct is divided into six distinguishable regions, infundibulum, magnum, isthmus, tubular shell gland, shell gland pouch, and vagina. Each region is morphologically different and involved in some way in the process of egg formation, i.e. in the process of formation of chalazae, thin and thick albumen, inner and outer shell membranes and a calcified shell

around the central mass of yolk (Gilbert, 1971). Warren and Scott (1935) determined the transit time of the egg through the oviduct.

Table 1.1: showing the length and time spent by egg in different parts of the oviduct

Organ	Length (cm)	Weight (gm)	Transit time (hrs)
Infundibulum	9	1	.25
Magnum	32	17.6	2 to 3
Isthmus	10	4.4	1.25
Shell gland pouch	11	13.5	18-20
Vagina	10	5	-

(Details of table taken from Warren and Scott, 1935; Aitken, 1971)

1.2.1 Infundibulum

Although not directly connected with the ovary, the infundibulum engulfs the ovum. The infundibulum is divided into three parts, the anterior segment or ampulla, the mid segment and the posterior segment known as the chalaziferous region. At the time of ovulation, the infundibulum plays an active role. The wrinkled fimbrial lip, owing to the presence of blood and lymph vessels, undergoes vascular engorgement expanding the ostium of the infundibulum. Further movement in longitudinal and transverse directions, along with ciliary beating in the ampulla, is responsible for movement of the ovum away from the ovary (abovarian direction) (Fujii *et al.*, 1981). Sometimes, the infundibulum fails to pick up the ovum and this leads to a condition known as internal ovulation. Such an internal egg is reabsorbed, possibly by the ovary itself (Sturkie, 1955). The proximal part of the infundibulum is less folded than the distal part. According to Solomon (1983), the anterior part of the infundibulum is lined by a tall columnar epithelium with a population of ciliated non-secretory cells. As the organ narrows, the mucosal folds become more dense and secondary and tertiary folds appear. The middle and caudal parts of the infundibulum are lined by both ciliated non-secretory and non-ciliated secretory cells. The epithelium of non-ciliated cells secretes mainly acidic mucus (Aitken, 1971) and both ciliated and non-ciliated cells are lined by microvilli. The caudal part of the infundibulum is lined by tubular glands packed with electron dense secretory granules. Fertilization of the ovum occurs in the

infundibulum and, according to Bain and Hall (1969), formation of the first layer of albumen and chalaza starts here, with this region being very activate during ovulation. Aitken and Johnston (1963) distinguished epithelial layers into four different types;
1) Non-secretory ciliated columnar cells with microvilli and little evidence of secretory granules, covering the tips and sides of the oviduct folds. These cells have numerous vacuoles and an apically placed nucleus. 2) Mucus-secreting ciliated cells containing dense granules and a basally situated nucleus, 3) Secretory cells in the glandular epithelium differ in shape from the mucus-secreting epithelial cells and contain fewer secretory granules, 4) Cells lining the tubular glands in the glandular epithelium present mainly in the caudal area of the infundibulum.

Knowledge of the infundibulum was later elaborated by Wyburn *et al.* (1970) who studied the infundibulum at various stages of egg formation and noticed changes in rough endoplasmic reticulum (RER) deposits of the surface and glandular epithelia. Makita and Kiwaki (1968) studied the ultrastructural details of the infundibulum of the quail and found large vacuoles in secretory cells. They suggested that secretory cells transport watery fluid to clean the surface of the ovum. The study by Wyburn *et al.* (1970) is the only study of the changes occurring in the infundibulum at different positions of the egg, but its findings were not supported by observation of changes occurring at the surface by scanning micrography. Knowledge of changes in the infundibulum at the cellular level, during various egg positions, employing a combination of microscopic techniques such as transmission and scanning electron microscopy would be an advantage.

1.2.2 Magnum

This part is known mainly as an albumen-secreting area of the oviduct and secretes a range of proteins making up the albumen. This is the largest and most conspicuous part of the oviduct in which the egg spends the most time prior to entering the shell gland. During the development of the oviduct, the magnum develops at a faster rate than the other regions (Solomon, 1983). When the hen reaches peak production, the magnum attains its maximum size, although there is no direct relationship between oviduct size and age of hen. Growth and development of the magnum is predominantly associated with hyperplasia which is characterised by a remarkable increase in total DNA (Yu and Marquardt, 1973).

The walls of the magnum consist of an outer muscular layer, hereafter referred to as the muscularis, and inner lamina propria containing tubular glands lined by secretory cells. The differentiation of tubular glands from the epithelial surface occurs at less than 4 months of age and albumen secretion in the tubular gland lumen occurs between 4 and 5 months of age (Yu and Marquardt, 1973). These authors have also reported an increase in the RNA/DNA ratio and a high concentration of cellular RNA in the magnum during the laying phase as compared to the moulting phase. This would be, of course, due to the high metabolic activity of the magnum during lay. Different peaks of metabolic activity in the magnum at various egg positions would be expected but, obviously, this needs confirmation and further investigation.

The epithelium of the magnum is clearly demarcated from the tubular gland by fibrous tissue. Mucosal folds are prominent and the mucosa is lined by a columnar epithelium, the height of which increases caudally. The epithelium of the magnum consists mainly of secretory and non-secretory cells (also referred as the granular cells). Bakst and Howarth (1975) observed that non-ciliated cells comprise 50 % of the surface epithelium of the magnum and their surface is covered by overlying cilia from the ciliated cells. However, these authors did not study scanning electron micrographs at different stages of egg formation. The secretory granules of the secretory cells bulge into the lumen, pushing the nucleus towards the base of the cell. The secretory granules are dense in the middle and caudal portions of the magnum, covering the area inside the cell from apex to base. The non-secretory ciliated cells typically have a broad apex and are the same all over the magnum. Makita and Sandbourne (1970) described the openings of tubular glands onto the surface epithelium, stating that these openings are difficult to locate by light or electron microscopy, although these openings were later observed by Wyburn *et al.* (1970) using light microscopy. The secretory gland cells in the magnum usually open onto the surface (Aitken, 1971). Later, Bakst and Howarth (1975) confirmed the presence of these openings by scanning electron microscopy. However, the appearance of these openings at various egg positions in the oviduct requires further explanation. In another scanning microscopic study conducted by Bakst (1978), the magnum in contact with the ovum appeared pale while the remainder appeared distended. Bakst showed that the secretion of thin albumen strands from glandular openings coalesced to form a sheet of albumen.

The mucus secreted by epithelial cells is acidic mucopolysaccharide. Wyburn *et al.* (1970) described three different types of tubular gland cells, A, B, and C. Type A gland cells contain electron dense granules of varying size with some pale granules, type B contain areas with a large amount of amorphous material. The type A and Type C gland cells are considered to be different phases of one cell type because type C gland cells are found after the passage of an egg. In other words, the type C cell appears to be the reparatory phase of a type A gland cell. The nucleus of the type C cell is pressed against the basal membrane and there are prominent Golgi complexes and reticular spaces.

The magnum contributes a range of proteins including ovalbumin, conalbumin (ovotransferrin), ovomucin, ovomucoid, flavoprotein, ovomacroglobulin, lysozyme and avidin. Of these proteins, ovalbumin contributes the largest amount, 54 % of the total egg white (Gilbert, 1971). The jelly-like property of egg white is attributed to ovomucin which is secreted by granular (non ciliated) cells of magnum. Gland cells type A secrete ovalbumin and gland cells type B contribute lysozyme (Wyburn *et al.*, 1970). Granular cells react strongly to alcian blue dye, which imparts a blue colour due to the presence of glycoproteins. Patchy or complete loss of this stain from the surface epithelium of the magnum was observed by Davidson (1984) in cases of watery whites. Such findings can be observed in the magnum of infectious bronchitis virus infected hens. (Randall and Reece, 1996; Butler *et al.*, 1972). Another egg white protein, avidin, has been localised amongst the secretory granules of tubular gland cells by immunocytochemical methods (Kami and Yasuda, 1984). Secretion of most of these proteins is mediated by hormonal action (O'Malley, 1967)

1.2.3 Isthmus

This is a small functional area lying between the magnum and shell gland. The magnum is clearly separated visually from the isthmus by a white marking (Aitken, 1971). The visual translucence of this area is due to lack of tubular glands (Sultana *et al.*, 2003). According to Mao *et al.* (2006) the asymmetrical and ellipsoid shape of the avian egg is attributed to the narrow width of the magnum-isthmus junction. The formation of the shell membranes starts in the isthmus (Stemberger *et al.*, 1977). Draper *et al.* (1972) divided the isthmus into two areas and named the proximal part the granular isthmus and the distal part the red region. The isthmus is lined by a pseudostratified columnar epithelium of ciliated and non-ciliated

cells (referred to as granular cells). Along with these types of cell, some mitochondrial cells are also present in the surface epithelium (Draper *et al.*, 1972). The ciliated cells have a proximally situated nucleus with scattered granules. The granular cells contain more secretory granules than ciliated cells and have a basally situated nucleus. The granular cells have a bulging appearance when they are full of secretory granules. The height of the epithelium is lower than the magnum. Intracisternal granules in the cisternae of the endoplasmic reticulum are a typical characteristic of the isthmus (Draper *et al.*, 1972) and, according to Solomon (1983), these intracisternal granules are the indicators of protein synthesis. However, their appearance at different phases of secretion during egg formation has not been described. The proximal part of the isthmus contains electron dense granules of variable density (Solomon, 1975). The free surface of the isthmus is lined by microvilli projecting into the lumen. According to Wyburn *et al.* (1973), the egg stays in this region for 15 to 30 min. Granular blebbing is dense and the secretory granules are diastase resistant PAS positive granules. The glands of the isthmus are convoluted. Solomon (1975) reported two types of gland cells in the isthmus, gland cells of type 1 and type 2. Gland cells type 2 are predominantly noticed after egg passage and the two gland cell types are thought to be the different phases of secretory activity. The cells of the glands are cuboidal, narrowing towards the lumen. Type 1 gland cells contain large secretory granules of different densities and distended reticular endoplasmic reticulum (RER). On the other hand, short cords of RER scattered throughout the cytoplasm have the appearance of type 2 gland cells of the isthmus. The secretory granules in the isthmus stain pink with eosin and are paler in the proximal part as compared to the distal region.

1.2.4 Tubular shell gland (TSG)

The isthmus extends to the tubular shell gland which is lined by a pseudostratified columnar epithelium. Most of the ultrastructural appearance of the tubular shell gland is similar to the isthmus (Solomon, 1991), although the function could be comparable to the shell gland pouch. The tubular shell gland was formerly known as the red isthmus. The epithelial cells of the TSG are involved mainly in the process of calcium transfer (Solomon, 1975), which begins initially in the granular isthmus (Stemberger *et al.*, 1977). The secretory granules and cellular structure of the TSG are also similar to those of the isthmus. The glycogen content of the cells in the TSG is high. Wyburn *et al.* (1973) found that the glucose content of the egg isolated from this area was high but these authors did not study this region of the

oviduct. Wyburn's observation was further stated by Solomon (1991). The high glucose content of eggs removed from this region may be attributed to the presence of glycogen granules in the gland cells of the TSG. According to the observations by Stemberger *et al.* (1977), mammillary cores are secreted by the epithelial cells of the tubular shell gland. The mammillary cores are seeding sites up on which shell crystallisation begins and are the initial templates for the calcified shell. Hence, any change in the morphology of mammillary cores could be responsible for shell structure defects. Initial calcification during the formation of the mammillary layer begins in the tubular shell gland and tubular gland cells in the TSG contribute calcium (Solomon *et al.*, 1975; Creger *et al.*, 1976). The time spent by the egg in this region is relatively short but is very important for the formation of a good egg shell.

1.2.5 Shell gland pouch (SGP)

The shell gland pouch is lined by a pseudostratified columnar epithelium which is composed of apical and basal cells. The uterine glands are lined by cuboidal epithelial cells with marked vacuolated cytoplasm (Johnston *et al.*, 1963). The apical cells are similar throughout the uterus but show changes during different stages of the laying cycle. Wyburn *et al.* (1973) described the two types of cells in the surface epithelium as ciliated and granular. The free surface of these cells is always interspersed with cilia and microvilli. The secretory granules in the anterior area are denser as compared to the middle and posterior areas (Johnston *et al.*, 1963). Vacuolation is usually found at all stages of laying cycle but is greater at the end of shell calcification. The intracellular space normally expands when the shell gland surrounds the shell membrane. According to Breen and Bruyn (1969), the vacuoles in secretory cells of the shell gland are sites for recycling of secretory granules. In their study, they showed the appearance of secretory granules disintegrating at the membrane of the vacuole. However, they failed to show any remnants of such granules inside vacuoles which would have been more confirmatory and there has been no further study of this. The secretory granules in the SGP are smaller than those in the isthmus. Most of the vacuoles in the glandular cells appear empty but some of them are filled with dense particles. An individual gland is made up of six wedge-shaped cells, each of which has a large nucleus at the basal surface. Each glandular cell has microvilli on the luminal surface (Wyburn *et al.*, 1973). However, Johnston *et al.* (1963) observed the appearance of dense particles only during the presence of a calcified shell in the SGP. This observation was

supported by Breen and Bruyn (1969) who stated that the gland cell does not show any prominent signs of secretory activity. Gland cells play a major role during calcification and microvilli of gland cells swell during calcification. Makita *et al.* (1973) described an increase in the number of secretory granules in the shell gland of laying hens and predicted that secretory granules have little role in eggshell matrix formation. However, their observation was based on the presence of similar density of secretory granules both in active and resting phases of the oviduct. Breen and Bruyn (1969) observed the Golgi complex to be the principle site of granule formation, although Makita *et al.* (1973) suggested the granule-packed mitochondria as a possible source. McCallion (1953), in his cytological observations, did not find any significant difference in the shell gland of hens producing thick and thin-shelled eggs.

Earlier, the mechanism of calcium secretion by the shell gland was studied by Eastin and Spaziani (1978a,b), who concluded that calcium secretion occurs by diffusion and active transport. The calcium secretion can be linked to the luminal concentration of HCO_3^-. In an electrochemical study, Mongin and Carter (1977) postulated that, during egg shell formation, calcium is derived from the epithelial linings of the shell gland. According to Simkiss and Taylor (1957), the fibrils of the organic shell matrix are secreted by the epithelial cells of the shell gland pouch.

1.3 Formation of the egg and cellular changes during egg formation

Formation of an egg is a complex phenomenon with respect to cellular and hormonal changes and many factors are involved in the process. Many factors influence the formation of eggs laid by the hen in her pullet year but the number of eggs laid by a hen is governed by the ability of the ovary to produce ova and the capacity of the oviduct to transform it to a complete egg. A typical egg weighs about 58 gm and contains about 7 gm protein, 6.7 gm fat, 0.3 gm of carbohydrate, 2.5 gm of mineral, 39 gm of water and 3 gm of non-metallic elements. The egg is mainly formed of three constituents yolk, egg white, and shell. The secretions of the oviduct are controlled mainly by a balance of oestrogen and progesterone (Gilbert, 1971).

Yolk proteins are mainly produced by the liver under oestrogenic stimulation. The proteins are then transported in the blood to the ovary (Husbands and Brown, 1965). Light and

hormonal levels have a prominent impact on the process of ovulation. Ovulation usually follows the laying of the previous egg within 30 minutes, except when the previous egg is laid in the late afternoon, in which case ovulation is delayed until the next morning. This is due to the well-known effect of light on ovulation (Conrad & Scott, 1938). Johnson (2000) stated that the time between oviposition and subsequent ovulation is 15 to 75 minutes. The average time required for the ovum to enter the oviduct is about 15 minutes (Warren & Scott 1935).The infundibulum becomes very active during the ovulation with the fimbria becoming extended due to vascular engorgement and grasping and engulfing the released ovum. A series of cellular changes occurs in the oviduct during ovulation. The number of secretory granules in the infundibulum increases which causes the bulging of the surface layer of the infundibulum. Similar findings were recorded in the glandular cells. Protein synthesis occurs mainly in the ribosomes, the synthesised protein then accumulates in granular endoplasmic cisternae and is packed and sequestered by the Golgi complex. The mature granules then migrate towards the apex of the granular cell to be discharged into the lumen by rupturing the cell membrane (Wyburn *et al.*, 1970). The epithelial cells in the caudal region of the infundibulum secrete the initial layer of albumen which is a thick gel-like secretion.

The magnum plays a major role in the secretion of albumen. However, to initiate its full secretory potential, the magnum requires progesterone and oestrogen (O'Malley, 1967). During the passage of the egg through the magnum, the height of the magnum epithelium increases with accumulation of dense secretory granules. The egg takes three hours to pass through the magnum, as thick and thin layers of albumen surround the yolk. The magnum contributes the majority of proteins including ovalbumin, conalbumen, ovomucin, lysozyme, ovomucoid and avidin. The thick albumen of the egg contains mainly ovomucin which is formed by the granular cells of the magnum and imparts a jelly-like consistency to the albumen (O'Malley, 1967). Wyburn *et al.* (1970) concluded that lysozyme may be contributed by B cells of the glands in the magnum although they could not provide evidence of this finding. Palmiter and Gutman (1970) observed that ovalbumin, ovomucoid, conalbumen and lysozyme are located in the tubular gland cells of the magnum. Their observations were based on immunofluorescence antibody technique in which they did not observe any immunofluorescence amongst the cells of surface epithelium. After spending 2-3 hours in the magnum, the egg enters the isthmus and stays there for a short time. According to Draper *et al.* (1972), three parts of the oviduct, isthmus, tubular shell gland

and shell gland pouch, are involved in the process of shell formation. The reticular formation starts depositing a continuous deposit of fibrous layer around the rotating egg mass. In the granular isthmus, the substance of the core and mantle of shell membrane fibres is deposited onto the egg. The process of fabrication occurs inside the gland cells of the granular isthmus. The gland cells of the functioning oviduct contain electron dense material as a filament.

The avian egg shell consists of organic and inorganic components. The organic component includes the shell membranes, mammillary cores, organic matrix and cuticle. The inorganic component includes the mammillary, palisade and surface crystal layers (Brackpool, 1995). In tubular shell gland, after formation of the mammillary cores, the egg enters the shell gland pouch with the shell membranes still relatively loose and stays there for at least 20 hours. The shell gland pouch starts pouring a watery secretion into the egg and this process is called "plumping" (Simkiss & Taylor, 1971). According to Simkiss and Taylor (1971), formation of egg shell pores may be the effect of this continual secretion. The process of shell formation is initiated in the isthmus with the formation of the shell membranes and continues with the formation of the mammillary cores in the tubular shell gland. The main part of shell mineralization occurs in the shell gland pouch. Most of the calcium used for shell formation is derived from the blood although a small amount may be present in the shell gland pouch (Hohman and Schraer, 1966). The mitochondria of the gland cells in the shell gland pouch accumulate sufficient calcium ions. Epithelial cells play a major role in active transport of calcium from the blood stream. Simkiss and Tyler (1957) suggested that the mammillary core surrounded by sulphated protein is a chelating agent which removes calcium from the blood (chelation is the union between a metallic ion and a chelating molecule). The carbonate ions may then displace the chelating agent from calcium to form a calcium carbonate complex. Calcium secretion is thought to be under the control of hormones (Eastin and Spaziani, 1978a). According to Simkiss & Taylor (1971), in the shell gland pouch during egg shell formation, calcium moves across the epithelial cells of the shell gland. The mitochondrial cells can store a small quantity of calcium ions in the absence of calcification (Hohman and Schraer, 1966). Calcium-binding protein, which is known to mediate the transport of calcium across the intestinal wall, has been found in the avian shell gland pouch (Rabon *et al.*, 1991). Diets containing 3.6 % calcium are the chief source of calcium during egg shell calcification. Moreover, the transport of calcium ions across the shell gland is also dependent on the healthy reproductive status of the hen.

Calcium ions migrate from serosal layer to mucosal layer during the presence of an egg in the shell gland (Simkiss and Taylor, 1971).

There is still controversy about the site of synthesis of shell pigment. Tamura *et al.* (1965) report that epithelial cells are responsible for pigment secretion. However, according to Baird *et al.* (1975), the shell pigment protoporphyrin is derived from the blood. The compounds responsible for pigmentation are protoporphyrin, uroporphyrin and coproporphyrin (Solomon, 1987).

The cuticle is the last layer to be deposited on the egg. It is a waxy substance which plays an important role in protecting the egg from bacterial penetration. According to Johnston *et al.* (1963), both apical and basal cells lining the pouch region are involved in this process and the cuticle is formed by non-ciliated secretory cells of the shell gland Solomon, (1991).

1.4 Infectious bronchitis virus (IBV)

As the name indicates, infectious bronchitis is primarily a respiratory syndrome, although the virus replicates in other organs also. The virus is a member of the coronaviridae family which comprises a single genus coronavirus. Coronaviruses are a group of lipid containing enveloped RNA viruses that have been classified together on the basis of their unique morphology and certain biochemical similarities (Tyrrell *et al.*, 1978).

IBV is pleomorphic but generally rounded. It possesses an envelop that is 90-200 nm in diameter with the club-shaped surface projections called spikes being about 20 nm in length (Mcintosh, 1974). All coronaviruses have a high molecular weight single stranded RNA genome polyadenylated at the 3' terminus and with seven polypeptides most of which are glycosylated (Tyrrell *et al.*, 1978). One of the unglycosylated polypeptides of molecular weight above 50,000 is associated with the genome and appears to form the nucleocapsid. IBV virions contain three virus specific proteins; the large spike (S or E_2), small matrix (M or E_1) and internal nucleocapsid (N) proteins. The S protein comprises two or three copies of each of two glycopeptides S1 and S2. Haemagglutination-inhibiting and most of the virus neutralizing antibodies are induced by the S1 glycopeptide (Cavanagh *et al.*, 1986). This observation was confirmed by Ignjatovic and Galli (1995) who found that only the S1

glycoprotein can induce protection (Cavanagh *et al.*, 1984); the other proteins M and N cannot.

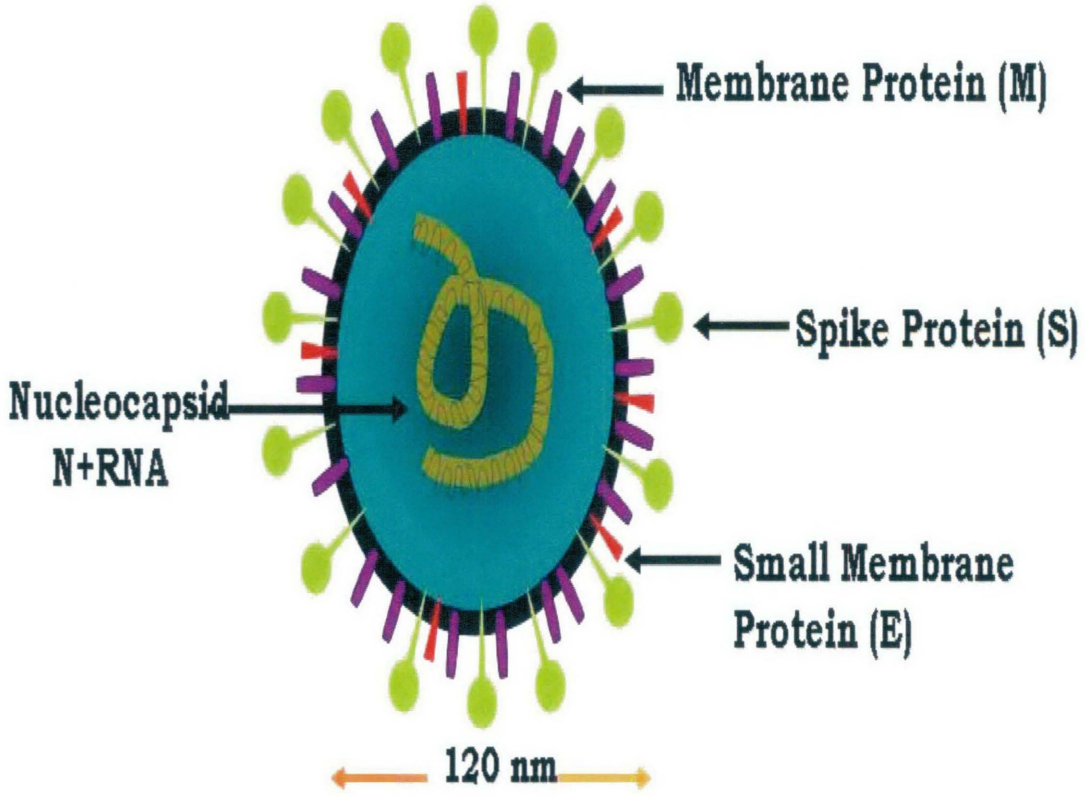

Plate 1.2: Schematic diagram showing the structure of Infectious bronchitis virus particle

The variation in S and N genes is believed to be important because of their role in immunity and virus replication. Both of these genes have been used regularly to study the emerging variants of IBV. There are four small non-structural proteins, 3a, 3b, 5a and 5b and a 3' untranslated region. The 3' untranslated region is responsible for initiation of negative strand RNA synthesis and has also been used to access the genotypic variation amongst the newly emerging IBV strains (Williams *et al.*, 1993).

1.4.1 Growth and Cultivation of virus

Virus can be grown very well in 9 to 12 day old embryonated eggs inoculated by the allantoic route. The yolk sac route of inoculation is unreliable because of the presence of

maternal antibodies in the yolk sac (Sharma and Adhlakha, 1996). Following inoculation in embryonated chicken eggs, the highest concentration of virus can be detected after 36 hrs. The virus titre may start decreasing if eggs are kept in the incubator after death of the embryo. (Cumming, 1967). Dwarfing and curling of embryos is a typical lesion of an IBV-infected embryo.

The virus can also be cultivated in cell culture. Both tracheal organ culture and kidney cell culture have been used in the past. Syncytia formation is the characteristic of infected cell cultures. Chubb and Ma (1974) pointed out that there can be rapid alterations in immunogenicity and pathogenicity of the virus during growth in cell culture. The egg inoculation method is the best for cultivation of the virus as it is a relatively simple technique.

1.4.2 Virus replication and transmission

According to Patterson and Bingham (1976), IBV usually develops in the cytoplasm by the process of budding into cisternae or vesicles. A later study by Tomley *et al.* (1987) found that IBV replicates in the cytoplasm. Virion formation occurs by a budding process at the membrane of the endoplasmic reticulum and not on the cell surface. The virion accumulates in the smooth vesicles. New virus appears 3 to 4 hrs after infection with maximum output per cell being reached within 12 hrs at 37°C. In poultry birds, the initial site of replication is the epithelium of the trachea where IBV can be detected at day one after the infection. From this site, the virus usually spreads to most other organs such as the lung, kidney, oviduct, ovaries, spleen, bursa of Fabricius, liver and most of the intestine. The infection may spread through the blood stream (Crinion *et al.*, 1971a) although Hofstad and Yoder (1966) found difficulty in isolation of virus from the blood stream. They found the highest titre of virus in the trachea and kidney at three and five days post-inoculation, respectively.

IBV spreads rapidly amongst the chickens in a flock. Susceptible birds kept in contact with infected chickens usually develop signs of disease within 48 hrs (Cunningham, 1970). Virus was isolated consistently from the trachea, lung, kidney and bursa. The frequency of isolation varies with the time frame but Alexander and Gough (1977) isolated virus from caecal tonsils and faeces up to 14wk and 20wks respectively. Jones and Ambali (1987)

isolated the G strain of IBV up to 28 wks of age in tracheal and cloacal swabs from birds infected at day-old. Cook (1971) reported isolation of virus from eggs of experimentally-infected laying hens between one and six weeks after infection. Virus was also isolated from the day-old chicks of these hens. Based on these findings Cook concluded that the virus can be transmitted vertically. She further found that the virus can also be isolated from the semen of cockerels for up to two weeks following inoculation, indicating the possibility that the oviduct of susceptible hens and eggs in the oviduct could have been infected with such semen. Cook's findings were supported by McFerran *et al.* (1971) who isolated IBV from newborn chicks and dead-in-shell embryos of infected layer birds. These authors also documented the pathological lesions of dwarfing and curling of embryos collected from the same birds and reported isolation of virus from eggs up to 43 days after recovery from infection. The eggs had been hatched from an infected flock and reared in an IBV free environment.

The nature and persistence of IBV infection remains undefined. The extended and intermittent shedding of virus from infected birds is a potential risk for flock-to-flock transmission via contamination of personnel or equipment. The frequency of airborne transmission is unknown but evidence of transmission of IBV over a distance of 1200 yards was reported (Cumming, 1970a). Contaminated water could also be a source of infection as IBV has been found to survive in water (Jordan and Nassar, 1973). The role of vehicle transmission in the epidemiology of IBV has not been clearly established. IBV can be transported either symptomatically or asymptomatically by some galliform and non galliform birds (Cavanagh, 2005).

The incubation period of IBV is 18 to 36 hrs but mostly it depends upon the dose and route of infection. Natural spread requires about 36 hrs or more (Hofstad, 1967). The rate of mortality from the disease depends mostly upon the virulence of the infecting serotype, age and immunity status of the hens and presence or absence of secondary bacterial infection. However, Hofstad (1978) reported it to be 25 % in chickens less than 6 wks of age.

Fabricant and Levine (1951) isolated virus from yolk up to 36 days post infection, but this could be less important as antibodies in yolk can neutralize any virus present in yolk. However, these authors predicted the risk of breakage of infected eggs and subsequent virus infection in newly hatched chicks. However, maternal antibodies in day-old chicks can

protect them very well under such circumstances. Moreover, the vertical transmission of IBV is of little importance as maternal antibodies in chicks can easily neutralize the transmitted virus from an infected mother. Cook and Garside (1967) found that progeny hatched from IBV-infected hens were free from IB based on serum neutralization titres. This experiment did not clearly explain whether true egg transmission of IB can occur or not, as the infected dams used in this experiment were reinfected. Virus reinfection could have stimulated antibody production in the dams and these antibodies might have neutralized any virus deposited in the eggs. Cook (1971) also established the fact that transmitted virus can neither initiate infection nor protection.

1.4.3 Host range

The chicken is considered to be a natural host for infectious bronchitis virus infection and chickens are susceptible at all ages. However, there are reports of isolation of IBV from pheasants with respiratory signs and drops in egg production (Gough *et al.*, 1996; Spackman &Cameron, 1983). Hence, the transmission of virus infection from pheasants to poultry birds is possible. Earlier, Cumming (1969b) made an attempt to study the possible role of magpies in virus transmission and found it of little importance. The virus does not appear to be of public health significance. Bronchitis virus isolated from humans is morphologically similar, but antigenically different, from IBV (Cavanagh, 2003).

Recently, Liu *et al.* (2005) isolated from peafowl a coronavirus having close genomic identity with an infectious bronchitis virus vaccine strain. He found the virus isolated from teal to be extremely nephropathogenic in chickens and concluded that these wild birds have the potential to transmit virus to susceptible chicken populations. Ito *et al.* (1991) reported the isolation of a coronavirus from guinea fowl, which was antigenically related to IBV. In Australia, coronavirus was isolated from racing pigeons suffering from respiratory distress (Barr *et al.*, 1988). This virus showed typical IBV lesions in embryos but, after reinoculation of healthy pigeons and chickens, chickens were affected but not the pigeons. This suggests that the pigeons might have suffered from another concurrent infection which caused respiratory distress. However, the transmission of virus infection from pigeons (sometimes as symptomless carriers) to poultry birds cannot be ruled out. The coronavirus isolated from turkeys was genetically and antigenically related to IBV (Guy *et al.*, 2000). However, due to differences in their sequences of S proteins, these viruses were considered

a separate species. The number of cases of coronavirus in avian species has doubled in recent years (Cavanagh, 2005). The wide host range may be responsible for rapid mutation of this virus.

1.4.4. Strain variation in pathogenicity

Besides the respiratory tract IBV infects most of the epithelial linings in the kidney, alimentary tract, and oviduct. However, strains of IBV differ in virulence or pathogenicity for different systems. The majority of IBV strains do not induce mortality acting alone but variable mortality rates are obtained indicating the differing pathogenicity of the respective strains (Smith *et al.*, 1985). The virulence and severity of pathogenesis of IBV is not yet well defined, although the stasis of cilia in IBV infection is usually used to assess the severity of the infection. Cubillos *et al.* (1991) found variation in tracheal pathogenicity in birds infected by four IBV isolates isolated in Chile. On the other hand, Dhinakar Raj & Jones (1996a) and Cook *et al.* (1976) found little or no difference in IBV infected tracheal organ cultures. Most of the literature clearly indicates that most of the IBV strains, along with Massachusetts serotype, produce predominantly a respiratory syndrome (Cavanagh and Naqui, 1997). However, most of these strains may not act alone in infection but may be accompanied by secondary bacterial infections.

Most of the IBV strains affecting the respiratory tract can occasionally cause kidney damage (Jones, 1974). The high predilection of some IBV strains towards the kidney and their virulence was first reported in Australia by Cumming (1962), who reported the T strain of IBV to be highly nephropathogenic. T-strain can also cause a mild respiratory syndrome by an independent effect (Ratanasethakul and Cumming, 1983a). Clinical signs were more prevalent in the respiratory tract of IBV–M41 infection as compared to IBV–T infection due to severe tracheal damage and inflammatory cell infiltrate (Fulton *et al.*, 1993). According to Ignatovic *et al.* (2002), IBV can be classified into two major categories as either nephropathogenic or respiratory strains, and nephropathogenic strains of IB differ in their pathogenicity. It has been reported that the T and Belgian strain of IBV are nephropathogenic and can induce high mortality of up to 50 % in day-old specific pathogen free (SPF) chickens (Chong and Apostlov, 1982; Meulemans *et al.*, 1987). Several strains of IBV have been reported to be isolated from the intestine such as ECV2 strain from the oesophagus (Lucio and Fabricant 1990), 793/B (Dhinkar Raj and Jones ,1996 c) and G

strain which is classified as enterotropic due to its prolonged persistence in the intestinal tract (El Houadfi *et al.*, 1986). However, all isolated strains were mildly pneumotropic.

Roberts (2005) found increased antibody titres in most of the unvaccinated birds infected with T strain as compared to N1/88 strain of IBV where only some birds became positive for IBV antibodies. Variation in the ability to cause disease and loss of egg production and quality were reported by Guittet *et al.* (1988). Cook and Huggins (1986) found that the same variant strain of IBV caused only a small decrease in egg production but marked effect on egg shell colour. However, Roberts (2005) found mild effects of IBV challenge on internal egg quality and egg shell quality. Pathogenicity of IBV strains also varies in oviduct infection. M41 and T strains of IBV are more pathogenic as compared to Connecticut and Iowa in day-old chicks (Crinion and Hofstad, 1972a) but strain variation in pathogenesis on the oviduct in adult laying hens is not yet defined.

In Australia, the antigenic diversity of IBV strains was clear following the work of Wadey and Faragher (1981), who established the antigenic relationship between 17 Australian IBV strains by dividing them into 9 subtypes. However, this study did not state pathological variation. Ratanasethakul and Cumming (1983a) studied the variation between pathogenicity of vaccine strains for kidney and trachea. Of the two vaccine strains currently used in Australia, Vic S affected both kidney and trachea but A3 replicated only in the kidney. There has been a major antigenic (Ignjatovic *et al.*, 1997) and pathogenic (Ignjatovic *et al.*, 2002) variation in recently-isolated IBV strains which may be attributed to suppression of similar antigenic field strains by effective vaccines.

Recent molecular studies have shown that the spike glycoprotein "S" has a major role in the tissue tropism and emergence of a large number of IBV serotypes. Out of the two glycopeptides S1 and S2 of the S protein, an amino acid sequence in S1 mutates more frequently which is mostly believed to be responsible for serotype variation (Cavanagh and Davis, 1988). Although it has been believed that the antigenic variation in infectious bronchitis virus is due to change in amino acid residues within the S glycoprotein, variation in the N protein has also been recorded in Australian strains of IBV (Sapats *et al.*, 1997a). This region is considered to be highly conserved.

1.5 Tissue tropism of IBV

As the name indicates, initially IBV was thought to be the virus causing a respiratory syndrome but extensive research in later years proved that IBV infects a wide range of epithelial surfaces of poultry birds. Thus, the virus is pantropic and has been associated not only with bronchitis but also with nephritis, drop in egg production and inflammatory changes in the alimentary tract. The variable tissue tropism of IBV is due to the different strains of IBV. The tissue tropism and pathogenesis of IBV infection has been studied in different organs by various methods including histopathology, immunohistochemistry, and electron microscopy.

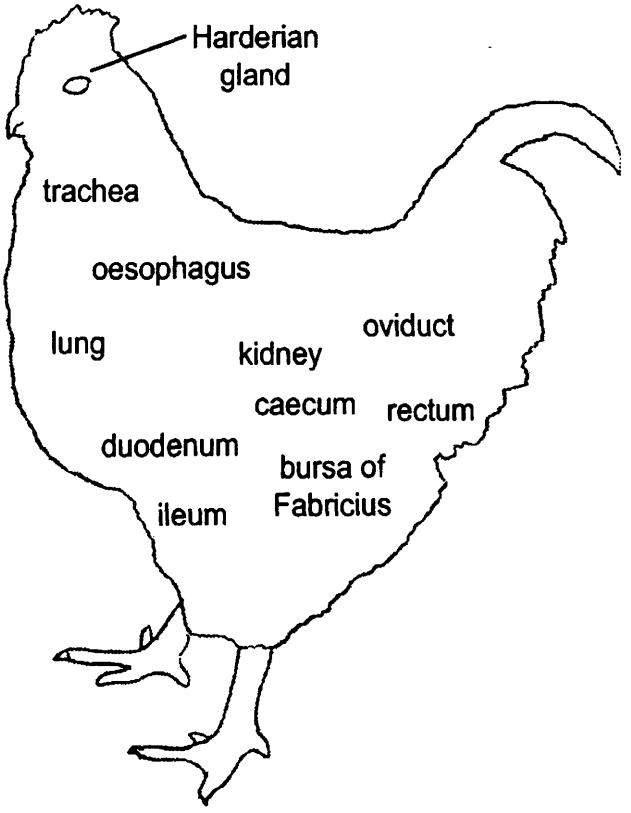

Plate1.3: Schematic diagram showing the susceptible organs in hen's body likely to be affected by various strains of IBV.

1.5.1 Harderian gland

The Harderían gland, which is situated in the posteriomedial aspect of the eyeball, plays a vital role in offering local immunity to conjunctiva and the upper respiratory tract. The gland is covered with connective tissue and divided into lobes (Wright *et al.*, 1971). This tubuloacinar structure produces mostly merocrine secretion. The gland consists of primary and secondary collecting ducts, a main collecting duct and acini. The collecting duct epithelium is lined by cuboidal epithelium whereas the acinar epithelium is columnar. The gland takes up antigen and produces local antibody with the help of resident plasma cells (Randall and Reece, 1996). This gland is responsible for activation, proliferation and differentiation of B cells (Scott *et al.*, 1993).

The Harderian gland plays a very important role in the process of immune responses during IBV infection. Many studies with respect to IBV as an antigen and vaccine have been carried out by investigating the response of this gland. Davelaar and Kouwenhoven (1976) studied the infection of vaccine and field strains of IBV in the Harderían gland of birds and found that a virulent field strain of IBV was responsible for degeneration of plasma cells and lack of lymphoid foci, whereas the vaccine strain increased their number. Hence authors concluded that lymphoid foci and plasma cells were the main cells playing a major role in conferring immunity. Survashe *et al.* (1979) confirmed this view and reported a progressive increase in the number of plasma cells and lymphocytic nodules in the Harderian gland. However, plasma cells with Russel bodies were observed but not counted. Baba *et al.* (1990) investigated the role of the Harderian gland in immunoglobulin production and found that the Harderian gland cells mostly produce IgA, whereas IgM and IgG were in low concentrations. Transforming growth factors and interleukin (IL-6) play a major role in formation of IgA antibodies from plasma cells. IgA protects the mucosal surface from surface antigen by the process of immune exclusion. Increased IgA levels in lacrimal fluid have been reported after IBV vaccination by Toro *et al.* (1997) and also after experimental IBV infection with Massachusetts strain M41 via the ocular-nasal route (Ganapathy *et al.*, 2005). In another study by Toro *et al.* (1996), persistence of vaccine strain virus has been observed in the stroma (interlobular space) of the Harderían gland. These authors predicted that the persistence of plasma cells and lymphoid foci could be an expression of immunocompetence. In all the above studies, unfortunately, no effort has been made to analyse, quantitatively, cells which have a major significance in immune function (plasma

cells, Russel bodied plasma cells, globular leucocytes). Some efforts to quantitate lymphoid cells were, however, made by Survashe *et al.* (1979) but the technique which was used was not fully quantitative.

1.5.2 Trachea

IBV, as the name indicates, has a primary predilection towards the respiratory system and the trachea is the most affected organ in the system. Much research has been conducted on the respiratory tract during IBV infection. Most of the clinical signs such as gasping, snicking or coughing appear during the first or second week of infection, although infection time may be prolonged in the presence of further complications such as secondary bacterial infections.

IBV is mainly epitheliotropic and enters epithelial cells by viropexsis (Patterson and Bingham, 1976) which has been widely explored by Nakamura *et al.* (1991). The virus replicates at high concentrations in the respiratory tract followed by lung, kidney, bursa of Fabricius and alimentary tract (Hofstad and Yodder, 1966). These authors further found that the highly passaged virus did not multiply in the respiratory tract. The pathology of the respiratory tract has been investigated extensively by many workers (McDougall, 1968; Nakamura *et al.*, 1991, Arshad *et al.*, 2003) who observed severe loss of cilia, mucus glands and goblet cells, desquamation of the mucosal epithelium, oedema in the sub-epithelium and occasional heterophilic exudate in the tracheal lumen after 48-72 hrs of infection. Regeneration and chronic inflammatory lesions were observed after 7 days p.i. although lymphoid nodules still persisted in the submucosal region (Purcell and McFerran, 1972). The ultrastructural study conducted by Arshad *et al.*, (2003), observed that Malaysian strains of IBV can cause severe cytopathology in the trachea on day 3 p.i. and regeneration on day 12 p.i. Microscopic respiratory lesions are symptomatically characterised by coughing, sneezing, rales and dyspnoea. In layers, sometimes respiratory signs may not be prominent and could be easily unnoticed (Ignjatovic and Sapats, 2000).

The pathogenicity of IBV strains for the respiratory tract varies greatly. Because of the virus's ability to damage cilia in the trachea both *in-vivo* and *in-vito*, a ciliary movement score has been used to assess the extent of damage caused by virus infection. Both vaccine and field strains of IBV can also enhance the incidence of bacterial infection such as *E coli*

in the respiratory tract (Smith *et al.*, 1985; Matthijs, *et al.*, 2003) which could potentiate mortality in younger chickens. The probable cause of this mortality could be the loss of cilia and depletion of mucus-secreting glands. Mucociliary action plays a vital role in combating against foreign bodies (Ross and Corsin, 1974). A similar effect can also be observed during mixed infection of mycoplasma and IBV (Hopkins and Yoder, 1984; Ganapathy and Bradbury, 1999). According to the findings of Landman and Feberwee (2004), IBV infection can also aggravate the incidence of arthritis in layers. This could be due to spread of *Mycoplasma* to the joints through the IBV-damaged respiratory tract. The intensity of respiratory lesions varies among genetic lines of chickens (C and 15I) (Nakamura *et al.*, 1991). Although Williams *et al.* (1985) observed high mortality amongst chickens infected with a mixture of IBV and E. coli, Vandekerchove *et al.* (2004) observed that mortalites resulting from colibacillosis amongst layers under field conditions are not necessarily associated with IBV. However, Williams *et al.* (1985) used 9 day old chickens in the experiment hence age differences are a possible explanation for such different interpretations.

1.5.3 Kidney

Although the respiratory tract is the first site of predilection for infectious bronchitis virus, many strains of the IBV are nephropathogenic. The strains with higher nephropathogenicity are always associated with heavy mortality (Chong and Apostovlov, 1982). The greater inclination and virulence of virus towards the kidney as well as the respiratory tract was first reported by Cumming (1962) in Australia. IBV can replicate rapidly and can cause severe nephropathogenicity if inoculated through the cloaca (Uneaka *et al.*, 1998). The nephritic syndrome is characterised by increased water consumption, wet droppings and excretion of large amount of urates through the faeces (Cumming, 1963). According to the study conducted by Afanador and Roberts (1994), the loss of water (a diuresis) during infection with IBV could be associated with lower urine osmolality and higher rates of urinary excretion of sodium, potassium and calcium. Australian T strain of IBV is responsible for elevation of plasma uric acid levels and their greater absolute tubular excretion (Afanador and Roberts, 1994) which perhaps could be the cause of passage of large amount of urates with the faeces. Infected birds tend to consume a lot of water. In cold weather, IBV-infected birds consume more water, which leads to cold stress, which could be an augmenting factor for mortality during IBV infection (Cumming, 1963). In kidneys, the virus maintains its

epitheliotropic property and multiplies in the proximal convoluted tubule (PCT), distal convoluted tubule (DCT), and collecting ducts (CD) (Chen and Itakura, 1996). The virus multiplication is microscopically characterised by necrosis of proximal convoluted tubules, distension of distal convoluted tubules, necrotic foci, infiltration of heterophils and lymphocytes in the interstitial space, oedema of Bowmans capsule, urate and granulocytic casts in collecting ducts and heavy interstitial oedema. (Cumming, 1963; Chong and Apostolov, 1982; Owen, 1991; Jolly, 2001). The repair of epithelial cells in the kidney is associated with interstitial lymphocyte infiltration and lymphoid nodules (Chen *et al.*, 1996). Kidney weight relative to body weight has been reported to be reliable index of kidney damage during IBV infection (Afanador and Roberts, 1994).

According to immunohistochemical analysis, epithelial cell proliferation in collecting ducts starts at the stage of degeneration and the process continues until regeneration (Tsukamoto *et al.*, 1996). Tsukamoto *et al.* (1999) with the help gicerin (a novel cell adhesion molecule belonging to the immunoglobulin superfamily) further studied the regeneration process in the kidney. They observed that gicerin expression regulates only during embryonic development and during the phase of cell repair after injury which was initiated by IBV infection in the kidney. Chong and Apostolov (1982) demonstrated virus antigen in all segments of the kidney tubule, although staining was mainly confined to the proximal convoluted tubule. Condron and Marshall (1986) further observed that cytopathological alterations such as change in volume density of mitochondria, change in membrane structures of the proximal convoluted tubule and dilatation of endoplasmic reticulum could disturb the transport of ion and water. As explained by Chen and Itakura (1996), renal failure also could be due to the impairment of fluid and electrolyte transport as a result of IBV induced damage in the lower nephron. Renal lesions after infection with a nephropathogenic strain of IBV may be delayed due to immunosuppressance. In one study, Chen and Itakura (1997) studied the mixed renal infection in IBV + IBD infected birds and found that renal lesions in dual infected birds were long-lasting. Similar findings were also recorded by Chandra (1988) in bursectomized and IBV-infected birds.

Chen and Itakura (1996), in their ultrastructural study, showed virus particles in proximal, distal tubules, collecting tubules, Henle's loop and collecting ducts. Urate and granulocytic casts in the collecting ducts are potent indicators of urolithiasis, which is a common manifestation of IBV infection in layer birds (Cavanagh and Naqui, 1997). The

nephropathogenicity of IBV varies greatly according to strain of virus. Chen and Itakura (1996) observed that the intensity of infection of IBV may vary from region to region in the same organ. Australia has a history of nephropathogenic strains since the 1960s, although many respiratory strains have been isolated recently (Ignjatovic *et al.*, 2002). These authors further speculated that the change in pathogenicity could have been contributed to by the widespread use of infectious bronchitis vaccines based on nephropathogenic strains.

1.5.4 Intestine

The multiplication of IBV in enteric tissues has been demonstrated. The T strain of IBV has been isolated from cloacal swabs and faeces (Alexander and Gough, 1977). Massachusetts serotype vaccine virus has been isolated also from cloacal swabs at 63 days p.i. (Naqui *et al.*, 2003) although faeces or cloacal swabs used for virus isolation could possibly have been contaminated by the urine/urates of infected birds. Isolation of virus from the gut contents may provide information regarding the persistence of tropism of this virus for the intestine. Virus of the Massachusetts strain M41 has been isolated from oesophagus, proventriculus, duodenum, jejunum and caecal tonsils (Lucio & Fabricant, 1990). Strains of IBV can be enteropathic by virtue of their prolonged persistence in the gut, as compared to other organs. Certain strains of IBV such as G strain (Jones and Ambali, 1987) are enterotropic rather than pneumotropic and cause diarrhoea only in broilers. Despite several reports of viral persistence in gut tissue, detailed histopathological and gross changes have not been clearly elucidated. McDonald *et al.* (1983) isolated virus only rarely after crop inoculation with H52 and H120 vaccine strains. Physiological factors such as various digestive enzymes and variation in pH may be major constraints for the growth of virus in the intestinal tract.

Escorcia *et al.* (2002) observed virus particles of Mexican strains of IBV in the proventriculus and gizzard of specific pathogen free embryos. However, the only microscopic change observed in this study was significant reduction of mucus thickness. Escorcia *et al.* (2000) also detected virus from the caecal tonsils of broiler chickens. It would be, however, interesting to study whether Australian strains can multiply in the intestine with similar effects. Differences among viral strains of IBV most likely explain differences regarding the tissue tropism. Alvarado *et al.* (2006) detected Arkansas (Ark) and Massachusetts (Mass) serotypes of IBV in caecal tonsils in commercial broiler flocks after vaccination at day one by coarse spray. The Massachusetts type was detected in the early

stages whereas the Arkansas strain was recovered at later stages of infection. However, whether both strains were persistent, with or without pathology, was not studied. The distribution of virus in different regions of the body is given in table 1.2.

Table 1.2: Titres and distribution of infectious bronchitis virus in different organs of chicks

Tissue	Virus titre (Log $_{10}$) on days following inoculation					
	Day 1	Day 3	Day 5	Day 7	Day 10	Day 14
Trachea	5	5	5	5	3	2
Kidney	3	4	5	5	4	3
Proventriculus	5	3	3	3	3	3
Duodenum	4	4	3	3	3	3
Jejunum	0	2	2	0	0	0
Rectum	3	5	6	4	4	4
Bursa	2	3	5	4	3	3
Caecal tonsils	2	3	4	4	4	4
Ileum	3	5	3	3	3	3
Oviduct	-	3.34	-	-	-	-
Ovary	-	3.20	-	-	-	-

(Details of above table are taken from Ambali & Jones, 1990 and Crinion *et al.*, 1971b)

1.5.5 Muscle

Gough *et al.* (1992) isolated 793/B strain of infectious bronchitis virus from cases of pectoral myopathy but, according to Dhinkar Raj and Jones (1997a), the serum creatinine level, which is indicative of muscle damage in the myopathic birds, was unchanged so they further concluded that immune complexes forming in the muscle area could be causing pectoral myopathy.

1.5.6 Oviduct

Isolation and multiplication of infectious bronchitis virus in epithelial linings and tubular glands of the oviduct is still under debate. Despite the fact that the deleterious effect of IBV on the egg industry is well known all over the world, very few studies have been undertaken regarding the details of pathogenesis of IBV in the fully functional oviduct between 1957 and 2007. This could be due to the amount time required, maintenance costs and difficulty in keeping birds under strict isolation. Unfortunately, in today's world, there is a dearth of funds for such basic field studies, and reliance on molecular work has increased irrespective of its practical significance in the field.

After finding virus in the yolk of freshly laid eggs and in a fully formed egg in the oviduct of a mature hen, Fabricant and Levine (1951) attempted to isolate IBV from the egg yolk under controlled conditions. These researchers isolated virus from the 2nd to the 6th week post-infection. Cook (1971) studied the vertical transmission of IBV by isolating virus up to the 6th week post-infection from the vitelline membrane of eggs laid by infected hens and for up to 4 weeks from their chicks. However, it is important to note that a hen was infected through contact with an infected cockerel. Hence the study does not explain whether the virus had actually entered the egg through an infected oviduct or via semen. Further, McFerran *et al.* (1971) reported IBV lesions in newborn chicks and dead-in-shell embryos. There are several possibilities here: considering the findings of Cook (1971), embryos had not been protected by maternal antibodies, respective hens had been infected before 3 weeks (nothing has been mentioned about time of infection in McFerran's study) or there may have been external contamination of embryos by infected semen. However, nothing is mentioned about cockerels in McFerran's study. Had the virus been transmitted from an infected oviduct, it could have been confirmed by histopathological studies or by isolation of virus from the oviduct of the hen. Unfortunately, in both of the studies, no such attempts were made.

Sevoian and Levine (1957) and Butler *et al.* (1972) studied the pathology of in the fully functional oviduct using the Massachusetts strain of IBV. Both of these studies reported glandular dilatation in the magnum, although no virus isolation was attempted in either experiment. Also, Sevoian and Levine (1957) failed to keep a control flock IBV free, but found uniform changes in the oviduct of infected hens. Pathology of the oviduct was also

studied by Crinion *et al.* (1971a) in chicks. Later, after maintaining that infected flock until birds were in lay, Crinion found 17 % patent and 83 % non-patent oviducts (Crinion *et al.*, 1971b).

Crinion & Hofstad (1972b) observed pathogenicity of 7 and 55 times passaged IBV in chickens at different ages from day old to 29 days and found that low passage virus was infective at all ages. Crinion & Hofstad (1972a) also compared the pathogenicity of three different American serotypes of IBV and the Australian T strain. These authors regarded T strain as pathogenic for younger chickens, although less pathogenic than the Massachusetts strain. Jones and Jordan (1970& 1972), after infecting day old chickens, recorded developmental abnormalities in the oviduct. However, how the virus made the oviduct abnormal remains unexplained. It was not possible to support the findings pathologically due to the limited amount of oviduct tissue in chicks. Electron microscopic studies would have been helpful to understand this and can be conducted with a very small sample of tissue. These authors could not isolate virus from the abnormal oviducts.

In another study, Jones and Jordan (1971) successfully isolated virus from various parts of the oviduct on 6^{th} day post-infection. However, after 6^{th} day p.i. they could not isolate or locate the viral antigen with fluorescent antibody technique in the oviduct of all infected hens. Again, details of pathogenesis remained unexplored. Lucio and Hitchner (1970) failed to isolate the IB virus by immunofluorescence in the oviduct of infected birds. McMartin and McLeod (1972) observed abnormal oviducts in naturally-infected laying hens and supported the view of Broadfoot *et al.* (1956) that younger birds are more likely to be affected than older birds. Despite these findings in the field, Peters *et al.* (1979), using oviduct organ culture, showed that oviduct explants prepared from chickens aged 1 to 36 weeks were equally susceptible to the H52 vaccine strain of IBV. However, he observed that resistance of kidney explants increased with the age of the chickens. The IBV vaccine strain (H52) used in the above experiment has been reported to be pathogenic to the oviduct of young chickens, with the potential to induce permanent damage (Duff *et al.*, 1971). However, it should be noted here that the extent of pathogenicity of the H52 strain has not been studied in laying hens *in-vivo*.

Isolation of IBV from the oviduct during field outbreaks in layers has been reported from India. Pradhan *et al.* (1982) attempted isolation of IBV from 50 oviduct samples collected in

the field from chicks aged 1 to 6 weeks. They isolated IBV from 3 and Newcastle disease virus (NDV) from 24 samples. The NDV finding was not surprising as all chicks in this study had been vaccinated against NDV, indicating that NDV can remain in the oviduct for a considerable amount of time. However, such findings regarding IBV have yet to be confirmed. Moreover, the strains isolated were identified as the Massachusetts type by virus neutralization test. Isolation of IBV from clinical cases was also reported by Maiti *et al.* (1986) who isolated virus from cloacal swabs and reproductive organs of hens of 6 to 16 months of age with a history of drops in egg production. Five out of 30 cloacal swabs and 2 out of 22 pooled ovaries and oviduct samples were positive for IBV. In this study, the isolation of IBV from clinical cases indicates the long term IBV carrier status of the infected hens. Another report of IBV isolation from field cases was described by Christopher *et al.* (1996). Pradhan *et al.* (1984) compared the sensitivity of tracheal and oviduct organ culture and concluded that oviduct organ culture was more efficient when compared to tracheal organ culture. The idea of stimulating development of the premature oviduct (the term used was "precociously-developed oviduct") was firstly employed in this experiment. Day-old chickens were injected with oestrogen prior to collection of oviduct tissue for organ culture. Histopathology of oviduct organ culture tissue showed loss of cilia and epithelial degeneration on the 6^{th} day after virus inoculation. Similar histopathological changes in the oviduct were recorded on the 10^{th} day p.i. by Sevoian and Levine (1957) in their *in- vivo* studies with White Leghorn laying hens.

Dhinakar Raj and Jones (1996a) compared the virulence of different IBV strains in tracheal and oviduct organ cultures. The extent of virulence was detected using the relative ciliary score, immunofluroscence (IF), calmodulin assay and virus titres. The highest virus titre in the oviduct organ culture was recorded at 2 and 3 days after inoculation. On other hand, relative ciliary score was low at 5 and 6 days after inoculation. The results regarding timing of ciliostasis differed from the earlier studies of Pradhan *et al.* (1984). This method is an excellent way to check the virulence of different IBV strains within a short time, and with relatively little labour. However, further studies are essential to test their virulence in commercially hens in full lay, although these authors mentioned this as a major constraint.

Some *in-vitro* experiments have been carried out using oviduct organ cultures. Dhinakar Raj and Jones (1996b) showed that both field and vaccine strains of infectious bronchitis virus can multiply in organ cultures prepared from precociously developed oviducts. This

suggests that the virus has the capability of multiplying in the mature oviduct. However, from this study it would be difficult to predict the interaction between IBV and the fully functional oviduct, as many other factors such as bird age and breed, temperature, and other environmental factors can influence the pathogenicity of the virus. Also, both at the cellular and functional levels, there can be major variation between precociously-developed oviducts and the naturally developed fully-functional oviduct of hens.

Dhinakar Raj and Jones (1996c) described IBV specific antibodies against the G strain of IBV and a significant correlation between titres of oviduct washings and egg production. Infectious bronchitis virus causes severe pathological lesions such as cilia loss, glandular dilatation, oedema and inflammatory cells in the subepithelium, lymphoid cell infiltration around blood vessels in the muscularis layer, features which were also reported during infection with American strains of IBV in hens by Sevoian and Levine (1957) and in young chickens by Crinion and coworkers (Crinion *et al.*, 1971 b; Crinion & Hofstad, 1972 b).

There are few reports available regarding isolation of virus from field outbreaks (Maiti *et al.*, 1985). Histopathological effects such as loss of cilia in the trachea and oviduct could increase the incidence of secondary bacterial infection and could induce mortality in young chickens, although this finding needs to be confirmed. The effects of mixed infection in the oviduct need further attention and vaccine strains need to be considered for any potential to damage the oviduct. No detailed study has been conducted to study the effects of vaccine strains of IBV on the fully functional oviduct.

It has been also documented in the past by McMartin (1968b) that different serotypes of IBV may vary in their pathogenicity for the reproductive tract. This was later confirmed in chickens by Crinion and Hofstad (1972a). McMartin found that, after severe drops in egg production, production may increase after two to three weeks but reaches only suboptimal levels. Areas of glandular hyerplasia in the oviduct may be a contributory factor to changes in albumen proteins (Butler *et al.*, 1972) which impart thick constituency to the albumen. Due to this effect, the proportion of thin and thick albumen changes and causes watery whites. The severity of IBV infection for the reproductive tract varies from hen to hen in the same flock (McMartin, 1968b) and perhaps with respect to age of laying hen (Broadfoot, 1956, McMartin and McLeod, 1972). However, *in-vitro* studies with oviduct organ cultures (Peters *et al.*, 1979) proved that the oviduct of all ages is susceptible. This was further

initially studied by experimental inoculation of hens by Sevoian and Levine (1957) who observed effects of virus infection in fully functional oviducts.

The fact that IBV causes a decrease in egg production has been known for at least 56 years, during which time many new strains of IBV have evolved. It is, however, interesting and surprising that very little effort has been either made since 1973 to conduct field experiments and that there are many facts such as thin-shelled eggs, temporary or permanent cessation of egg production and watery whites during IBV infection that remain unresolved. However, watery whites have been explained to some extent (Butler *et al.*, 1972, Davidson, 1986)

1.6 Effects of IBV on male reproductive system

Severity of IBV infection can be greater in males as compared to females (Cumming, 1967). Live attenuated IBV vaccination in roosters can cause epididymal stones, and decreased sperm production and serum testosterone levels (Boltz *et al.*, 2004). IBV has been also isolated from the sperm of infected roosters (Cook 1971). This highlights the possibility of sexual transmission of IBV in breeder flocks. Mahecha *et al.* (2002) observed a negative correlation between the incidence of epididymal stones and presence of IBV antibodies. It would be interesting to study the effects of pathogenic strains of IBV on the male reproductive tract.

1.7 IBV vaccines and oviduct

As new strains of IBV are evolving continuously throughout the world, development of protective vaccines and protocols to achieve a standard level of immunity and cross protection against various strains has become a challenge to poultry researchers. Scientists have developed various vaccines and protocols to prevent this viral disease but the severity of the disease varies from place to place and flock to flock. Although there is continuous evolution of the virus, vaccination is the only reliable way to prevent production losses. Two types of vaccine have been tried in various experiments, live attenuated and inactivated vaccines. The main type of vaccine used in commercial flocks is live attenuated vaccine. Many studies have been conducted using live attenuated or inactivated vaccines, investigating protection offered either to the trachea (Winterfield *et al.*, 1972; Gough and Alexander, 1979; Darbyshire and Peters, 1984 and Cook *et al.*, 1999) or kidney

(Ratnasethkul and Cumming, 1983b; Pensaret and Lamberchts, 1994; Cook *et al.*, 2001) or both (Ignjatovic and Galli, 1994; Song *et al.*, 1998) for the extent of protection offered by current vaccines. This information indicates that most of the vaccines and vaccination programs for laying and breeding birds have been assessed by the level of resistance of either the respiratory or urinary systems rather than the reproductive system. Investigation of the response of the oviduct to inactivated IBV vaccine was studied by McMartin (1968a) in birds infected with *Mycoplasma gallisepticum* and challenged with IBV.

Dhinakar Raj and Jones (1996c) found IgA and IgG antibodies in IBV infected oviduct washes. They correlated the haemagglutination and ELISA titres of oviduct washes with egg production and suggested this as a possible way of assessing the cause of a decline in egg production. Collection of oviduct washes would be impracticable in the field situation and little is known about the extent of virus clearance by antibodies generated locally in the oviduct. It would be interesting to study the titres of oviduct washes in vaccinated, challenged hens to assess the extent of protection of the fully functional oviduct under field conditions. Also, such studies should include histopathology or ultramicroscopic studies to confirm the findings.

Dhinakar Raj and Jones (1996b) conducted an *in-vitro* challenge by a pathogenic strain of IBV in immunised chickens with a precociously-developed oviduct and found that immunisation offered little protection based on virus titre and ciliary activity. Oestrogen-treated precociously-developed oviducts are an elegant way to assess the efficacy of vaccines and the extent of damage caused by pathogenic strains to the oviduct. This technique saves time and the effort required to raise an IBV-free flock to maturity. However, the effect of oestrogen on the individual immune response cannot be neglected. Long term experiments are essential in commercial layer flocks to study the protection and pathogenicity levels of vaccine and virulent strains.

Dhinakar Raj and Jones (1997b) observed that vaccine strains multiply in the oviduct without any evidence of ciliostasis. One would expect attenuated vaccine to work like this. Although this is a helpful method of assessing the efficacy of vaccines *in-vitro, in-vivo* trials are needed to assess the potency of vaccines. Moreover, ciliary activity in precociously-developed oviducts during this experiment was not uniform which could influence the findings. These authors found that the virus is capable of multiplying in the embryonic

oviduct, although the extent of pathogenicity was not described. At this stage of development, the oviduct should not be damaged to a great extent owing to the presence of maternal antibodies.

Jolly (2005) reported mild pathology in the magnum and shell gland of hens vaccinated in the rearing phase and challenged in early lay, although she reported a transient decrease in Haugh Units. This could be because hens were sacrificed at 2 weeks post infection and by that time tissue might have been in the reparatory phase. Had the birds been killed in the initial phase of infection, the histological changes in the oviduct may have been more pronounced. Also, it would not be prudent to arrive at any conclusion regarding the protection offered by vaccine strains for the oviduct because of the absence of an unvaccinated challenge group in the study.

In another study, Sulaiman *et al.* (2004) observed that regular revaccination in laying hens can negatively affect production and egg shell quality. In his study, however, there was no histopathological backing for this finding. If shell quality was deteriorating along with production as the direct effect of IBV vaccination, there may have been pathology in the oviduct. This underlines the need to study the effect of vaccine strains on the oviduct, as little is known about the extent of damage or effect on development of the oviduct caused by currently-available vaccine strains.

Chew *et al.* (1997) studied the effect of attenuated infectious bronchitis virus and a vaccine strain on the oviduct after inoculating virus in 18 day old embryos or newly-hatched chicks. The study was based on histological scoring and electron microscopy. There was no pathology in the oviduct, probably due to the presence of maternal antibodies. These authors further stated that the vaccine strain of IBV was not pathogenic for the oviduct. However, had the birds been maintained up to sexual maturity, it would have provided more information regarding the impact of viral infection on the development of the oviduct. Also, vaccination of embryos requires skilled labour and is time consuming as compared to other routes of infection.

Gough and Alexander (1978) were unable to isolate virus in the oviduct of hens vaccinated at three weeks of age and challenged with the M41 strain at 22 weeks after vaccination. Hens were killed on the 4^{th} day p.i. It is difficult to draw conclusions about the protectivity

of the vaccines for the oviduct as, according to earlier studies, virus has been isolated from the oviduct between days 5 to 11 p.i. (Jones and Jordan, 1972). It would have been more informative if the experiment of Gough and Alexander had sampled birds later than 4 days p.i.

Box *et al.* (1988) stated that live IBV vaccines should be avoided on layer farms owing to their deleterious effects on egg production. Jolly (2005) concluded that rearing phase vaccination is capable of protecting egg internal and external quality. Despite these facts, regular IBV vaccination during the laying cycle is a common practice. IB revaccination could be disadvantageous (Roberts *et al.*, 2004) and the extent to which regular IBV revaccination or intercurrent IBV infection can induce microscopic pathology in the oviduct of the mature laying hen needs further investigation.

1.8 Egg quality and production during IBV infection

The definition of egg quality varies but, in general, it can be defined as the visual and physical characteristics of an egg which affects its acceptability in the market or hatchability in breeder bird flocks (Roberts and Ball, 2004). Along with disease, there are many other factors which could be responsible for poor egg quality (Roberts, 2004) but only virus (disease) effects will be discussed here.

From its formation up to its supply to the consumer, many factors can influence the quality of an egg. Consumers in some countries including Australia prefer brown shelled eggs, in contrast to the United States where white shelled eggs are the norm, so maintaining colour uniformity is an additional challenge for the producer.

In layers, IBV reduces egg production and causes deterioration of internal quality and egg shell quality. (Sevoian and Levine, 1957; McDougall, 1968; Munner *et al.*, 1986). Besides IBV, there are many other diseases such as Newcastle disease caused by a paramyxovirus, and egg drop syndrome caused by an adenovirus which can affect egg production. Some other infectious agents such as *Mycoplasma* can enhance the effects of IBV (McDougall, 1968). IBV is known to cause a decrease in or cessation of egg production but the mechanism is still unknown (Dhinakar Raj and Jones, 1997a). Some research has been done to study the possible causes of inferior internal quality during IBV infection. Jones and

Jordan (1972) established the possibility that some virus strains have the potential to damage the cells of the upper part of the oviduct which could be a cause of watery albumen or "watery whites". One possible cause of watery white or thin albumen as described by Butler *et al.*, (1972) is dilatation of glands or loss of mucopolysaccharides from the surface epithelium of the magnum. The surface epithelial cells of the magnum (particularly the mucus-secreting cells) secrete ovomucin which imparts a thick consistency to the albumen Wyburn *et al.*, (1970). Davidson (1986) also reported the loss of mucopolysaccharides from the surface epithelial cells of the magnum in hens producing watery whites. IBV tends to multiply in mucus-secreting cells (Dhinakar Raj and Jones, 1997 a). Jones and Jordan (1971) demonstrated IBV in the surface epithelium of the oviduct by immunofluorescent staining. Hence, loss of mucopolysaccharides from the surface epithelium could be a virus induced phenomenon. The loss of mucopolysaccharides from the surface epithelium and gland dilatation in the magnum has also been reported by Butler *et al.*, (1972) during IBV infection in White Leghorns. However, the mechanism by which virus is causing loss of mucopolysaccharides from individual cells is not yet known.

IBV also affects egg shell quality with infection in layer flocks resulting in thin, corrugated and misshapen eggs (Hill and Lorenz, 1956, Sevoian and Levine, 1957, Cook, 1971). However, how IBV causes the formation of poor quality of egg shells is not yet clear. Nephropathogenic strains of IBV have been reported to cause extensive damage to the tubular epithelium of the collecting duct and distal convoluted tubule (Cordon and Marshall, 1986, Chen and Itakura, 1996). Jolly (2005) suggested that inferior shell quality was due to lower plasma calcium levels resulting from reduced calcium ion reabsorption due to distal tubular damage during IBV infection. However, low plasma calcium levels during IBV infection have not been widely reported, in fact they have not been evaluated in most instances. Also, the etiology of deterioration of egg shell quality during infection with nephropathogenic strains of IBV does not explain the reason behind poor shell quality during infection with respiratory strains of IBV.

Thin shelled eggs, however, could not be considered as a pathognomic lesion during IBV infection, as poor shell quality could be the result of other respiratory diseases (Hill and Lorenz, 1956). Also, other factors such as age of hen (Roberts 2004), stress (Brackpool, 1995, Leary 1999), mycotoxins (Zaghini *et al.*, 2005) and dietary composition (Ronald *et al.*, 1985) are responsible for poor shell quality. Physiological stress induced by IBV

infection could affect egg shell quality, although it is essential to study and confirm the exact mechanism by which IBV affects egg shell quality. Sevoian and Levine (1957) observed pathology in the surface epithelium of the tubular shell gland and shell gland pouch of IBV infected White Leghorn hens. It is possible that damage to the surface epithelium of the tubular shell gland could have altered the formation of mammillary cores which are the foundation of the egg shell (Brackpool, 1995). Also, pathology in the shell gland could have been responsible for alteration in calcium deposition during egg shell formation.

Another peculiar finding during IBV infection in layers is cessation of egg production (McMartin and Macleod, 1972) either temporarily or permanently (Dhinakar Raj and Jones, 1997a) the reason of which is still not known (Jolly, 2005). However, different strains of IBV can produce varying effects from loss of shell colour without drop in egg production (Cook and Huggins, 1986) to egg production drops of up to 50% (Hopkins and Beard, 1985). The cessation of egg production due to decreased feed and water intake during IBV infection has been reported by Sevoian and Levine (1957) indicating an indirect action of virus on the oviduct. Crinion (1972) studied the changes in egg quality of hens infected at an early age.

1.9 IBV in Australia

Australia has a history of nephropathogenic strains of IBV which were first described from the state of New South Wales. After the first report of IBV isolation from cases of uraemia in hens by Cumming (Cumming, 1962), Cumming conducted a range of experiments to explore this virus infection in detail. He firstly made an attempt to infect other species (Cumming, 1969b) and concluded that further study is essential in this context because of reports of IBV infection in other avian species in other countries. Cumming (1969a) surveyed layer flocks serologically and found that 65% of flocks were IBV positive in New South Wales at that time. Cumming (1970b) evaluated the response to inactivated vaccine in IB-infected chickens and found that the Massachusetts-type inactivated vaccine strain offered good protection to T strain-induced nephrosis. He also found in the same study that cold stress exacerbated the effect of IBV. His research was limited to cockerels. Nevertheless, frequent reports of IBV outbreak in vaccinated flocks were reported by Chubb *et al.* (1976).

A field trial assessing the efficacy of IBV vaccines was conducted by Ratanasethakul and Cumming (1983a) with two vaccines strains, A3 and Vic S. These two vaccine strains are currently in use in the Australian poultry industry. Vaccine strain A3 was developed from the field strain N1/62 isolated in Armidale in 1962 which was passaged 25 times. Vic S vaccine was developed after the 20^{th} passage in chicken embryos of a strain isolated in Victoria (Ratanasethakul and Cumming 1983b). The efficacy of these vaccines in protection of the trachea and kidney was studied following vaccination by various routes (Ratanasethakul and Cumming 1983a, 1983b). However, no research has yet been conducted regarding the pathology of vaccine strains in the oviduct and the extent of protection offered by vaccine strains to the oviduct using different vaccination protocols.

A3 seemed to more protective against nephropathogenic strains than respiratory strains (Ratanasethakul and Cumming 1981). However, Vic S can initiate pathology in the trachea and kidney and appears to protect the kidney of broilers against nephropathogenic T strain at 15 days of age (Afanador and Roberts, 1994). Ignjatovic and McWaters (1991) first reported the strain N1/88 which was isolated from a vaccinated broiler flock. Using monoclonal antibodies developed against three structural proteins of IBV, these authors divided the strains of IBV into five antigenic groups. Later, the two Australian strains of IBV, T and N1/ 88, were placed into different genotypic groups by Sapats and coworkers as the strains vary both in the nucleocapsid (Sapats *et al.*, 1996a, 1996c) and glycoprotein sequences (Sapats *et al.*, 1996a). Protective effects of vaccine in layers have not been studied extensively in Australia. Sulaiman *et al.* (2004) observed deleterious effects of regular revaccination on shell quality and production and concluded that revaccination or revaccination at the time of moulting could be a disadvantage. In this study, other factors such as handling stress or moulting stress might have influenced egg quality. Jolly *et al.* (2005) studied the effects of IBV challenge in vaccinated birds and confirmed that, while regular revaccination could be helpful, careful vaccination during the rearing phase may be a more practical approach.

New variants are still evolving in Australia more than 40 years after the first isolation of IBV. During isolation of IBV from recent out breaks in New South Wales, Ignjatovic *et al.* (2006) found the strains to be different from the earlier two subgroups. All strains were isolated from vaccinated broiler flocks The glycoprotein (S1) gene sequences of these recently isolated virus are markedly different although nucleocapsid (N) gene sequences are

similar to IBV strains from subgroup 1. However, the extent of pathogenicity of these strains for layer flocks is not known.

The uterotropism of Australian strains of IBV in still speculative. It is known world-wide that IBV can be devastating to the layer industry by having negative effects on internal and external egg quality. In Australia, IBV is regarded as a problem for the egg industry. Despite a lack of knowledge about the extent of pathogenicity of Australian strains for the oviduct, and IBV being a potential threat to egg industry, a low priority has been given to studying the effects of IBV on layers. Vaccination/challenge studies are necessary to investigate the impact of recently-isolated virus strains on layers.

The details summarised in Table 1.3 clearly indicate that IBV has been isolated regularly since 1962 and has kept changing its antigenicity. This could be due to constant mutation and recombination of the virus. Although it would be an impossible task to conduct field trails to assess utetropism of all of the above strains, there is a need to assess the extent of pathogenicity of different subtypes of virus with broad tissue tropism. Also it is important to conduct *in-vitro* trials to assess the extent of protection conferred by the current IB vaccines to the oviduct.

Table 1.3. Details of Infectious bronchitis virus isolated in Australia.

Number	Name of the strain/Isolate	Subtype	Tropism	Reference
1	Q1/61	ND	Respiratory/ Nephropathogenic	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
2	T (N1/62)	C	Respiratory/ Nephropathogenic/	Cumming (1962), Ignjatovic *et al.* (2002), Crinion *et al.* (1971),
3	A3 (N2/62)	C	Nephropathogenic	Ratanasethakul and Cumming (1983)
4	Vic S	B	Respiratory/ Nephropathogenic	Ratanasethakul and Cumming (1983), Sapats *et al.* (1996)
5	N3/62	J	Respiratory	Ignjatovic and McWaters (1991)
6	Q1/63	ND	Respiratory/ Nephropathogenic	Ignjatovic *et al.* (2002)
7	Q1/64	ND	Respiratory/ Nephropathogenic	Ignjatovic *et al.* (2002)
8	Q1/65	C	Respiratory/ Nephropathogenic	Ignjatovic *et al.* (2002)
9	Q2/66	ND	Proventric	Ignjatovic *et al.* (1997)
10	Q1/67	ND	Nephritis	Ignjatovic *et al.* (1997)
11	Q1/69	ND	Nephritis	Ignjatovic *et al.* (1997)
12	V1/71	G	Respiratory	Wadey and Faragher (1981), Ignjatovic *et al.* (2002)
13	V2/71	F	Respiratory	Wadey and Faragher (1981), Ignjatovic *et al.* (2002)
14	Q1/73	E	Respiratory/ Nephropathogenic	Klieve and Cumming (1988), Ignjatovic and

				McWaters (1991), Ignjatovic *et al.* (2002)
15	N8/74	C	Respiratory/ Nephropathogenic	Wadey and Faragher (1981), Ignjatovic *et al.* (2002)
16	N9/74 (Appin)	D	Respiratory/ Nephropathogenic	Klieve and Cumming(1988), Sapats *et al* (1996)
	N1/75	I	Respiratory/ Nephropathogenic	Wadey and Faragher (1981), Ignjatovic *et al.* (2002)
17	N2/75	H	Respiratory/ Nephropathogenic	Sapats *et al.* (1996)
18	Q1/76	B	Respiratory/ Nephropathogenic	Wadey and Faragher (1981), Ignjatovic *et al.* (2002)
19	N1/81	P	Nephritis	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
20	N4/81	ND	Respiratory/ Nephropathogenic	Ignjatovic *et al.* (2002)
21	N1/82	B	Nephropathogenic	Ignjatovic *et al.* (1997)
22	N2/84	C	Nephropathogenic	Ignjatovic *et al.* (1997)
23	N3/87	B	Respiratory	Ignjatovic *et al.* (1997)
24	N25/87	N	Respiratory	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
25	Q1/88	M	Respiratory	Ignjatovic *et al.* (1997)
26	N1/88	L	Respiratory	Sapats *et al.* (1996)
27	N3/88	L	Respiratory	Ignjatovic *et al.* (1997)
28	N6/88	L	Respiratory	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
29	Q3/88	M	Respiratory	Sapats *et al.* (1996)

30	N1/89	L	Respiratory	Ignjatovic *et al.* (1997)
31	N2/89	L	Respiratory	Ignjatovic *et al.* (1997)
32	N3/89	ND	Respiratory	Ignjatovic *et al.* (2002)
33	Q1/89	ND	Respiratory	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
34	NT2/89	O	Nephritis	Ignjatovic *et al.*, (1997), Ignjatovic *et al.* (2002)
35	V5/90	B	Respiratory	Sapats *et al.* (1996)
36	N2/90	L	Respiratory	Ignjatovic *et al.* (1997)
37	N5/90	L	Respiratory	Ignjatovic *et al.* (1997)
38	V4/90	B	Respiratory	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
39	V7/90	B	Respiratory	Ignjatovic *et al.* (1997)
40	Q4/91	C	Nephropathogenic	Ignjatovic *et al.* (1997)
41	V18/91	Q	Respiratory	Ignjatovic *et al.* (1997)
42	V19/91	Q	Respiratory	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
43	V6/92[a]	B	Respiratory	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
44	V6/92[b]	R	Respiratory	Ignjatovic *et al.* (1997)
45	V9/92	R	Respiratory	Ignjatovic *et al.* (1997)
46	V1/93	ND	Respiratory	Ignjatovic *et al.* (1997)
47	V2/93	S	Respiratory	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
48	V3/93	S	Respiratory	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
49	N1/94	L	Respiratory	Ignjatovic *et al.* (1997), Ignjatovic *et al.* (2002)
50	N6/94	T	Respiratory	Ignjatovic *et al.* (1997)
51	N12/94	C	Nephropathogenic	Ignjatovic *et al.* (1997)
52	Q1/99	ND	Not determined	Mardani *et al.* (2006a)
53	Q4/99	ND	Not determined	Mardani *et al.* (2006b)
54	Q1/02	ND	Not determined	Mardani *et al.* (2006b)

55	V1/02	ND	Nephropathogenic	Mardani *et al.* (2006a)
56	V2/02	ND	Nephropathogenic	Mardani *et al.* (2006a)
57	V3/02	ND	Nephropathogenic	Mardani *et al.* (2006b)
58	N4/02	ND	ND	Ignjatovic *et al.* (2006)
59	N1/03	ND	Respiratory	Mardani *et al.* (2006a)
60	N4/03	ND	ND	Ignjatovic *et al.* (2006)
61	N5/03	ND	ND	Ignjatovic *et al.* (2006)
62	Vac1	A	Respiratory/ Nephropathogenic	Ignjatovic *et al.* (2002)
63	Vac5	B	Respiratory/ Nephropathogenic	Ignjatovic *et al.* (2002)

ND is not determined

1.10 Economic impact of IBV on Layer Industry in Australia

In Australia, 13.175 million poultry layer birds produce 203 million dozen eggs per annum. The gross value of production is $ 288 million per annum (Australian Bureau of Statistics, 2006). The egg industry in Australia loses in excess of $10-15 million dollars per year through downgrading of eggs. Diseases affecting the reproductive tract of adult laying flocks such as IB and egg drop syndrome could contribute to these losses. A better understanding of the pathogenesis of these diseases, which influence internal and external egg quality, will assist development of strategies to reduce the incidence of downgrading. Currently, in Australia, approximately 85 per cent of all eggs produced are sold in shell form, primarily through grocery chains. The balance is processed into liquid, frozen and dried egg products for use in the food service and processed food sectors.

In Australia, currently, all pullets reared for egg production are vaccinated by live attenuated strains of infectious bronchitis virus. It is usual practice to administer three doses in the rearing phase before point of lay. Jolly *et al.* (2005) observed that, when birds vaccinated in the rearing phase were infected with IBV, there were deleterious effects on internal and external quality of eggs laid. Many producers utilise the practice of boosting immunity regularly during the lay cycle. However, questions have been raised about possible negative effects of attenuated vaccines in adult layer birds (Sulaiman *et al.*, 2004). As mentioned earlier in the literature, detailed information about the pathogenesis of IBV in the oviduct of layers is conspicuous by its absence. It is essential to carry out studies of the impact of IBV on egg quality in commercial laying flocks. All around the world, Australia has a reputation for producing food of very high quality. The challenges faced by the Australian poultry industry are reflected in poultry industries around the world. Research needs to be focused on improved welfare standards, improved production systems and health management programs that will not only assist the Australian poultry industry to meet these challenges, but establish the Australian poultry industry as the universal benchmark in the world. Although the deleterious effect of IBV on egg production and quality is well known, very little research has been conducted on this aspect.

1.11 Detection Techniques for IBV

IBV replicates in a range of organs although the preliminary site of replication is the respiratory tract. From a diagnostic point of view, the most complicating factor is individual variation in the response of birds to IBV infection. There are several studies reporting the isolation of IBV over long periods (2 to 7 months) from vaccinated or infected flocks (Alexander and Gough, 1977; Alexander *et al.*, 1978). Cook (1968) suggested that a possible cause of long term virus excretion could be the cross-infection within the infected or vaccinated flock.

There are a number of assays available for diagnosis of IBV such as virus isolation, immunofluorescence assay, virus neutralization, immunoperoxidase essay, and viral RNA isolation. IBV infection can also be diagnosed serologically by detecting antigen specific antibodies against infection by Haemagglutination Inhibition (HI), Agar Gel Precipitation Test (AGPT), and Enzyme Linked Immunosorbent Essay (ELISA). All these tests vary in their specificity and sensitivity. However, for successful and confirmed diagnosis, detailed knowledge about the pathogenesis of the virus is essential. Haemagglutination Inhibition assay could be used for IBV detection (King and Hopkins, 1984) although, due to the high level of cross reactivity that can occur amongst IBV strains, the test is not recommended (Cook *et al.*, 1987) The most common and cheap method of virus isolation is by using embryonated chicken eggs. However, this test takes time to detect field strains of virus as the virus may not adapt to produce lesions until is has been passaged five times.

1.11.1 Cell culture

Cell or organ culture for virus isolation is the gold standard technique and tracheal organ culture is the most sensitive and suitable method for virus isolation (Cook *et al.*, 1976). However, it is laborious and needs special infrastructure in laboratories. In such organ cultures, ciliary movements are the indicators of virus infectivity. The choice between tracheal organ culture and chicken embryo inoculation has been a matter of debate. Cunningham (1970) suggested that adaptation to embryonated eggs is necessary whereas Cook (1976) observed it as an unnecessary step. The latter observation resulted from the isolation and titration of a field strain (HV-10) of IBV in tracheal organ culture but not in chicken embryos. The requirement of chicken embryo adaptation was further confirmed by

Cowen and Hitchner (1975) who performed more than 40 serial passages of IBV Clark 333 strain in chicken embryos to produce cytopathic effects upon inoculation into cell culture. Further, they found that frequent monitoring of cell culture fluid infectivity was essential (which was performed by chicken embryo inoculation) to avoid the loss of virus titre. The use of tracheal organ cultures in propagation of non-ciliostatic strains of IBV would be again a matter of debate. Yachida *et al.* (1981) observed that virulence of IBV strains *in ovo* and in organ culture can vary depending on the temperature. Otsuki *et al.* (1979) used primary chicken embryo kidney cells for IBV replication. Pradhan *et al.* (1984) observed that oviduct organ culture was more efficient than tracheal organ culture, however the tedious process of maintaining oviduct organ culture could be a major limitation. Peters *et al.* (1979) compared the susceptibility of chicken kidney and oviduct organ cultures, and observed that kidney organ cultures are more likely to be resistant as compared to oviduct organ cultures as age advances. In this study also, the two strains of virus were passaged in embryonated eggs before inoculation into organ culture. It has been also been reported that kidney and lung cells are the most susceptible cells for IB virus growth (Lukert, 1965). Chubb and Ma (1974) were able to grow one out of five chicken embryo adapted strains of Australian nephritis-inducing isolates of IBV and found the tissue culture propagated virus to be less pathogenic *in-vivo*. This was not surprising as virus growth in eggs and its further adaptation in tissue culture can reduce its pathogenicity (Cunningham, 1970). Ferreira *et al.* (2003) studied the growth of IBV in a chicken embryo-derived cell line. However, this cell line required several passages to adapt the virus strain which could be a major limitation for its use.

From these studies in the past, it can be concluded that cell culture could be an efficient technique for some strains of IBV, although the time required for virus cultivation is less than that required for chicken embryo inoculation. However, as chicken embryo inoculation is cheaper and less laborious than cell culture, and convenient to adapt most of the IBV strains, it would be the method of choice.

1.11.2 Serological tests

Serological techniques have been used extensively in the past. Traditionally, the virus neutralisation (VN) test, conducted in embryonated eggs, was used to isolate and identify IBV infections, but is now rarely used because of its limitations including the requirement

for a large number of embryonated chicken eggs. Enzyme-Linked Immunosorbent Assay (ELISA) for either antigen or antibody has been reported to be a successful test for detection of IBV. In an antigen ELISA, the antigen can be captured by anti-IBV antibodies coated on microtitre plates (Nagano *et al.*, 1990; Ignjatovic and Galli, 1995; Lougovskaia *et al.*,2002) but the quantity of virus required for detection is high and the sensitivity for detection of virus infection from chicken organs is low (Naqui 1990). IBV infection can also be diagnosed by detecting the appearance or rise in IBV specific antibody titres. However, many factors such as age at vaccination or infection (Ignjatovic & Galli, 1995), presence of maternal antibodies at the time of vaccination (Dewitt *et al.*, 1998), immunity derived from prior infection (Dewitt, 2000; Cook *et al.*, 1999) and cross reaction between serotypes, as dozen of serotypes are present in the field, may influence the results. Similarly, immunosuppression caused by various intrinsic and extrinsic factors may affect the humoral response to IBV vaccination and give varying results.

Mostly the enzyme linked immunosorbent assays are developed to detect antibodies in susceptible flocks and also the status of antibody titres after vaccination. Dewitt *et al.* (1997) suggested that antibody ELISA can be a very effective method for checking the IBV antibody negative or positive status of the flock. Dewit *et al.* (1998) also further reported that there can be individual variations in titres amongst the birds in the time post-infection. Bronzoni *et al.* (2001) first developed the antigen competitive ELISA for detection of IBV (detection limit was $10^{4.1}$ EID_{50} /well). However, their test could not detect the vaccine strain because of a lower dose level. Also, this test did not differentiate between vaccine and virulent strains or differentiate between IBV serotypes.

1.11.3 RNA isolation for IBV detection

Currently, reverse transcriptase polymerase chain reaction (RT-PCR) is commonly used for diagnosis of IBV (Jackwood *et al.*, 1997; Keller *et al.*, 1998; Ramneek *et al.*, 2005). The use of PCR in molecular diagnostics has increased to the point where it is now accepted as a gold standard for detecting nucleic acid from a number of origins and it has become an important tool in research laboratories. PCR for virus detection is displacing all the standard techniques such as cell culture and various serological essays (Niubo *et al.*, 1994). In many instances, the primary aim is to detect the serotype of the virus. However, during detection of IBV from clinical samples, in many instances, samples have to be passaged in eggs.

Genotyping of the virus is also an effective method, not only to detect the virus, but also for grouping them on the basis of their nucleotide or amino acid sequences. For IBV, various genotyping methods include PCR (Keller *et al.*, 1998), RNA finger printing (Kusters *et al.*, 1987), RT-PCR and restriction fragment length polymorphism (Mardani *et al.*, 2006 a & b) and S1 or N gene sequencing (Sapats *et al.*, 1996a, b). Variation in the S1 (Callison *et al.*, 2001) and nucleocapsid gene sequences (Ignjatovic *et al.*, 2006) has proved to be of critical importance for the emergence of new variants as both of these genes are responsible for virus replication and immunity. However, the 3' UTR region which is involved in initiation of negative strand RNA has also been used to study variation amongst the emerging IBV strains (Williams *et al.*, 1993). Using RT-PCR, IBV has been also isolated directly from clinical samples such as the trachea (Handberg *et al.*, 1999) or kidney (Mardani *et al.*, 2006a). However, there are no reports of IBV isolation from the fully-functional oviduct by RT-PCR.

Until now, a combination of PCR and detection assay has been used to obtain quantitative data with promising results. However, these approaches have disadvantages of the laborious post-PCR handling steps required to handle the amplicon (Guatelli *et al.*, 1989). The detection of amplified DNA relies upon the electrophoresis of nucleic acid in the presence of ethidium bromide and visualization of resulting bands under UV light (Kidd *et al.*, 2000). Southern blot detection of the amplicon is also time consuming and requires multiple PCR product handling steps. This further risks the spread of amplicon throughout the laboratory (Holland *et al.*, 1991). Alternatively, PCR-ELISA may be used to capture the amplicon onto the solid phase using biotin or digitoxin labelled primers and oligonucleotide probes (Wateinger, 2001). Once captured, the amplicon can be detected using enzyme labelled avidin. This approach has become the foundation of real-time PCR. The monitoring of accumulating amplicon in real time has been made possible by labelling of the primers, probes or amplicon with fluorogenic molecules (Matthews and Kricka 1988). Recently Jackwood *et al.* (2003) developed the syber green real-time reverse-transcriptase polymerase chain reaction for detection of infectious bronchitis virus. They observed that the reaction is very rapid and cheap as compared to conventional PCR and detected the presence of a minimum amount of viral RNA (0.01 pg RNA) from allantoic fluid. However the syber green dye used in the development of real time PCR can also bind to the unspecific primer dimmers by intercalating double stranded DNA (Hara *et al.*, 2003). The use of Taq man probe solves these problems and, recently, a quantitative real-time PCR test

has been developed by Callison *et al.*, (2007) using tracheal swabs. In the field level, it would be practical to test samples such as faeces or eggs by employing such molecular tests.

Real time PCR offers significant improvements to the quantification of viral load because of its enormous dynamic range that can accumulate at least eight $\log^{10}$ copies of the nucleic acid template (Ishiguro *et al.*, 1995; Locatelli *et al.*, 2000; Moody *et al.*, 2000). Based on the above facts, distribution of virus in various parts of the oviduct after IBV challenge needs to be assessed to ascertain whether the virus of itself is capable of causing pronounced drops in egg production and quality usually observed in the field. The reliability of this test in the field for detection of IBV needs to be determined, bearing in mind the frequent pathogenic and genetic drift in IB viruses. Because of the continuous emergence of new pathogenic strains, the chicken embryo inoculation test is still important for pathotyping.

1.12 Introduction to current study

The present research was conducted to explore some of the unanswered questions regarding deterioration of egg production and quality during IBV infection in layer flocks. Over the last 50 years, the interaction of IBV with the mature oviduct has received little attention despite the well known effects of IBV on egg quality. Even in Australia, there are many perceptions, without confirmatory evidence, regarding the effects of IBV on layer flocks. The nephropathogenicity and respiratory tropism of IBV have been widely studied but the extent of uterotropism of Australian strains of IBV in still not known. Hence, the present study was designed to investigate the effects of two phylogenetically different IBV serotypes on the oviduct. Currently, all commercial laying hens in Australia are vaccinated against IBV although the extent of protection offered to the oviduct with currently available vaccines is not known. The present study will also investigate the effects of IBV on the oviduct of unvaccinated hens, as a model for birds which have not been properly vaccinated. Also, until now, kidney, faeces and caecal tonsils were known to be the principal candidates for excretion of virus after IBV infection. The possibility that the oviduct is also able to excrete virus will be studied by virus isolation from the oviduct, along with other organs such as kidney, trachea and gut contents. Isolation of virus over the post infection period using the traditional method of virus isolation in embryonated eggs will be compared with detection using the reverse transcriptase polymerase chain reaction.

Chapter 2

Materials and Methods

2.1 Virus

The two strains of virus T and N1/88, used through out all the experiments, were obtained from Dr. Jagoda Ignjatovic, CSIRO, Geelong, Australia. Each challenged bird received virus at the dose rate of 2 x 10^5 EID_{50} intraocularly and the control birds were inoculated with normal saline.

2.2. Bleeding and blood analysis

All hens were bled at the 28^{th} week of age (2 weeks before infection), 35^{th} week (5 weeks post infection) and 40^{th} week (10^{th} weeks post infection). The hens which were killed by carbon dioxide asphyxia, at regular intervals post infection during the experiment, were also bled before killing. Three mL blood samples were collected in heparinised syringes and stored on ice. The blood electrolytes ionised calcium, sodium and potassium were measured using an AVL 983 Electrolyte Analyser (with ion selective electrodes) and results were presented in mmol/mL. Each blood sample was then used to fill duplicate haematocrit capillary tubes and centrifuged at the speed of 13,000 rpm for 3 min. The haematocrit reading was then taken using a haematocrit reader. The remaining sample was centrifuged at 6000 rpm for 10 min and plasma was collected and stored frozen for later use.

2.3 Histopathology

Tissues collected at the time of the main experiment were processed by standard histological techniques for examination of microscopic pathology.

2.3.1 Tissue fixation and processing

All tissue samples were fixed in 10 % neutral buffered formalin where they remained until processing commenced. Formalin fixed samples were dehydrated through ascending grades of alcohol, 50 %, 70 %, 90 % and three changes in absolute alcohol. The tissue was then cleared with three washes of xylene and finally placed into two changes of molten wax (Paraplast, Oxford Labware, Saint Luis, USA), the latter was processed under vacuum to enhance the penetration of wax through the entire thickness of the tissue. All tissue processing for histology was carried out in an automated histokinette (Shandon Citadel 2000 Tissue processer) and took 16 hrs to complete.

2.3.2. Embedding

After processing in the histokinette, the cassettes containing tissue were transferred to molten wax in a Paraffin Embedding Centre (Leica EG 11660, Jung Histology Products). The tissues were removed from the tissue cassettes and placed in metal moulds of appropriate size. The mould was filled with molten wax. The labelled part of the tissue cassette was placed on the metal mould containing the tissue sample to identify the particular sample. The moulds were then cooled rapidly on the cooling platform of the embedding station. The metal moulds were removed, leaving the tissue embedded in a block of wax. The excess wax around the mould was then trimmed using a blunt knife to allow placement of the tissue block on the microtome.

2.3.3 Sectioning

The tissue block was fixed on the microtome facing towards the microtome blade (Feather Safety Razor Co. Ltd., Medical Division). Ribbons of sections were cut and transferred to a water bath at a temperature of 45-50°C. Intact sections were collected and transferred onto five slides (Objekttrager microscopic slides, HD Scientific) per sample. The slides were then placed on a hotplate at 42°C for 4 hrs. Each slide was numbered for identification. The slides were then stored in microscope slide boxes

and kept until staining. The slides were kept in the box for at least 3 days prior to staining to ensure sections were properly adhered to the slides.

2.3.4 Staining

All slides were stained with haematoxylin & eosin stain (H & E). This stain stains nuclei a dark blue colour and cytoplasm and connective tissue a pink colour. Some of the kidney and magnum slides were stained with alcian blue stain also. Alcian blue stain makes it easy to identify medullary areas in the kidney and imparts a blue colour to the mucopolysaccharides in the surface epithelium of the magnum. The staining solutions were prepared, and the staining was conducted, as described in Appendix 1.

2.3.5 Microscopy

All stained slides were observed under a light microscope (Nikon, Alphashot-2 YS2). The required fields were selected and the images captured using a digital camera (Canon, EOS 300D) and adapter (LM- Scope digital SLR adapter with canon bayonet C mount thread) in conjunction with the microscope. All stained sections of trachea, kidney, infundibulum, magnum, isthmus, tubular shell gland and shell gland pouch were examined by light microscopy.

2.3.6 Histopathological lesion scoring and statistical analysis

All stained sections of trachea, kidney, infundibulum, mid-magnum, isthmus, tubular shell gland and shell gland pouch were examined by light microscopy and the most prominent lesions were scored as no change (0), mild (1), moderate (2) or severe (3), after the methods of Nakamura *et al.* (1991). The average lesion score was obtained by counting affected cells in five randomly-distributed microscopic areas at x200 magnification (field diameter 920 μm). The mean lesion score from two hens was then calculated.

The Mann Whitney U test was used to test for main effects of IBV challenge strain in hens and to test for differences between hens and cockerels. The Kruskal-Wallis test was used to test for main effects of time post-infection. Interactions between IBV

strain and days p.i. were not investigated statistically owing to small sample sizes. All statistical analyses were conducted using Statview® version 5.0.1 for windows (SAS Institute Inc. Copyright © 1992- 1998).

2.4 Transmission electron microscopy (TEM)

2.4.1 Tissue fixation and washing

The oviduct was removed under strict aseptic conditions. Small pieces of infundibulum, magnum, isthmus, tubular shell gland (TSG) and shell gland pouch (SGP) were pinned onto cardboard and then immersed in 5 mL fixative (2% Para formaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 5.5). Each tissue sample was fixed for 2.5 hrs. After fixation, all the tissues were washed twice in 0.1M phosphate buffer for 20 mins to wash the glutaraldehyde traces from tissues. During washing, the vials were kept rotating to enhance the washing of tissue.

2.4.2 Tissue Cutting and Processing

After washing, tissues were removed from the cardboard and placed into a petri dish. Tissues were then cut into 1mm pieces for TEM and approximately 4 mm pieces for SEM with a sharp razor blade under a dissecting microscope (Kyowa Model SDZ-P, Tokyo, Japan). The tissues for scanning microscopy were preserved in 0.1 M buffer until the following day. After cutting, all tissues were post-fixed with osmium tetroxide (1% osmium tetroxide, buffered with 0.1M phosphate buffer), with rotation, for 2 hrs. The samples were again washed twice in 0.1 M buffer and then dehydrated in ascending grades of alcohol (50%, 70%, 80%, 90% and two washes of absolute alcohol). After dehydration, tissues were transferred to a mixture of alcohol and resin (2:1) for 2.5 hrs, Samples were then left overnight in alcohol and resin (1:1) followed by 1:2 for 6 hrs. All samples were then transferred to full strength resin and kept overnight with rotation.

2.4.3 Embedding

The tissues left overnight in full strength resin were cut under a microscope and embedded in plastic moulds with freshly-made epoxy resin. One sample was embedded in six replicates. The moulds were incubated at 70°C for 24 hrs and blocks were then removed and stored for sectioning.

2.4.4 Sectioning and staining

Blocks were trimmed in an hexagonal shape using a sharp blade and fitted on the ultramicrotome (Ultra cut E, Reichert- Jung) facing towards a diamond knife (Diatome, size 24/10, Diatome Bienne Switzerland).

Semi-thin sections of 60µm, from 5 blocks of each tissue from an individual hen, were collected on thin glass strips. The glass strips were kept on a hot plate for drying. The glass strips were later stained with toluidine blue and then washed with 50% alcohol and distilled water. The sections were observed under a light microscope (Nikon, 6 YS2-H, China) and the required area was selected. The block was further fine-trimmed and ultra-thin sections (80 to 90 nm) were then collected on grids. The ultra-thin sections collected were left for drying overnight and kept protected from dust.

All grids with ultra thin sections were stained with a saturated solution of uranyl acetate (2% of 4% stock solution diluted in 100% ethanol) for 30 min. and washed with distilled water. The sections were then stained with filtered lead citrate for 7 min in a carbon dioxide free environment and washed in CO_2 free water (distilled water boiled and stored in air tight container). All stained grids were kept for drying. Extreme care was taken to avoid contamination with dust particles throughout the processing. Sections were examined under a transmission electron microscope (Joel, JEM- 1200 EX).

2.5 Scanning electron microscopy (SEM)

The samples were fixed and cut as described above. After dehydration in ascending grades of alcohol (50%, 70%, 80%, 90% and two washes of absolute alcohol), samples were placed in an aluminium boat. At all times after dehydration, the samples were kept immersed in alcohol. The aluminium boat along with samples immersed in alcohol was transferred to the dryer chamber in stainless steel thimbles for critical point drying (Polaron E3000). Before transferring samples, the dryer chamber was kept chilled with all valves open. After loading samples into the dryer, all valves were closed and samples were flushed for 10 min with liquid CO_2 to remove ethanol. The tissue samples were allowed to sit in liquid CO_2 for 1 hr and the flushing process was repeated. The flow of CO_2 after this process was reduced and the dryer was slowly heated from 15 ^{o}C up to 40^{o}C (P= 1200 psi). Pressure was then slowly released and dried samples were removed from the chamber. Critical point dried samples were mounted on aluminium stubs with an appropriate orientation. The mounted samples were sputter gold coated (Polaron E5100) and observed under a scanning electron microscope (Joel, JSM -5800LV)

2.6 Egg production and quality

Egg production records of laying hens were kept daily throughout each experiment. Group records for production were converted to daily egg production percentages (eggs/hen/day as a percentage). Eggs were collected daily as described in individual experiments. After collection, eggs were analysed both for internal and external quality. Egg weight was measured in grams on a weighing balance (Ohaus). Shell reflectivity (indicator of lightness of shell colour) was measured using a reflectometer (measures amount of light reflected from the shell surface). Egg shell breaking strength was measured in Newtons and deformation was measured in mm by quasistatic compression using the Q/C- SPA machine. All eggs were analysed for the internal quality parameters albumen height, Haugh Units and yolk colour score. Albumen height was measured in mm, and from this measurement, Haugh Units were calculated using the formula described earlier by Haugh (1937).

$$\text{Haugh units} = 100\text{LOG}\left[H - \frac{\sqrt{G}\,(30W^{0.37} - 100)}{100} + 1.9\right]$$

H= albumen height in mm

G= 32.2

W= Weight of whole egg in grams

Yolk score was measured with a QCC yolk colour meter calibrated to the Roche scale. External egg quality was measured. The shell percentage was calculated as the ratio of shell weight to egg weight expressed as a percentage. All the equipment used for egg quality analysis, except for egg shell thickness, was supplied by Technical Services and Supply, UK. All egg shells were washed and left for drying overnight. The air dried egg shells were weighed in grams. Egg shell thickness was measured by taking shell pieces from three equidistant points on the equator with the shell membrane intact. Shell thickness was measured using a gauge constructed from a Mitutoyo Dial Comparator gauge Model 2109-10. Length and breadth was measured using a digital caliper (Mitutoyo digimatic caliper, Japan) and values were used to calculate the shape index (breadth x 100/ length).

2.7 ELISA

Blood plasma samples were analysed for infectious bronchitis virus antibodies using commercially available ELISA kits (IDEXX Laboratories Ltd.). The samples were processed as per the manufacturer's instructions. The microtitre plates were read on a Microplate reader (Benchmark, BIO-RAD) used in conjunction with Microplate Manager ^{TM} Computer program (BIO-RAD). The absorbance values were calculated as an S/P ratio from which the positive/ negative titre endpoint is calculated for each plate assayed.

2.8 Kidney weights

After euthanasia, both the kidneys from each hen were taken out and weighed individually. Kidney size was then expressed as right, left and total kidney weight as a percentage of body weight.

2.9 Statistical analysis

All data were analysed by Statview® version 5.0.1 for windows (SAS institute Inc. copyright© 1992-1998). Analysis of variance was used to test for effects of variables with significance indicated by probability of less than 5 %. Fishers protected LSD test was used to distinguish differences between means.

2.10 Virus isolation

Virus isolation was attempted from the trachea, kidney, oviduct and intestinal contents from each individual hen. One gm of kidney sample and 1 gm of lower intestinal contents was weighed and mixed into 1 mL of nutrient broth containing an antibiotic mixture (Appendix 2). Kidney tissue was forced through a sterile syringe before mixing with nutrient broth. Oviduct samples (all segments) were scraped using sterile scalpel blades and 1 gm of oviduct scraping was mixed with 1 mL antibiotic-containing nutrient broth. A 1 cm length of trachea was also scraped and the scrapings were mixed with antibiotic-containing nutrient broth. All the samples mixed in broth were shaken vigorously and centrifuged at 4000 rpm for 15 min in a refrigerated centrifuge. The supernatant was transferred to clean sterile vials and frozen immediately at -20°C. Two gm faecal samples, collected from 1 to 10 weeks post-infection from five hens of each group, were weighed and mixed with antibiotic-containing nutrient broth. The samples were then processed as mentioned above.

The sample extracts were inoculated into the allantoic cavity of 9-day old chicken embryos. Eight eggs were used per sample and the eggs were incubated at 37°C. The eggs where the embryo died within 24 hrs of inoculation were discarded. Two days after virus inoculation, three out of eight eggs were removed from the incubator, chilled and allantoic fluid was collected. The collected allantoic fluid was immediately frozen. The remaining five eggs were opened one week after virus inoculation to check for lesions in the embryos (death/dwarfing and curling). Strict aseptic conditions were maintained to avoid any chance of cross contamination. Sissors, forceps and trays were washed with boiling water after processing of each sample and wiped with alcohol before further use. Bench space was wiped with bleach and alcohol after each processing. Sterile gloves were used all the times.

2.11 Extraction of viral RNA from allantoic fluid

Extraction and purification of RNA was performed using a Qiagen RNAeasy kit, according to manufacturer's instructions, with some modifications. A 400 µL sample of allantoic fluid was mixed with 300 µL of RTL buffer and 8 µL of mercaptoethanol was then added. This mixture was vortexed and kept at room temperature for 15 min. After incubation at room temperature, 700 µL of 70% ethanol was added and the solution was transferred to a spin column in two steps. Washing and elution was performed as per instructions on the kit. The elution volume was 30 µL. Extracted RNA was quantified and stored at -70°C until used. A cold chain was maintained throughout the extraction to avoid the degradation.

2.12 Primers

For the detection of T strain IBV from the oviduct samples, the polymerase chain reaction was carried out using a universal primer pair (UTR1 and UTR 2) as described earlier (Adzher *et al.*, 1996). These primers correspond to the untranslated region of the IBV genome which gave a 298 base pair product after agarose gel electrophoresis and ethidium bromide staining. The above universal primers, however, could not detect the IBV strain N1/88. Another primer pair binding within the nucelocapsid gene of N1/88 (Nucleotide 668 to 790) was selected and the nucleocapsid sequence was retrieved from GeneBank accession number U52599 (Sapats *et al.*, 1996a). Thus two designed primers, forward primer (AGATGGCTGAGCGTAAGTAC), reverse primer (CCTCCTCAATCATCTTGTCATC) amplified a 123 base pair product. The primer pair for IBV strain N1/88 was designed using Beacon designer (Version 5, Premier Biosoft international, Palo Alto, USA).

2.13 Single step reverse transcription polymerase chain reaction

Reverse Transcriptase PCR was carried out using a one step RT-PCR kit (Qiagen) as per the manufacturer's instructions. The reaction was carried out in 25 µL reaction mixture containing 5µL of 5x reaction buffer, 1µL of dNTP mix (containing 10mM

each of dATP, dCTP, dGTP and dTTP), 1 μM of each oligonucleotide, 1 μL of enzyme mixture (Qiagen single tube RT-PCR kit utilises recombinant heterodimeric enzymes expressed in *E. coli*), 5 μL of RNA template and 8 μL of RNase free water. All the mixtures were kept on ice until transferred to the thermal cycler. Both reverse transcription (RT) and PCR were carried out in a single tube. Cycle 1, 30 min at 50 °C; cycle 2, 15 min at 95°C; cycle 3 to 30, 1 min at 94 ^{0}C, 1 min at 56°C (57°C for N1/88 strain) and 1 min at 72°C. The final extension was performed at 72°C for 10 min.

2.14 Agarose gel electrophoresis

The PCR products were confirmed by 1% agarose gel. The edges of a clean electrophoresis tray were sealed with adhesive tape and the tray placed on a flat surface. The comb with eight teeth (5 x 1.5) was clamped 0.5 to 1 mm above the surface of the tray to form sealed wells. The 1% agarose was prepared in Tris-EDTA buffer (TE buffer, Appendix3). The slurry was heated in a microwave until the agarose was dissolved completely. The agarose was cooled under running water and poured into a tray to form a 2 to 3 mm thick gel slab and allowed to set for 30 min. The combs and adhesive tapes were removed. Each PCR product was mixed with 2 μL of gel loading dye and 5μL of TE (Appendix 3). The mixture was loaded into the sealed wells in the tray submerged in TE buffer in an electrophoresis trough. The electrophoresis was carried out at 100V for 45 mins. The gel was stained with ethidium bromide and photographed using ultraviolet transillumination. The base pair size of amplified product was derived from the running standard marker adjacent to all the testing samples.

Chapter 3

Preliminary studies on comparative histopathology of two serotypes of infectious bronchitis virus (T & N1/88) in White Leghorn hens and cockerels

3.1 Introduction

IBV strains vary greatly in their tissue tropism. IBV may occur as a respiratory syndrome, with clinical signs being difficulty in breathing, rales, coughing, or sneezing with or without nasal discharge (Parsons *et al.*, 1992, McMartin, 1993). Maximum IBV antibody titres were recorded in the trachea between 5 and 10 days post infection (p.i.) (Ambali and Jones, 1990, Otosuki *et al.*, 1990). Different IBV strains can grow at many epithelial surfaces in addition to the respiratory tract, including the kidney, oviduct and parts of the gastrointestinal tract (reviewed by Dhinakar Raj & Jones, 1997a). Besides a primary predilection towards the respiratory tract, certain strains of IBV have been found to be severely nephropathogenic. The nephropathogenicity of IBV was first reported in Australia (Cumming, 1962), followed by the USA and many parts of Europe (Butcher *et al.*, 1990, Picault *et al.*, 1988, Zanella, 1988), Japan (Shimakura and Hirai, 1971) India (Bayry *et al.*, 2005) and China (Liu *et al.*, 2005). A range of histopathological studies in IBV infection has been carried out in the past in either broilers or in pullets. In addition, these studies were restricted mainly to individual organ systems. However, very little information is available on the histopathological changes occurring in a range of tissues over time, in hens and cockerels challenged with IBV. Hence, the present study was designed to investigate the details of pathological changes occurring in various tissues in hens and cockerels challenged with the nephropathogenic T-strain, as compared with the more respiratory N1/88-strain.

3.2 Materials and Methods

3.2.1 Chickens

All chickens were kept unvaccinated against IBV and reared under strict isolation conditions with *ad libitum* feed and water. The IBV antibody-free status of hens and cockerels was maintained for 65 weeks for hens (51 chickens) and 10 weeks for cockerels (50 chickens). All the chickens used for the histopathological study described in this chapter had been used earlier for different experiments. The hens had been used for the production of fertile eggs and the cockerels resulted from eggs hatched for the production of the hens. The oviduct segments, magnum, tubular shell gland and shell gland pouch, were collected to study the effects, if any, of IBV on the oviduct of unvaccinated hens. This was a preliminary study to determine if the two Australian strains of IBV had trophism for the main oviduct regions.

3.2.2 Experiment 1

White Leghorn hens at 65 weeks of age were divided into two groups of twenty-two hens, each of which was challenged with either T or N1/88 strains of IBV. Seven hens were kept unchallenged as a control. Three hens from each challenge treatment group and one hen from the control group were killed and examined on days 3, 6, 10, 13, 16 and 21 post-infection (p.i.). Harderian gland, trachea, caecum, kidney, magnum, tubular shell gland and shell gland pouch (Johnston *et al.*, 1963, Solomon, 1975, Stemberger *et al.*, 1977) were collected for examination.

3.2.3. Experiment 2

White Leghorn cockerels were divided into two groups each of twenty chickens which were challenged with the same strains of IBV mentioned above. Eight chickens were kept as a control. Four chickens from both treatment groups and two chickens from the control group were killed and examined on days 2, 4, 6, 8, 10 post-infection (p.i.). Harderian gland, trachea, caecum and kidney were collected for examination.

All the tissue samples collected during the experiment were subjected to histopathological examination as described in chapter 2 section 2.3. The lesions were scored and data was analysed statistically.

Table 3.1: Lesions scored for each tissue examined following infection with T and N1/88 strain of IBV

Tissue	Lesions/ affected cells scored
Harderían gland	degeneration of collecting duct epithelium, lymphocyte infiltration in septa, heterophils in subepithelium, exfoliative epithelial cells in collecting tubules
Trachea	cilia loss, epithelial degeneration, hypertrophy of alveolar mucous glands, oedema in mucosa, lymphocyte infiltration
Caecum	No lesions recorded
Kidney	oedema of Bowman's capsule, urate casts, necrotic foci, granulocytic casts, lymphocyte infiltration, duct and tubular dilatation, necrosis of proximal convoluted tubule
Magnum	loss of mucopolysaccharides from epithelial cells, tubular gland dilatation, lymphocyte infiltration, edema in submucosa
Tubular shell gland and shell gland pouch	loss of cilia, tubular gland dilatation, lymphocyte infiltration, oedema in submucosa

3.3 Results

3.3.1 Harderian gland

In control hens, the main features were some plasma cells (mature B lymphocytes that are specialized for antibody production) in the subepithelium, intact collecting duct epithelium, and occasional lymphocyte infiltration around the blood vessels in the glandular interstitium. Similar findings were recorded in the control cockerels.

In hens, there were no statistically significant main effects of IBV strain on the histopathological lesions in the Harderian gland. However, there was a significant main effect of days p.i. on all but one of the lesions investigated (Table 3.2). In T

strain infected hens, on day 3 p.i., there was infiltration of plasma cells and globular leukocytes in the subepithelium and the collecting duct epithelium was severely damaged. On 6, 10, and 13 days p.i., plasma cells and lymphocytes around blood vessels in the septa (interlobular space) were frequently observed. On 16 and 21 days p.i., most of the collecting duct epithelium had regenerated, but the lymphocyte infiltration in the interstitium and globular leukocytes in the subepithelium were still extensive (Plate 3.1). Exfoliative epithelium, along with inflammatory cells, was seen occasionally in the collecting duct lumen from days 3 to 16 p.i. Migration of heterophils into the subepithelium was mild at 6 and 10 days p.i. In N1/88 infected hens, most of the lesions were similar to those of T stain infection but the lesions were less severe.

For cockerels, there was no statistically significant main effect of IBV strain on any of the lesions and there was a significant main effect of days p.i. only for the incidence of lymphocyte infiltration in septa (Table 3.3). In addition, there was a significant main effect of sex of chicken only for the incidence of heterophils in the subepithelium. In cockerels, degeneration of collecting duct epithelium, heterophilic infiltration in the subepithelium and debris of exfoliated epithelium in the collecting duct lumen was mild at 2 days p.i. These changes were mild to moderate at 4 and 6 day p.i. and, on days 8 and 10 p.i., most of the collecting duct epithelium had regenerated. However, mild to moderate infiltration of heterophils in the subepithelial space continued. There was an increase in the number of plasma cells from 2 days p.i. which was constant throughout the experiment. Lymphocyte infiltration in the septal area was extensive throughout the experiment. The T strain of IBV was more pathogenic as compared to N1/88.

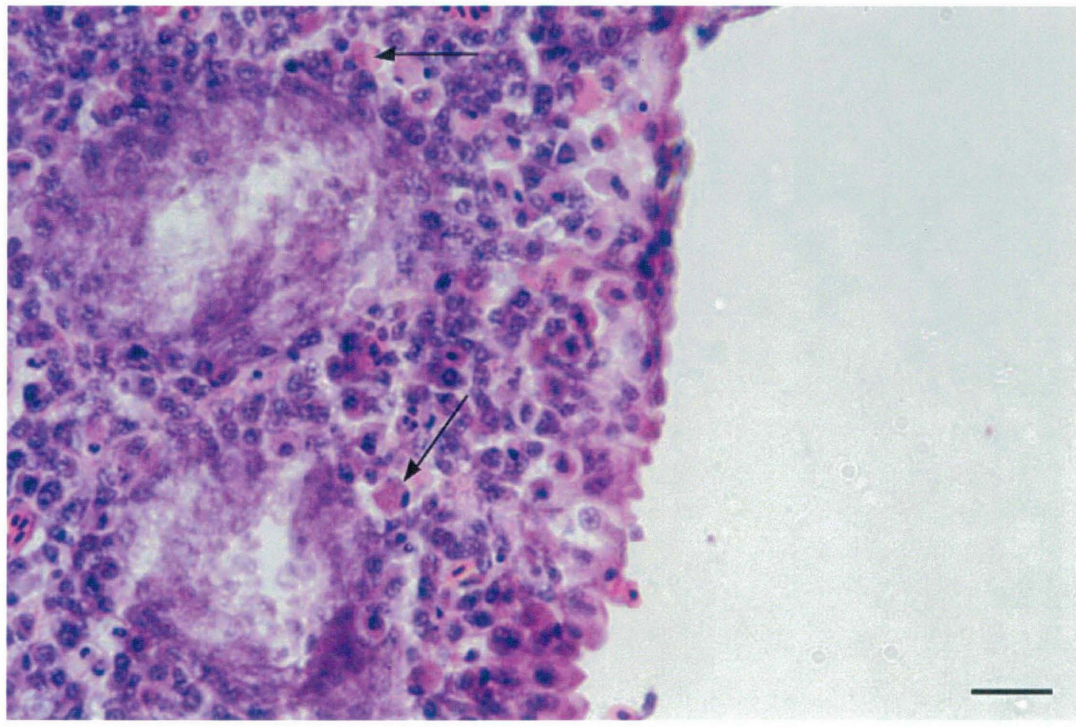

Plate 3.1: Globular leukocytes in Harderian gland (arrows) of T strain infected hen on day 21 p.i. H &E x 400. Scale bar represents 25 µm

Table 3.2: Comparative histopathology of Harderian gland in hens infected with T and N1/88 strain of IBV

	Day post inoculation of IBV													
	3		6		10		13		16		21		P value	
Lesions in Harderian gland	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	Treatment	Days p.i.
Degeneration of collecting duct epithelium	3	2	2	3	1	2	1	1	0	0	0	0	NS	0.003
Lymphocyte infiltration in septa	1	1	1	2	3	2	3	2	3	3	3	1	NS	0.040
Heterophils in sub epithelium	0	0	1	0	1	1	0	0	0	0	0	0	NS	0.019
Exfoliative epithelial cells in collecting tubules	1	1	2	1	2	1	0	1	1	1	0	0	NS	NS

0, No change; 1, Mild; 2, Moderate; 3, Severe.
Mean of lesion score from three hens
Significance assumed at $p<0.05$. NS is not significant
Main effect of IBV strain was determined by Mann-Whitney U test
Main effect of days p.i. was determined by Kruskal-Wallis test

Table 3.3: Comparative histopathology of Harderian gland in cockerels infected with T and N1/88 strains of IBV

	Day post inoculation of IBV												
	2		4		6		8		10		P value		
Lesions in Harderian gland	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	Treatment	Days p.i.	H v C
Degeneration of duct epithelium	1	1	0	0	1	1	0	0	0	0	NS	NS	0.053
Lymphocyte infiltration in septa	2	2	3	3	3	3	3	3	3	3	NS	0.022	NS
Heterophils in sub epithelium	1	1	1	2	1	1	1	0	1	2	NS	NS	0.004*
Exfoliative epithelial cells in collecting tubules	1	1	1	1	1	1	2	0	0	0	NS	NS	NS

, H v C- Hen versus cockerel
0, No change; 1, Mild; 2, Moderate; 3, Severe.
Significance assumed at $p<0.05$. NS is not significant
Mean of lesion score from four cockerels
Main effect of IBV strain was determined by Mann-Whitney U test
Main effect of days p.i. was determined by Kruskal-Wallis test
Hen versus cockerel score was compared by Mann-Whitney U test

3.3.2 Trachea

Normal tracheal epithelia, with healthy cilia and mucus glands, were seen in the control hens and cockerels (Randall and Reece, 1996).

In hens, there were no statistically significant main effects of IBV strain on any of the histopathological lesions investigated but there was a significant main effect of days p.i. on all the lesions scored (Table 3.4). There were no microscopic changes at three days p.i., in either T- or N1/88-infected hens. Severe pathology occurred mainly from day 6 p.i. in the form of loss of cilia and mucus glands, changes in the mucosal epithelium from columnar to squamous, oedema in the subepithelium and occasional heterophilic exudate in the tracheal lumen. Most of the above lesions persisted in moderate to mild form in both infected groups on day 10 p.i. On day 13 p.i., most of the cilia and the epithelium had regenerated in the N1/88 group. The hypertrophied glands were normal with occasional exudate in the lumen. Goblet cells appeared to be less frequent in both the T- and N1/88-infected groups on days 3-10 p.i. but, after day 10, this appeared to be the case only in the N1/88-infected group. On days 16 and 21, most features of the tracheas appeared normal. However, thickening of the mucosa with infiltration of lymphocytic nodules persisted from days 13 to 21 p.i.

In cockerels, there were no statistically significant main effects of IBV strain on any of the histopathological lesions investigated but there was a significant main effect of days p.i. on all the lesions scored (Table 3.5). In addition, there was a significant main effect of sex of chicken only for the incidence of hypertrophy of glands and oedema in the submucosa. In cockerels, cilia loss along with degeneration of the epithelium was severe to moderate in T-infected chickens and moderate to mild in N1/88-infected chickens on 4 and 6 days p.i. Lesions were moderate in both IBV groups on the 8th day p.i. Moderate to severe hypertrophy of glands, with an increased number of lymphocytes and mucosal oedema was persistent from 4 to 10 days p.i. The severity of lesions in both challenge groups, in hens as well as cockerels, was similar. However IBV seemed to be more pathogenic in the trachea of males as compared to females.

Table3. 4: Comparative histopathology of trachea in hens infected with T and N1/88 strain of IBV

	Day post inoculation of IBV													
	3		6		10		13		16		21		P value	
Lesions in Trachea	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	Treatment	Days p.i.
Cilia loss	0	0	3	3	3	1	1	0	0	0	0	0	NS	0.004
Epithelial degeneration	0	0	3	3	2	1	1	0	0	0	0	0	NS	0.004
Hypertrophy of alveolar mucous glands	0	0	2	1	2	2	1	1	0	0	0	0	NS	0.001
Oedema in mucosa	0	0	3	3	2	2	0	1	0	0	0	0	NS	0.001
Lymphocyte infiltration	0	0	0	1	1	1	2	2	2	2	3	2	NS	0.001

0, No change; 1, Mild; 2, Moderate; 3, Severe.
Mean of lesion score from three hens
Significance assumed at $p<0.05$. NS is not significant
Main effect of IBV strain was determined by Mann-Whitney U test
Main effect of days p.i. was determined by Kruskal-Wallis test

Table 3. 5: Comparative histopathology of trachea in cockerels infected with T and N1/88 strains of IBV

	Day post inoculation of IBV												
	2		4		6		8		10		P value		
Lesions in Trachea	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	Treatment	Days p.i.	H v C
Cilia loss	0	0	3	2	3	1	2	2	0	0	NS	<0.001	NS
Epithelial degeneration	0	0	3	2	2	1	2	2	0	0	NS	<0.001	NS
Hypertrophy of alveolar mucous glands	0	0	3	2	2	2	3	2	1	0	NS	<0.001	0.031
Oedema in mucosa	0	0	2	2	3	2	3	2	2	0	NS	0.002	0.033
Lymphocyte infiltration	0	0	3	3	2	2	2	2	2	2	NS	<0.001	NS

H v C- Hen versus cockerel
0, No change; 1, Mild; 2, Moderate; 3, Severe.
Mean of lesion score from four cockerels
Significance assumed at $p<0.05$. NS is not significant
Main effect of IBV strain was determined by Mann-Whitney U test
Main effect of days p.i. was determined by Kruskal-Wallis test
Hen versus cockerel score was compared by Mann-Whitney U test

3.3.3 Kidney

The kidneys of the chickens from the control groups were normal.

In hens, there was no statistically significant main effect of IBV strain on any of the lesions investigated but there was a significant main effect of days p.i. on 4 out of the 7 lesions scored (Table 3.6). In hens, the main kidney lesions consisted of necrosis of proximal convoluted tubules, distension of distal convoluted tubules (not scored), necrotic foci, infiltration of lymphocytes in the interstitial space, oedema of Bowmans capsule, urates and granulocytic casts in collecting ducts. The lesions were more apparent on the 10^{th} day p.i. in both T- and N1/88-infected hens (Plate 3.2). The pathology continued up to day 13 in the infected groups. Granulocytic casts and oedema of Bowmans capsule in the T-infected group persisted until the 16^{th} day p.i. Infected tissue was cleared out by inflammatory cells with diffuse lymphocyte infiltration in the cortex as well as the medulla from day 13 until the end of experiment in the T-infected group.

For cockerels, there was a statistically significant main effect of IBV strain only for lymphocyte infiltration. However, there was a significant main effect of days p.i. for all lesions except duct and tubular dilatation (Table 3.7). There were no significant main effects between hens and cockerels. In both groups of infected cockerels, pathology was observed mainly from day 4 and 6 p.i. On the 8^{th} day p.i., most of the changes in the N1/88 group had subsided but granulocytic and urate casts, along with necrotic foci, were evident in the T-infected group. On the 10^{th} day p.i., no changes were recorded except for lymphocyte infiltration, which was a consistent finding from day 4 p.i.

3.3.4 Caecum

No changes were observed in the caecum in any of the groups of either hens or cockerels.

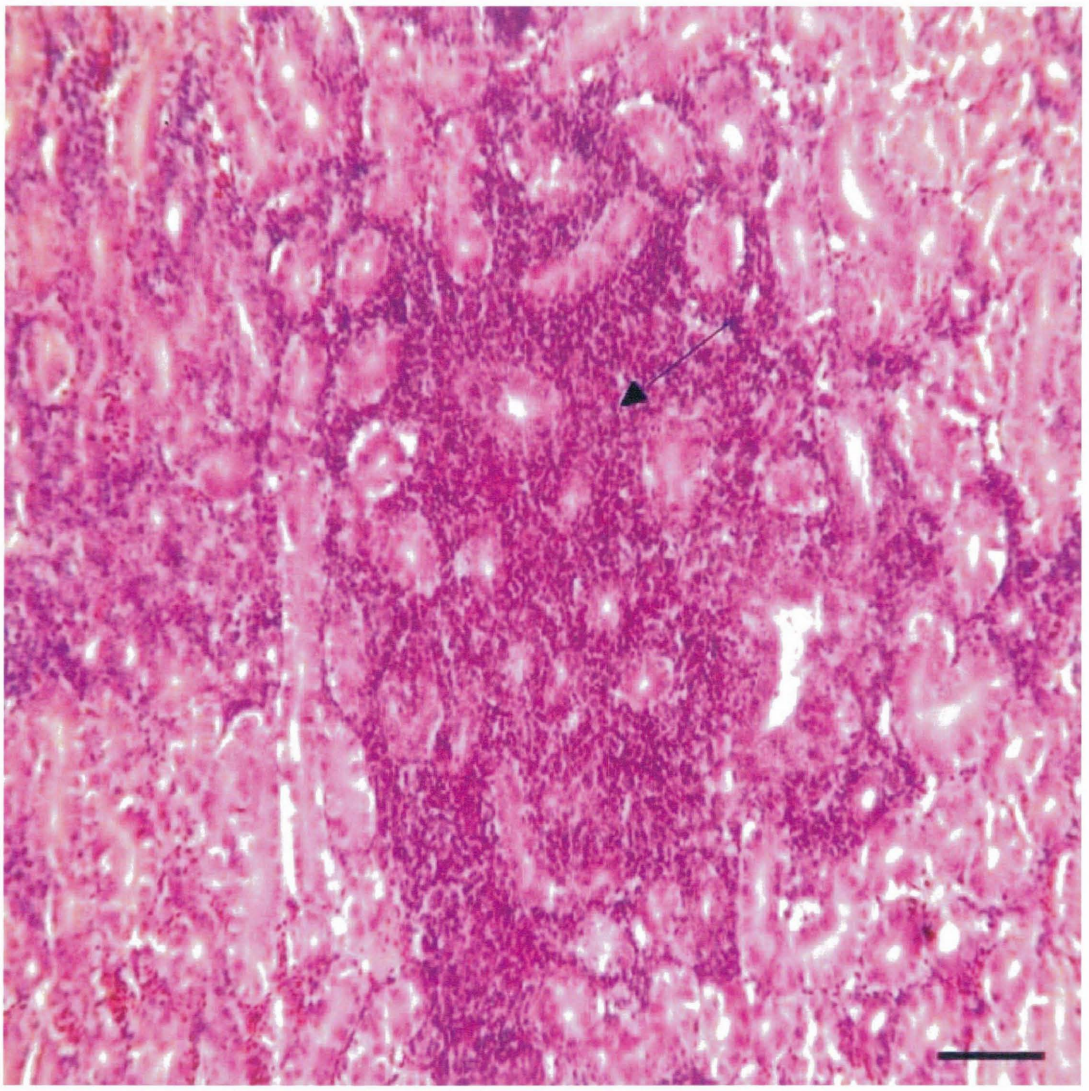

Plate 3.2: Lymphocyte infiltration in kidney (arrow) of N1/88 strain infected hen on day 10 p.i. H&E x 200. Scale bar represents 50 µm.

Table 3. 6: Comparative histopathology of kidney in hens infected with T and N1/88 strain of IBV

	Day post inoculation of IBV													
	3		6		10		13		16		21		P value	
Lesions in kidney	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	Treatment	Days p.i.
Oedema of Bowman's capsule	1	0	0	0	2	1	2	1	2	0	0	0	NS	0.0311
Urate casts	0	0	0	1	2	1	1	0	0	0	0	0	NS	NS
Necrotic foci	0	0	0	1	2	0	2	0	0	0	0	0	NS	NS
Granulocytic casts	0	0	2	0	1	1	0	2	1	0	0	0	NS	0.027
Lymphocyte infiltration	0	0	0	0	0	0	2	1	3	2	2	0	NS	0.004
Duct and tubular dilatation	0	0	0	0	1	1	2	1	0	0	1	0	NS	NS
Necrosis of Proximal convoluted tubule	0	0	0	0	1	1	1	1	0	0	0	0	NS	0.0520

0, No change; 1, Mild; 2, Moderate; 3, Severe.
Mean of lesion score from three hens
Significance assumed at $p<0.05$. NS is not significant
Main effect of IBV strain was determined by Mann-Whitney U test
Main effect of days p.i. was determined by Kruskal-Wallis test

Table 3.7: Comparative histopathology of kidney in cockerels infected with T and N1/88 strains of IBV

	Day post inoculation of IBV												
	2		4		6		8		10		P value		
Lesions in kidney	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	Treatment	Days p.i.	H v C
Oedema of Bowman's capsule	0	0	2	0	1	2	0	0	0	0	NS	0.032	NS
Urate casts	0	0	1	2	1	0	1	0	0	0	NS	0.002	NS
Necrotic foci	0	0	0	2	2	0	1	0	0	0	NS	0.041	NS
Granulocytic casts	0	0	2	2	2	1	1	0	0	0	NS	0.001	NS
Lymphocyte infiltration	0	0	2	1	2	1	3	1	2	1	0.0102	0.007	NS
Duct and tubular dilatation	0	0	0	0	1	1	0	0	0	0	NS	NS	NS
Necrosis of Proximal convoluted tubule	0	0	0	0	0	0	0	0	0	0	-	-	NS

H v C- Hen versus cockerel
0, No change; 1, Mild; 2, Moderate; 3, Severe.
Mean of lesion score from four cockerels
Significance assumed at $p<0.05$. NS is not significant
Main effect of IBV strain was determined by Mann-Whitney U test
Main effect of days p.i. was determined by Kruskal-Wallis test
Hen versus cockerel score was compared by Mann-Whitney U test

3.3.5 Oviduct

Different parts of oviduct, magnum, tubular shell gland (TSG) and shell gland pouch (SGP) were examined separately.

In the magnum, there was no statistically significant main effect of IBV strain but there were significant main effects of days p.i. for all the lesions scored (Table 3.8). In the magnum, on the 10^{th} p.i., severe gland dilatation and complete absence of mucopolysaccharides from epithelial cells in the N1/88-infected group was the prominent finding (Plate 3.3). This finding was moderate in T-infected hens. Lymphocyte cell infiltration in both T-and N1/88-infected groups was mild on the 10^{th} day p.i. and was mild to moderate at days 13 to 21 p.i. In both infected groups, oedema in the submucosa was moderate on the 10^{th} day p.i. and moderate to mild on day 13 p.i.

In the TSG and SGP, there was no statistically significant main effect of IBV strain but there were significant main effects of days p.i. for all the lesions scored except oedema in the submucosa (Table 3.9). In the TSG and SGP of both infected groups, on day 10 p.i., cilia loss, changes in the epithelium, gland dilatation, oedema in the submucosa and lymphocyte infiltration were the main findings. Glandular dilatation was severe in the T group and moderate in the N1/88 group on the 10^{th} day p.i. (Plate 3.4). However, glandular dilatation was mild in the T-infected group and moderate in the N1/88-infected group on day 13 p.i. The dilated glands were filled with homogenous pink-stained material which was also observed on the 13^{th} day p.i. in T-infected hens. Oedema in the submucosa was moderate on day 10 p.i. but mild on day 13 p.i. The intensity of infiltration of inflammatory cells was greater in the TSG and SGP of T-infected hens on days 16 and 21 p.i. From 13 to 21 days p.i., lymphocyte infiltration in the lamina propria and muscularis layers was moderate in the T-infected group but mild in the N1/88 infected group. Most of the tissues had regenerated with the appearance of mitotic figures at 21 day p.i. All the parts of oviduct in the control hens appeared normal throughout the experiment.

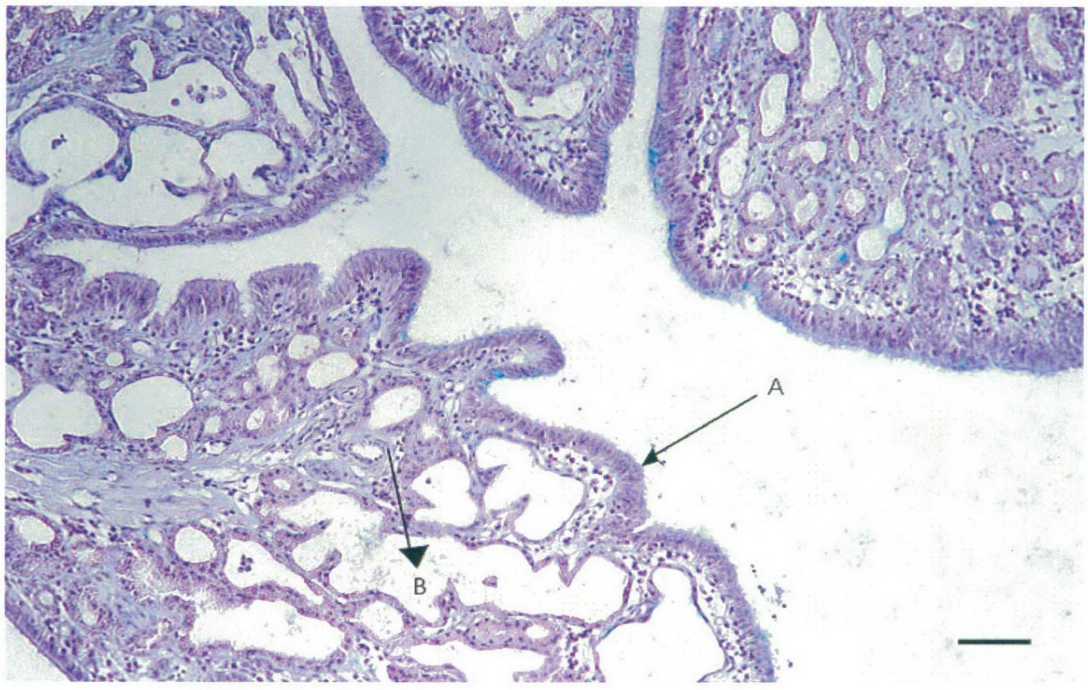

Plate 3.3: Magnum of N1/88 strain infected hen on day 10 p.i. Alcian blue stain showing unstained epithelial cells (arrow, A) and dilatation of glands (arrow, B). H & E.x200. Scale bar represents 50 µm. Egg in upper magnum.

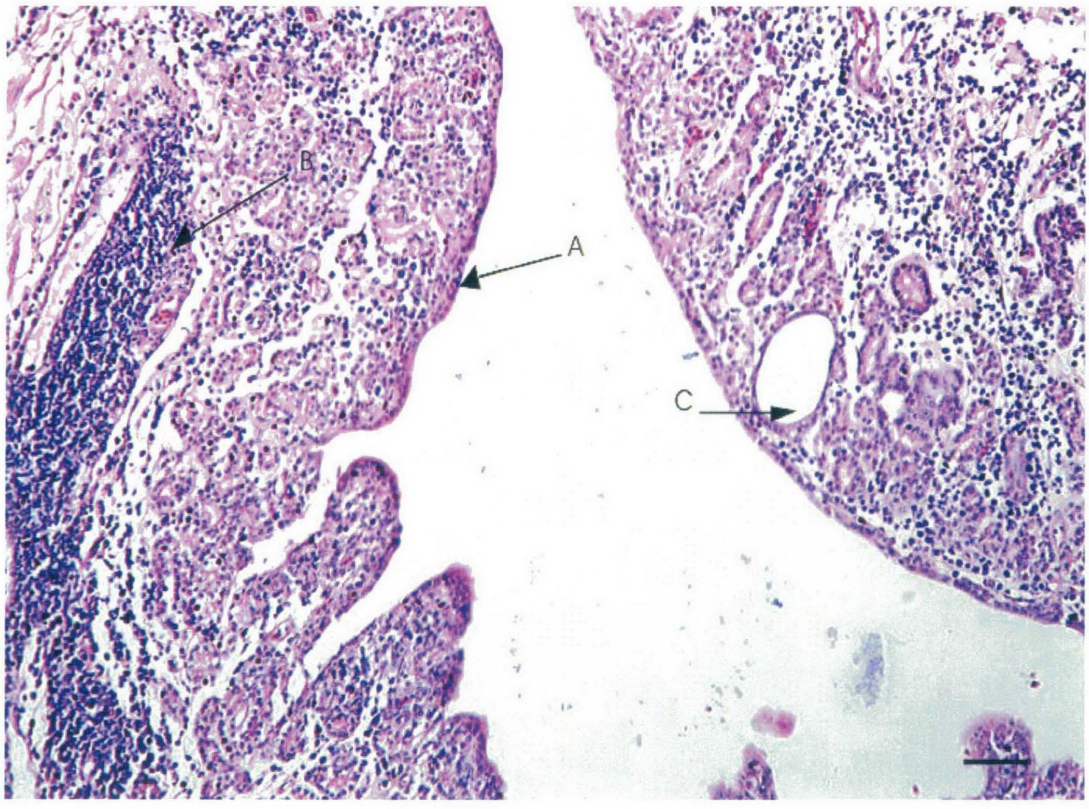

Plate 3.4: Shell gland pouch of T strain infected hen showing loss of cilia (arrow, A), infiltration of lymphocytes (arrow, B) and glandular dilatation (arrow, C) on day 10 p.i. H& E x 200. Scale bar represents 50 µm. No egg in the oviduct

Table 3.8: Comparative histopathology of magnum in hens infected with T and N1/88 strain of IBV

	Day post inoculation of IBV													
	3		6		10		13		16		21		P value	
Lesions in oviduct	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	Treatment	Days p.i.
Loss of cilia	0	0	0	0	2	3	1	2	2	1	0	0	NS	<0.001
Tubular gland dilatation	0	0	0	0	2	3	1	3	0	0	1	0	NS	0.028
Lymphocyte infiltration	0	0	1	0	1	1	2	2	2	1	2	2	NS	0.044
Oedema in sub mucosa	0	0	0	0	2	2-	2	1	1	0	0	0	NS	0.005

0, No change; 1, Mild; 2, Moderate; 3, Severe.
Mean of lesion score from three hens
Significance assumed at $p<0.05$. NS is not significant
Main effect of IBV strain was determined by Mann-Whitney U test
Main effect of days p.i. was determined by Kruskal-Wallis test

Table 3.9: Comparative histopathology of TSG and SGP in hens infected with T and N1/88 strain of IBV

	Day post inoculation of IBV													
	3		6		10		13		16		21		P value	
Lesions in oviduct	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	Treatment	Days p.i.
Loss of cilia	0	0	0	0	3	2	2	2	1	1	0	0	NS	0.002
Tubular gland dilatation	0	0	0	0	3	2	1	2	0	0	1	0	NS	0.001
Lymphocyte infiltration	0	0	+	-	1	1	2	2	2	1	2	1	NS	0.009
Oedema in sub mucosa	0	0	0	0	2	2	1	1	0	0	0	0	NS	NS

0, No change; 1, Mild; 2, Moderate; 3, Severe.
Mean of lesion score from three hens
Significance assumed at $p<0.05$. NS is not significant
Main effect of IBV strain was determined by Mann-Whitney U test
Main effect of days p.i. was determined by Kruskal-Wallis test
Hen versus cockerel score was compared by Mann-Whitney U test

3.4 Discussion

In challenged groups of hens (T and N1/88), the severity and time frame of lesions in the Harderian gland were very similar which indicates that both IBV strains are equally pathogenic for the Harderian gland. However, regeneration occurred more quickly in the N1/88 infected chickens than the T strain chickens. Our finding regarding regeneration of the ductal epithelium agrees with that of Toro *et al.* (1996). In cockerels, a similar pattern of damage and regeneration was observed. However, the severity index of lesions in the hens was higher than for the cockerels. Globular leukocytes originate from mast cells or granulocytic eosinophils. The occurrence of globular leukocytes in the subepithelium of the Harderian gland in the infected chickens could be the result of active immunostimulation episodes. However, the function of globular leukocytes is not fully understood. Our finding regarding a significant difference in lymphocyte infiltration around the blood vessels in the septa, at different days post inoculation, agrees with Survashe *et al* (1979) who found a similar trend in the Harderian gland of chickens challenged with the H120 vaccine strain.

In the past, the histopathology of IBV in trachea has been extensively described but most of the studies were limited to young chickens. In the present study, histopathological lesions and the time course of infection observed in the trachea after IBV challenge can be compared with the findings of Nakamura and coworkers who compared the histopathology of IBV in the trachea of two chicken lines using the M41 IBV strain (Nakamura *et al.*, 1991). However, these authors report time frames for the various lesions that differ between the two lines of chickens studied and also differ from those found in the present study. Fulton *et al.* (1993), using respiratory tract lavage in two-week old chickens, found increasing numbers of inflammatory cells at different time intervals up to four days following M41 and T-strain IBV infection. In addition, more total cells and more inflammatory cells were recovered for the M41-infected chickens than for the T-strain infected or the control chickens. However Fulton studied the response at 2, 8, 24, 48, 72 and 96 hours p.i. in two week-old chickens, whereas our findings are from adult hens and cockerels at 2-3 day intervals. A similar trend of increasing numbers of inflammatory cells was found in the present study up to 13 to 21 days p.i. In addition, lesions were similar for both

IBV strains, indicating a similar predilection of both strains for the trachea. Lesions were more severe and persistent in both the groups of cockerels as compared to the hens, which suggests that virus is highly pathogenic for the trachea of young cockerels as evidenced by a higher incidence of hypertrophy of glands and oedema in the mucosa. It is likely that the severity and time course of lesions is influenced by strain of IBV, strain of chickens and age of chickens.

The histopathological changes observed in the kidney match previous findings (Purcell *et al.*, 1976; Chen *et al.*, 1996) except for the microscopical lesion of interstitial oedema which was not observed in the present study. The T strain IBV was more nephropathogenic (Chong and Apostolov, 1982) as compared to N1/88 in hens and similar observations were recorded in the younger cockerels. Granulocytic casts and urate casts could be a manifestation of urolithiasis and Cavanagh and Naqi (1997) also reported an increased incidence of casts following IBV infection.

Most of the changes in the oviduct were noticeable on the 10^{th} day p.i., a finding that is in accordance with Sevoian & Levine (1957). Glandular dilatation and loss of mucopolysaccharides in the epithelial layer of the magnum may be the contributory factor in albumen thinning (Butler *et al.*, 1972). The moderate inflammatory cell debris in the lumen of the oviduct may lead to the presence of meat spots in egg albumen as reported by McDougall (1968). The duration and severity of effects suggest that T strain has more affinity and pathogenicity for the tubular shell gland and shell gland pouch but N1/88 is more pathogenic for the magnum of the oviduct. This finding suggests that the fully functional oviduct is susceptible to IBV infection. The significant difference in lesion score in different parts of the oviduct with respect to time could be compared with studies in infected oviduct cell culture by Pradhan *et al.* (1984) who studied ciliary movement in oviduct cells. However, there is a dearth of literature regarding *in-vivo* quantitative assessment of other lesions in the oviduct which precludes comparison of our findings with those of other workers. Further investigation is required to fully describe the effects of IBV on the shell forming regions of the oviduct, tubular shell gland and shell gland pouch, including the mechanisms causing misshapen and soft shelled eggs, and cessation of (or reduced) egg production.

In both the IBV infected hen and cockerels, the intensity of lesions in the kidney was not as severe and persistent as reported in earlier literature. However, the reverse was observed for lesions in the trachea which were both persistent and severe in both T and N1/88 infected chickens. The T strain (N1/62) has been regarded as highly nephropathogenic (Cumming, 1962, Klieve and Cumming, 1988) and has been reported as replicating in the trachea with mild lesions (Ignjatovic *et al.*, 2002). Our findings suggest that, besides being nephropathogenic, the T strain of IBV has an ability to produce severe pathology in the trachea. Similarly, Sapats *et al.* (1996b) reported that N1/88 did not replicate in kidney. However, our findings were that N1/88 can produce kidney lesions in hens as well as in cockerels. In addition, the N1/88 strain has also been isolated from the kidney by Roberts (2005)

At the same time, the possibility of an intrinsic factor such as bird age, influencing the pathogenesis for kidney (Albassam *et al.*, 1986) and oviduct (Crinion *et al.*, 1971 a & b) cannot be ignored. After experimental challenge with IBV, the sequential observations by histopathology suggest that the IBV replicates first in the Harderian gland, then the tracheal mucosa and then simultaneously replicates in the kidney and oviduct. On the other hand, in cockerels, the IBV first replicates in the Harderian gland and then simultaneously in the trachea and kidney.

Chapter 4

Preliminary studies on histopathology of two serotypes of infectious bronchitis virus in laying hens vaccinated in the rearing phase.

4.1 Introduction

Infectious bronchitis virus (IBV) has a great economic impact on the layer industry as it affects egg production and quality. Vaccination frequency and time has been always the topic of debate for the egg industry. At present all over the world, a range of vaccines is available commercially, with various administration protocols recommended. According to Cavanagh and Naqui (1997) the vaccine protocol for laying pullets includes one or two revaccinations during rearing and boosters during lay at 8-10 week intervals. Sulaiman *et al.* (2004) reported that regular revaccination during lay has a deleterious effect on egg production and quality. However, when hens vaccinated during rearing were challenged in mid- and late-lay, there were negative effects on production and quality of eggs (Jolly 2005). Relatively little information is available from previous studies regarding histopathological changes occurring in challenged birds. The present study was designed to investigate the extent of protection offered by vaccination on various organs in hens vaccinated during rearing and challenged at 110 weeks of age with two phylogenetically different Australian strains of IBV.

4.2 Materials and methods

In total, 36 Hyline Grey (Hy-Line Australia Pty Ltd., Maitland, New South Wales) hens were used in this experiment. All birds were vaccinated with commercial vaccine at the age of day old, four weeks and twelve weeks and had been used previously as a fertile flock. All the vaccinated hens were kept physically separated from the unvaccinated White Leghorns discussed in Chapter 3. Twelve HyLine birds from two groups at the

age of 110 weeks were challenged with two different Australian strains of virus, T and N1/88 (virus source and dose as described in chapter 2, section 2.1). Six birds were kept as a control and maintained throughout the experiment while another six control birds were sacrificed. Two birds from each treatment and one bird from the control group were sacrificed on days 3, 6, 10, 13, 16, and 21 post-infection (p.i.). Harderian gland, trachea, kidney and different regions of oviduct, magnum, tubular shell gland and shell gland pouch (Solomon, 1991) were fixed in 10% neutral buffered formalin. The tissues were processed by standard histological procedures, (as described in chapter 2 section 2.3). Lesions were not scored.

4.3 Results

4.3.1 Harderian gland

In control birds, the main features were some plasma cells in the sub-epithelium, an intact collecting duct epithelium and acinar epithelium, and migratory infiltration of lymphocytes in the interstitium.

On the 3rd day p.i., in both T- and N1/88-infected birds, the collecting duct epithelium displayed moderate damage. On 6, 10, and 13 days p.i., lymphoid cells around blood vessels and globular leukocytes in the subepithelium were numerous. On 16 and 21 days p.i., most of the ductal epithelium had regenerated, the number globular leukocytes was reduced, but the lymphocyte infiltration in the interstitium was at a peak. Migration of lymphocytes into the sub-epithelium was mild on 3, 13 16 and 21 days p.i. but moderate at 6 and 10 days p.i. In N1/88-infected hens, most of the lesions were similar to birds infected with T-strain IBV. Exfoliative epithelium, along with a few inflammatory cells, was a constant finding in all infected birds, as well as control birds, throughout the experiment.

4.3.2 Trachea

All changes in the trachea were mild to moderate. In both T- and N1/88-infected groups, there was occasional loss of cilia on day 6 p.i., with moderate distortion of the epithelium. Cilia in most parts of the trachea appeared normal for the remainder of the experiment. The hypertrophy of glandular cells was noted on day 6 p.i. and continued up to day 13 p.i. However, the severity of glandular hypertrophy was greater in N1/88-infected birds than for T-infected birds. On the 21st day p.i., most of the tissue appeared normal but there was extensive thickening of mucosa due to heavy infiltration of lymphocytes in both the infected groups.

In vaccinated control birds, there were no changes except persistent heavy lymphocytic infiltration in the mucosal layer. (Plate 4.1)

4.3.3 Kidney

In control birds, occasional focal infiltration of lymphocytes and granulocytic casts granulytic casts were observed.

In both the infected groups, granulocytic and urate casts in the collecting duct and occasional necrotic foci were persistent throughout the experiment, beginning at day 6 p.i. In the T-infected group, there was lymphocytic infiltration in the interstitial space at 16 and 21 days p.i., and heavy lymphocytic infiltration was also recorded in one bird from the N1/88-infected group killed at the 16th day p.i. Moderate oedema in Bowmans capsule was observed in the T-infected group, but oedema was mild in N1/88-infected birds, on 10th and 13th day p.i. The remainder of the tissue appeared normal.

4.3.4 Oviduct

As compared with challenged vaccinated birds, in control vaccinated birds, there was no prominent pathology in any part of the oviduct including the magnum, tubular shell gland or shell gland pouch, but there was mild infiltration of lymphocytes around blood

vessels in the muscularis area. A small number of plasma cells were also recorded in the interglandular space.

In the magnum of T-infected birds, there was moderate loss of cilia on day 10 p.i. which continued up to the 16th day p.i. However, occasional cilia loss was also observed in one bird killed on the 21st day p.i. In the magnum of N1/88-infected birds, most of the epithelial cells were devoid of mucopolysaccharides on the 10th day p.i. (Plate 4.2). In contrast, the epithelium of control birds showed continuous staining (Plate 4.3). There was occasional loss of cilia until the 16th day p.i. Lymphocytic infiltration in the subepithelial space was persistent in the magnum of both the infected groups.

In T- and N1/88-infected birds, the findings and time frame of the effects of IBV infection in both the tubular shell gland and shell gland pouch were similar. There was occasional loss of cilia in both the tubular shell gland and shell gland pouch on the 10th day p.i. which continued up to the 21st day p.i. in the shell gland of T-infected birds (Plate 4.4). Most of the blood vessels in the muscularis area were accompanied by lymphocytes although infiltration was occasional and moderate in the subepithelium. Moderate dilatation of glands was noted in the shell gland of T infected birds on the 10th day p.i. ands continued up to the 16th day p.i.

4.4 Discussion

The exfoliative epithelium in the Harderian gland, observed in all three treatment groups, may result from exposure to a naturally occurring irritant such as dust. In both challenged groups (T and N1/88) of vaccinated birds, the severity and time frame of lesions in the Harderian gland were almost the same which indicates that both strains are equally pathogenic for the Harderian gland although regeneration occurred more quickly in the N1/88-infected birds. Our findings agree with those of Davelaar & Kouwenhoven, (1976) and Toro *et al.* (1996). The presence of globular leukocytes originating from mast cells and eosinophilic granulocytes could be the result of active immunostimulation episodes.

For the trachea, lesions were similar for both IBV strains, indicating a similar predilection of both strains for the trachea. However, most parts of the trachea appeared normal by the end of experiment. This indicates that the trachea has been protected to a moderate extent by the vaccination protocol used. Our findings regarding loss of tracheal cilia in the initial phase of IBV challenge in vaccinated birds agree with the results of Di-Matteo *et al*, (2000). The cause of the extensive thickening of the mucosal layer, due to infiltration of lymphocytes in both control and treated birds, needs further investigation.

The histopathological changes observed in the kidney of the challenged groups matches previous findings (Ratanasethakul and Cumming, 1981). Severity of kidney lesions was greater in T- as compared to N1/88-infected hens. Jolly (2005) also reported lymphocytic infiltration in the kidneys of T-infected birds.

Most of the changes in the oviduct of the challenged groups were noticeable on the 10^{th} day p.i., as was recorded also by Sevoian & Levine (1957). However, the moderate pathology of T strain in the tubular shell gland and shell gland pouch, and severe pathology of N1/88 particularly in the magnum, indicates the disparity between the strains of virus and their ability to induce pathology in different parts of the oviduct. Our finding regarding loss of mucopolysaccharides from epithelial cells in the magnum is in accordance with Davidson (1986) who reported a similar observation in the magnum of hens producing watery whites.

The histopathological findings in the trachea and kidney of both T- and N1/88-infected vaccinated birds suggest that the vaccination protocol has offered moderate protection in both of these organs. On the other hand, the extent of the histopathology in the oviduct indicates that the vaccination protocol has offered only limited protection for the oviduct against infection by both the strains of infectious bronchitis at a very late stage of lay. The significant pathology and affinity of both IBV strains for the oviduct of laying hens suggests that, despite vaccination, the fully functional oviduct can be affected, which could influence egg production and egg quality. However, intrinsic factors such as bird age could also influence the pathogenesis of IBV effects on the

oviduct (Crinion *et al.*, 1971) as well as egg production and quality (Roberts, 2004). It is difficult to compare our results of the effects of IBV on the oviduct of vaccinated hens with those of other researchers owing to a scarcity of literature regarding the response of the normal and fully functional oviduct during IBV infection. This study focused on a detailed study of the pathogenesis of IBV in birds in full lay, to form a basis for further studies. Despite the fact that IB revaccination could be disadvantageous (Roberts *et al.*, 2004), the extent to which regular IBV revaccination or intercurrent IBV infection can induce microscopic pathology in the oviduct of the mature laying hen needs further investigation.

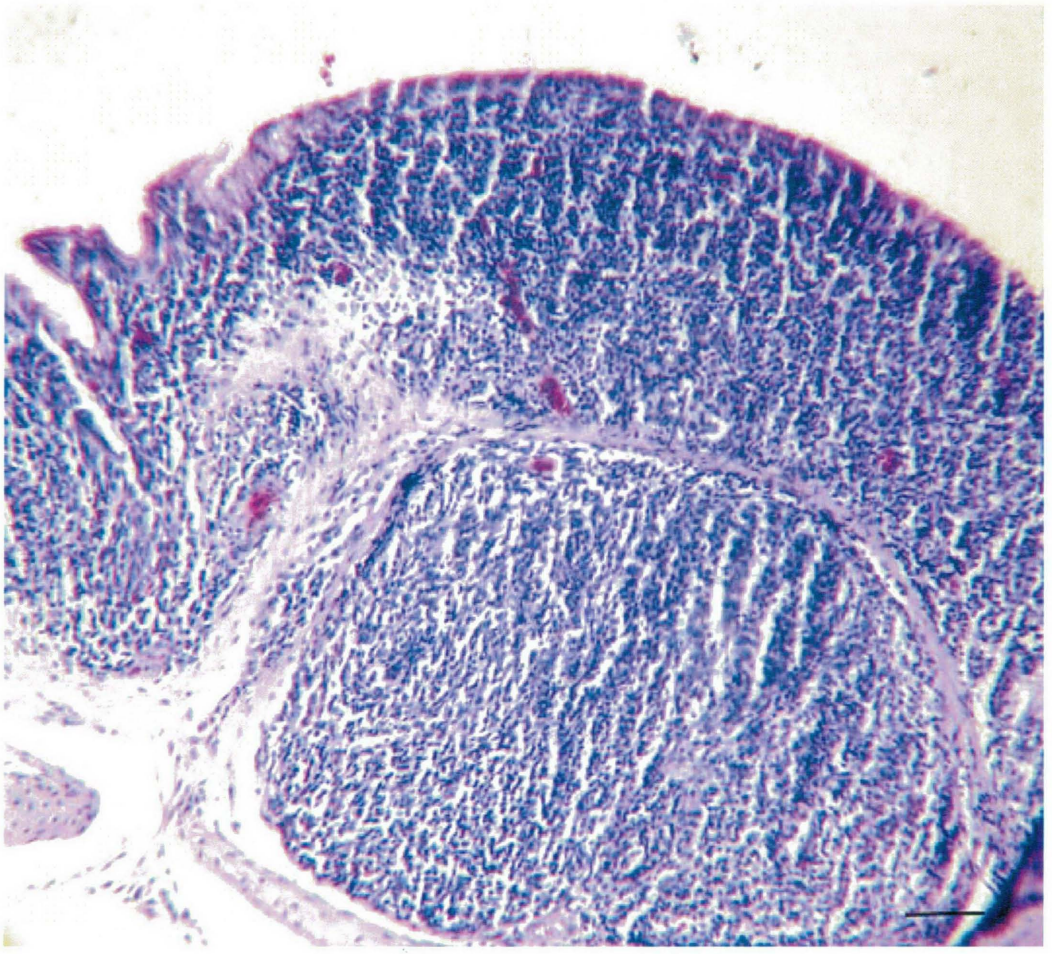

Plate 4.1: Trachea of HyLine hen from control group. Note intense thickening of tracheal mucosa along with the lymphocyte nodule. H & E. x 100. Scale bar represents 100 µm

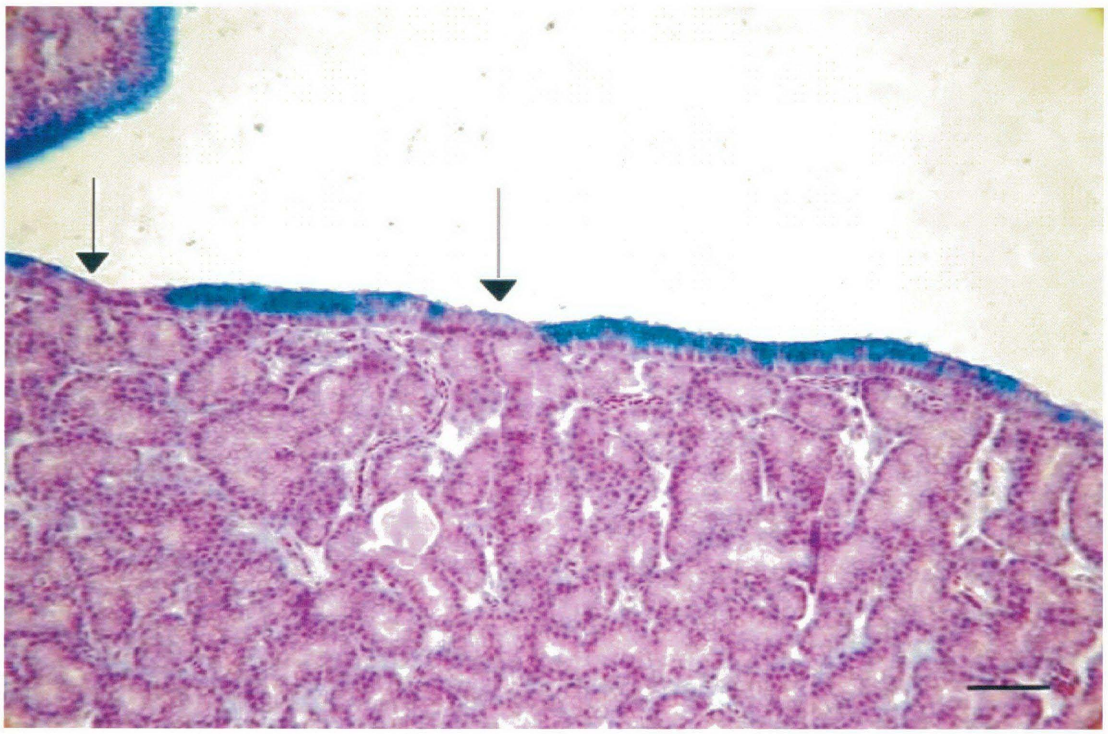

Plate 4.2: Magnum of N1/88 infected Hyline hen. Alcian blue x 400. Scale bar represents 25 µm. Soft shelled egg in shell gland pouch of oviduct.

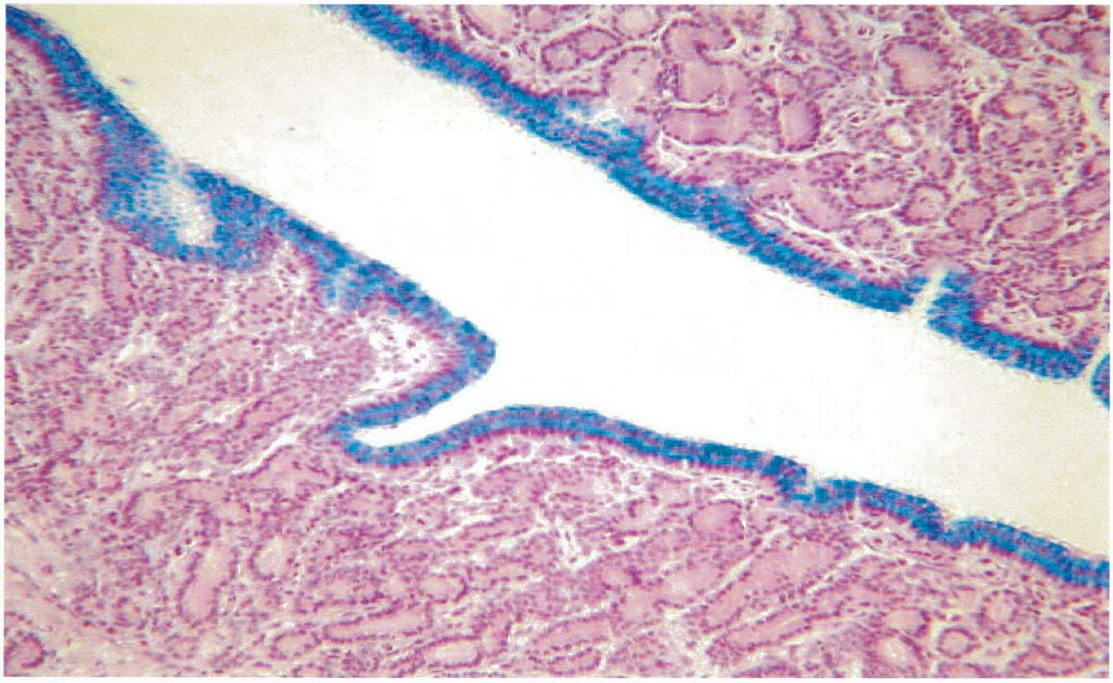

Plate 4.3. Magnum of control HyLine hen. Alcian blue x 400. Scale bar represents 25 µm. Soft shelled egg in shell gland pouch of oviduct.

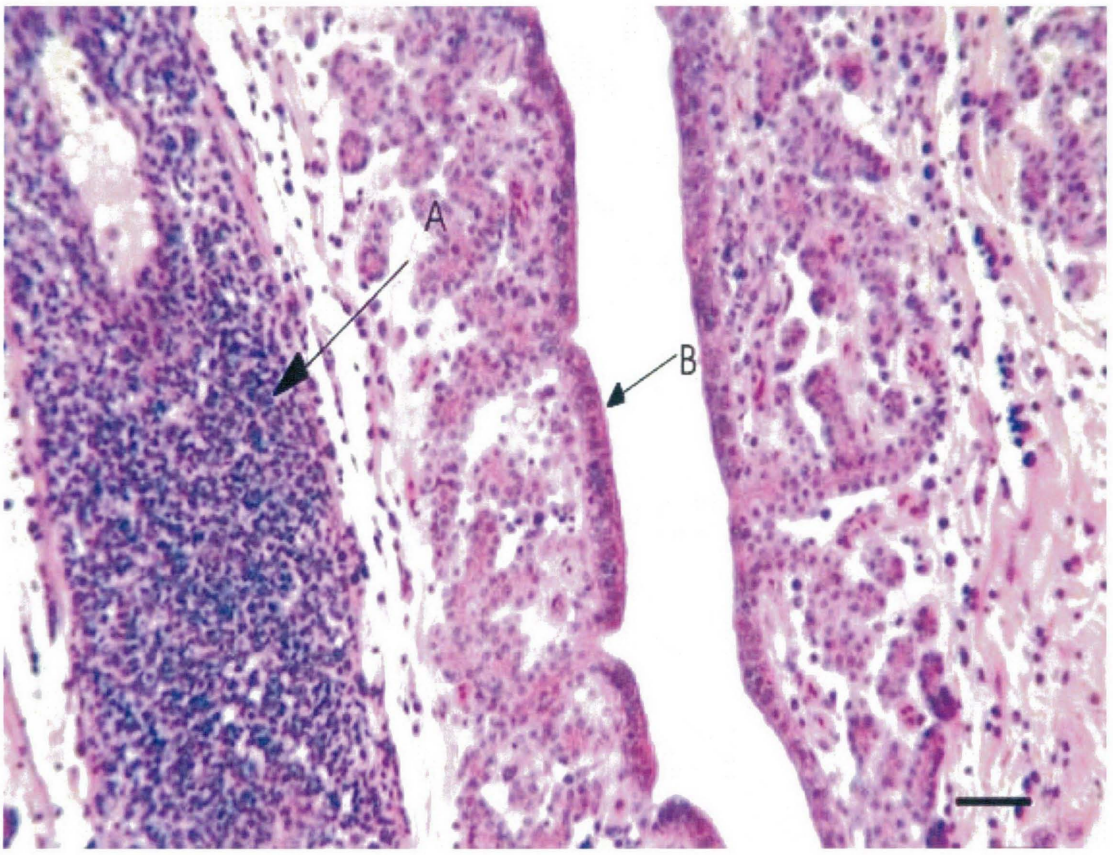

Plate 4.4: Shell gland pouch of T infected HyLine hen. H & E x 200. Scale bar represents 50 µm. Egg in lower magnum. Note lymphocyte infiltration in lamina propria (Arrow A) and loss of cilia from the surface epithelium (Arrow B)

Chapter 5

Physiological and histopathological observations during infection of Isa Brown laying hens with two strains of infectious bronchitis virus

5.1 Introduction

During the preliminary studies with unvaccinated White Leghorn hens and cockerels, it was observed that the Australian strains of infectious bronchitis virus (IBV) have a capacity to infect many of the epithelial surfaces in chickens. The present study was designed to investigate the pathological changes occurring in various tissues such as the Harderian gland, trachea and kidney in unvaccinated Isa Brown hens challenged with the T and N1/88 strains of IBV at 30 weeks of age.

Birds inoculated with infectious bronchitis virus were found to have significantly increased consumption and loss of water, with negative sodium and potassium balances (Heath, 1970; Condron and Marshall, 1985). Negative sodium balance could be due to a reduction in reabsorption of filtered sodium in the proximal convoluted tubule and increased output of this ion. The negative potassium balance could be due to the decreased intake of this ion (Condron and Marshall, 1985). Kidney weight as a percentage of body weight has been found to be a reliable indicator for the assessment of kidney damage caused by IBV (Wideman and Cowen, 1987, Afanador and Roberts, 1994). In the present experiment, besides detailed histopathological examination of the Harderian gland, trachea and kidney, kidney weights, IBV antibody titres, plasma electrolytes and body weight were measured and compared for Isa Brown hens in full lay, infected with two strains of IBV. Isa brown hens were selected because they are the most commonly used commercial laying hens in Australia. The samples collected during this experiment were also processed for electron microscopy, virus isolation and RT-PCR and the additional results are

described subsequently in chapters 6 to 11. Details regarding internal and external egg quality changes are described in chapter 9.

5.2 Materials and methods

5.2.1. Experimental chickens

Day-old Isa Brown pullets (150) were obtained from the Baiada Winton hatchery near Tamworth, New South Wales. All chickens received Rispens vaccine against Mareks disease at the hatchery but no other vaccinations. Birds were weighed every two weeks during rearing and were beak-trimmed at the age of 15 weeks. Hens started laying at the age of 16 weeks at which time they received 13hrs of light per day. This was progressively increased by half an hour per week to 16 hrs from 22 weeks onwards. At 25 weeks of age, hens were divided into three groups, one control with 48 hens and two treatment groups with 51 hens each. Pathogen-free status of the hens was maintained by isolation and strict biosecurity. All the hens were found negative for IBV antibodies prior to the virus challenge.

5.2.2. Housing

Pullets were raised in isolation sheds at the University of New England campus. All equipments, sheds and clothing were fumigated with formaldehyde gas before use. All the other equipment necessary for dealing with the hens was washed with antiseptic and wiped or sprayed with ethanol before use. Hens were transferred from floor pens to individual cages in different isolation sheds at 25 weeks of age and remained there until the end of the experiment. Specific protective clothing was used for each shed and entry of personnel was restricted to the two researchers only. Birds were initially fed commercial broiler starter to 3 weeks of age, then chick starter to 5weeks followed by pullet grower to 16 weeks and layer mash thereafter, until the end of experiment. Feed was not sterilized. The birds reared as the uninfected control group were always visited first and IBV-infected chickens were housed in different isolation sheds, separated from one another.

5.2.3. Experimental procedure

At 30 weeks of age, all Isa Brown hens in a treatment group were challenged intraocularly with one of two different strains of IBV, T or N1/88. Hens in the control group were inoculated intraocularly with sterile normal saline solution as a sham treatment. Two hens from each challenge group and one hen from the control group were euthanased at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 days post-infection (p.i.). At the time that the hens were sacrificed, the position of the egg in the oviduct was noted. Hens from the control group were deliberately killed with the egg at different positions in the oviduct to enable changes in the oviduct due to the position of the egg to be taken into account. Two hens which were out of lay from the T infected group were killed on the 30^{th} day p.i.

Prior to euthanasia, each hen was bled as described in chapter 2 (section2.2). After euthanasia, hens were weighed before being opened for collection of tissues. Kidneys from all the hens were taken out carefully and excess fat around the kidneys was removed. Left and right kidneys from each hen were weighed. Kidney size was then expressed as right kidney, left kidney and total kidney as a percentage of body weight. The data collected on different days in a week were pooled on a weekly basis. All the hens were again bled at 5 and 10 weeks p.i. The blood samples were collected and electrolytes analysed as described in chapter 2 (Section 2.2). The plasma samples were also used to determine the antibody titres. From the day of infection to 10 weeks p.i., 24 hens from each group were weighed at 2 weekly intervals. Experiment was terminated at 10 weeks post infection. Harderian gland, trachea and kidney were fixed in formalin and processed for histopathology as described in chapter 2 (section 2.3). The lesions scored from the trachea and kidneys are described in Table 5.1. Some Harderían gland samples were preserved for further stereological studies.

Table 5.1: Lesions scored from each tissue examined following infection with T and N1/88 strains of infectious bronchitis virus.

Tissue	Lesions/ affected cells scored
Trachea	cilia loss, epithelial degeneration, hypertrophy of alveolar mucous glands, oedema in mucosa, lymphocyte infiltration
Kidney	oedema of Bowman's capsule, urate casts, necrotic foci, granulocytic casts, lymphocyte infiltration, duct and tubular dilatation, necrosis of proximal convoluted tubule

5.3 Results

5.3.1 Clinical findings

Thirteen hens from the T-infected group and 15 hens from the N1/88-infected group showed coughing, rales and sneezing between the 3rd and 9th days p.i. All the respiratory signs had disappeared by day 10 p.i. None of the hens in either infected group died following infection. There was no drop in egg production in either of the groups. Albumen height and Haugh units were low in the N1/88 infected group in the 2nd, 5th and 6th weeks p.i. and in the T-infected group from the first week until the end of experiment (details of egg quality results are presented in chapter 9). Eggs with yolks that separated from the albumen during egg breakout were observed mostly between the 10th and 16th days p.i. in the N1/88 and T-infected groups.

One hen from the N1/88 infected group went out of lay from day 12 p.i. and was killed at the end of experiment on the 24th day p.i. Another two hens from the T-infected group, which stopped laying from 13th day p.i., were killed on the 30th day p.i. No symptoms were observed in control group.

5.3.2 Body and kidney weights

Body weight in the T- and N1/88-infected groups tended to be lower than the controls from the 2nd (32 weeks) to the 6th (36 weeks) weeks post infection (Fig. 5.1). Left and right kidney weights, percentage left and right kidney weights in relation to body

weight and percent total kidney weight in relation to body weight did not vary over the weeks of experiment. These parameters did not vary among the challenge treatment groups and also there was no interaction between treatment group and week of experiment (Table 5.2).

5.3.3 ELISA antibody titre

All hens were found negative for IBV antibodies prior to the virus challenge. Overall, there were significant differences among treatment groups ($P< 0.0001$) and weeks p.i. ($P< 0.0001$) for antibody titres. Also, there was an interaction between treatment group and week p.i. ($P< 0.0001$). Hens from the control group were negative for IBV antibodies throughout the experiment. Compared to the control, antibody titres in both the T- and N1/88-infected groups were significantly higher from 2 to 10 weeks p.i. (Figure 5.2).

5.3.4 Blood analysis

There was a significant main effect of time in relation to challenge on all blood electrolyte and haematocrit measurements (Table 5.3). There was no significant effect of virus challenge treatment on plasma sodium, potassium or ionised calcium. Also, there was no effect of virus challenge on haematocrit. However, there was a significant interaction between weeks post infection and virus challenge treatment on plasma ionised calcium levels.

Table 5.2: **Effect of IBV on kidney weights over 4 weeks post infection. Values are Mean ± SEM. K is control, N1/88 and T indicate viral strain of IBV. G is group, W is week post infection and NS is not significant.**

		Weeks post infection					P value		
	Group	1	2	3	4	All	Group	Weeks	G*W
Left kidney	K	6.37± 0.34	6.33± 0.18	5.52± 0.23	6.95± 0.60	6.29± 0.22	NS	NS	NS
Weight	N1/88	6.18± 0.34	5.73± 0.35	5.71± 0.31	5.49± 0.35	5.78± 0.16			
(LKW)	T	6.06± 0.08	6.00± 0.22	5.57± 0.24	6.09± 0.12	5.93± 0.09			
	All	6.17± 0.14	5.96± 0.17	5.61± 0.15	6.02± 0.22				
Right	K	6.02± 0.29	6.19± 0.10	5.50± 0.07	6.65± 0.51	6.09± 0.17	NS	NS	NS
kidney	N1/88	6.09± 0.26	5.67± 0.23	5.66± 0.25	5.38± 0.26	5.70± 0.13			
Weight	T	5.81± 0.20	5.60± 0.13	5.45± 0.18	5.95± 0.18	5.70± 0.09			
(RKW)	All	5.96± 0.14	5.74± 0.12	5.54± 0.12	5.86± 0.19				
LKW %	K	0.336± 0.008	0.337± 0.005	0.303± 0.021	0.346± 0.017	0.331± 0.008	NS	NS	NS
Body	N1/88	0.344± 0.021	0.317± 0.015	0.323± 0.011	0.305± 0.019	0.322± 0.009			
Weight	T	0.353± 0.009	0.324± 0.015	0.316± 0.014	0.333± 0.008	0.332± 0.006			
	All	0.346± 0.009	0.324± 0.008	0.316± 0.008	0.324± 0.010				
RKW %	K	0.318±0.010	0.330± 0.007	0.302± 0.007	0.331± 0.013	0.320± 0.005	0.016	NS	NS
Body	N1/88	0.339± 0.017	0.314± 0.009	0.322± 0.015	0.299± 0.016	0.318± 0.007			
Weight	T	0.338± 0.014	0.303± 0.011	0.309± 0.006	0.326± 0.012	0.319± 0.006			
	All	0.334± 0.009	0.313± 0.006	0.312± 0.007	0.316± 0.009				
Total	K	0.654± 0.017	0.667± 0.009	0.605± 0.026	0.677± 0.030	0.651± 0.013	NS	NS	NS
Kidney	N1/88	0.683± 0.035	0.631± 0.023	0.645± 0.026	0.604± 0.034	0.641± 0.015			
Weight %	T	0.691± 0.021	0.627± 0.025	0.625± 0.019	0.659± 0.020	0.650± 0.012			
Body weight	All	0.680± 0.016	0.637± 0.014	0.629± 0.013	0.640± 0.018				

Table 5.3: Effect of IBV on plasma electrolytes over 10 weeks post infection. Values are Mean ± SEM. K is control, N1/88 and T indicate viral strain of IBV. G is group, W is week post infection and NS is not significant.

		Weeks post infection								P value		
	Group	-2	1	2	3	4	5	10	All	Weeks	Group	G*W
Na	K	147.0± 0.39	146.6± 1.62	148.8± 0.81	148.3± 0.56	150.0± 1.35	148.2± 0.46	149.7± 0.46	148.2± 0.25			
mmol/L	N1/88	147.8± 0.53	149.0± 1.62	148.5± 0.52	148.8± 1.72	147.5± 1.47	148.8± 0.50	150.0± 0.56	148.6± 0.29	<0.0001	NS	NS
	T	148.0± 0.44	144.6± 3.35	148.8± 1.23	146.7± 3.12	149.8± 0.76	147.8± 0.40	149.9± 0.54	148.3± 0.31			
	All	147.6± 0.26	146.7± 1.52	148.7± 0.52	147.8± 1.37	148.9± 0.73	148.2± 0.26	149.9± 0.29				
K	K	4.56± 0.04	4.97± 0.35	5.14± 0.18	4.82± 0.09	5.06± 0.12	5.00± 0.04	5.09± 0.07	4.86± 0.03			
mmol/L	N1/88	4.55± 0.04	4.89± 0.13	5.00± 0.16	5.21± 0.33	4.89± 0.10	4.97± 0.05	5.11± 0.07	4.84± 0.03	<0.0001	NS	NS
	T	4.54± 0.06	4.53± 0.17	4.83± 0.23	4.95± 0.17	4.92± 0.09	4.89± 0.08	5.01± 0.05	4.76± 0.04			
	All	4.55± 0.03	4.73± 0.11	4.96± 0.11	5.03± 0.14	4.94± 0.06	4.95± 0.03	5.07± 0.03				
Ca	K	1.54± 0.02	1.53± 0.09	1.62± 0.13	1.54± 0.03	1.49± 0.06	1.64± 0.01	1.66± 0.02	1.60± 0.01			
mmol/L	N1/88	1.55± 0.02	1.37± 0.08	1.47± 0.01	1.53± 0.04	1.52± 0.06	1.59± 0.01	1.64± 0.02	1.58± 0.01	<0.0001	NS	0.0195
	T	1.62± 0.01	1.29± 0.06	1.48± 0.05	1.46± 0.03	1.52± 0.05	1.58± 0.01	1.68± 0.01	1.59± 0.01			
	All	1.57± 0.01	1.37± 0.05	1.50± 0.03	1.50± 0.02	1.51± 0.03	1.61± 0.01	1.66± 0.01				
Hct	K	28.22± 0.52	29.66± 0.88	28.83± 0.72	28.66± 0.88	27.50± 1.75	27.61± 0.37	25.69± 0.50	27.40± 0.27			
%	N1/88	27.50± 0.42	27.66± 1.14	29.58± 1.08	27.50± 1.23	27.75± 2.78	26.42± 0.47	26.21± 0.58	27.12± 0.27	<0.0001	NS	NS
	T	28.33± 0.39	30.25± 0.70	29.66± 1.10	28.66± 1.35	27.41± 0.93	26.96± 0.42	25.83± 0.52	27.60± 0.25			
	All	28.02± 0.25	29.10± 0.61	29.46± 0.60	28.20+ 0.72	27.65± 1.13	27.05± 0.24	25.89± 0.30				

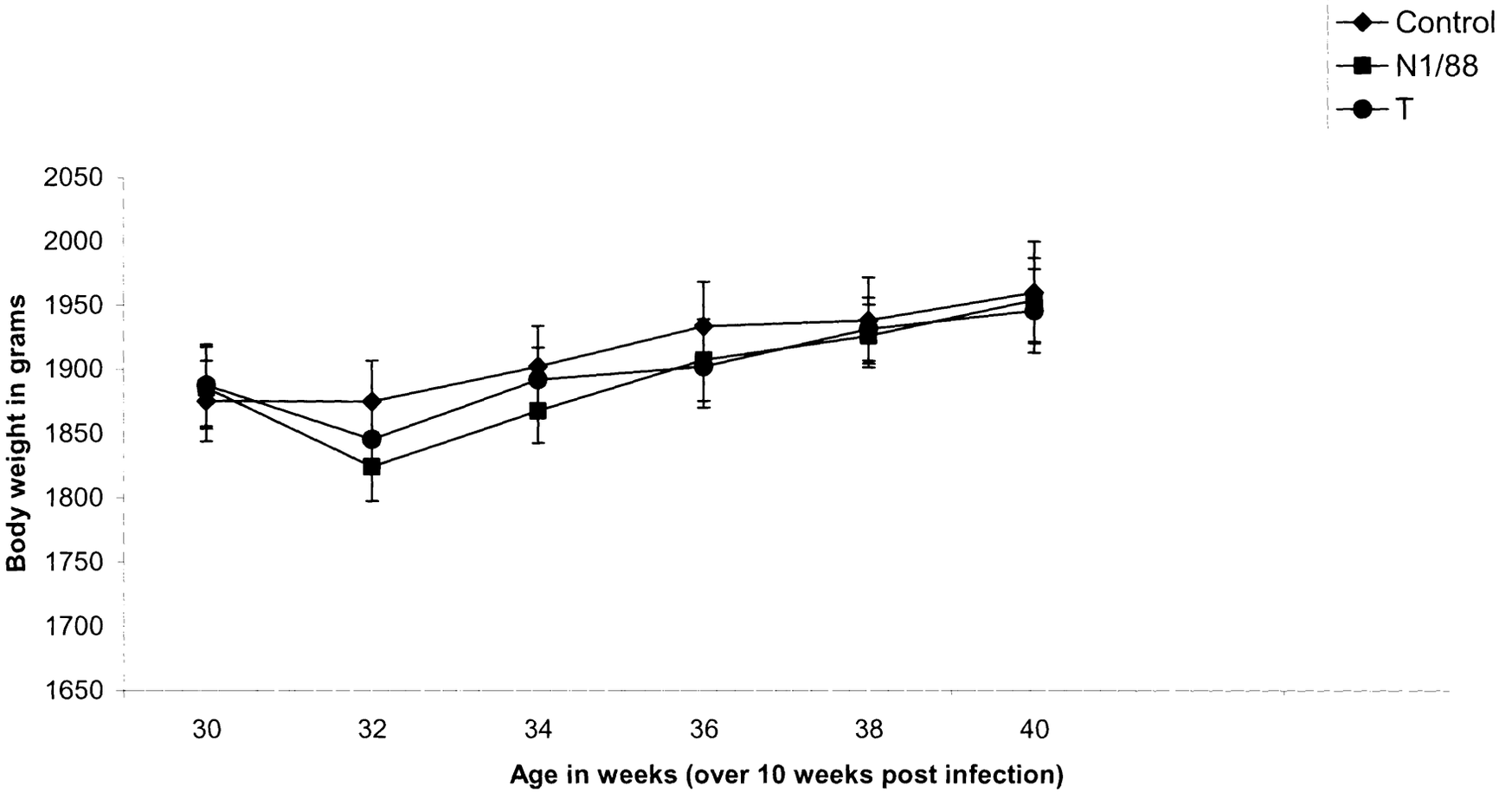

Figure 5.1. Body weight of Isa brown hens over 10 weeks post infection

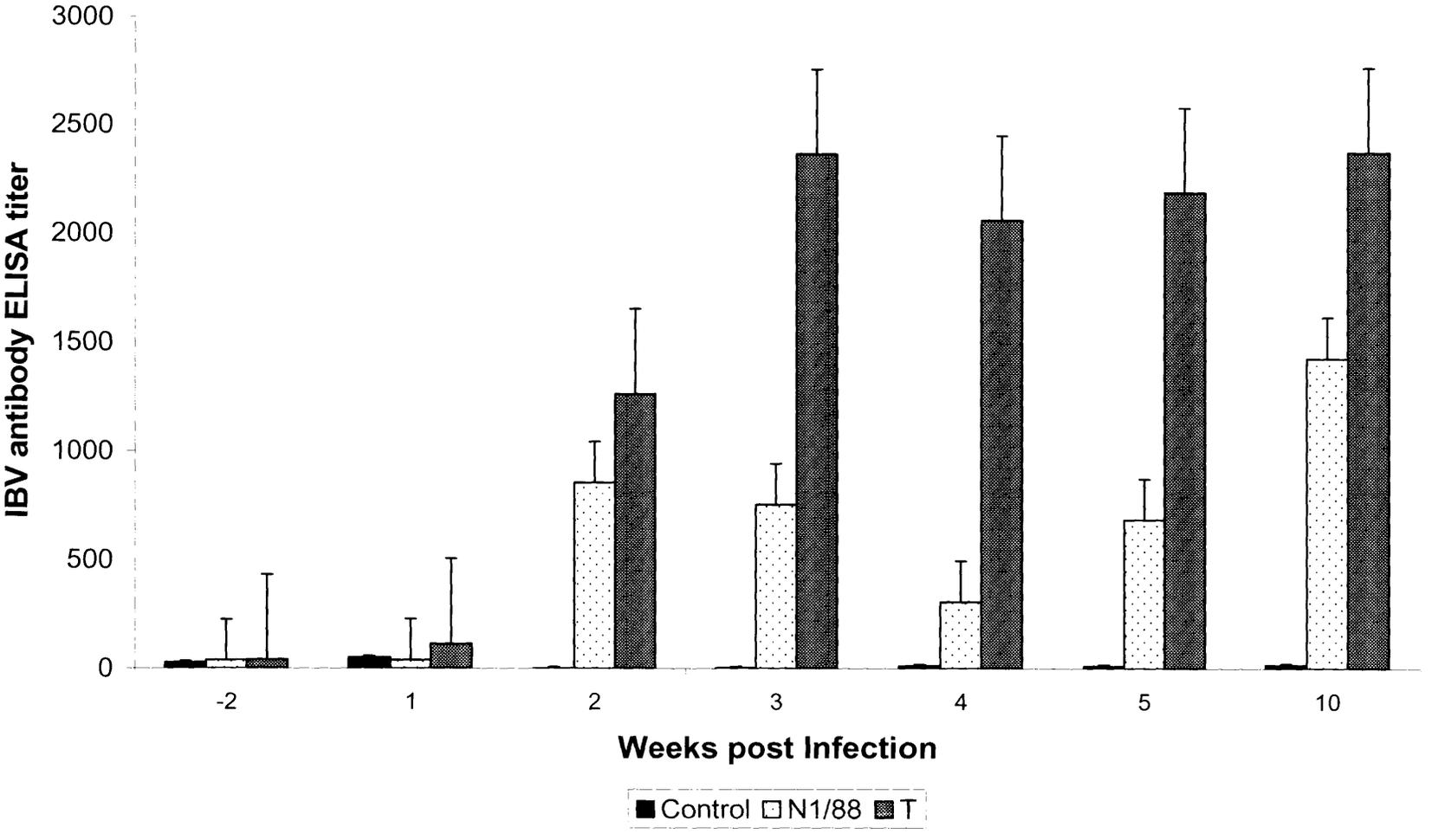

Figure 5.2 Antibody titers of the control and infected hens across the period of infection

5.3.5 Histopathology

5.3.5 (i) Harderian gland

In control hens, the main features were some plasma cells (mature B lymphocytes that are specialized for antibody production) in the subepithelium, intact collecting duct epithelium, acinar epithelium and occasional lymphocyte infiltration around the blood vessels in the glandular interstitium (Plate 5.1).

In T strain-infected hens, on day 3 p.i., there was infiltration of plasma cells and globular leukocytes in the subepithelium and the collecting duct epithelium was severely damaged. There was necrosis in the primary collecting duct of the Harderian gland of some hens (Plate 5.2). On 2, 4, 6, 8, and 12 days p.i., plasma cells and lymphocytes around blood vessels in the septa (interlobular space) were frequently observed. On 14 day p.i. and onwards, most of the collecting duct epithelium had regenerated, but the lymphocyte infiltration in the interstitium and globular leukocytes in the subepithelium were still extensive (Plate 5.3). Lymphocyte nodules were observed in the subepithelium of the Harderian gland of one hen killed on the 16^{th} day p.i. (Plate 5.4). Exfoliative epithelium, along with inflammatory cells, was seen in the collecting duct lumen from days 2 to 14 p.i. In N1/88 infected hens, most of the lesions were similar to those of T stain infection but the lesions were less severe.

5.3.5 (ii) Trachea

Normal tracheal epithelia, with healthy cilia and mucus glands, were seen in the control hens (Randall and Reece, 1996). (Plate 5.5)

In hens, there were no statistically significant main effects of IBV strain on any of the histopathological lesions investigated but there was a significant main effect of days p.i. on all the lesions scored (Table 5.4). In N1/88-infected hens killed on the 2^{nd} day p.i., there was mild to moderate oedema along with cilia loss. The cilia loss in both the challenge groups was severe on the 6^{th} day p.i. (Plate 5.6) and continued up to the 8^{th} day p.i. in the N1/88-infected group, while hens from the T infected group showed

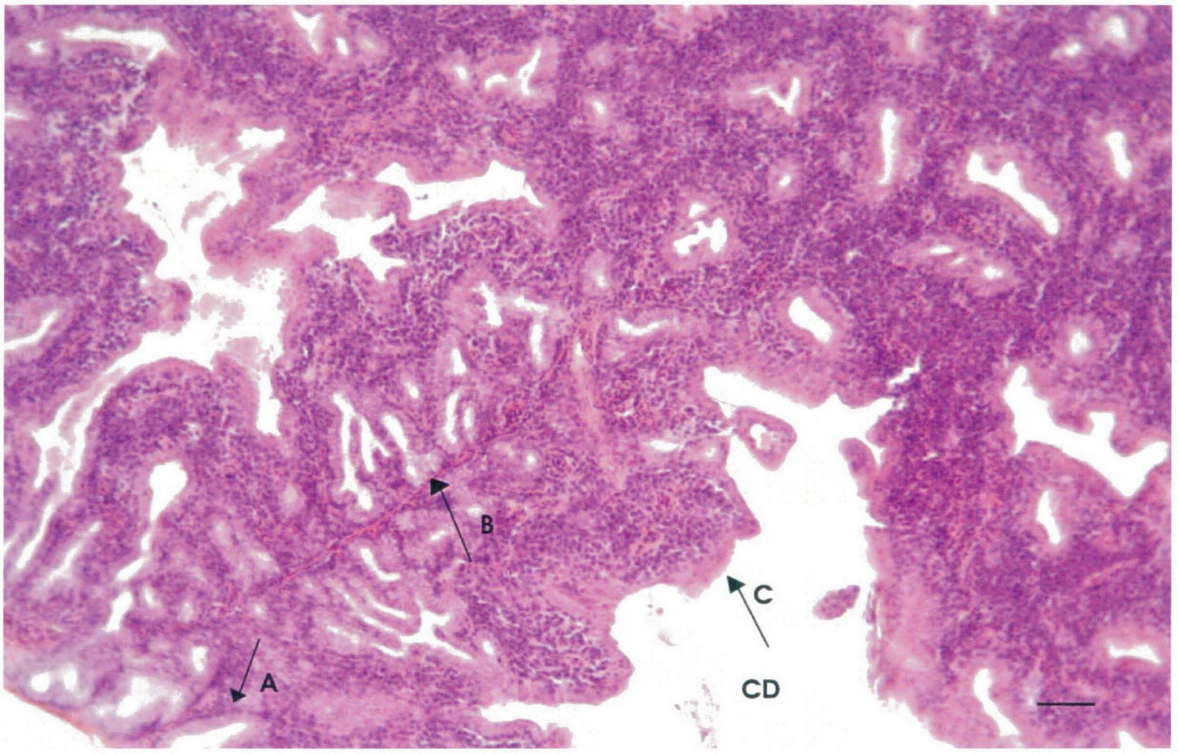

Plate 5.1: Harderian gland of hen from control group at 14 days p.i. Acini (Arrow A), interlobular septa (Arrow B), duct epithelium (Arrow C), Collection duct (CD). Scale bar represents 100 µm. H & E

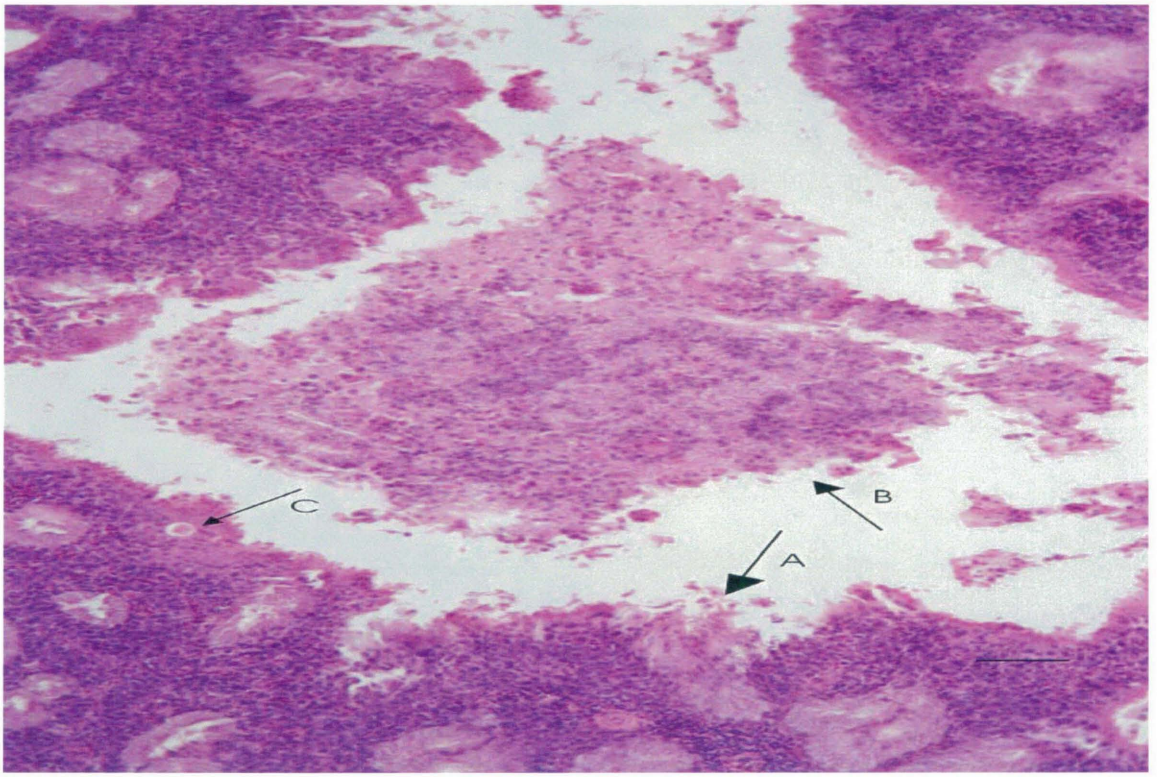

Plate 5.2: Harderian gland of hen from T infected group at 4 days p.i. disrupted duct epithelium (Arrow A), epithelial debris in duct lumen (Arrow B), necrosis in secondary collecting duct (Arrow C). Scale bar represents 100 µm. H & E

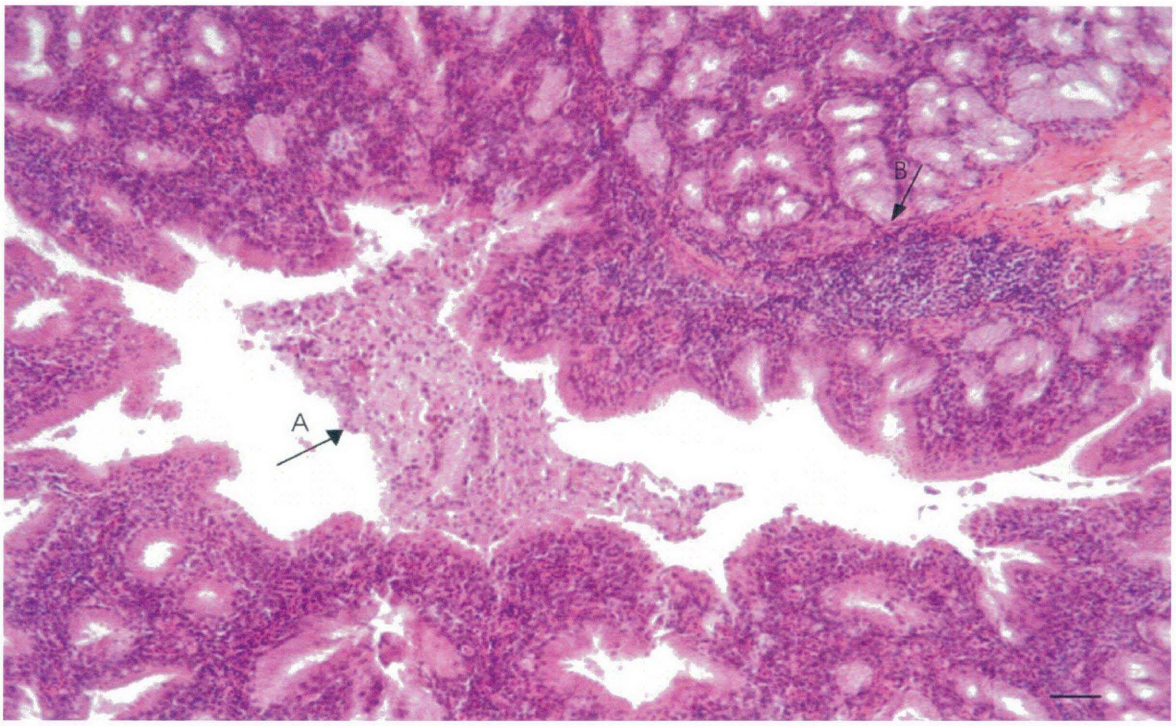

Plate 5.3: Harderian gland of hen from N1/88 infected group at 4 days p.i. epithelial debris in duct lumen (Arrow A), lymphocyte infiltration in the interlobular septa (Arrow B), Scale bar represents 100 µm. H & E

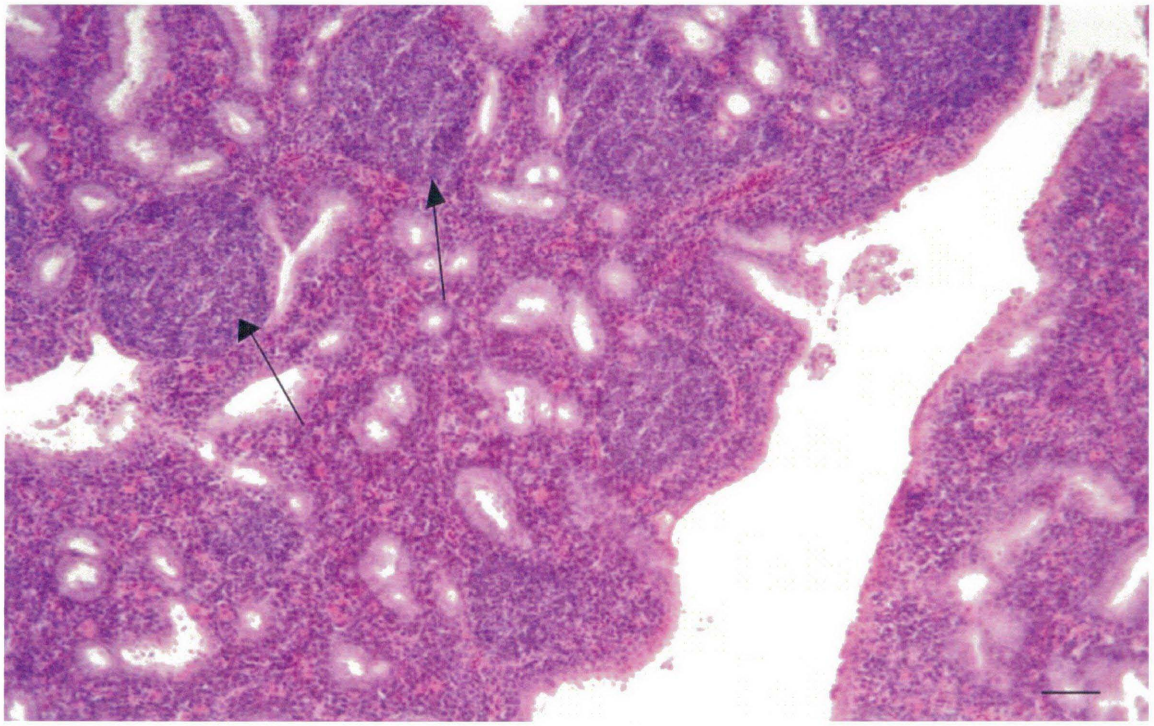

Plate 5.4: Harderian gland of hen from T infected group at 16 days p.i. Lymphoid nodules in the interlobular septa (Arrows). Scale bar represents 100 µm. H & E

moderate to severe cilia loss to 8 days p.i. The cilia loss in the T-infected group was mild from days 12 to 16 and on the 20th day p.i. and was not observed in the trachea of hens killed on days 22 and 24 p.i. In the N1/88 infected group, the cilia loss was moderate from 10 to 22 days p.i. but was absent on day 24 p.i. The epithelial degeneration in the T-infected group was severe during 4 to 8 days p.i., mild from 10 to 16 days p.i. and was not observed from days 18 to 24 p.i. In the N1/88-infected group, epithelial degeneration was moderate on days 2 to 4 p.i., severe from 6 to 8 days p.i. and mild from 10 to 12 days p.i. Epithelial degeneration was not observed in this group from day 14 until the end of experiment.

In the T-infected group, alveolar mucus glands were hypertrophied to a moderate extent on days 4 to 8 p.i. The hypertrophy was mild from 10 to 16 days p.i. and completely absent from day 18 onwards until the end of the experiment. In the T-infected group, oedema in the submucosa was moderate from days 4 to 8 p.i.Oedema was mild on the 10th day p.i. and was not observed from day 12 until the end of the experiment. In the N1/88-infected group, oedema was mild to moderate from 2 to 12 days p.i. but was not observed in any of the N1/88 infected hens from 14 to 24 days p.i.

In T-infected hens, lymphocyte infiltration was moderate to severe from days 4 to 16 p.i. and also in the hens sacrificed on day 24 p.i. However, it was mild from days 18 to 22 p.i. In the N1/88-infected hens, lymphocyte infiltration was mild at the onset of infection on days 2 and 4 p.i. and during the last phase of experiment on days 22 and 24 p.i. but was severe to moderate from days 6 to 20 p.i.

Goblet cells were less conspicuous in both the T- and N1/88-infected groups on days 4 to10 p.i. but, on days 12 and 14 p.i., this appeared to be the case only in the N1/88-infected group. From days 20 to 24 p.i., inflammation accompanied by mitotic figures was absent from most parts of the trachea although lymphoid nodules persisted.

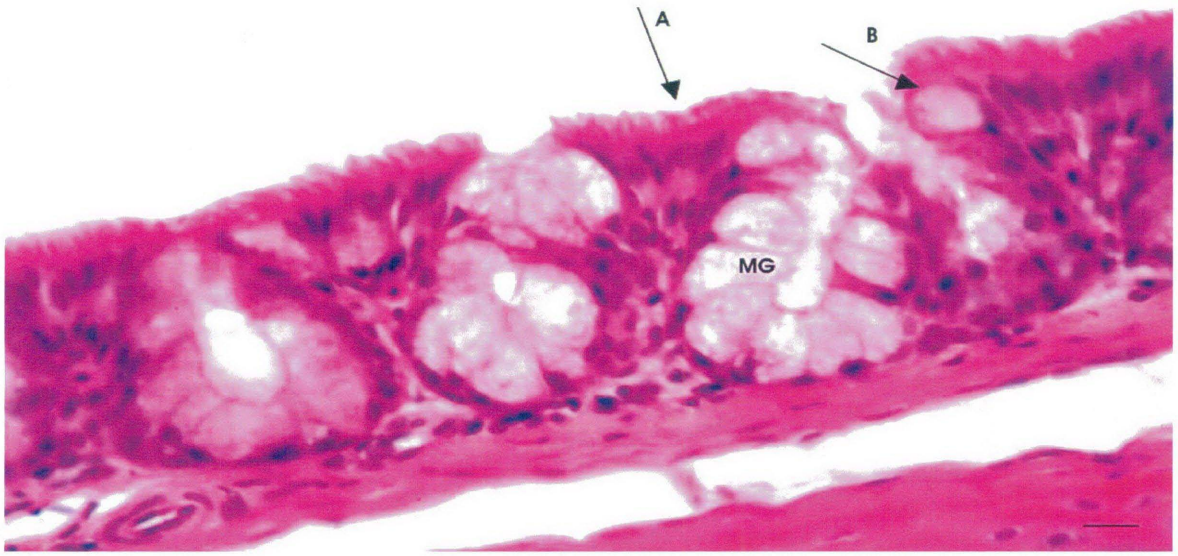

Plate 5.5: Trachea of hen from control group at 4 days p.i. Cilia (Arrow A), goblet cells (Arrow B), Mucus gland (MG).x 400 Scale bar represents 25 µm. H & E

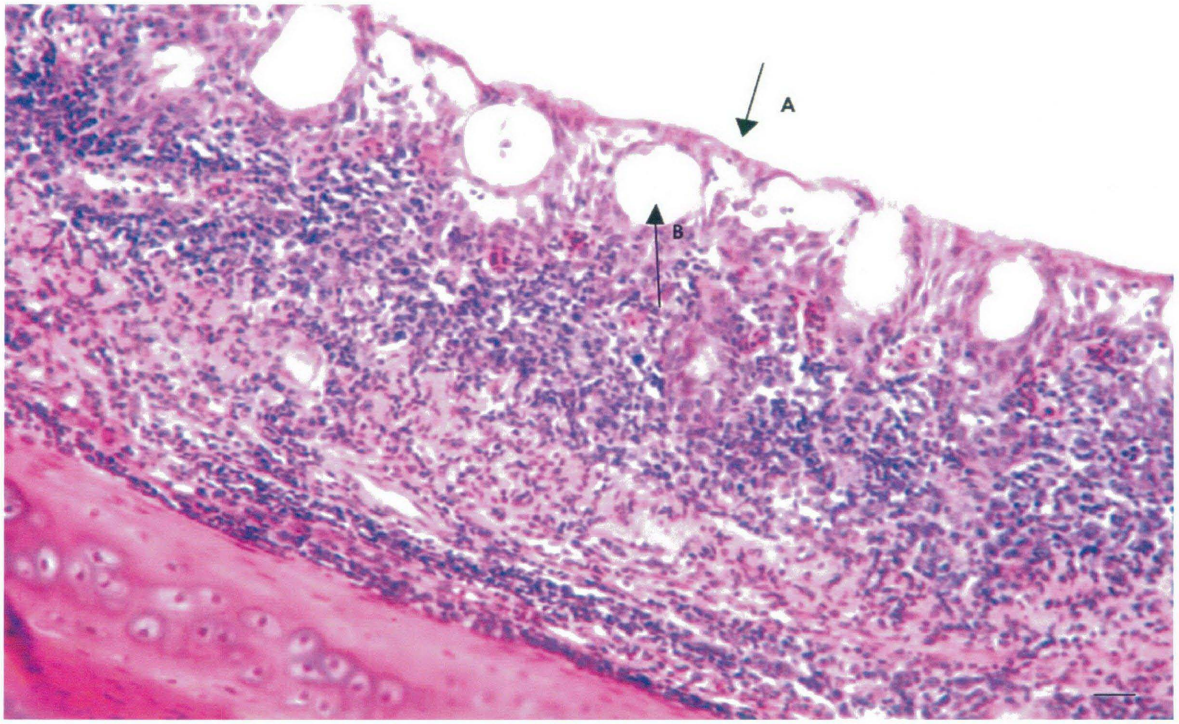

Plate 5.6: Trachea of hen from N1/88-infected group at 6 days p.i. Cilia loss from surface epithelium (Arrow A), hypertrophied mucus glands with cellular debris in the lumen (Arrow B). Lymphocyte infiltration with thickening of submucosa. x 400 Scale bar represents 25 µm. H & E

Table 5.4: Comparative histopathology of trachea in hens infected with T or N1/88 strain of IBV

Days post inoculation																							**P value**	
	2		4		6		8		10		12		14		16		18		20		22		24		Treatment	Days p.i.
Lesions in Trachea	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88		
Cilia loss	0.0	1.5	3.0	1.5	3.0	3.0	2.5	3.0	1.0	1.0	1.0	1.0	1.0	0.5	0.5	0.0	0.0	0.5	0.5	0.5	0.0	0.5	0.0	0.0	NS	0.0025
Epithelial degeneration	0.0	1.5	3.0	1.5	3.0	3.0	2.5	2.5	1.0	1.0	1	1.5	1.5	0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NS	0.0034
Hypertrophy of alveolar mucous glands	0.0	1.5	1.5	1.5	2.0	2.5	2.5	1.5	0.5	0.5	1.5	1.5	1	0.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NS	0.0058
Oedema in mucosa	0.0	1.5	2.0	1.0	2.0	1.5	2.0	2.0	1.0	1.5	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NS	0.0013
Lymphocyte infiltration	0.0	1.0	3.0	1.0	2.5	3.0	3.0	3.0	2.0	2.5	2.5	2.5	2.5	2.0	2.5	2.0	1.0	2.0	1.5	2.5	1.0	0.5	2.5	0.5	NS	0.0121

0 , No change; 1, Mild; 2 , Moderate; 3 , Severe.

Values are Means of lesion score from two hens

Significance assumed at $p<0.05$. NS is not significant

Main effect of IBV strain was determined by Mann-Whitney U test

Main effect of days p.i. was determined by Kruskal-Wallis test

5.3.5 (iii) Kidney

The kidneys of the hens from the control group were normal. (Plate 5.7)

Of the lesions investigated, there was a statistically significant main effect of IBV strain on granulocytic casts and lymphocyte infiltration. There was a significant main effect of days p.i. on 2 out of the 5 lesions scored, oedema of Bowman's capsule and urate casts (Table 5.5). The main kidney lesions consisted of necrosis of proximal convoluted tubules, infiltration of lymphocytes in the interstitial space of T- (Plate 5.8) and N1/88-infected hens (Plate 5.9), urates, granulocytic casts in collecting ducts (Plate 5.10) and oedema of Bowmans capsule.

Oedema of Bowman's capsule was not observed in the any of the hen from control group (Plate 5.11). Hens from the T-infected group showed mild to moderate oedema of Bowman's capsule from days 8 to 16 p.i. (Plate 5.12) but this was mild on day 18 p.i. In the T-infected group, oedema in Bowman's capsule was not observed from the 20^{th} to the 24^{th} day p.i. In the N1/88-infected group, oedema was mild from days 8 to 16 p.i. and was not observed from 18 to 24 days p.i. In both T- and N1/88-infected groups, urate and granulocytic casts were observed to a mild to moderate extent throughout the infection except on day 2 p.i., although granulocytic casts were not observed in either of the infected groups on days 20 and 24 p.i.

Lymphocyte infiltration was moderate in the T-infected group from days 6 to 14 and on the 18^{th} and 24^{th} days p.i. Extensive infiltration of lymphocytes was observed on days 20 and 22 p.i. in this group. Large lymphoid nodules were observed in the kidneys of both the T strain-infected hens killed on the 30^{th} day p.i. In the N1/88-infected group, lymphocyte infiltration was mild from 8 to 24 days p.i. The infiltration of lymphocytes in the N1/88 group was focal while, in T infected hens, it was diffuse. Intense infiltration of lymphocytes was common in the cortex of kidneys of T-infected hens.

Mild necrosis of the proximal convoluted tubule was observed only in the kidneys of T-infected hens on days 8 and 20 p.i.

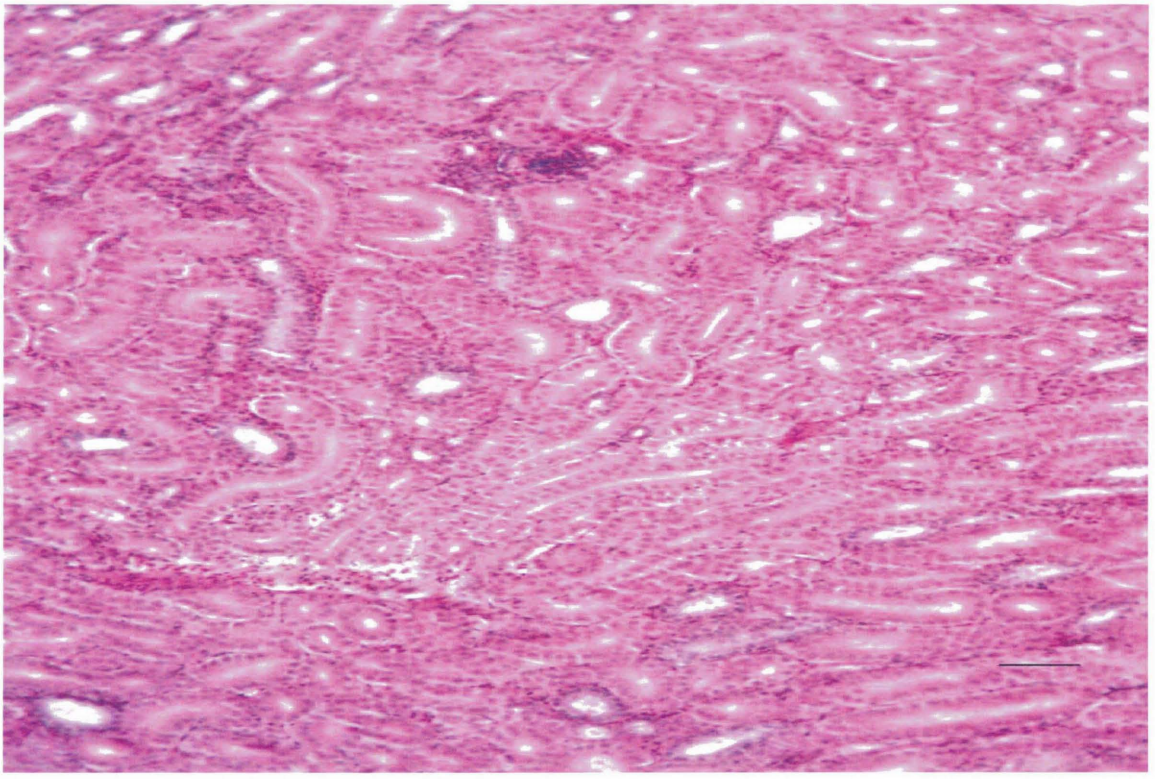

Plate 5.7: Kidney of hen from control group at 8 days p.i. x 200 Scale bar represents 50 µm. H & E

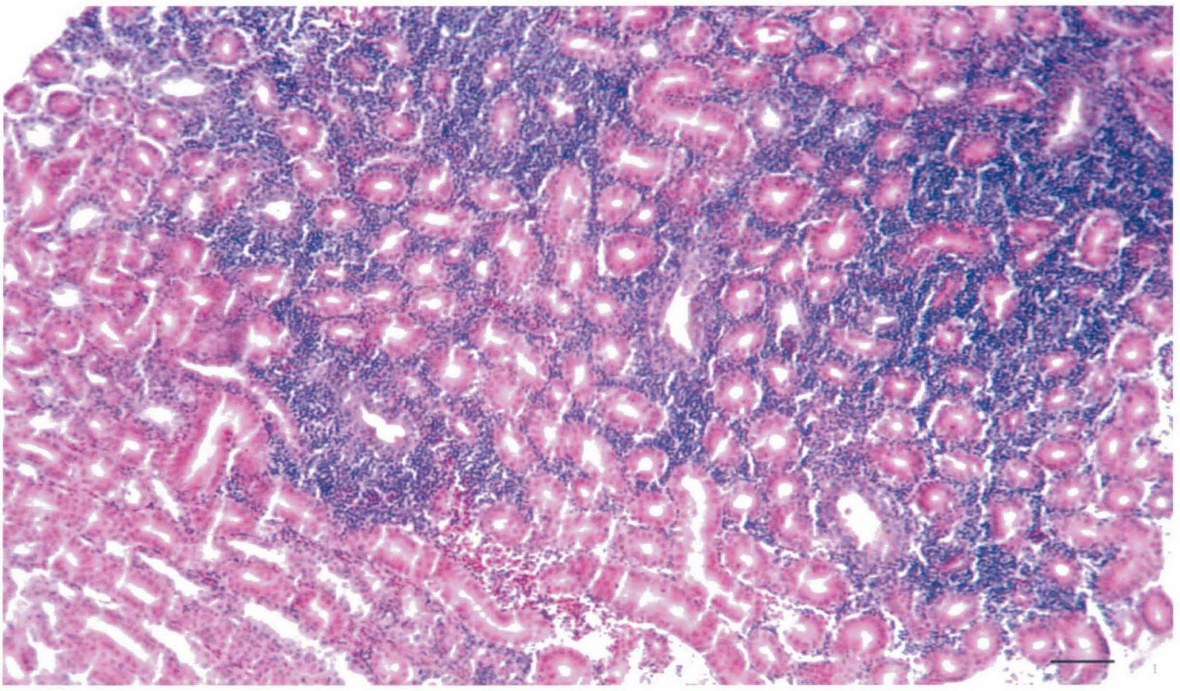

Plate 5.8: Kidney of hen from T strain-infected group at 8 days p.i. x 200 Scale bar represents 50 µm. H & E

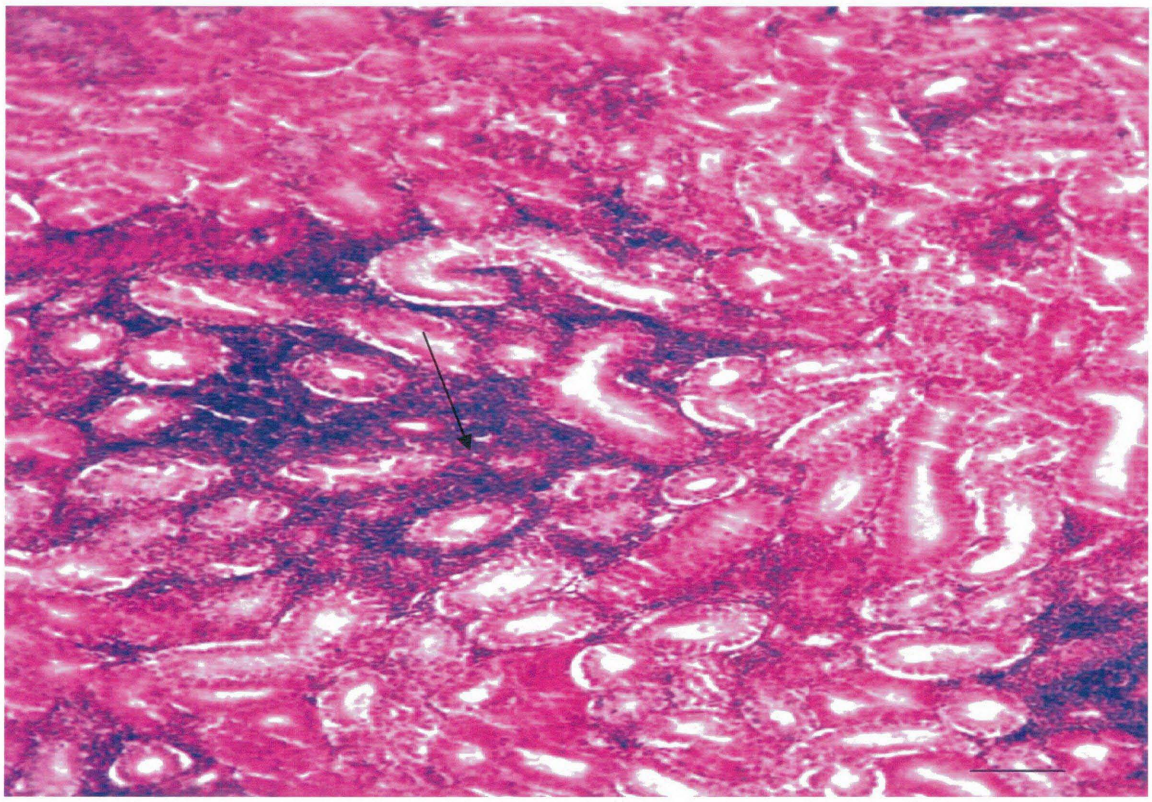

Plate 5.9: Kidney of hen from N1/88 strain-infected group at 8 days p.i. Lymphocyte infiltration (Arrow) x 200 Scale bar represents 50 µm. H & E

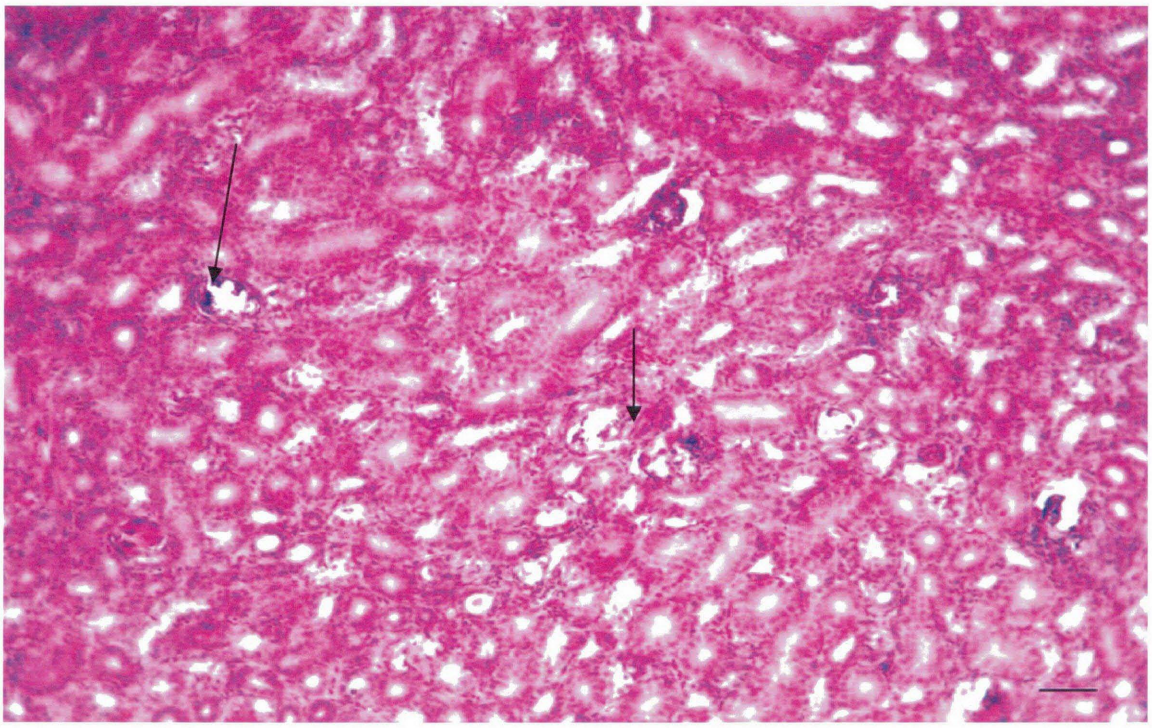

Plate 5.10: Kidney of hen from T strain-infected group at 12 days p.i. Urates in collection duct (Arrows) x 200 Scale bar represents 50 µm. H & E

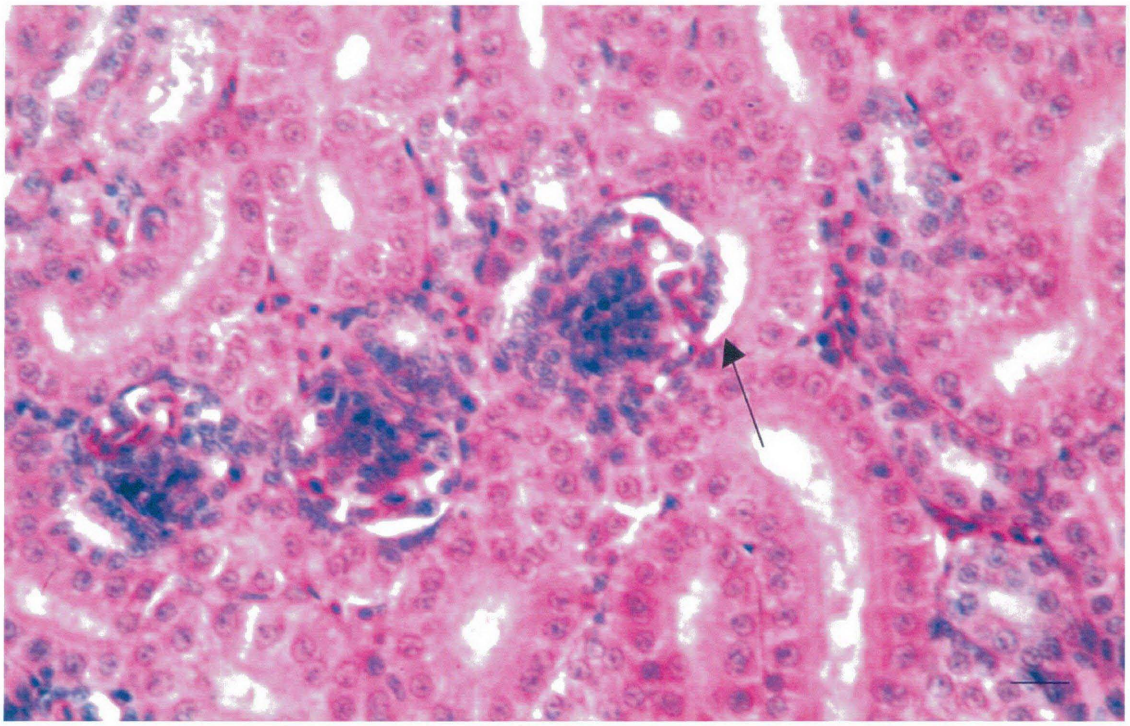

Plate 5.11: Kidney of hen from control group at 8 days p.i. x 400 Scale bar represents 25 µm. H & E . Note the normal Bowmans capsule.

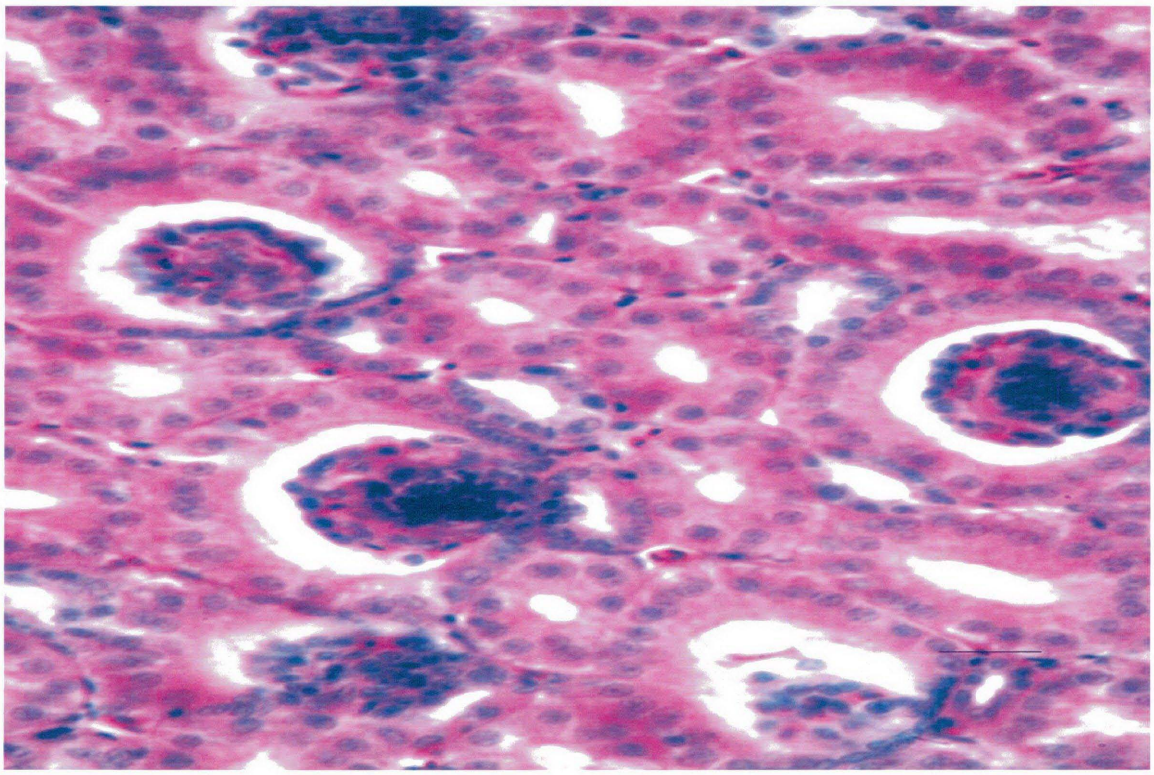

Plate 5.12: Kidney of hen from T strain infected group at 8 days p.i. Note the oedema of Bowmans capsule x 400 Scale bar represents 25 µm. H & E

Table 5.5: Comparative histopathology of kidneys from hens infected with T and N1/88 strains of IBV

Days post inoculation																							**P value**	
	2		4		6		8		10		12		14		16		18		20		22		24		Treatment	Days p.i.
Lesions in kidney	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88	T	N1/88		
Oedema of Bowman's capsule	0.0	0.0	0.0	0.0	0.0	0.0	2.0	1.5	2.5	1.5	1.5	1.5	1.5	0.5	1.5	0.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NS	0.0017
Urate casts	0.0	0.0	1.5	0.5	1.5	0.5	1.0	1.0	2.5	1.0	1.0	0.5	0.5	0.5	0.5	0.5	0.5	0.0	0.0	0.0	0.5	0.5	0.0	0.0	NS	0.0245
Granulocytic casts	0.0	0.0	1.0	1.0	1.5	1.5	2.0	0	1.0	1.0	2.0	1.0	1.0	0.5	0.0	1.0	0.5	0.5	1.0	1.0	2.0	0.0	2.0	1.0	0.0178	NS
Lymphocyte infiltration	0.0	0.0	0.0	0.0	2.0	0	2.0	1.5	2.0	0.5	2.5	1.0	2.0	1.5	1.5	0.5	2.5	0.5	3.0	0.5	3.0	0.5	2.0	1.5	<0.0001	NS
Necrosis of Proximal convoluted tubule	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.5	0.0	0.0	0.0	NS	NS

0 , No change; 1, Mild; 2 , Moderate; 3 , Severe.

Values are Means of lesion score from two hens

Significance assumed at $p<0.05$. NS is not significant

Main effect of IBV strain was determined by Mann-Whitney U test

Main effect of days p.i. was determined by Kruskal-Wallis test

5.4 Discussion

All the hens from the control group were negative for IBV antibodies throughout the experiment. The progressive increase in antibody titer in both T- and N1/88-infected groups indicates that both groups were exposed to a sufficient dose of virus.

Plasma electrolyte and haematocrit parameters varied significantly over the weeks of the experiment. An increase in blood electrolytes with hen age was recorded earlier by Jolly (2005). Haematocrit and plasma electrolyte values were compared amongst the control, T and N1/88 infected hens. There were no significant differences between the control and either of the infected groups for haematocrit or plasma electrolytes. It was expected that, in the present experiment, T strain of IBV being more nephropathogenic would show changes in plasma electrolytes across the challenge period. However, no significant changes were recorded. Earlier, Condron and Marshall (1986) concluded that structural alteration in the tubular epithelial cells during IBV infection can cause electrolyte imbalance. However in the present study, despite pathology in kidney, no such electrolyte imbalance was observed. However, it must be noted that there was no necrosis in the proximal convoluted tubules (PCT) in the kidneys of T-infected hens. Mild necrosis was observed in one hen on day 8 p.i. Severe necrosis in the PCT was observed by Condron and Marshall (1985) and Jolly (2005) in kidneys of T strain-infected SPF chickens or cockerels. Earlier, Heath (1969) had observed high plasma potassium and haematocrit values in young White Leghorn cockerels. In the present trial, the lack of change in these parameters could be due to bird age or breed differences. Also, as observed by Cumming (1967), cockerels are more susceptible to IBV nephritis as compared to hens.

Although not statistically significant overall, the plasma calcium levels were lower during the 1st week after infection in the T-infected group, a finding that needs further investigation. Calcium excretion by the kidneys is regulated largely by the action of parathyroid hormone on the distal convoluted tubules (Goldstein and Skadhauge, 2000). However, in the present experiment, pathology was not found in the distal convoluted tubule (DCT) of the kidneys of any of the hens killed (such pathology in DCT would be visible under the light microscope) so reduced calcium levels cannot be linked to pathology of the DCT. Also, there was no deterioration of egg shell

quality during that week. So the results suggest no link between poor eggshell quality and inability of the kidneys to maintain calcium homeostasis. Severe disruption of the tubular epithelium of the distal convoluted tubule and collecting duct during IBV infection has been reported by (Pohl, 1974) although plasma calcium levels were not studied during Polh's experiment.

There was no significant variation in body weight over the weeks post infection or among the treatment groups and there was no significant interaction between treatment group and time post infection. This indicates that the virus had little effect on body weight in this trial. Although not significantly different, the kidney weights in both the infected group tended to be lower compared to the control. Wideman and Cowen (1987) found a decrease in kidney weights with IBV infection in birds receiving a high calcium diet. On other hand, Afanador and Roberts (1994) observed an increase in kidney weights with acute infection of T strain of IBV in cockerels.

In both T- and N1/88-infected Isa Brown hens, the severity and time frame of lesions in the Harderian gland were similar which indicates that both IBV strains are equally pathogenic for the Harderian gland of Isa Brown hens. Our finding regarding regeneration of the ductal epithelium agrees with that of Toro *et al.* (1996). Globular leukocytes, which originate from mast cells or granulocytic eosinophils, were also observed in the subepithelium of the Harderian gland in the infected hens. Lymphocyte infiltration around the blood vessels in the septa, at different days post inoculation, agrees with the findings of Survashe *et al* (1979) who reported a similar trend in the Harderian gland of chickens challenged with the H120 vaccine strain.

The pathology of the trachea during IBV infection has been extensively studied because the trachea is regarded as the primary target (Fulton *et al.*, 1993; Nakamura *et al.*, 1991; Arshad *et al.*, 2003). In the present study, the histopathological changes appeared earlier in the N1/88-infected hens as compared to the T-infected hens and the lesions in the N1/88-infected hens were more consistent. N1/88 strain has been reported to have a strong predilection towards the trachea (Sapats *et al.*, 1996b). Respiratory signs were observed mainly from 3 to 9 days post infection. This supports the view of McMartin (1993) that, during uncomplicated IB infections, respiratory symptoms are usually gone by 10 to 14 days although secondary bacterial infections

may prolong the recovery (Williams *et al.*, 1985). Both the strains of IBV used in the present study can cause severe pathology in the trachea of Isa Brown laying hens which pose the threat of secondary bacterial infections.

The histopathological changes observed in the kidney match previous findings (Purcell *et al.*, 1976; Chen *et al.*, 1996) except for the microscopical lesions of interstitial oedema and necrosis of proximal and distal convoluted tubules which were not observed in the present study. The T strain IBV was more nephropathogenic compared to N1/88 in Isa Brown hens. Granulocytic casts and urate casts could be a manifestation of urolithiasis and Cavanagh and Naqi (1997) also reported an increased incidence of casts following IBV infection.

During earlier experiments with White Leghorn hens, it was observed that T strain is capable of inducing pathology both in the kidney and trachea. Similar findings were recorded in the present experiment with Isa Brown hens although pathology was more severe in the kidneys of White Leghorns, as compared to Isa brown hens. Earlier, Cumming and Chubb (1988) reported that the extent of nephropathogenicity can vary between two breeds of birds. The results of the present study indicate that White Leghorns are more susceptible to IB nephritis than Isa Brown hens. However, the White Leghorns were challenged at a later stage of life (65 weeks) compared with the Isa Brown hens (30 weeks). Inverse age resistance of chicken kidneys to infectious bronchitis virus has been reported by McDonald *et al.* (1980). These authors observed that mortality was greater in 10 week old chickens compared to 3 weeks old. However, for adult hens, strain of bird appears to be of greater importance than bird age. Our findings suggest that, besides being nephropathogenic, the T strain of IBV has an ability to produce severe pathology in the trachea.

After experimental challenge with IBV in Isa brown hens, sequential observations by histopathology suggest that IBV replicates simultaneously in the Harderian gland and trachea followed by kidney.

Chapter 6

Ultrastructural changes in the oviduct of the laying hen during the laying cycle

6.1 Introduction

The avian oviduct is a complex biological organ that undergoes a series of hormonal, neural, biochemical and cellular changes during the formation of an egg. After ovulation, several layers are deposited around the yolk from different parts of the oviduct, this process has been studied in the past but with few ultrastructural and immunocytological studies. The avian oviduct is of special interest to the layer industry. Any alteration or deviation in the function of the oviduct of a laying hen can cause direct effects on egg and egg shell quality. Deterioration in egg and egg shell quality costs the egg industry millions of dollars. The avian oviduct is divided into five regions; infundibulum, magnum, isthmus, tubular shell gland and shell gland pouch. The tubular shell gland was initially known as the red region of the isthmus but because of its role in egg shell formation, it was later named the tubular shell gland (Solomon, 1975).

Many researchers have investigated the series of changes during egg formation in the infundibulum (Aitken and Johnston, 1963; Wyburn *et al*, 1970), magnum (Wyburn *et al.*, 1970), isthmus, tubular shell gland (Draper *et al.*, 1972; Johnston *et al.*, 1963, Solomon, 1983) and shell gland pouch (Wyburn *et al.*, 1973; Breen and Bruyn, 1969). Scanning micrographs of the oviduct have been studied by Bakst and colleagues (Bakst and Howarth, 1975; Bakst, 1978).

During the course of study of the effects of infectious bronchitis virus on the reproductive tract of laying hens, scanning and transmission electron microscopic features of the different parts of the oviduct were studied in hens from the control group. The present study observed the simultaneous changes occurring on the surface

as well as in the cells of the oviduct during the passage of the egg. These results assisted with the interpretation of any changes that were observed following challenge with infectious bronchitis virus.

6.2 Material and methods

The details of the rearing and management of hens are described in chapter 5 section 5.2. Thirteen hens at 30-40 weeks of age were sacrificed at different egg positions in the oviduct (Table 1). The position of the egg was noted, the oviduct was opened and small pieces of infundibulum, middle part of magnum, isthmus, tubular shell gland and shell gland pouch were collected for histology and electron microscopy.

Table 6.1: The hens killed at different egg positions and placed in groups

Group	Position of egg	Number of hens
1	Egg in Infundibulum	1
2	Egg in top magnum	2
3	Egg in mid magnum	2
4	Egg in lower magnum	1
5	Egg in isthmus	2
6	Soft shelled egg in shell gland Pouch	1
7	Hard shelled egg in shell gland pouch	3
8	No egg	1

6.2.1 Histology and Electron microscopy

The details of preparation and observation of tissue for histology, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are described in chapter 2.

6.3 Results

The avian oviduct is lined by non-secretory ciliated and secretory non-ciliated cells (also called granular cells). The ciliated cells had both cilia and microvilli on the apical surface, whereas non ciliated cells have only microvilli. The glandular tissue is lined by cuboidal epithelial cells. Scattered lymphocytes were present in all parts of the oviduct but t mainly in the lamina propria and muscularis regions. Plasma cells were also observed in these areas.

6.3.1 Changes in Infundibulum

The infundibular wall is made up of outer longitudinal and inner circular layers of muscularis. The lamina propria contains tubular glands lined by secretory cells which are most numerous in the posterior segment. Internally, the surface epithelium of the anterior segment of the infundibulum (ampulla) is lined by ciliated non-secretory cells with occasional groups of granular cells (Plate 6.1). The middle and posterior segments of the infundibulum (chalaziferous region) are lined by ciliated non-secretory and non-ciliated granular cells (Plate 6.2).

The surface of the infundibulum was covered with secretory material in the hen from Group 1 (Plate 6.3). This was further confirmed by TEM in which secretory blebs were observed on the granular cell surface. The middle and posterior segments of the infundibulum (chalaziferous region) were lined by ciliated and granular cells. Under the scanning electron microscope, the anterior segment of the infundibulum was covered mostly by ciliated cells. The distal segment was covered with ciliated cells and an increased number of non-ciliated cells. The mucosal folds in the distal region were more voluminous than in the proximal region. A similar appearance was observed in hens from Groups 2, 3, 4, 5, 6 and 7. In the hens from Groups 8 and 1, the granular cells appeared to bulge on the surface and ciliated cells appeared embedded between the bulging granular cells. The proximal and distal surfaces of the infundibulum were covered with mucus in the hen from Group 1. Glandular openings were not observed in the distal region of the infundibulum. The granular cells had abundant microvilli at the apical surface.

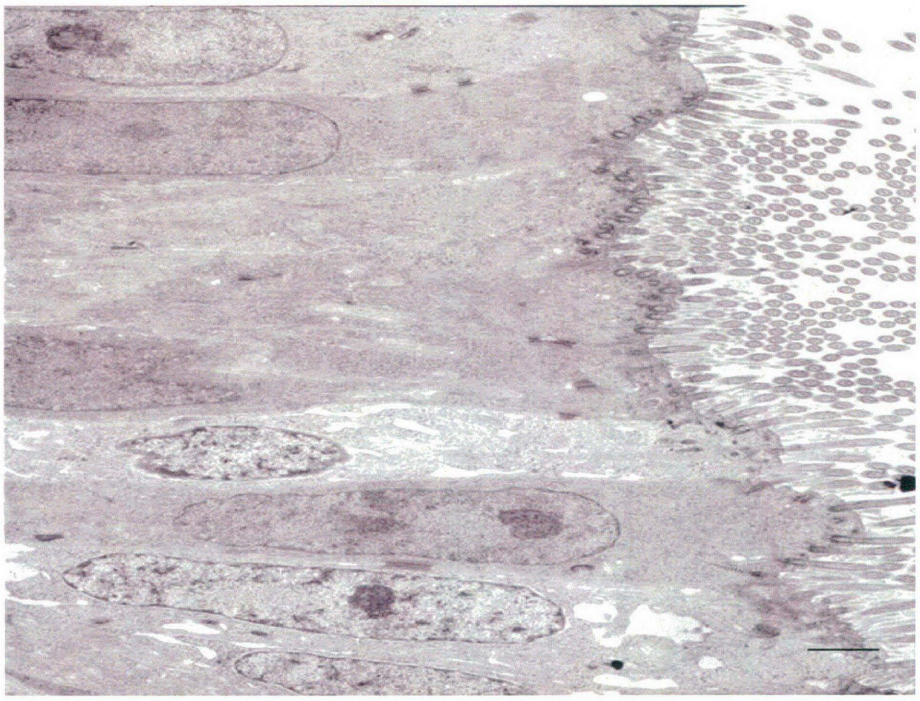

Plate 6.1: TEM of proximal infundibulum. No egg in the oviduct. X 4000 Scale bar represents 5 µm. Egg in the isthmus

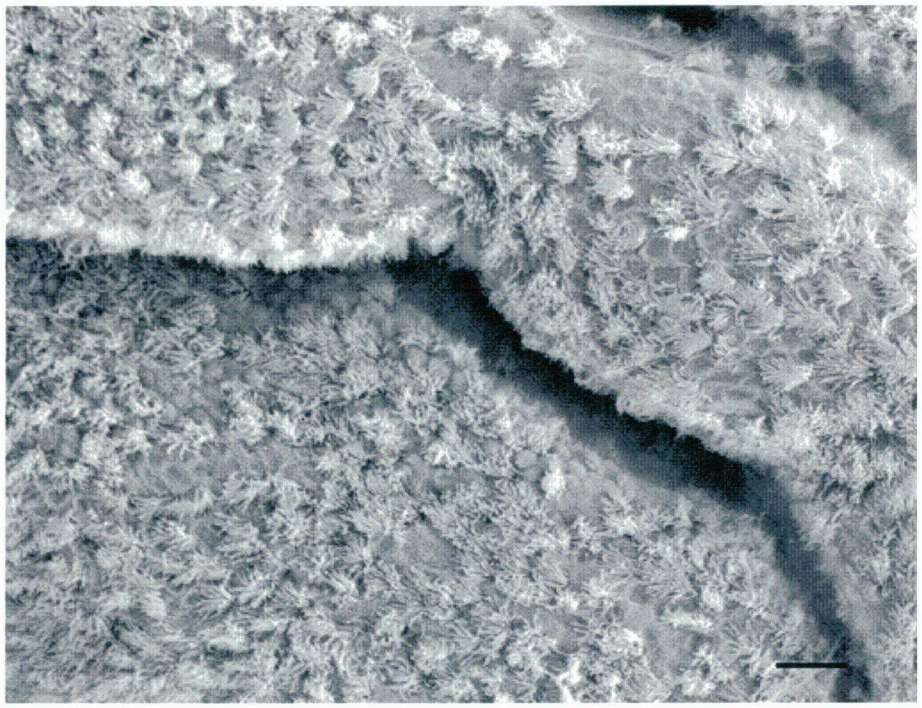

Plate 6.2: SEM of distal infundibulum lined by ciliated and non-ciliated cells. X 900. Scale bar represents 20 µm. Egg in the isthmus

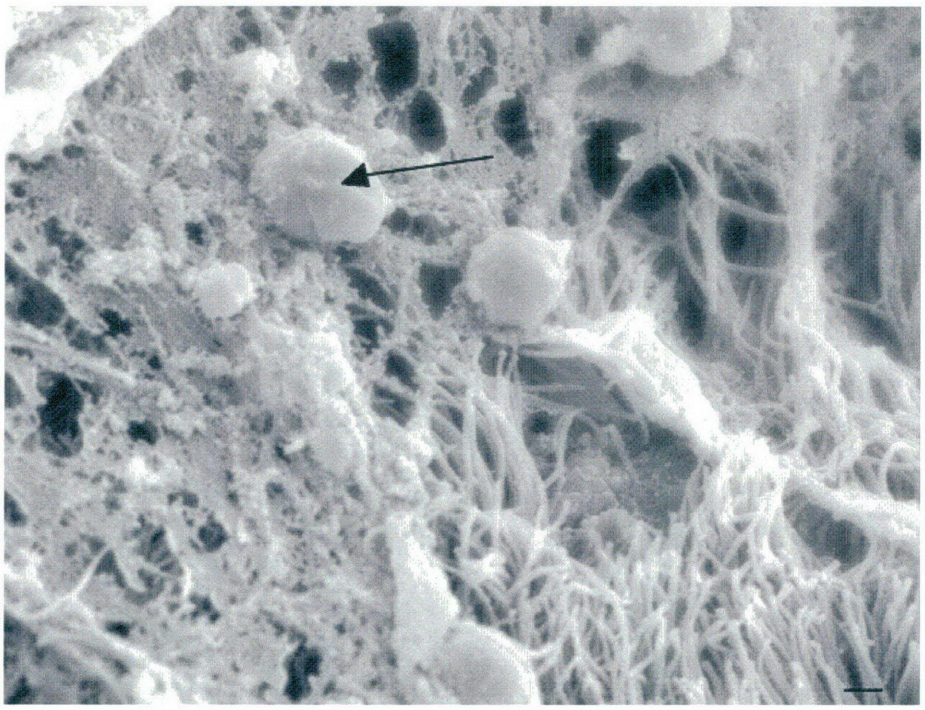

Plate 6.3: SEM of distal infundibulum covered with secretion. Note the secretory granule (Arrow). Egg in the infundibulum. X 10,000. Scale bar represents 1 μm. Egg in the infundibulum

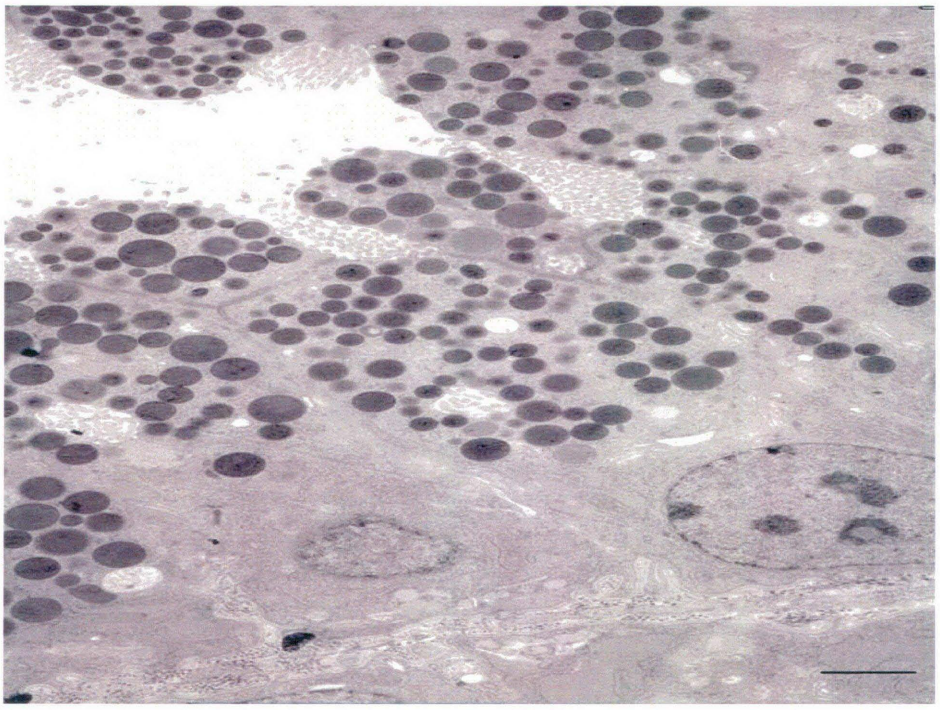

Plate 6.4: TEM of infundibulum. X 4000. Scale bar represents 2 μm. Egg in the infundibulum and hard-shelled egg in the shell gland pouch.

Under the transmission electron microscope, secretory granules were observed in the granular cells of the distal region of the infundibulum in the hens from all the groups.

The secretory granules were most numerous in the hens from Groups 7 and 1 (Plate 6.4). In the hen from Group 8, secretory granules were observed chiefly at the surface of individual cells. The microvilli of the secretory cells were anatomising, branching and swelling at the tip The Rough Endoplasmic Reticular (RER) cisternae in the surface and gland cells were dilated in the hens from Groups 7 and 1. In the hens from the remaining groups, the RER deposits appeared shrunken. Golgi bodies were most numerous in gland and surface epithelia of the hens from Groups 8 and 1. The Golgi complex appeared more compact in ciliated epithelial cells than non-ciliated cells at all positions of the egg in the oviduct. The density of secretory granules in glandular and granular cells varied, being greatest in the hens from Groups 8 and 1. The ciliated cells in the anterior and posterior regions of the oviduct contained fewer secretory granules which resembled the granules in the secretory cells. Plasma cells were regularly observed in both proximal and distal portions of the infundibulum in all hens.

6.3.2 Changes in Magnum

The walls of the magnum consist of an outer muscular layer (muscularis) and inner lamina propria containing tubular glands lined by secretory cells. There are three types of gland; A, B and C, at different levels of the magnum. Glands A and C are of one cell type but different phases of secretory activity whereas gland B is of a different cell type. The surface epithelium of the magnum is lined by ciliated non-secretory and secretory non-ciliated (granular) cells. Under the scanning electron microscope, most of the granular cells were prominent and bulged on the luminal surface of the magnum of all hens except those from Groups 3, 4 and 5 (Plate 6.5).

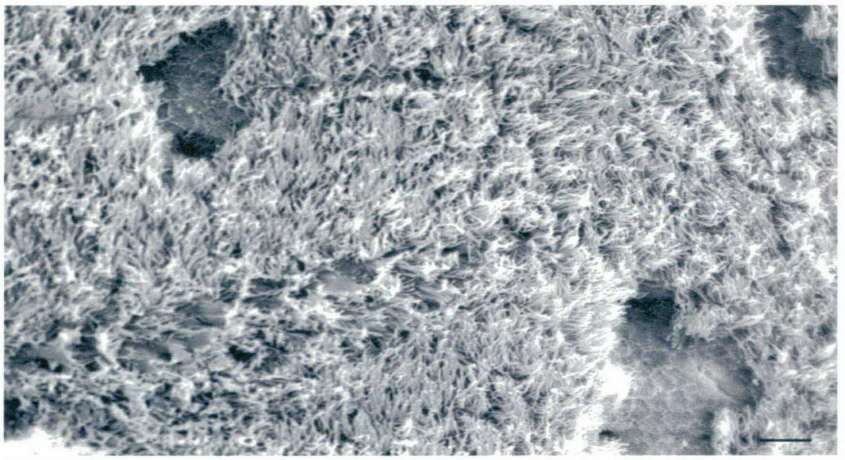

Plate 6.5: SEM of ciliated and non ciliated cells of magnum. X 900. Scale bar represents 20 µm No egg in the isthmus

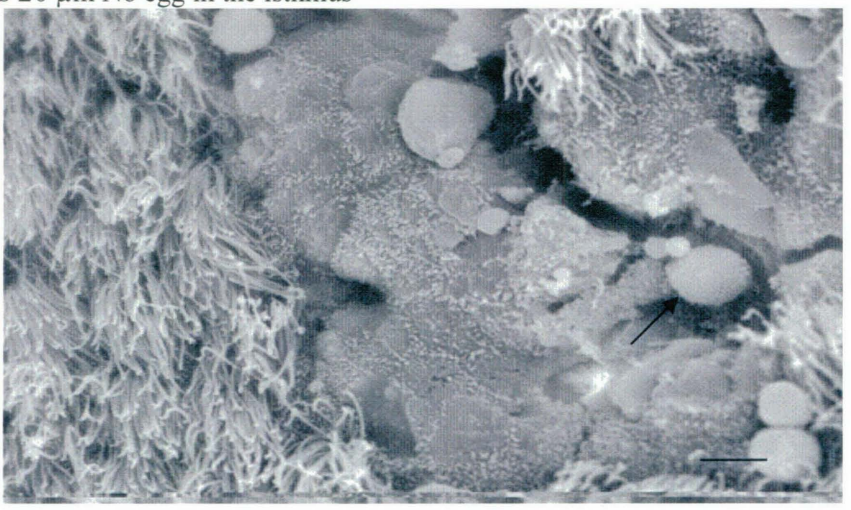

Plate 6.6: SEM of glandular opening of magnum. Note the secretory granule at the opening of gland. X 3000. Scale bar represents 5 µm. Egg in the mid magnum

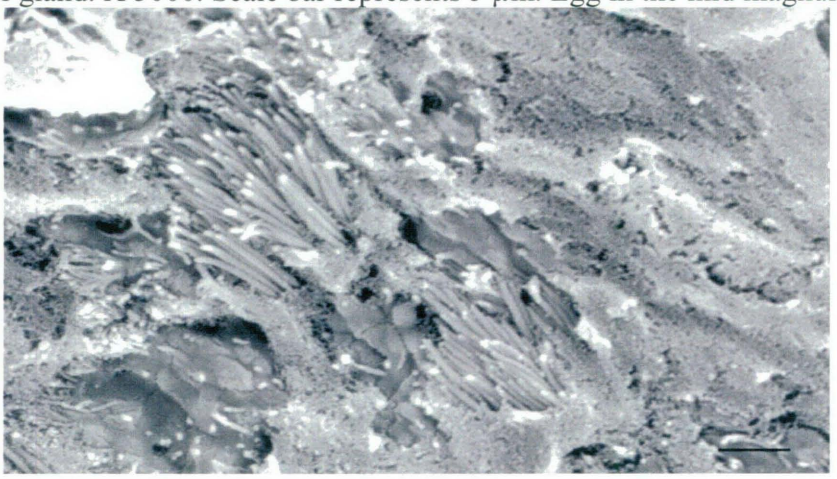

Plate 6.7: SEM of mid magnum covered with secretion. X 6000 Scale bar represents 2 µm. Egg in the lower magnum

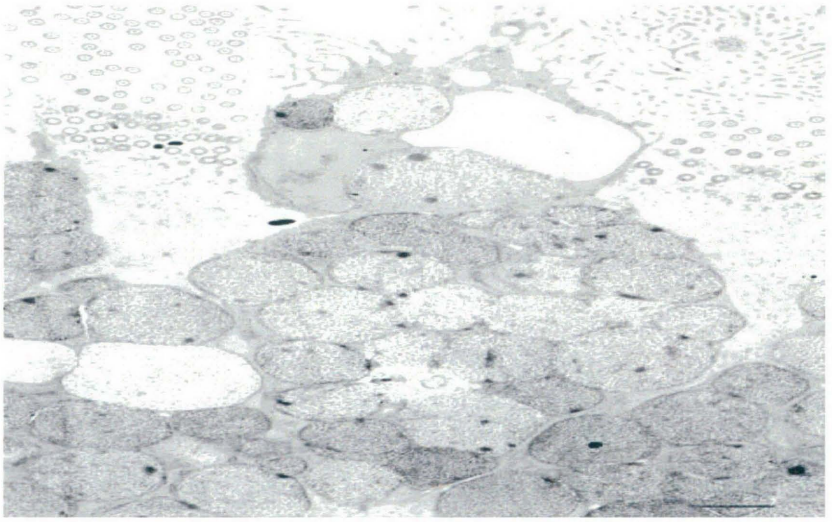

Plate 6.8: TEM showing secretory granules separating from the granular cells of mid magnum. X 5000. Scale bar represents 1 μm. Egg in the mid magnum

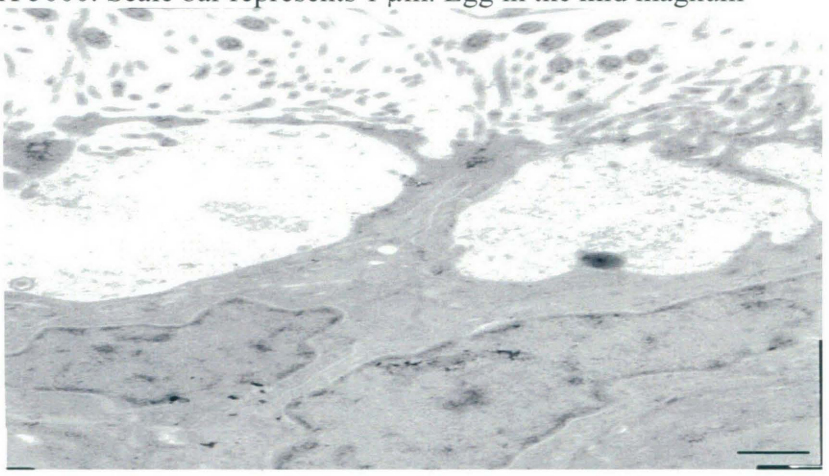

Plate 6.9: TEM showing empty pockets in the granular cells of mid magnum after emptying secretory granules in the lumen. X 6000. Scale bar represents 1 μm. Egg in the lower magnum

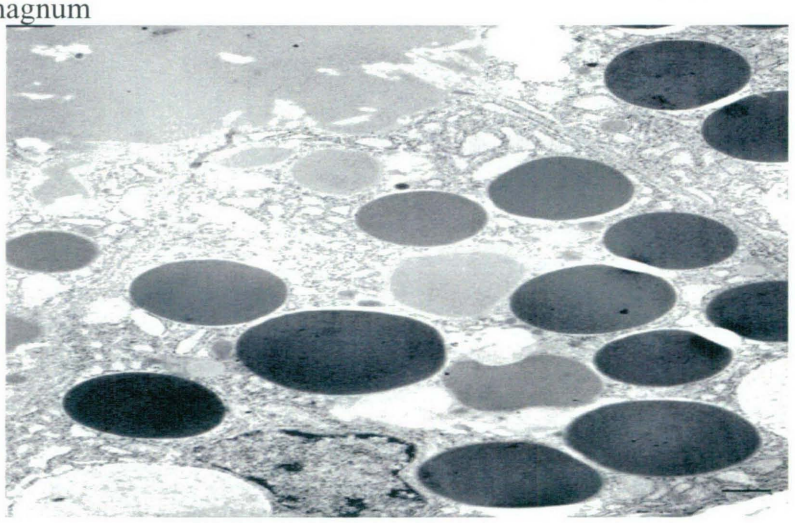

Plate 6.10: TEM showing gland cell type A in the lower magnum. X 6000. Scale bar represents 1 μm. Hard shelled egg in the shell gland.

Plate 6.11: TEM showing gland cell type C from the mid magnum. X 4000. Scale bar represents 2 μm Egg in the lower magnum.

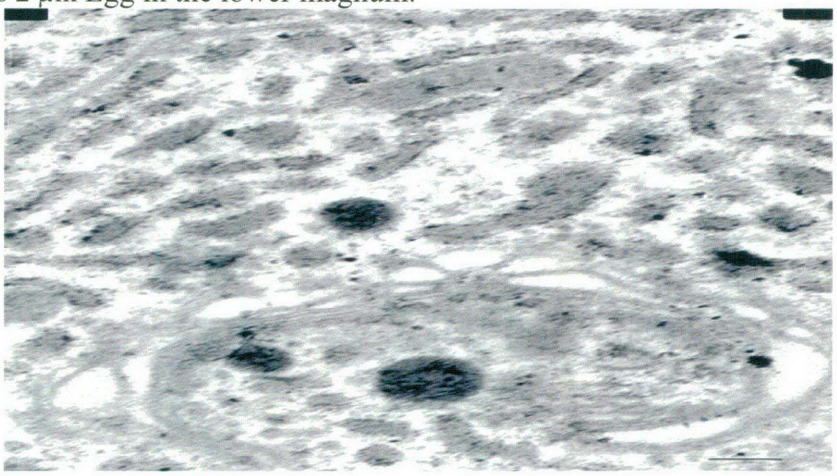

Plate 6.12: TEM showing widespread reticular endoplasmic reticulum with the spreading Golgi complex in type C gland cell of the mid magnum. X 20,000. Scale bar represents 20 nm. Egg in the lower magnum

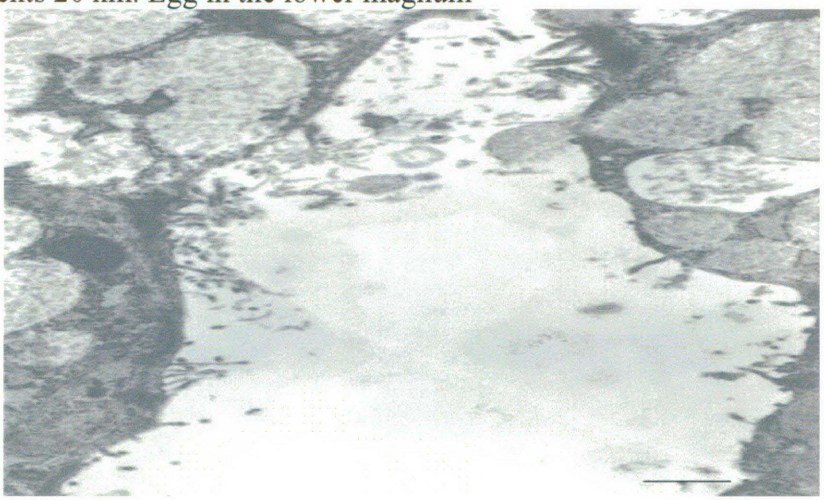

Plate 6.13: TEM showing type B gland cell in the magnum. X 6000. Scale bar represents 1 μm. Soft shelled egg in the shell gland.

Areas of granular cells bulging into the lumen were evident in between ciliated cells. At the time of peak secretion of albumen, secretory granules were observed at the surface glandular openings (Plate 6.6). Also, during the presence of egg in the mid magnum (hen from Group 3) the surface was mostly covered with secretion. Most parts of the magnum were covered by a layer of albumen even after passage of the egg (Plate 6.7). This was further confirmed by TEM observations. The secretory granules in the magnum were of different densities. The microvilli of the bulging granular cells were short. The surface of the magnum appeared ciliated in the hens from Groups 3, 4 and 7, although in the magnum of these hens, the granular cells were located in groups. At the time of peak secretion of albumen, the individual granular cells were actively producing secretory granules which were then released into the oviduct lumen (Plate 6.8). The endoplasmic reticular cisternae were present around to the nucleus. The Golgi complex was well developed with elongated sacules. The Golgi complex was situated above the nucleus. The granules with low electron density were observed adjacent to the Golgi complex. After the egg had passed, empty granular cell pockets were observed at the surface of the most of the cells of the magnum in hens from Group 4 (Plate 6.9). Secretory granules were still evident in some granular cells of the magnum in this hen. During the presence of an egg in the isthmus (hen from Group 5), most of the granular cells contained secretory granules. A similar picture was observed in the magnum of hens from Groups 6, 7, 8, 1 and 2. Granules were not recorded in ciliated cells of the magnum at any egg position in the oviduct. Mitochondria were scattered throughout both ciliated and granular cells.

Type A gland cells in the magnum were conspicuous during the presence of a hard shelled egg in the shell gland pouch. The glands were also conspicuous just after the passage of an egg. The scanning micrograph of the magnum revealed a group of non-ciliated cells bulging into the lumen. Type A gland cells in the magnum were observed also in the oviduct without an egg in it (Resting phase) and electron dense granules were a prominent feature of this gland cell. The granules varied in size and some pale granules were also observed (Plate 6.10). Type C gland cells in the magnum were evident mostly after passage of the egg through the magnum. Short cords of endoplasmic reticulum and small vesicles budding off from Golgi saccules were also observed (Plates, 6.11, 6.12). Gland cells type C were seldom seen when the egg was in the isthmus. The endoplasmic reticular deposits in type C gland cells

were enlarged when an egg was in the isthmus but were rare during the presence of a soft-shelled egg in shell gland. No cyclical changes were recorded in type B gland cells of the magnum (Plate 6.13) and the secretory granules in gland cell type B were amorphous.

6.3.3 Changes in Isthmus

The isthmus is separated from the magnum by a visible white line of demarcation which is a non-glandular zone. The surface epithelium of the isthmus and tubular shell gland is lined by ciliated, non ciliated and mitochondrial cells.

Under the scanning electron microscope, there was a predominance of ciliated cells on the surface in hens from Groups 6, 7, 8 and 1 (Plate 6.14). The non-ciliated secretory cells were most predominant and bulged on the luminal surface of the isthmus of hens from Groups, 2, 3, 4 and 5. In the isthmus of hens from Group 5, the secretory granules were mostly adhered to the outer surface of non-ciliated cells (Plate 6.15).

Under the transmission electron microscope, the granular cells of the isthmus were filled with supranuclear secretory granules with different densities. Granular cells showed blebbing in the hens from Groups 1, 2, 3 and 4 (Plate 6.16). In the hen from Group 5, the blebs were detached from the granular cell and located in the lumen (Plate 6.17). In hens from all the groups, ciliated cells appeared with sparse granules. Supranuclear cytoplasmic spaces were not observed in the granular cells of the isthmus of any hen. The granules in the ciliated cells were not in different phases of secretion as in granular cells. Different stages of granule formation were observed in the granular cell. The mitochondrial cell which is the third cell type in the surface epithelium besides ciliated cell and granular cells, was characterised by a large number of mitochondria in the hens from Groups 4 and 5. The mitochondrial cells bulged into the lumen of the isthmus in hens from Groups 4 and 5.

The type 1 gland cells described earlier by Solomon (1975) were evident in hens from Groups 3, 4 and 5 and also in the hen from Group 8. These glands were packed with secretory granules and the lumen was full of dense material in hens from Groups 4

and 5. The endoplasmic reticulum in type 2 cells appeared beaded (Plate 6.18). The gland cell type 1 was prominent in hens from group, 7, 1, 2. The cells were filled with secretory granules when the oviduct was empty. Reticular endoplasmic reticulum with intracisternal granules was a prominent feature in type 2 tubular gland cells of the isthmus.

6.3.4 Changes in tubular shell gland and Shell gland pouch

The ultrastructural morphology and cyclic changes in the surface epithelium of the tubular shell gland were similar to that of the shell gland pouch, except that the cytoplasm of gland cells of the tubular shell gland were filled with numerous glycogen granules in all the hens (Plate 6.19). Glycogen granules were dispersed throughout the cell cytoplasm. Glycogen granules were also recorded in the bulbous tips of the microvilli of the gland cells during the presence of an egg in the isthmus (Group 5)

The mucosal surface of the shell gland was lined by equal number of ciliated and non-ciliated cells (Plate 6.20). These cells have also been referred to as apical and basal cells by Aitken and Johnston (1963). The ciliated cells of the shell gland contained secretory granules which were numerous in the shell gland of hens from Groups 5 and 6. The small granules budding off from the Golgi complex were observed in such cells. Mitochondria also appeared long and scattered throughout the cell. The cilia of the ciliated cells of shell gland of the hen from Group 6 were interconnected forming net-like projections. In all other hens, secretory granules were present but fewer in numbers. The mitochondria were round in shape.

Secretory granules in non-ciliated cells were abundant in the shell gland of hens from Groups 5 and 6. Mitochondria were scattered throughout the cells. The appearance of the Golgi complex was similar to that of the ciliated cells of the shell gland but the secretory granules were smaller than granules in the ciliated cells. Large vacuoles were observed in the non-ciliated cells at all egg positions, although they were less common in the granular cells of the shell gland of the hens from Groups 5 and 6. In the shell gland of hens from all groups, the secretory granules were attached to the membrane of the vacuoles and the secretory granules appeared to shed their contents

in the vacuoles (Plate 6.21). Regression of the vacuoles, the cisternae of endoplasmic reticulum were prominent and extensive.

Disintegrated material, in the form of flocculent material, was also observed in the vacuoles (Plate 6.22). The cisternae of the endoplasmic reticulum were fewer when the vacuoles were at their maximum and, during the The ciliated cells of the shell gland appeared to be sandwiched between non-ciliated cells in the shell gland of the hen from Group 5 (Plate 6.23a). In the hens the other groups, the ciliated cells were more prominent and conspicuous on the surface of the shell gland (Plate 6.23b). In the shell gland of the hen from Group 6, white coloured flakes were observed (Plate 6.24) although it was not clear what caused this appearance.

The gland cells in the hens from Groups 5 and 6 were filled with secretory granules, pushing the nucleus towards the base of the cell. The lumen of the gland cell was narrowed but secretory granules were not recorded in them. The microvilli of the gland cells were enlarged with rounded tips. The lumen of the gland cells from the shell gland of the remaining hens was larger with empty circular bodies in the lumen (Plate 6.25). Mitochondria in these gland cells had closely packed cristae, distributed from apex to base of the cell. There were membrane-bound granules with the particulate material in them. Wyburn *et al.*, (1973) described these membrane bound granules as fixation artefacts.

Plate 6.14: SEM of ciliated and non-ciliated cells of the isthmus. X 6000. Scale bar represents 2 µm. No egg in the oviduct

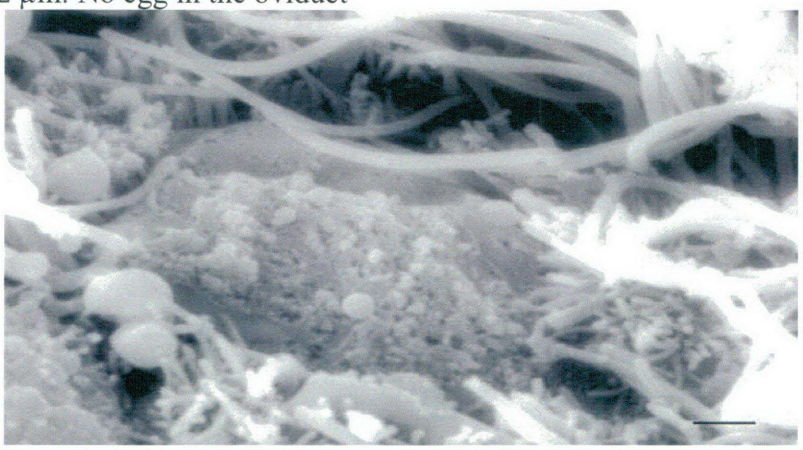

Plate 6.15: SEM of the secretion on the surface of non-ciliated cell in the isthmus. X 15000. Scale bar represents 1 µm. Soft shelled egg in the shell gland

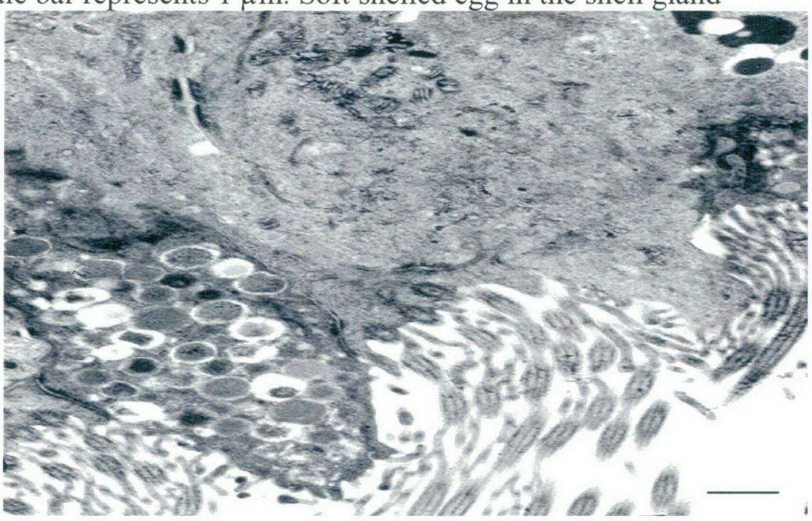

Plate 6.16: TEM showing blebbing of non-ciliated cells in the isthmus. Also note the bulging of the mitochondrial cell into the lumen. X 6000. Scale bar represents 1 µm

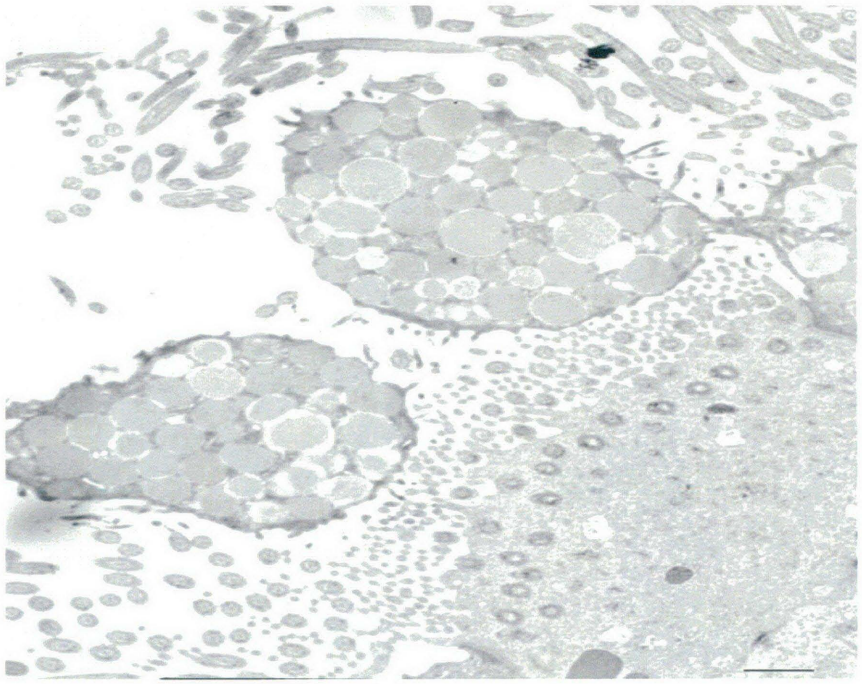

Plate 6.17: TEM showing release of secretory blebs from granular cells of isthmus. X 6000. Scale bar represents 1 µm. Egg in the isthmus

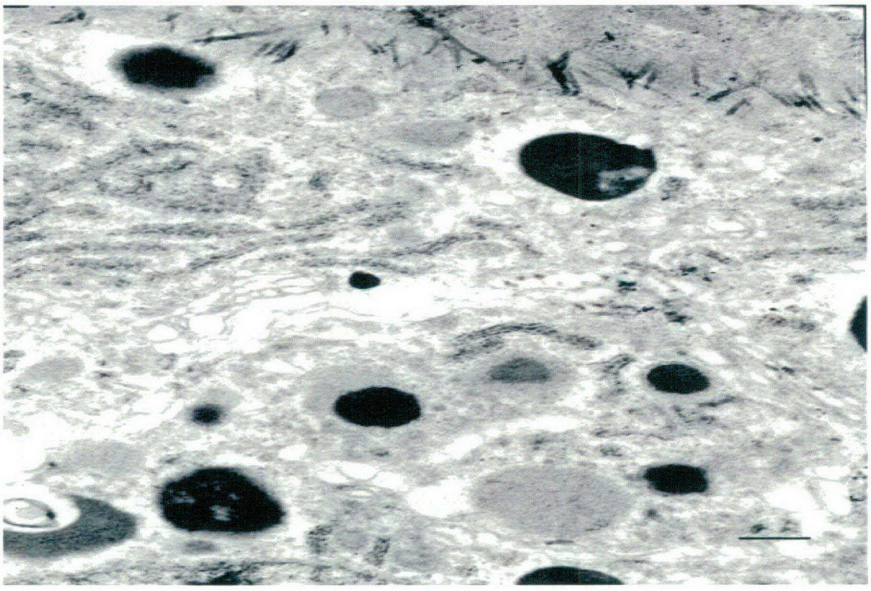

Plate 6.18: TEM showing reticular endoplasmic reticulum in the form of short cords in type 2 gland cells of the isthmus. X 10,000. Scale bar represents 500 nm. Hard shelled egg in the shell gland

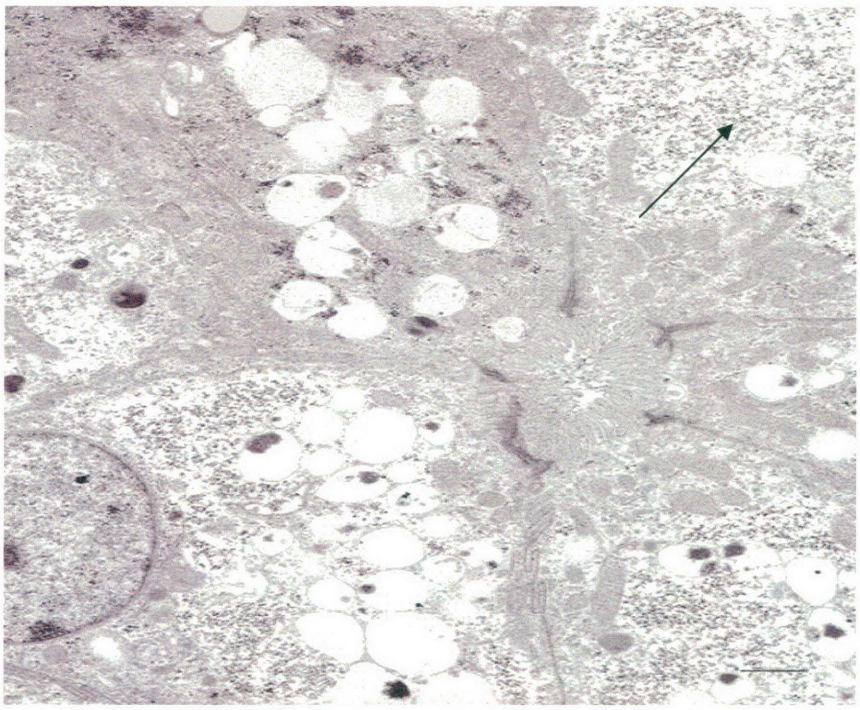

Plate 6.19: TEM of gland cell of tubular shell gland. Note the glycogen deposits (Arrow). X 5000. Scale bar represents 1 μm. No egg in the oviduct

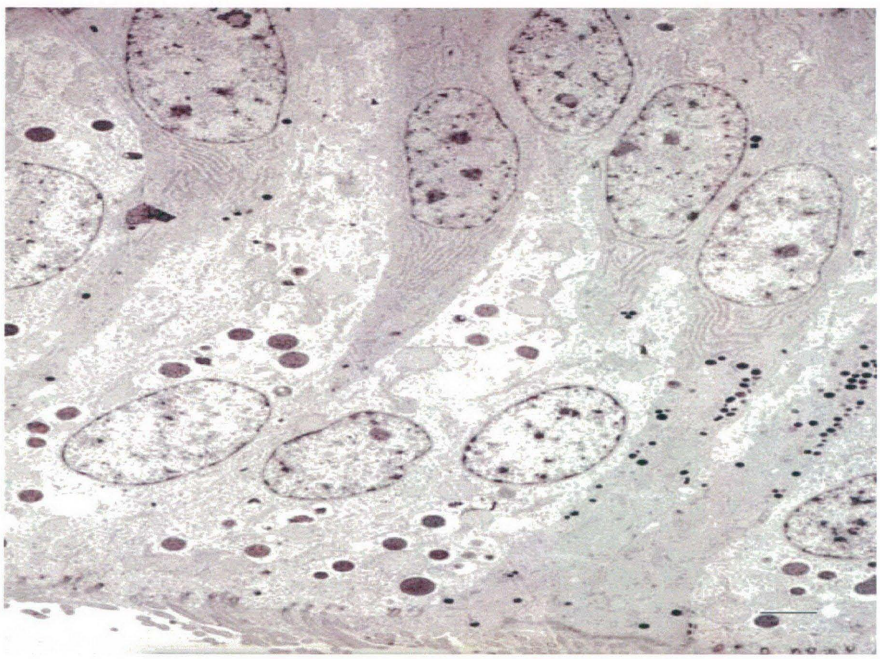

Plate 6.20: TEM of surface epithelium of shell gland pouch. Note apical and basal cells. X 2500. Scale bar represents 2 μm. Egg in mid magnum

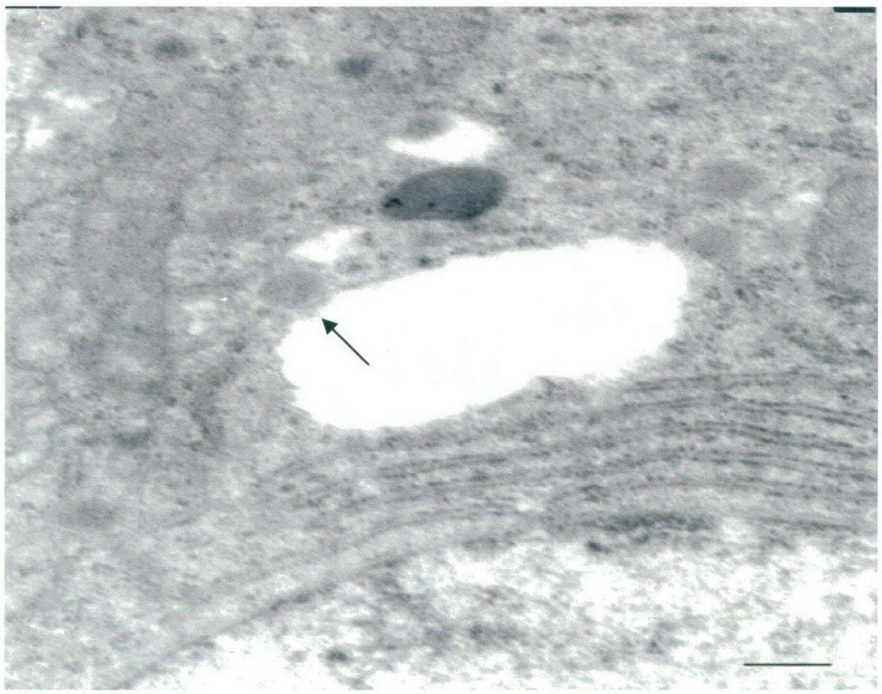

Plate 6.21: TEM of non ciliated cell of the shell gland showing shedding of a secretory granule into the vacuole. X 20000. Scale bar represents 200 nm. No egg in the oviduct

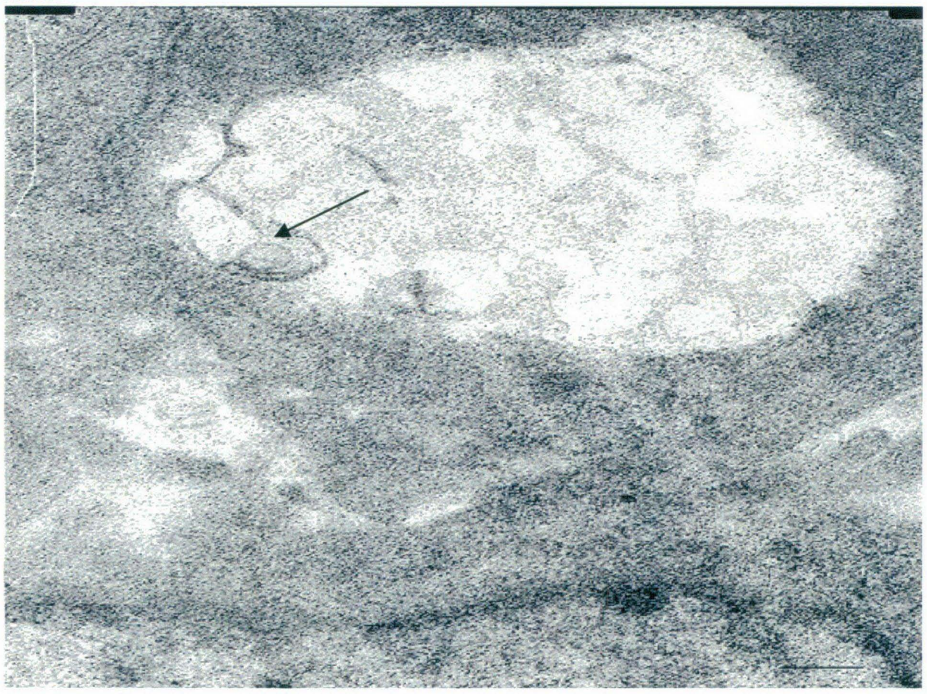

Plate 6.22: TEM of vacuole in the non-ciliated cell of the shell gland pouch. Note the disintegrated material (Arrow) probably derived from the secretory granule. X 25000. Scale bar represents 200 nm. No egg in the oviduct.

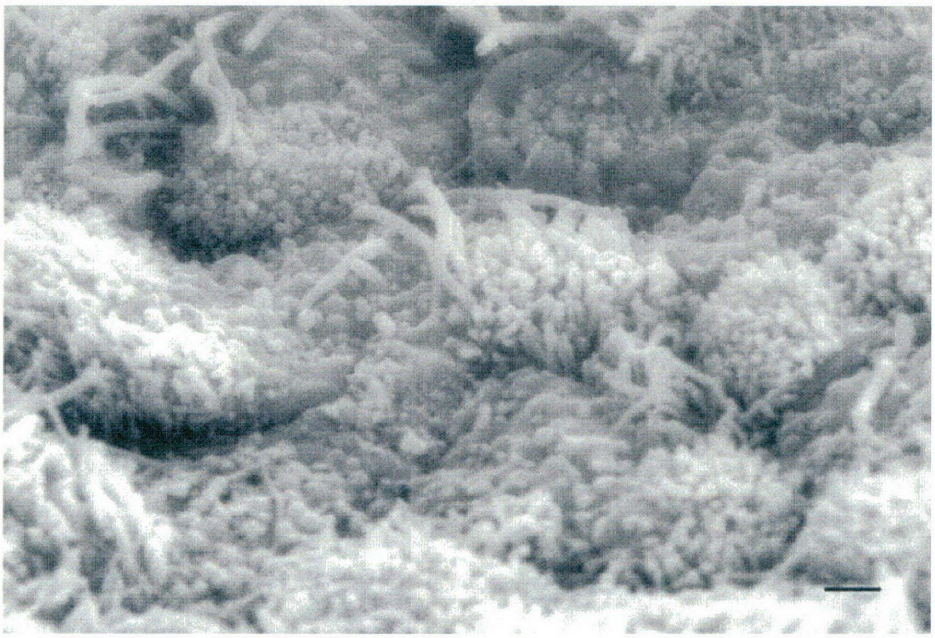

Plate 6.23a. SEM of ciliated and non-ciliated cells of the shell gland. Note the ciliated cells embedded in non-ciliated cells due to bulging of non-ciliated cells. X6,000. Scale bar represents 1 μm. Egg in the isthmus

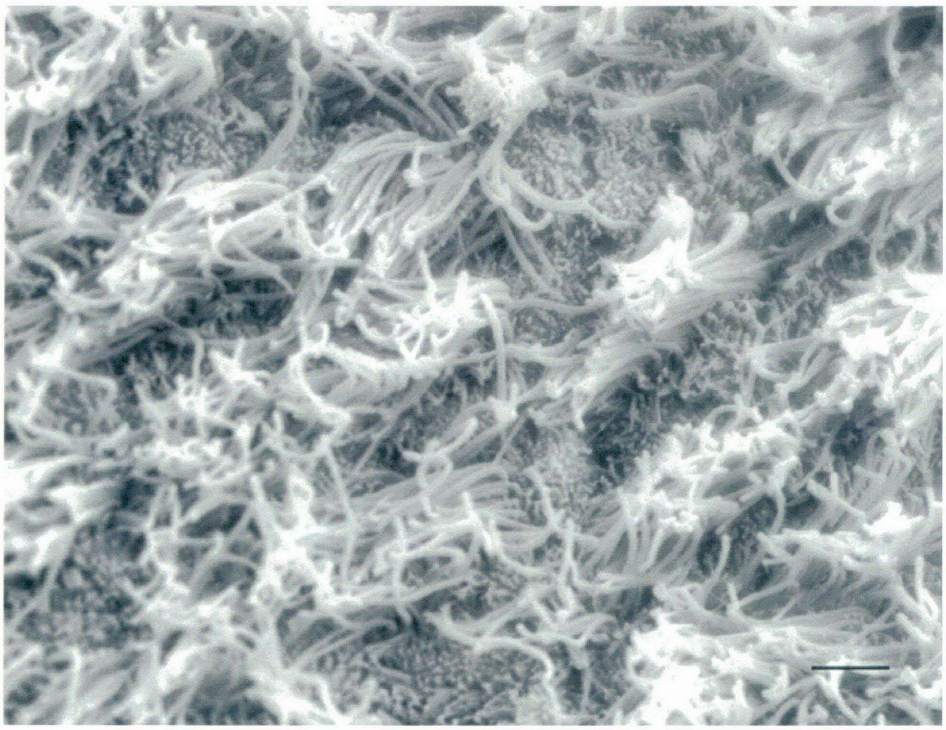

Plate 6.23b. SEM of ciliated and non-ciliated cells of the shell gland. Note the prominent ciliated cells against non-ciliated cells. X 6,000. Scale bar represents 1 μm. No egg in the oviduct

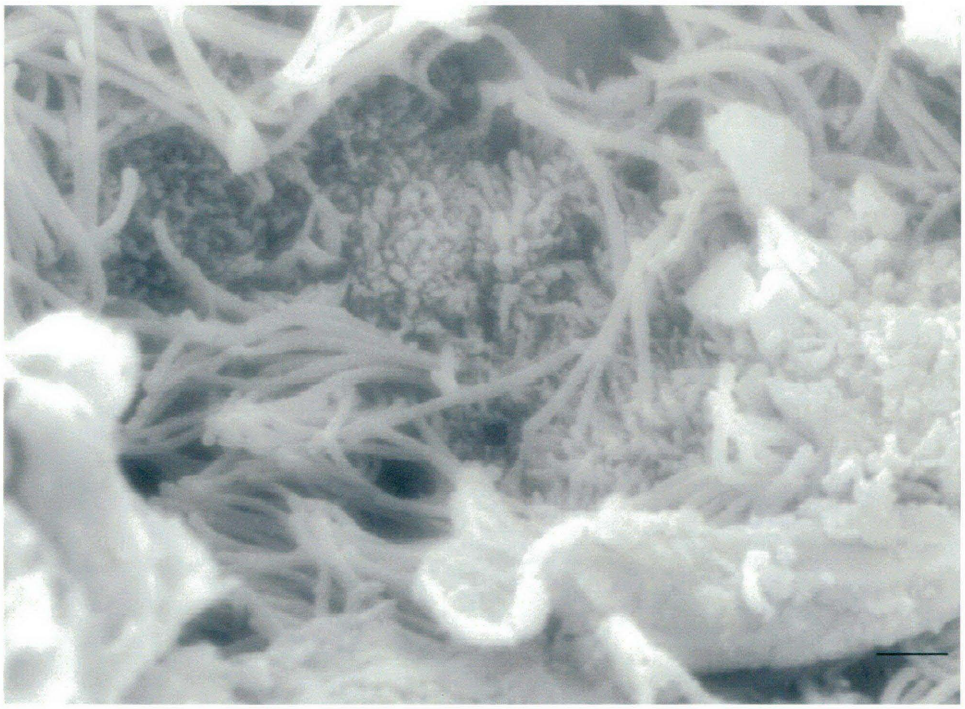

Plate 6.24. SEM of the shell gland. Note the white flakes on the surface. X 10,000. Scale bar represents 1 μm. Soft shelled egg in the oviduct.

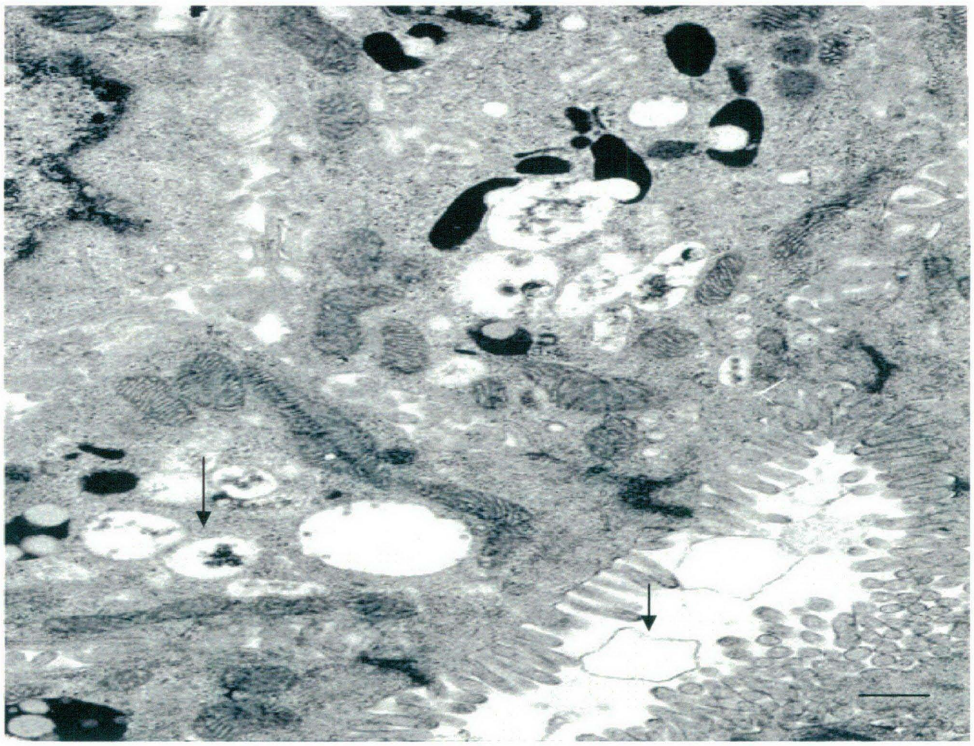

Plate 6.25: TEM of gland cell of the shell gland pouch. Note the membrane bound granules with granular material (Arrow). Also note the numerous mitochondria. X 8000. Scale bar represents 1 μm. No egg in the oviduct.

6.4 Discussion

During the main trial, it was inevitable that hens would be sampled when eggs were in different regions of the oviduct. Previous studies reported that the position of the egg in the oviduct influences the electron microscopical appearance of the oviduct. The results reported in this chapter provided information on the effects of the position of the egg in the oviduct on the ultrastructure of the cells in the different parts of the oviduct. This allowed effects of infectious bronchitis virus exposure to be distinguished from the intrinsic effects of egg position in the oviduct.

Most of the findings regarding the histology and transmission electron microscopy of the infundibulum were in accordance with the results of Wyburn and colleagues (Wyburn *et al.*, 1970). The finding regarding the presence of lymphocytes and plasma cells in all parts of the oviduct agrees with the results of Biswal (1954). The infundibulum was lined by equal numbers of ciliated and non-ciliated cells except for the anterior part which was predominately ciliated cells. This was also observed by Solomon (1983). The anterior part of the infundibulum was covered with secretory material in the hen from Group 1, which could be due to the presence of the ovum in this portion. However, when viewed under transmission electron microscopy, secretory granules were not recorded in the ciliated cells of this portion which explains its non secretory activity. Cells of the distal part of the infundibulum contribute to the egg contents and this correlates with the presence of tubular glands and granular cells in the distal infundiblum. Prominent changes were not observed. There were no marked effects of an egg passing through this region except for the secretory material in the anterior part of the infundibulum in the hen from Group 1 under SEM and deposits of RER and secretory granules in the hens from Groups 1 and 8 under TEM. Increased RER deposits and secretory granules in glandular cells of the infundibulum were also reported by Wyburn *et al.*, (1970).

Most of the microscopic findings regarding the structure of the magnum were in accordance with Wyburn *et al.*, (1970), although simultaneous scanning electronic microscopic changes during laying cycle have been described in the present study. The scanning micrographs regarding the glandular openings on the surface epithelium are similar to those reported by Makita and Sandborn (1970). These glandular

openings were difficult to locate under light microscopy and TEM but were clearly visible under SEM. Round secretory granules were observed on the surface of the granular cells and glandular openings which could be the secretory granules observed by TEM. It has been stated by Wyburn *et al* (1970) that, after releasing all the contents from the cell, the endoplasmic reticulum occupies most the cellular area, with a series of developing secretory granules adjacent to the Golgi complex. This can be seen in the form of small electron dense vesicles budding off from Golgi saccules. Such separated electron dense vesicles then gather together to form a secretory granule which then migrates to the apex. Similar cyclic events were recorded in the granular and gland cells of the magnum although not in gland cell type B. The cyclic changes recorded in gland cells type A and C are consistent with the account of Wyburn *et al.*, (1970). After migration of granules to the apex, the granules then discharge into the lumen, replacing the ruptured cell membrane, although in some cells the ruptured empty pockets were observed (Plate 6.9). Such structures were observed in the granular cells of the magnum of the hen from Group 4 where the egg had passed a few minutes before. This indicates that the granular cells of the magnum act as goblet cells adding their contents to the egg.

The densities of granules present in the granular cells of the infundibulum and magnum were different (Plate 6.4 and Plate 6.8). The role of the infundibulum in formation of the chalazae is a matter of conjuncture as work done in the past has shown that the chalazae become apparent only after entry of the ovum into the caudal magnum (Aitken, 1971). Aitken and Johnston (1963), however, predicted that enzymes secreted by infundibular glands could have some role in chalazae formation. Makita and Kiwaki (1968) stated that secretion from the granular cell has a role of cleaning the surface of the ovum.

In the past, it has been reported that albuminous material secreted by the albumen secreting cells of the magnum releases in to the lumen without being processed or concentrated in the Golgi region Zeigal and Dalton (1962), Fertuck and Newstead (1970) observed that Golgi complex has active role in granule formation. The result from the present experiment agrees with the findings of Fertuck and Newstead (1970).

The transmission electron micrographs of isthmus and tubular shell gland in the present study were in accordance with Draper *et al.* (1973) and Solomon (1975) and the scanning microscopic findings were consistent with those of Bakst (1978). Intracisternal granules were present in the gland cells at all egg positions as observed by Solomon (1975). Intracisternal granules are the means of regulating protein synthesis (Solomon, 1983). More information is needed regarding the role of mitochondrial cells in the isthmus, as changes were not recorded in those cells at the ultrastructural level. Immunocytological studies would probably be helpful to clarify the role of these cells.

The TEM changes observed in the tubular shell gland and shell gland pouch were consistent with the reports of Aitken and Johnston (1963), Breen and Bruyn (1969) and Wyburn *et al.* (1973). The present study confirms the findings of Breen and Bruyn (1969) that vacuoles in the non-ciliated cells of the shell gland recycle the secretory product. The endoplasmic reticulum in close proximity to the vacuole is the chief site for protein synthesis. Considering the prominent appearance of vacuoles in the absence of endoplasmic reticulum and vice versa, it is possible that, during the resting phase or after egg shell formation, the unutilised secretory granules from the granular cells of the shell gland are shed into the vacuoles. The degraded material in the vacuole could be reutilised by the endoplasmic reticulum for production of new secretory granules. Thus the secretory granule in the non-ciliated cells undergoes degradation instead of remaining intact in the cell cytoplasm.

The chicken oviduct appears to be highly complex. It would seem unlikely that traditional light and electron microscopy alone would be sufficient to identify the cellular changes in the oviduct. More recent and sophisticated techniques could contribute knowledge regarding the oviduct. However, it is important to study the naturally-occurring cellular changes while studying diseases that affect the chicken oviduct.