Chapter 7

Comparative histopathology and cytopathology of two strains of infectious bronchitis virus in the albumen-forming regions of the oviduct of Isa Brown laying hens.

7.1 Introduction

Previous studies have established the possibility that some strains of IBV cause physical damage to the cells of the upper oviduct (Jones and Jordan, 1972). However, the detailed pathogenesis is still unexplored and the mechanism by which IBV is capable of reducing or causing cessation of egg production is still unknown.

The infundibulum and magnum are complex structures of the avian oviduct and change their appearance at different positions of the egg in the oviduct (Wyburn *et al.*, 1970). The infundibulum is the site of fertilization and the presence of tubular glands in the posterior region of the infundibulum suggests a secretory role during egg formation. (Aitken, 1971). On the other hand, the magnum is the most conspicuous part of the oviduct and contributes a range of proteins during egg formation. Despite claims that IBV causes a deterioratation in egg quality, very few efforts have been made either to document or to investigate the details of the pathogenesis of IBV in the fully functional oviduct. Some studies have been conducted using the Massachusetts strain of IBV in hens in full lay (Sevoian and Levine, 1957, Cook 1971). Crinion and Hofstad (1972a) studied the comparative pathogenesis of four strains, including T strain, in chickens up to 29 days of age. However, Australian strains of IBV including T strain have not been studied in the fully functional oviduct of hens at the cellular level. Preliminary experiments showed that IBV strains T and N1/88 can cause severe pathology in the oviduct of unvaccinated White Leghorn hens and vaccinated Hyline Grey hens. In the present study, efforts have been made to define the cellular

pathogenesis of two Australian strains of IBV in the upper reproductive tract of Isa Brown hens in full lay, to investigate causes of thin albumen and also to study the cellular changes in the infundibulum and magnum during cessation of egg production.

7.2 Materials and Methods

The details of the experimental design have been explained in chapter 5. The processing of tissues for histology and electron microscopy was conducted as described in chapter 2 (Section 2.4). During the preliminary study (chapter 3) it was observed that both T and N1/88 strains of IBV can cause severe pathology in all parts of the oviduct. Therefore, the infundibulum and white isthmus, which had not been included in the preliminary trial, were collected for the examination during the challenge trial with Isa brown hens.

7.3. Results

7.3.1 Autopsy findings of the oviduct

Out of 24 hens killed from the N1/88 infected group, the oviduct and ovary of one hen, which was out of lay and sacrificed on the 24th day p.i., was atrophied. On the 18th day p.i., a fully formed egg was recorded in the infundibulum of another hen. The remaining 22 hens from this group had visibly normal and fully-functional oviducts and ovaries. In the T strain-infected group, out of 24 birds sacrificed, only two non-laying hens showed thin and inspissated yolk material in the oviduct. However their ovaries were functioning normally. No visible abnormality was recorded in the oviduct of remainder of the sacrificed hens except for the occurrence of meat spots in the magnum of 12 hens.

No hens from the control group showed any lesions.

7.3.2 Changes in infected cells of infundibulum

In control hens, all the histological and electron microscopic features at different positions of the egg in the oviduct were in accordance with those reported by Aitken and Johnston (1963) and Wyburn *et al.* (1970). Scanning electron micrographs of the

infundibulum were similar to those of Bakst and Howarth (1975). The infundibulum of control hens appeared normal under light (Plate 7.1) and electron microscopy (Plate 7.5).

Pathology was observed mostly in the infundibulum of T-infected hens. However, in the N1/88 group, pathology was observed only in the hen with an atrophied oviduct. There were lymphoid nodules in the lamina propria (Plate 7.2) and fibrosis was also observed (Plate 7.3). Mild gland dilatation was observed in the distal infundibulum of one hen on day 10 p.i. In the infundibulum, there was no statistically significant main effect of IBV challenge strain except for lymphocyte infiltration which was observed for the T group but not the N1/88 group. Also, there were no significant main effects of days p.i. for three lesions scored (Table 7.1). Lymphoid nodules were mild to moderate from days 10 to 24 p.i.

Scanning microscopy in T-strain infected hens revealed patchy loss of cilia from the anterior segment (ampulla) as well as the posterior segment (chalaziferous region) of the infundibulum in both the hens killed at 10 and 12 days p.i., and also in one hen killed at 14 days p.i. (Plate 7.4). Most of the cilia appeared normal from day 16 onwards. In the N1/88-infected group hen which was out of lay with an atrophied oviduct and sacrificed on day 24 p.i., the epithelium of both the anterior and posterior segments of the infundibulum appeared ciliated. However, in both hens which were out of lay in the T-infected group, patchy cilia loss was observed in the anterior and posterior segments of the infundibulum, even at day 30 p.i.

The loss of cilia from the ampulla and chalaziferous region was further confirmed by histological and electron microscopic observations (Plate. 7.6). There were no changes until day 10 p.i. except for a few plasma cells and lymphocytes in the mucosa.

Under the electron microscope, most of the cellular changes were observed in both the T-infected hens killed on days 10 and 12 p.i., and in one hen killed on day 14 p.i . Ultrastructural changes were also observed in the infundibulum of T-challenged hens sacrificed on day 30 p.i. On days 10 and 12 p.i., there were changes in the surface epithelium of anterior and posterior segments of the infundibulum. There were

increased deposits of rough endoplasmic reticulum (RER) in granular, ciliated and tubular gland cells irrespective of egg position (Plate 7.7). Virus particles were recorded in the RER spaces of some cells (Plate 7.8). Mitochondria appeared swollen in the granular, ciliated and tubular gland cells. The Golgi saccules were dilated and virus particles were noted in these dilated Golgi saccules of granular as well as ciliated cells The virus particles in dilated RER and Golgi saccules were recorded in one hen killed on days 10 and 12 p.i., but not in any hen on day 14 p.i. Virus particles were also observed in both the hens killed on the 30^{th} day p.i.

Phagocytic vacuoles in tubular gland cells were observed in one of each T-infected hen killed on day 14 p.i. There was no tubular gland dilatation in the posterior region of the infundibulum. Most of the Golgi bodies in mucus-producing non-ciliated cells and tubular gland cells of the posterior infundibulum in non-laying birds were completely inactive (without Golgi vesicles). The cisternae of RER in most of the epithelial and glandular cells appeared dilated with virus particles and there were many phagocytic vacuoles. In the hen from the N1/88 group with an atrophied oviduct, the granular cells were embedded in between ciliated cells but cilia loss was not observed.

No cellular pathology was observed in the remaining hens of either of the infected groups from 16 to 24 days p.i. except for lymphoid nodules in the muscularis area of the posterior region of the infundibulum of the T-infected group. Lymphoid nodules were also recorded in the infundibulum of one hen from the N1/88-infected group with an atrophied oviduct

No changes were recorded in the infundibulum of any of the control hens.

Table 7.1: Comparative histopathology of infundibulum of hens infected with T and N1/88 strains of IBV

	Days post inoculation																							
	2		4		6		8		10		12		14		16		18		20		22		24		P value	
Lesions in Infundibulum	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	G	D
Cilia loss	0	0	0	0	0	0	0	0	1.5	0	2	0	0.5	0	0.5	0	0	0	0	0	0	0	0	0	NS	NS
Gland dilatation	0	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NS	NS
Lymphocyte infiltration	0	0	0	0	0	0	0	0	2	0	1	0	0.5	0	1	0	0.5	0	1	0	0.5	0	0.5	1	0.003	NS

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

Mean of lesion score from two hens, G- Group, D- days p.i.

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

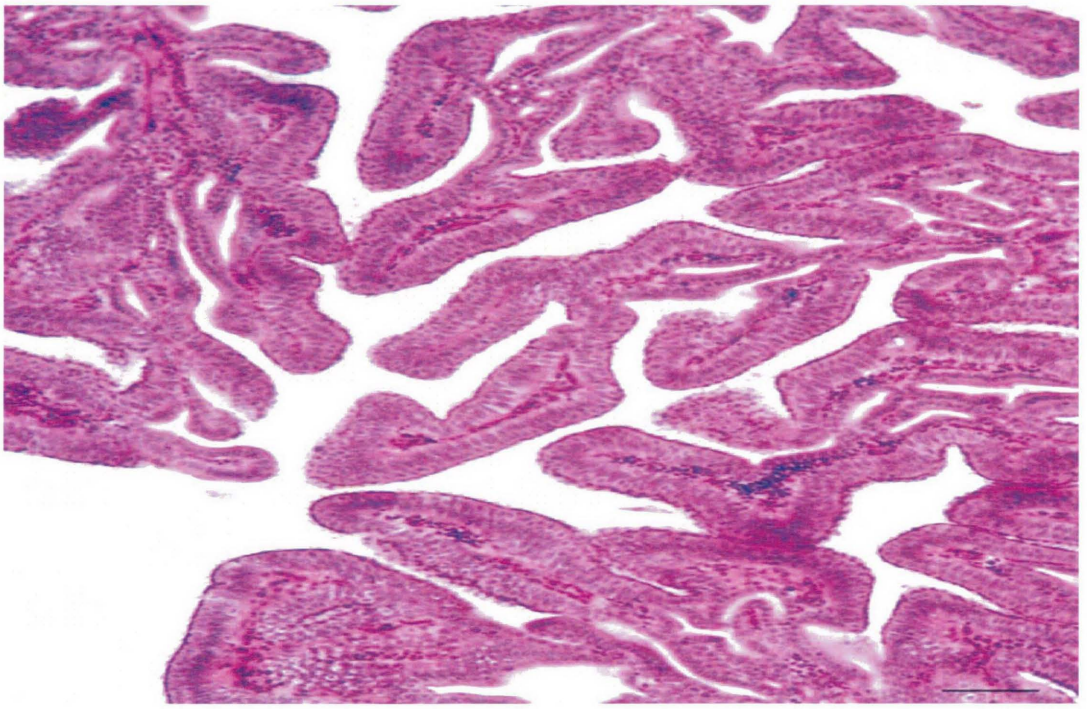


Plate 7.1: Anterior segment of infundibulum of control hen. H &E x 100. Scale bar represents 100 µm (hard shelled egg in shell gland)

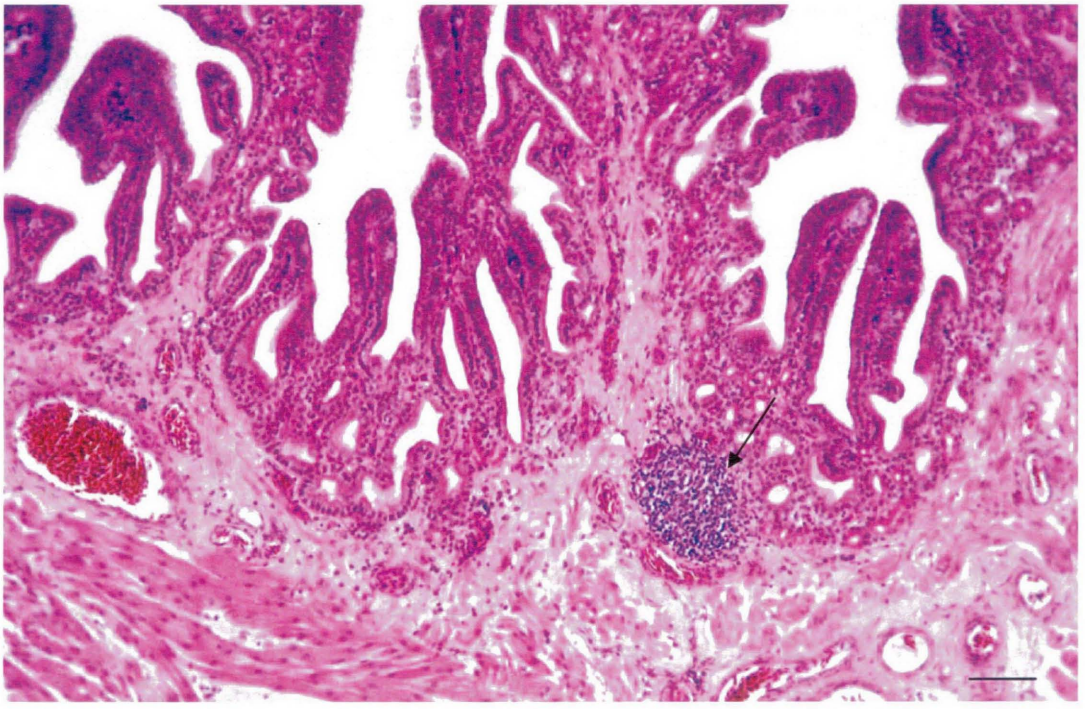


Plate 7.2: Anterior segment of infundibulum of T strain infected hen at day 24 p.i.. Lymphocyte nodules (Arrow). H &E x 100. Scale bar represents 100 µm (hard shelled egg in shell gland)

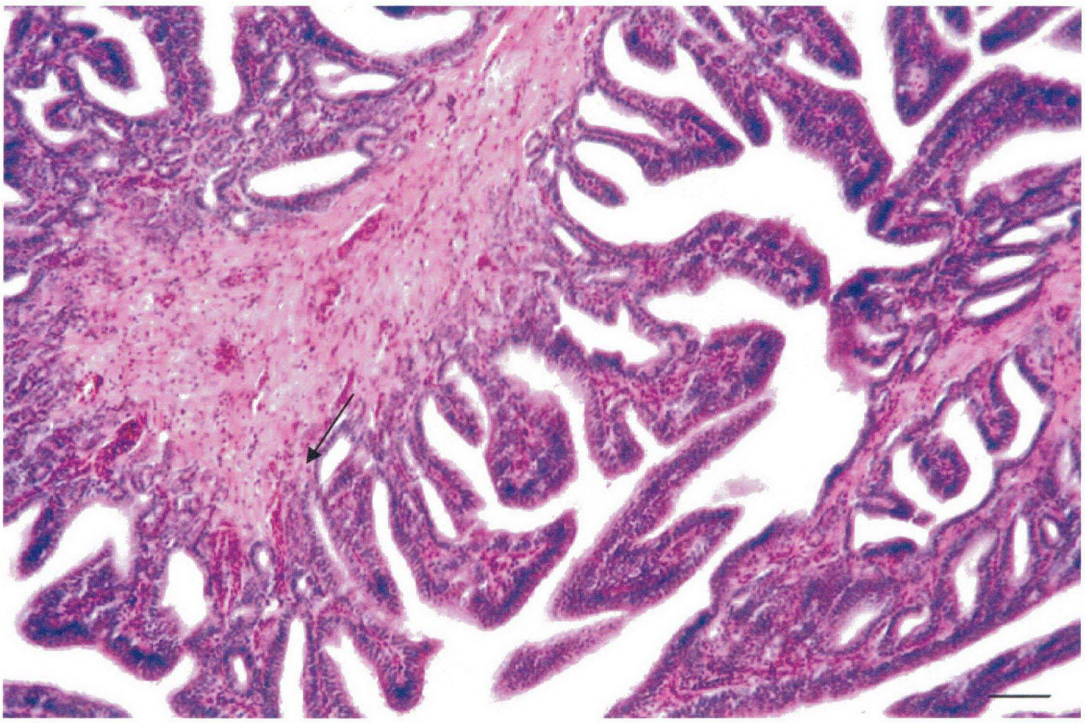


Plate 7.2: Anterior segment of infundibulum of N1/88 infected hen on day 24 p.i. Note the fibrosis in the lamina propria region (Arrow). H &E x 100. Scale bar represents 100 µm (hard shelled egg in shell gland)

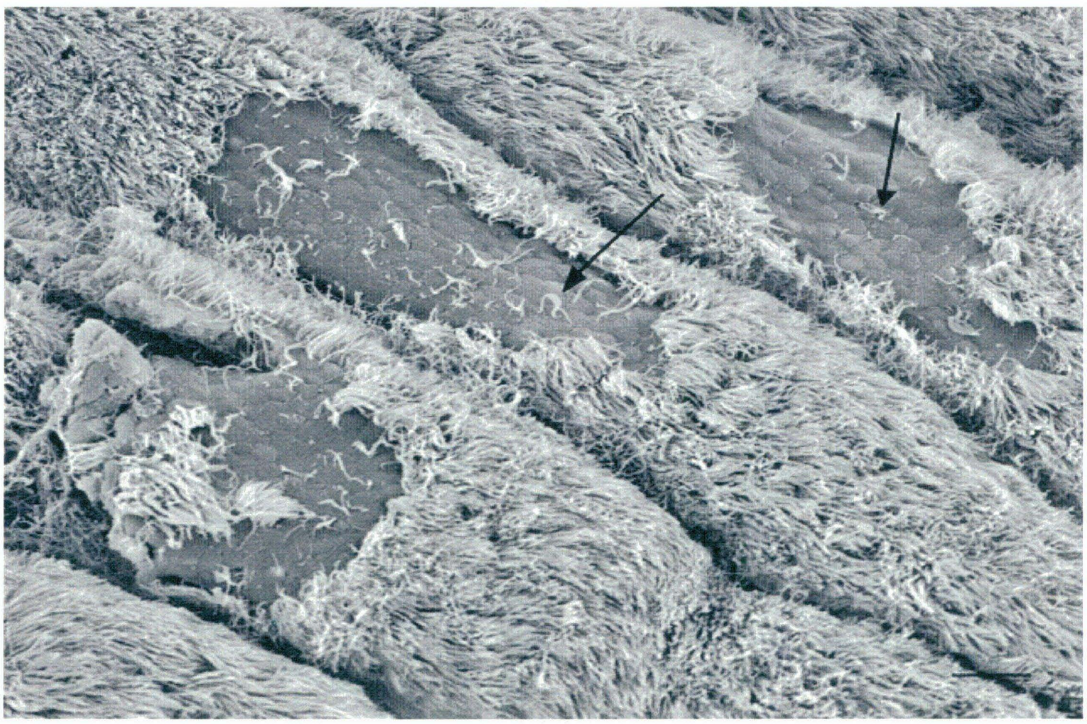


Plate 7.4: Scanning electron micrograph of anterior segment of infundibulum of T-strain infected hen at 10 days p.i. x 900. Scale bar represents 20 µm (hard shelled egg in shell gland). Note patchy loss of cilia (arrow)

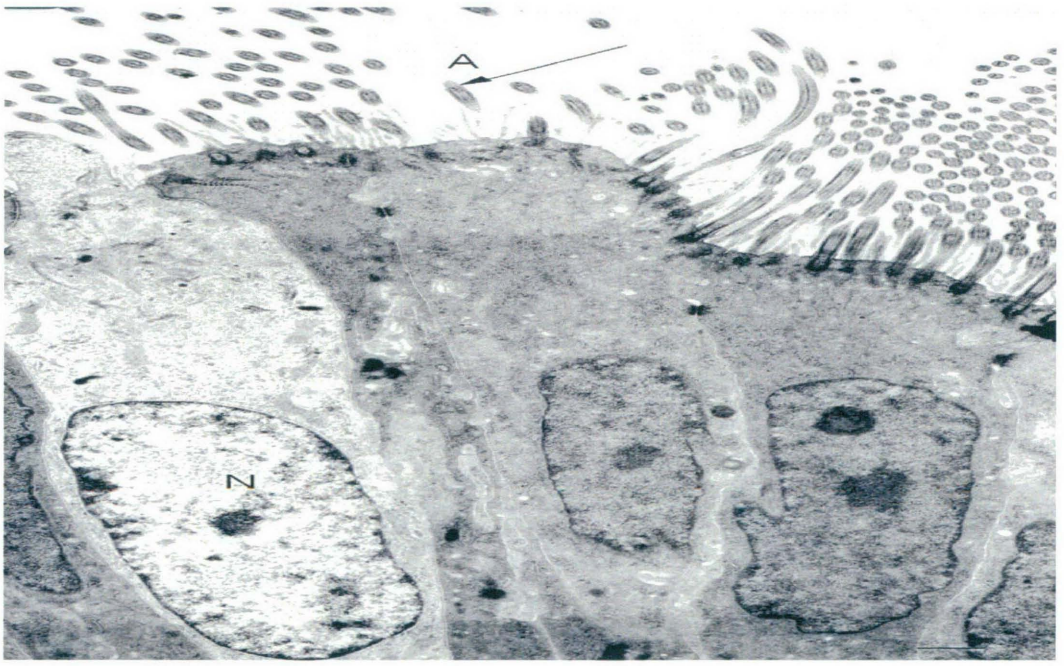


Plate7.5: Transmission electron micrograph of mid infundibulum of control hen. x 6000. Scale bar represents 1µm (hard shelled egg in shell gland). Note normal cilia (Arrow A) and nucleus (N)

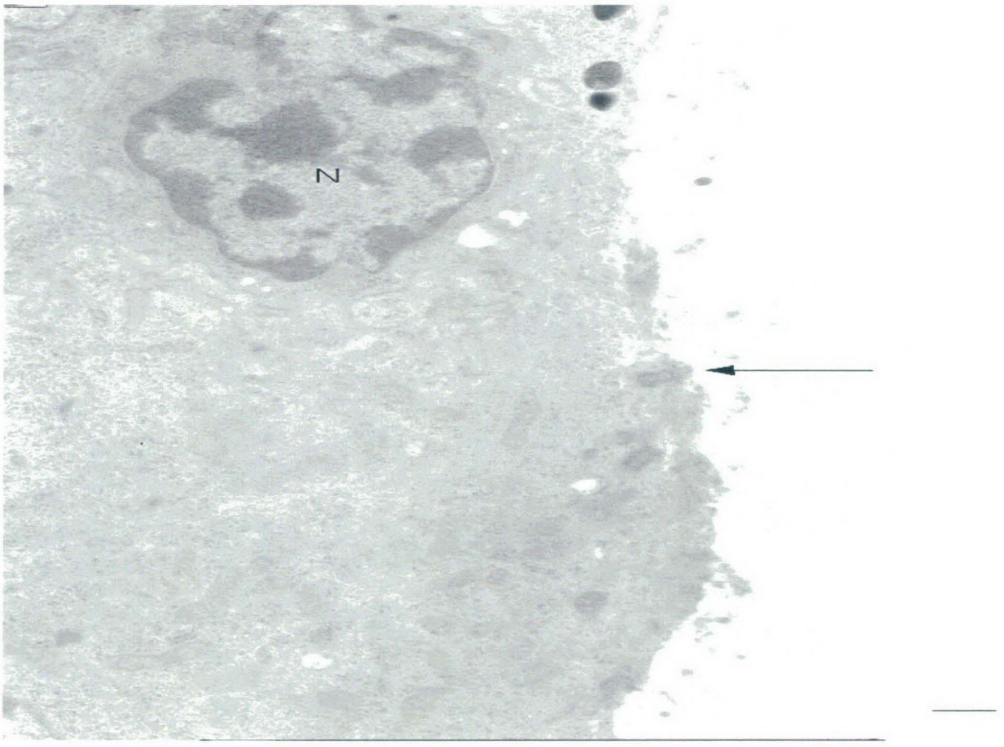


Plate7.6: Transmission electron micrograph of mid infundibulum of T-strain infected hen at 10 days p.i. x 6000. Scale bar represents 1 µm (hard shelled egg in shell gland). Note the loss of cilia (arrow), and nucleus (N)

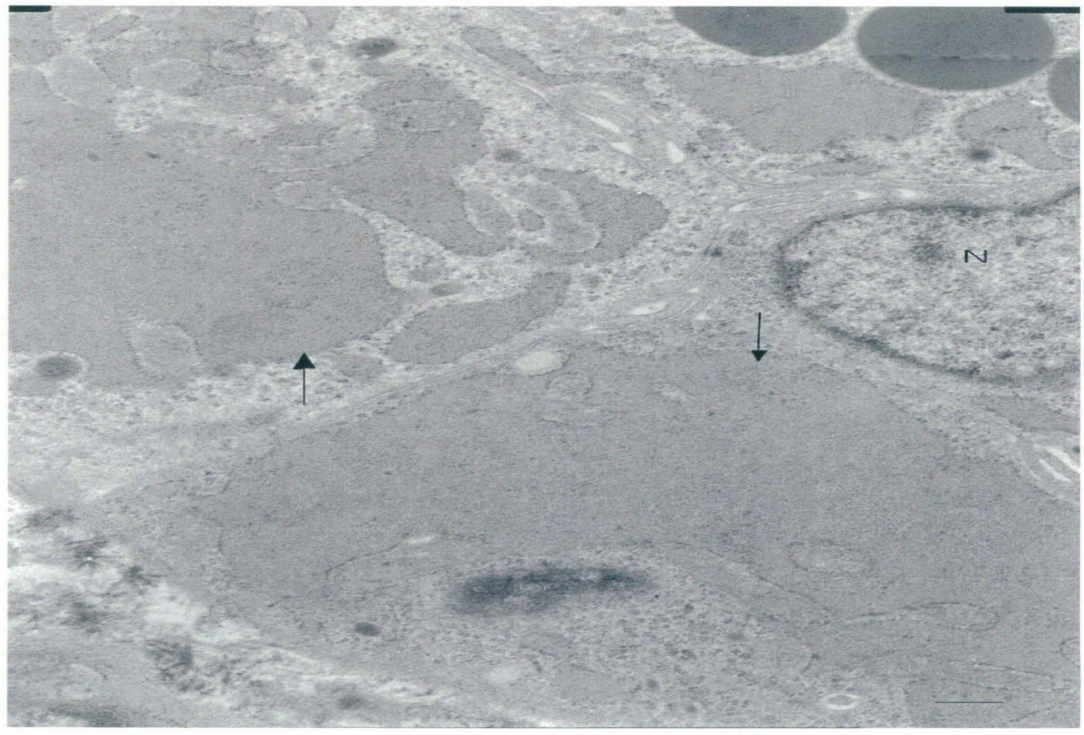


Plate7.7: Transmission electron micrograph of glandular cell of infundibulum of T strain infected hen at 30 days p.i. Note the increased RER deposits (arrow) and nucleus (N).x 8000. Scale bar represents 1 µm (no egg in oviduct)

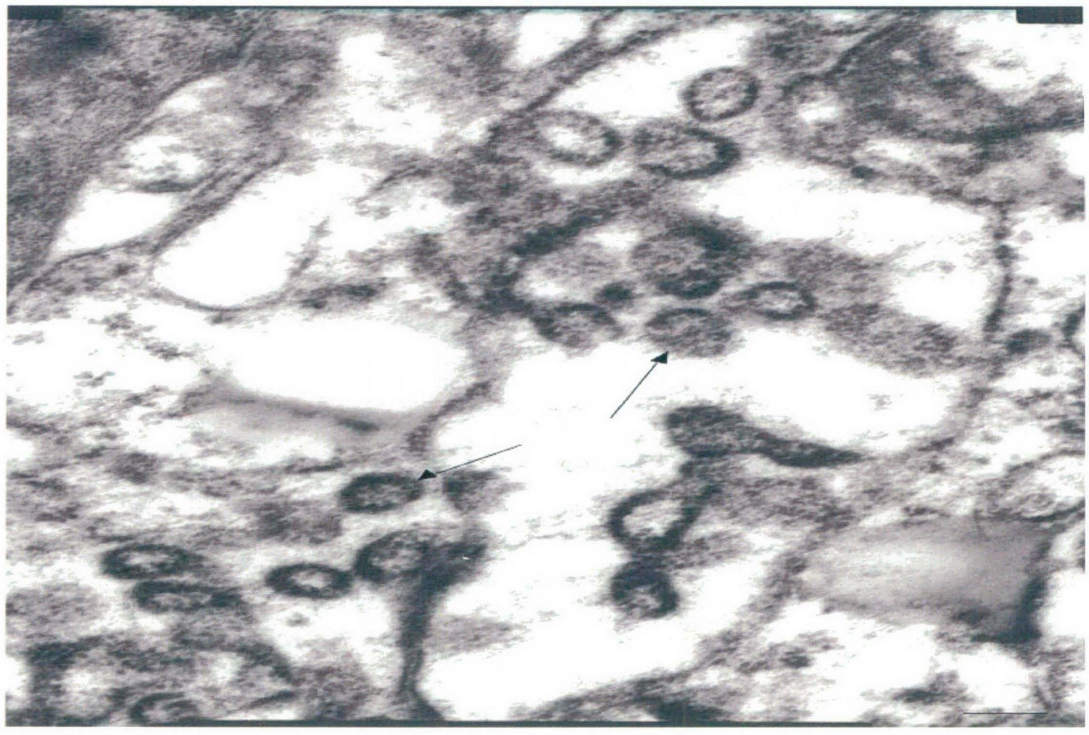


Plate 7.8: Virus particles in dilated cisternae of granular cell of infundibulum (arrow) of T-strain infected hen at 10 days p.i. x 30,000. Scale bar represents 200 nm (no egg in oviduct)

7.3.3. Changes in infected cells of magnum

Histology, scanning and transmission electron microscopic appearance of the magnum at different egg positions in the oviduct of control hens was in accordance with Bakst and Howarth (1975) and Wyburn *et al.*, (1970). (Plates, 7.9; 7.11; 7.13; 7.17)

Most of the histopathological findings were similar to our preliminary study (as described in chapter 3) except that tubular gland dilatation in the magnum of the N1/88 infected group was not recorded in this study. Tubular gland dilatation was mild in the hens from the T-infected group at 10 days p.i. The hens out of lay in the T-infected group showed intense gland dilatation (Plate 7.10), cilia loss from large areas and infiltration of lymphoid nodules. There was patchy loss of mucopolysaccharides and cilia in N1/88-infected hens, and complete loss of mucopolysaccharides from the surface epithelium of one T strain infected hen on day 16 p.i. (Plate 7.12).

In the magnum, there was no statistically significant main effect of IBV strain but there were significant main effects of days p.i. for two out of three lesions scored (Table 7.2). Cellular changes were observed in the magnum of both T and N1/88-infected hens. In T-strain infected hens, scanning electron microscopy revealed loss of cilia from large areas of the surface epithelium of the magnum of one out of two hens killed on days 10 and 12 p.i. However, cilia loss was patchy in the other two hens at 10 and 12 days p.i. and also in one hen on day 14 p.i. Cilia loss was also evident in both the hens killed at 30 days p.i. (Plate 7.14; 7.18). The loss of cilia was in patches in the magnum of N1/88-infected birds. (Plate 7.16). The microvilli of the surface epithelium in both the infected groups remained intact (Plate 7.14)

Under transmission electron microscopy, on day 10 p.i., the changes in infected cells were characterised by cellular swelling and reduced density of the cytoplasmic matrix. Endocytic vesicles with coated pits were observed in some granular cells of the magnum of two hens from the T-infected group and one hen from the N1/88-infected group on day 10 p.i. (Plate 7.19). In virus infected cells of the magnum, the nuclei in the granular cells were more conspicuous and granules were scanty or absent regardless of the egg position. The virus particles were observed mostly in both of the

T-infected hens killed on day 10 p.i. and one hen on day 12 p.i. In the N1/88-infected group, virus particles were noted in the magnum of one hen killed on 10 and 12 days p.i., but not in the hens killed on day 14 p.i. Virus particles were also detected in the magnum of both the hens killed on day 30 p.i. (Plate 7.20). The mitochondria in ciliated and granular cells were scattered throughout the cell and appeared either swollen or degenerated (Plate 7.21). In some of these cells, the Golgi complex was non-functional (Plate 7.22).

In virus infected cells of the magnum, the nuclei in the granular cells were more conspicuous and granules were scanty or absent regardless of the egg position. Virus particles were found mostly in dilated Golgi saccules of granular cells and cells of tubular gland type A. In some of these cells, the Golgi complex was without Golgi vesicles (Fig. 13). In cells of the surface epithelium and dilated tubular gland type A, the cisternae of RER were dilated with free polyribosomes in the cytoplasmic matrix. The budding of virus particles was observed in RER spaces. The type A glandular cells were hugely dilated with necrotic cells in the lumen.

In type A tubular glands, there were a few electron dense granules near the surface of cells in the supranuclear position. Type C tubular glands were not recorded in one T-strain infected hen on the 12^{th} day p.i. nor in the hens killed on day 30 p.i. There were numerous cytoplasmic vesicles of varying size and virus particles were occasionally noted in these cytoplasmic vesicles. The microvilli of some of the dilated glands were disrupted. Type B tubular glands of the magnum were occasionally dilated only in out-of-lay T-challenged hens sacrificed on day 30 p.i. Phagocytic vacuoles containing destroyed cellular organelles were found in one hen from the T-infected group on day 14 p.i. but virus particles were not recorded in them. The cellular organelles were mostly normal in both the infected groups after the 16^{th} day p.i. In N1/88-infected birds, all the above cytopathology was observed in one out of two hens on days 10 and 12 p.i. However, dilatation of tubular glands was not observed in the N1/88 group. In the magnum of the hen with an atrophied oviduct, the Golgi complex in granular cells was without Golgi vesicles and some secretory granules appeared near the surface of the cell. In both the hens from the T-infected group which were out of lay when sacrificed on day 30 p.i., there was degeneration of plasma cells with extreme dilatation of the RER and a large number of free ribosomes. A small number

of vacuoles in plasma cells contained membrane residues which were presumably derived from mitochondrial degeneration (7.23). There was intense infiltration of lymphocytes in the magnum of these hens.

There was no severe pathology in any of the magnum tissue of other infected hens of both groups sacrificed between 16 and 24 days p.i. although lymphoid nodules were observed in the interglandular space and muscularis area of the magnum in all hens from the T-infected group on days 16, 20 and 24 p.i. Also, in the N1/88 group, lymphoid nodules were recorded in the magnum of one hen on days 18, 22 and 24 p.i. Only a small number of secretory granules were found in granular cells of the magnum of T-infected hens at all egg positions from 16 to 24 days p.i.

No cellular pathology was observed in the magnum of any of the hens from the control group.

Table 7.2: Comparative histopathology of magnum of 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			
Lesions in Magnum	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	G	D.
Loss of Mucopoly-sacchrides	0	0	0	0	0	0	0	0	2	1	1.5	1	1.5	0.5	0	0	0	0	0	0	0	0	0	0	NS	0.0069
Gland dilatation	0	0	0	00	0	0	0	0	1.5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	NS	NS
Lymphocyte infiltration							0		1.5	1	1.5	1.5	1	0.5	0.5	0.5	1	1	0	0	0.5	0	0	1	NS	0.0036

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

Mean of lesion score from two hens, G- Group, D- days p.i.

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

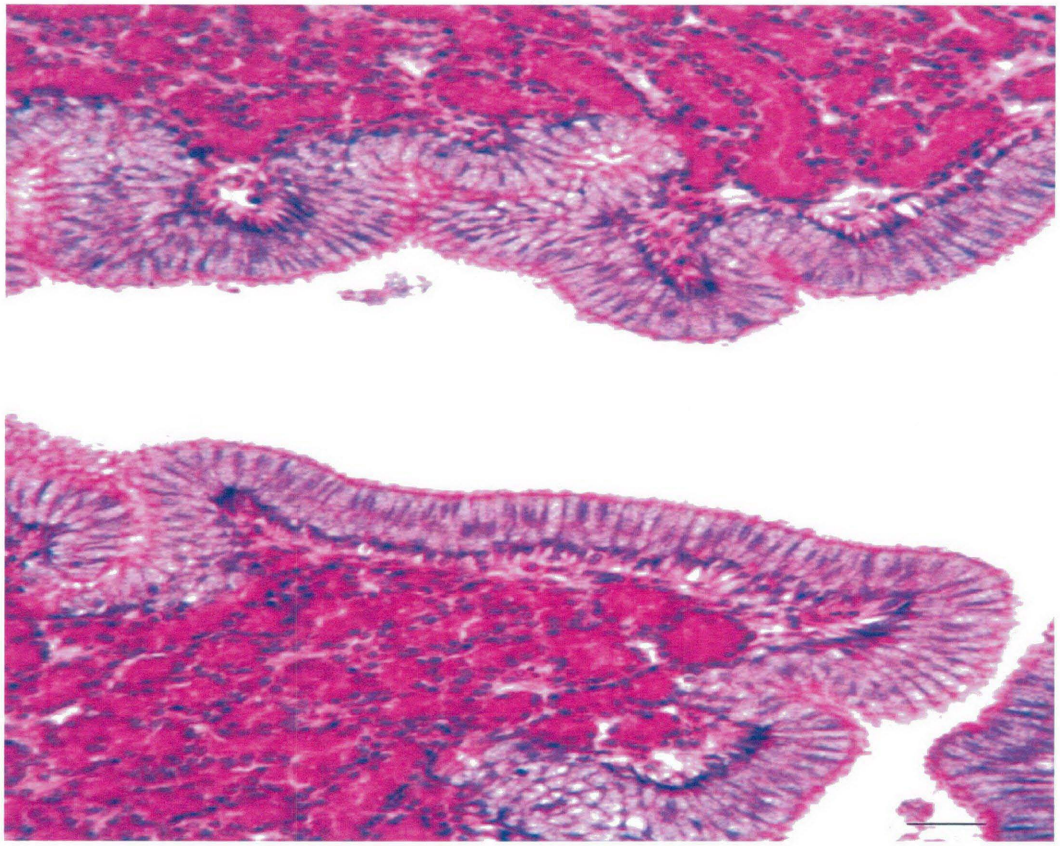


Plate 7.9: Magnum of hen from control group. H &E x 400. Scale bar represents 25 µm (hard shelled egg in shell gland)

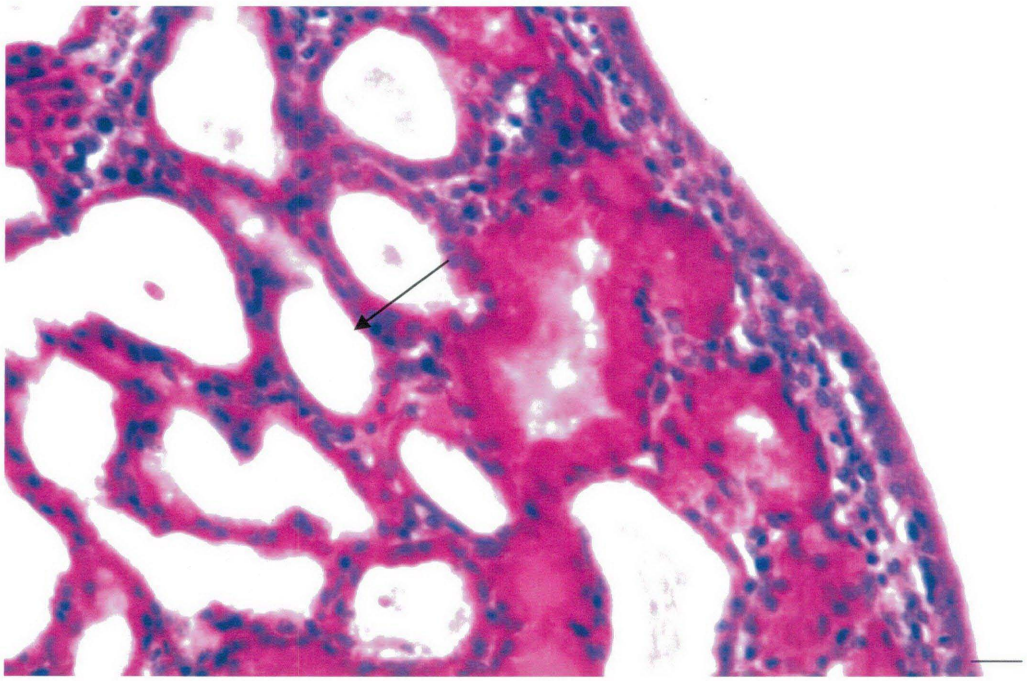


Plate 7.10: Magnum of hen from T strain-infected group at 30 days p.i. H &E x 400. Dilated tubular glands (Arrow) Scale bar represents 25 µm (no egg in shell gland)

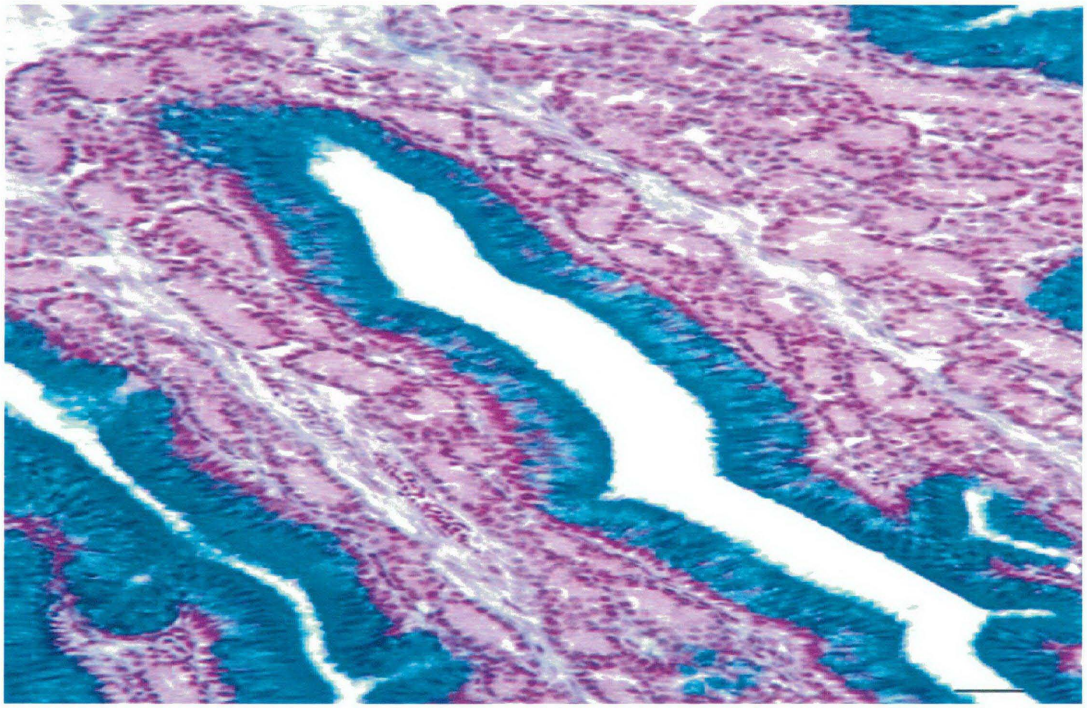


Plate 7.11: Magnum of hen from control group. Alcian blue x 400. Scale bar represents 25 µm (hard shelled egg in shell gland)

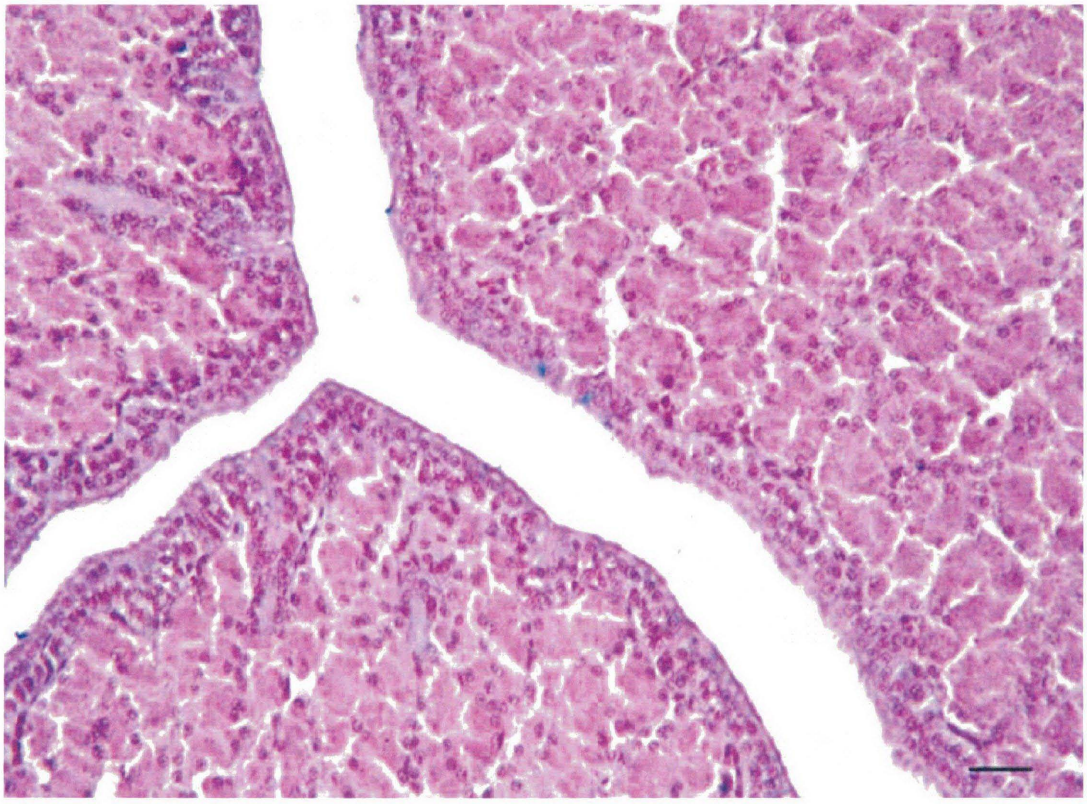


Plate 7.12: Magnum of hen from T strain infected group at 16 days p.i. Alcian blue x 400. Note the loss of blue colour from surface epithelium. Scale bar represents 25 µm (hard shelled egg in shell gland)

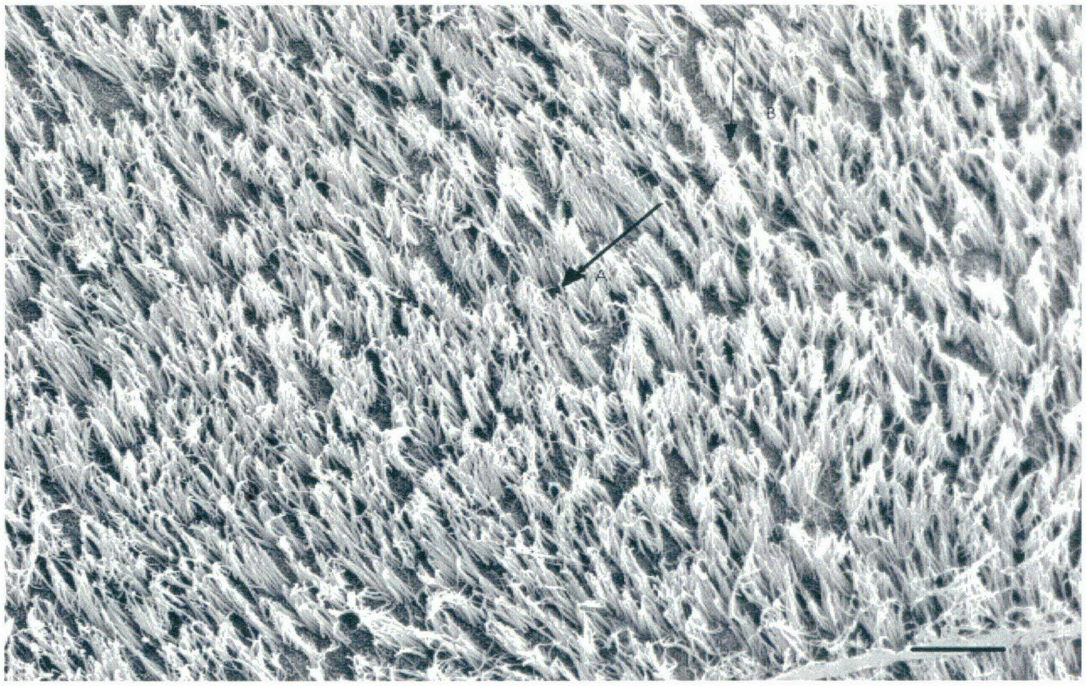


Plate 7.13: Scanning electron micrograph of magnum of control hen. x 900. Scale bar represents 20 µm. (No egg in oviduct). Note ciliated (arrow A) and non ciliated (arrow B) cells

Plate 7.14: Scanning electron micrograph of magnum of T-strain infected hen at 30 days p.i. Note loss of cilia with intact microvilli x 900. Scale bar represents 20 µm. (no egg in oviduct). Note the loss of cilia from surface epithelium

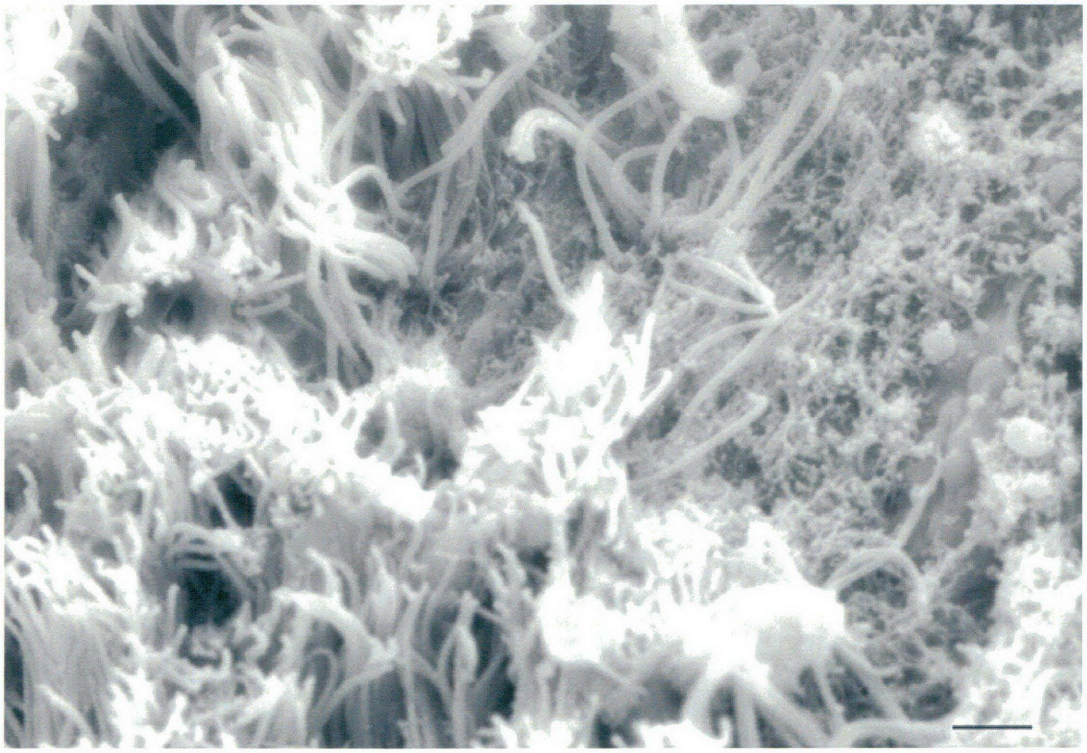


Plate 7.15: Scanning electron micrograph of magnum of control hen. x 6000. Scale bar represents 2 µm. (no egg in oviduct).

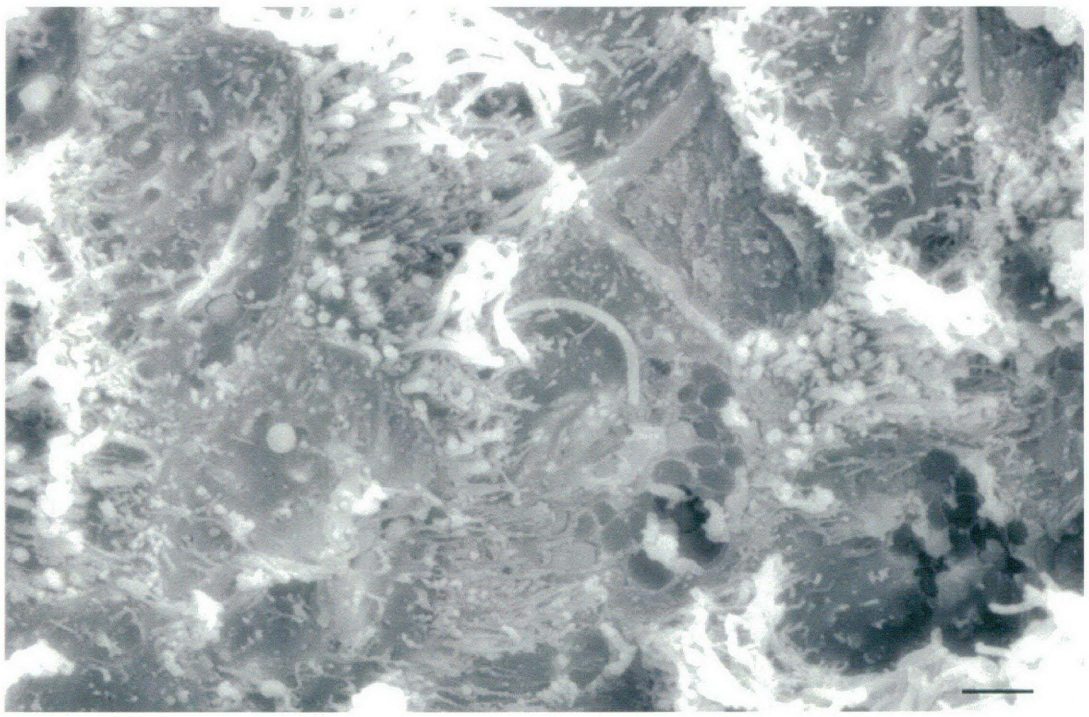


Plate 7.16: Scanning electron micrograph of magnum of a hen infected with N1/88 strain of IBV at 12 days p.i. x 6000. Scale bar represents 2 µm. (no egg in oviduct). Note the patchy loss of cilia from ciliated cells

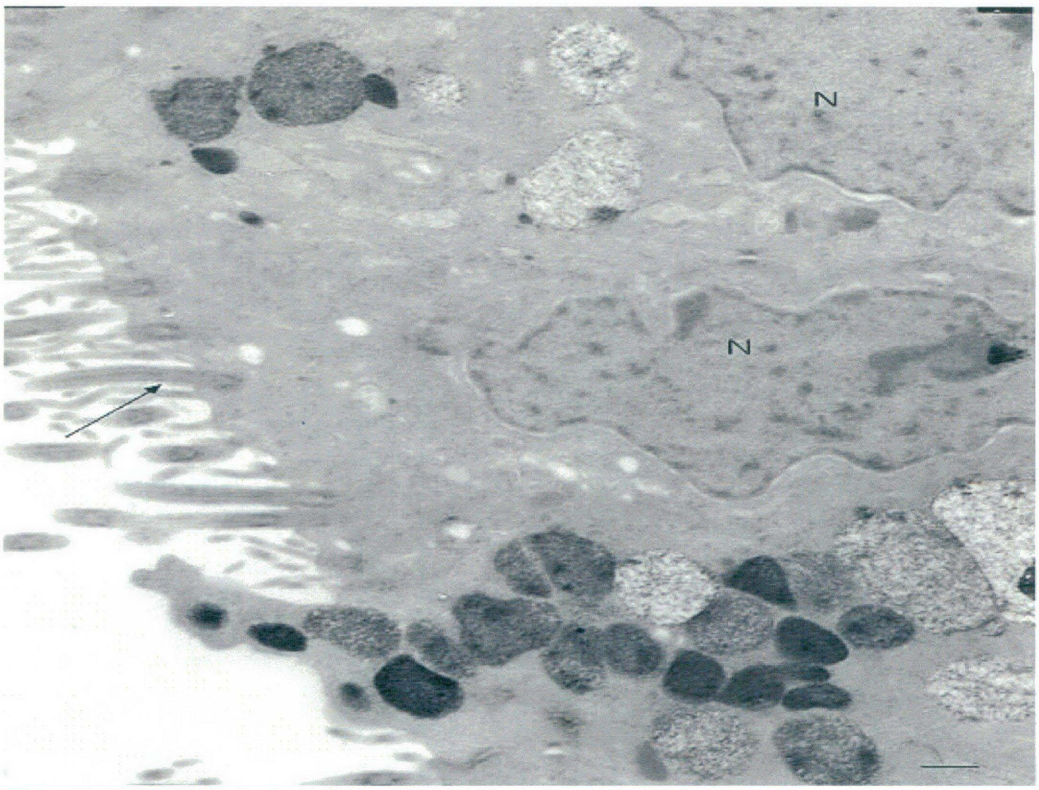


Plate 7.17: Transmission electron micrograph of magnum of control hen. x 8000. Scale bar represents 1 µm. (no egg in oviduct). Note normal cilia (arrow), nucleus (N)

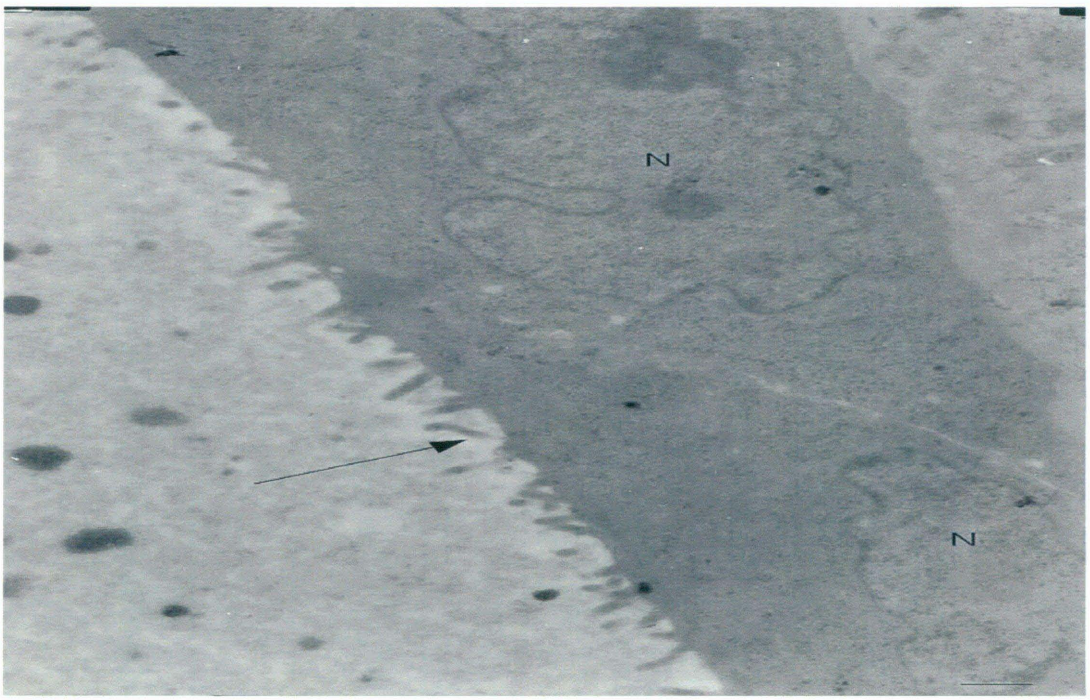


Plate 7.18: Transmission electron micrograph of magnum of T-strain infected hen at 10 days p.i. x 8000. Scale bar represents 1 µm. (no egg in oviduct). Note the loss of cilia from surface epithelium and change in morphology of surface epithelium (arrow) but intact microvilli. Nucleus (N)

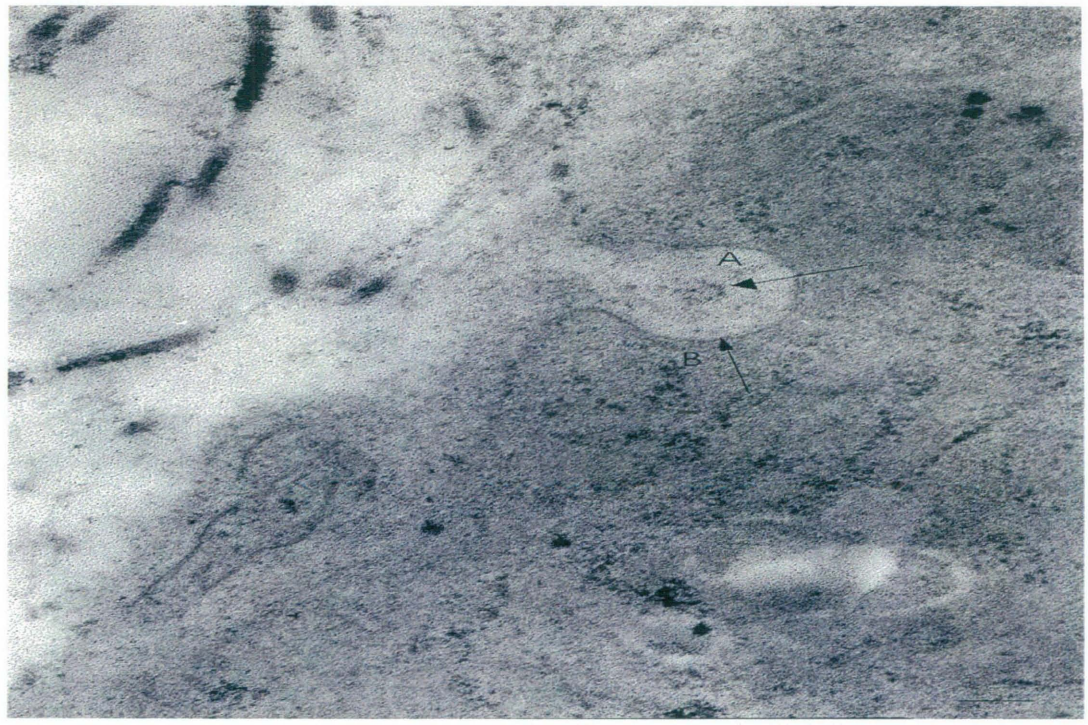


Plate 7.19: Endocytosis of virus particle in granular cell of magnum of N1/88 infected hen at 10 days p.i. x 25,000. Scale bar represents 200 nm (hard shelled egg in shell gland). Virus particle (arrow A), endocytic vesicle (arrow B)

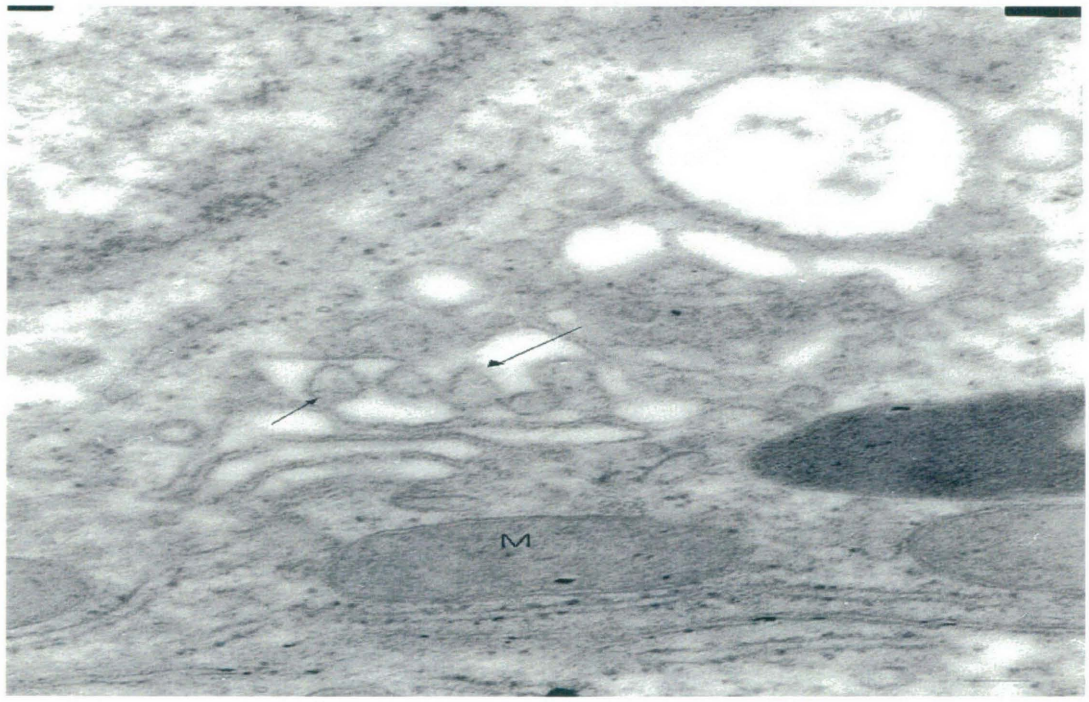


Plate 7.20: Virus particles in dilated Golgi saccules of granular cell of magnum (arrows) of N1/88 infected hen at 10 days p.i. x 30,000. Scale bar represents 200 nm (egg in upper half of magnum)

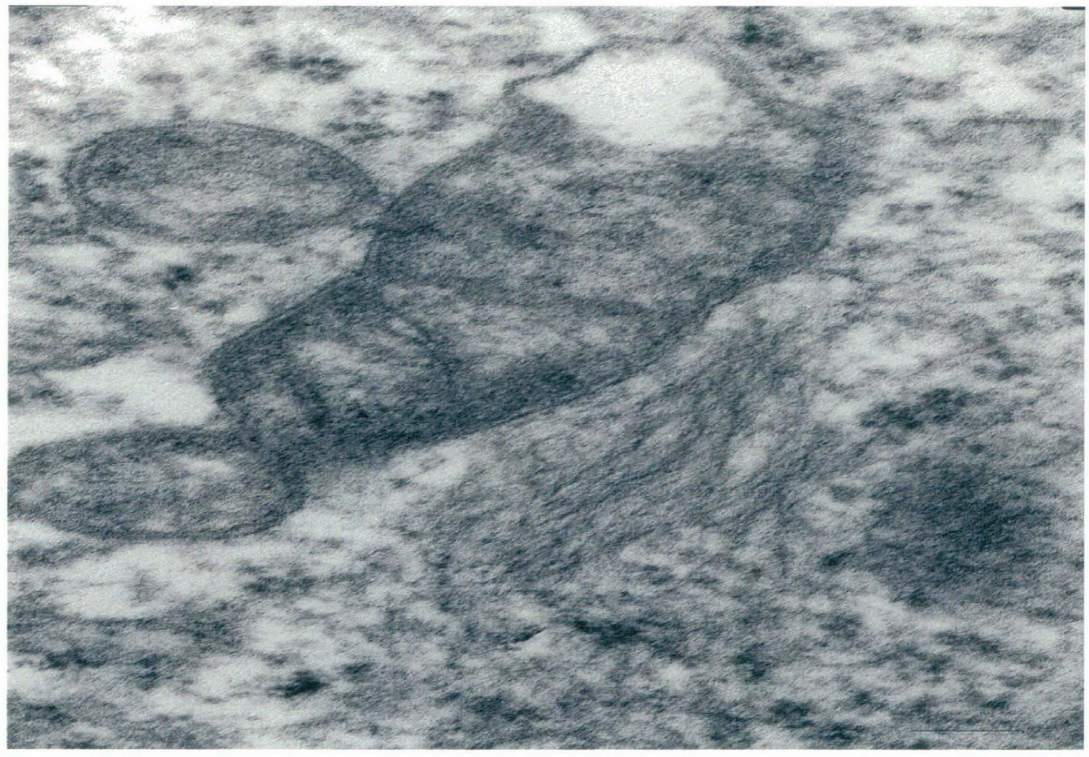


Plate 7.21: Degeneration of mitochondria in cell of tubular gland type A of magnum of T-strain infected hen at 14 days p.i. x 40,000. Scale bar represents 200 nm (hard shelled egg in shell gland).

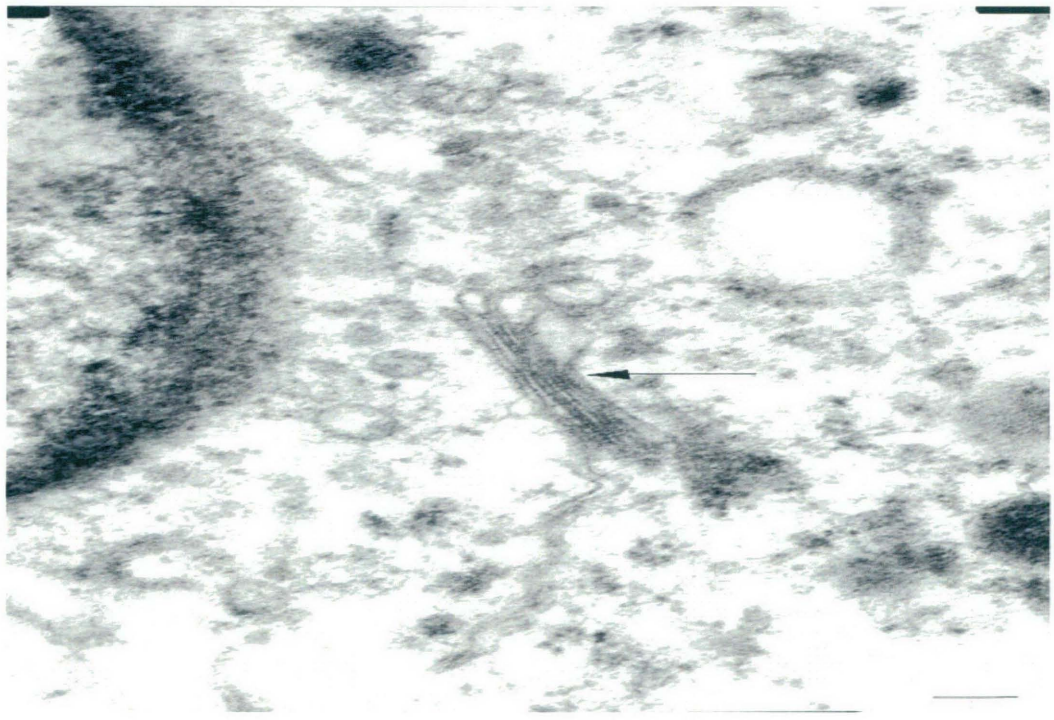


Plate 7.22: Inactive Golgi complex of granular cell (arrow) of T-strain infected hen at 30 days p.i. x 25,000. Scale bar represents 200 nm. (no egg in oviduct)

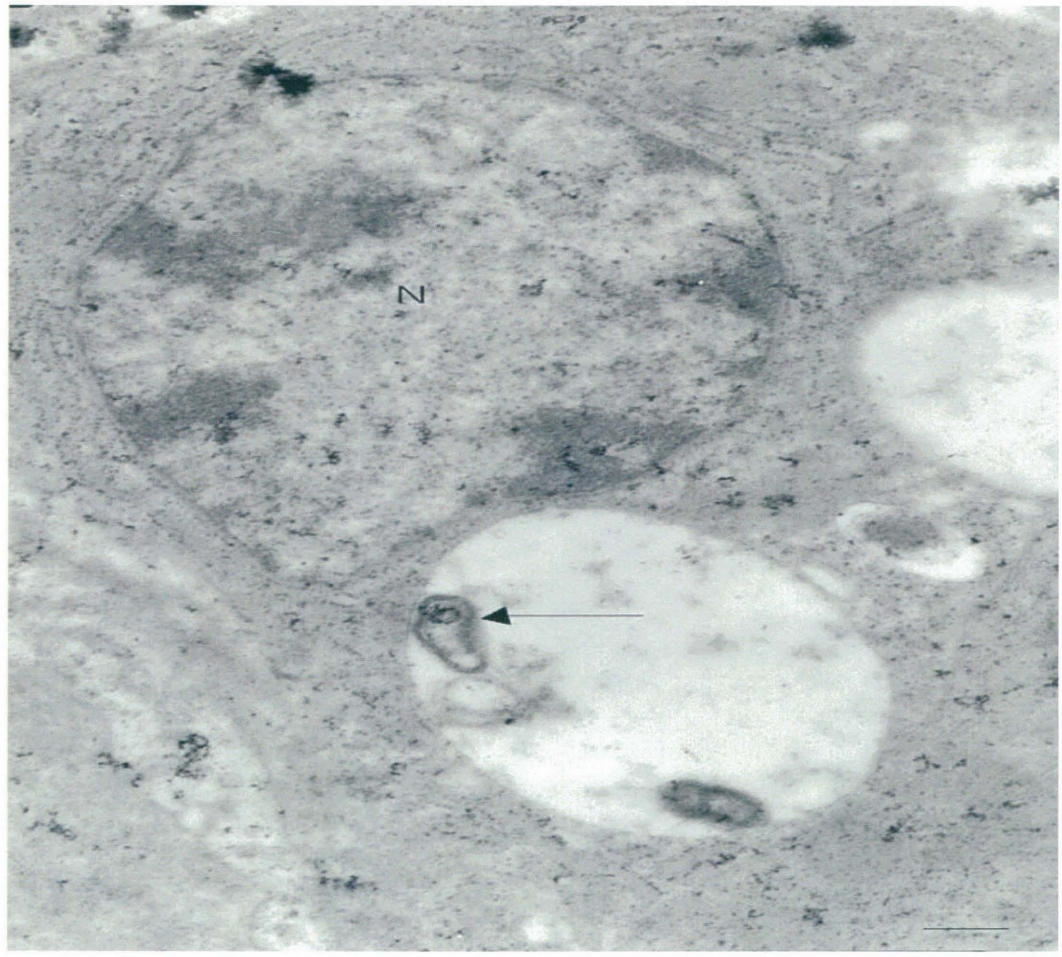


Plate 7.23: Vacuole containing membrane residues possibly derived from mitochondrial degeneration (arrow) in plasma cell of T strain infected hen in magnum at 30 days p.i. x 10,000. Scale bar represents 500 nm. (no egg in oviduct). Nucleus (N)

7.4 Discussion

Virus particles were found in both the infundibulum and magnum between the 10th and 12th days p.i. but not later than this until the 24th day p.i. However, virus particles were found in the hens which were out of lay and sacrificed on the 30th day p.i. Earlier, Jones and Jordan (1971) found the M41 Massachusetts strain of IBV in the magnum and shell forming regions in the oviduct on 6th day p.i. Jones and Jordan (1972) also isolated virus from the oviduct (regions not specified) of chicks infected at day old.

In the present study, we found that most of the hens recovered from infection and resumed laying but some hens stopped producing eggs and this was associated with presence of virus particles in the oviduct. Such hens, despite being of normal and healthy appearance, can act as persistent virus shedders. These findings support the view of McMartin (1968b) that response of individual hens during IBV infection varies greatly. In the present study, the effect of both the strains of IBV on the magnum was more intense than for the infundibulum, although virus particles and mild cellular pathology were observed in the infundibulum of T-infected hens. The marked increase in RER content in surface gland cells of the infundibulum and mid-magnum irrespective of egg position indicates the high susceptibility of these regions of the oviduct to virus infection.

Granular cells in the surface epithelium of the magnum secrete ovomucin while the type A tubular gland cells contribute ovalbumin which is the most common protein of egg white (Wyburn *et al.*, 1970). The severe pathology of type A tubular gland cells and absence of secretory granules from granular cells may result in thin and watery albumen, the condition known as "watery whites". Initially, this condition was studied using light microscopy by Butler *et al.* (1972), who found loss of mucopolysaccharides from surface epithelial cells and hyperplasia of tubular glands in the magnum. Tubular glands A and C are different phases of secretory activity of one cell type. During egg formation, type C glands appear following the release of secretory granules from type A gland cells (Wyburn *et al.*, 1970). The small number of type C tubular glands in the magnum of hens on the 10th and 12th days p.i., and their absence in hens which have stopped laying, indicates a lack of secretory activity

of type A gland cells. Moreover, between the 10^{th} and 14^{th} days p.i. and in non laying hens, virus particles were mostly detected in the Golgi complex and dilated cisternae of RER. The RER and Golgi complex together play an important role in the secretion of secretory granules in type A gland cells. (Wyburn *et al*, 1970). The former accumulates synthesised proteins after protein synthesis and the latter assembles, packs and sequesters the synthesised proteins into granules for secretion. We observed a similar sequence in granular cells of the magnum and infundibulum. The presence of IBV particles in those cellular organelles of granular and type A tubular gland cells could disturb the normal physiological rhythm of their function and this may stop these cellular organelles from making their contribution towards secretion either temporarily or permanently in some hens. Also, very few secretory granules in the granular cells of the magnum at all egg positions could be responsible for a continuous drop in albumen height and Haugh units in the T-strain infected group (Chapter 9, section 9.3.2).

In this study, there was no prolonged severe tubular gland dilatation in the magnum of N1/88 infected hens as was found in our preliminary study in Leghorn hens (Chapter 3) which could be due to breed and age differences. Cumming and Chubb (1988) observed that light breeds are more susceptible to IBV nephritis than heavy breeds. In the current experiment, out of 50 infected hens in each challenge group, only three hens, one from N1/88 with a non-patent oviduct and two from the T-infected group with patent oviducts, were out of lay. On the other hand, Crinion *et al.* (1971b), found abnormal oviducts in laying hens after infecting them at young age, which is in accordance with the view of McMartin and Macleod (1972) that the oviduct of younger chickens is more likely to be affected by IBV as compared to older chickens.

IBV has been isolated from vitelline membrane (Cook 1971) of eggs laid by IBV infected hens. Replication of IBV in the infundibulum and magnum suggests the need for isolation of virus from their secretions, such as egg albumen. In one of the hens which was out of production from the N1/88 group, both the ovaries and oviduct were atrophied which explains the reason for cessation of egg production. Regressed oviducts and ovaries during IBV infection were recorded also by Sevoian and Levine (1957). An inactive ovary with a normal oviduct was also recorded by McMartin and Macleod (1972) during natural infection with IBV. However, in the hens from the T-

infected group which were out of lay, the ovaries were still functional and yolk material was found in the oviduct, but it would still be difficult to conclude the reason behind the cessation of egg production. Studies regarding peristaltic movements of oviduct and hormonal levels during IBV infection would probably help to understand this process. However, the disturbance in the physiological rhythm of RER and Golgi complexes as well as inactive Golgi complex could be one of the contributory factors. The loss of cilia from the infundibulum and magnum could enhance the susceptibility of epithelial cells to secondary bacterial infection and may prolong the infection. However, the role of cilia during egg production and movement in the oviduct is still speculative and further investigations are essential in this context. Ciliary beating in the anterior segment of the infundibulum (ampulla) is partially responsible for movement of the ovum away from ovary (abovarian direction) (Fujii *et al.*, 1981). Also, cilia play a vital role in sperm transport (Johnson, 2000) so the loss of cilia could affect the ovum and sperm transport which may affect the fertility of breeder hens. Cellular changes in the chalaziferous region may interrupt the formation of the chalaziferous layer (layer consisting of pair of twisted strands which helps to hold the yolk in the centre of egg) and could be responsible for the tendency for yolk to separate from the albumen when eggs are broken out, as was observed in this study. However, cytochemical studies are essential to clarify this. Ultra structural patterns of plasma cell degeneration seen in the magnum may be related to disturbance of immunoglobulin secretion mechanisms and such degeneration was observed in Harderian gland of chickens by (Zicca *et al.*, 1982).

Overall, the T-strain of IBV was more pathogenic as compared to the N1/88 strain for the infundibulum and magnum of the fully functional oviduct, although N1/88 strain can affect the magnum to a moderate extent. This finding explains the uterotropism of Australian strains of IBV for the fully functional oviduct, irrespective of their pathogenic type or genetic group.

In the present study, although albumen heights were low in the T-infected group up to 30 days post-infection, we could not observe virus particles in the infundibulum or magnum of all of hens killed during that time. Also, there was no severe pathology except for a small number of granules in the granular cells of the magnum and lymphocyte infiltration. Hence further studies regarding virus isolation from these

tissues would be instructive and such studies are described in detail in chapter 11. Also, the cause of poor albumen quality may lie in the histopathology associated with the lower region of the magnum which was not studied during this experiment. Further research is required to describe the effects of the IBV on the lower magnum.

Chapter 8

Ultra structural observations on effects of infectious bronchitis virus in egg shell-forming regions of the oviduct of the commercial laying hen

8.1 Introduction

The egg industry all around the world loses millions of dollars a year as the result of egg quality problems and a disease such as IBV can contribute to these losses. It is reported in the literature that IBV is responsible for deterioration of egg shell quality (Sevoian and Levine, 1957; Cook, 1971; Munner *et al.*, 1986), although the effects of the Australian strains of IBV on the egg shell-forming regions of the oviduct of laying hens have not been studied or documented previously. Also, the causes of pale coloured egg shells and thin or soft egg shells during IBV infection are not yet clear. Information regarding cytopathology in the shell-forming regions of the oviduct of hens that had stopped laying during IBV infection, under experimental and field conditions, has not been reported previously. This study extends the earlier finding regarding ultrastructural cytopathology in the infundibulum and magnum of the fully-functional oviduct (Chapter 7). In the present study, the cellular pathogenesis of two strains of IBV was studied in the shell-forming regions, isthmus, tubular shell gland (TSG) and shell gland pouch (SGP) of the fully-functional oviduct of Isa Brown hens.

8.2 Materials and Methods

The details regarding the experiment have been described in chapter 5 (section 5.2). The shell-forming regions of the oviduct, isthmus, tubular shell gland and shell gland pouch, were collected at the time of sacrifice and processed for histology and electron microscopy as described in chapter 2 (Section 2.4). The histopathological lesions were Scored as described in chapter 2 (section 2.3.6).

8.3 Results

8.3.1 Changes in Isthmus

All parts of the isthmus of control hens appeared normal with no pathology being recorded.

No prominent microscopic lesions were recorded in the isthmus of the N1/88-infected group except for moderate infiltration of inflammatory cells in the submucosa and muscularis area in the hen with an atrophied oviduct on the 24th day p.i.

There were statistically significant main effects of IBV strain on two out of three lesions investigated but there was no significant main effect of days p.i. on any of the lesions scored (Table 8.1). Under the light microscope, mild loss of cilia was observed from days 8 to 12 p.i. in the T-infected group. There was glandular dilatation in one of the T strain infected hens on day 12 p.i. (Plate 8.1). The hens which were out of lay and killed on day 30 p.i. showed extensive infiltration of lymphocytes into the interglandular space (Plate 8.2)

Scanning micrographs of the isthmus were similar to those published by Bakst and Howarth (1975) (Plate 8.3).

In T-infected hens, there was loss of cilia in one hen killed on each of days 10 and 12 p.i (Plate 8.4). One hen killed on day 12 p.i. showed occasional tubular gland dilatation. Virus particles were observed in the isthmus of this hen only. Occasional lymphocyte infiltration was observed in the interglandular spaces in the isthmus of T strain-infected hens at 16, 20 and 24 days p.i.

Severe pathology was recorded in the isthmus of both the hens from the T-infected group which were out of lay when sacrificed on day 30 p.i., and large lymphoid nodules were seen in the muscularis area of the isthmus. There was loss of cilia from ciliated cells, phagocytic vacuoles, dilated cisternae and degeneration of mitochondria in surface and glandular epithelia. Virus particles were observed mostly in dilated cisternae (Plate 8.5) and only occasionally in virus-bearing and dilated rough endoplasmic reticulum (RER) (Figure 2). Virus particles were occasionally recorded

in ciliated cells. Some mitochondria in mitochondrial cells appeared to have degenerated. Type 2 gland cells were common in all parts of the isthmus and some of the glands in the isthmus of these hens appeared dilated. Necrotic cells, along with an elongated mass of secretion, were observed in the lumen of some dilated gland cells.

In T-infected hens, there was patchy loss of cilia in two hens on the 10^{th} and 12^{th} days p.i.. However, there was no glandular dilatation. Lymphoid nodules were observed in the submucosa in the isthmus of hens at 16, 20 and 24 days p.i. Severe pathology was recorded in the isthmus of hens from the T-infected group which had stopped laying. There was extensive infiltration of lymphocytes into the submucosa and large lymphoid nodules in the muscularis area. In granular cells, RER deposits were increased and some secretory granules were observed at the apex of individual cells. Virus particles were recorded in dilated cisternae of RER, dilated Golgi vesicles and cytoplasmic vesicles in surface and glandular epithelia. The scanning micrographs revealed patchy cilia loss from the surface Under the transmission electron microscope, virus particles were occasionally recorded in ciliated cells. Mitochondria appeared enlarged in both ciliated and mitochondrial cells. Type 2 gland cells were evident in all parts of the isthmus. The glands in the isthmus appeared dilated. Necrotic cells, along with an elongated mass of secretion, were observed in the lumen of some dilated gland cells. Phagocytic vacuoles were also observed in glandular cells.

Table 8.1: Lesion score in the Isthmus of hens infected with T and N1/88 strains of IBV

	Days post inoculation																							
	2		4		6		8		10		12		14		16		18		20		22		24		P value	
Lesions	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	G	D.
Cilia loss	0	0	0	0	0	0	1.5	0	1.5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0623	NS
Gland dilatation	0	0	0	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0	0		0	0	NS	NS
Lymphocyte infiltration	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0	1	0	0	0	2	0	0	0	0.5	1	0.0255	NS

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

Mean of lesion score from two hens, G- Group, D- days p.i.

Significance assumed at $p<0.05$. NS - 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

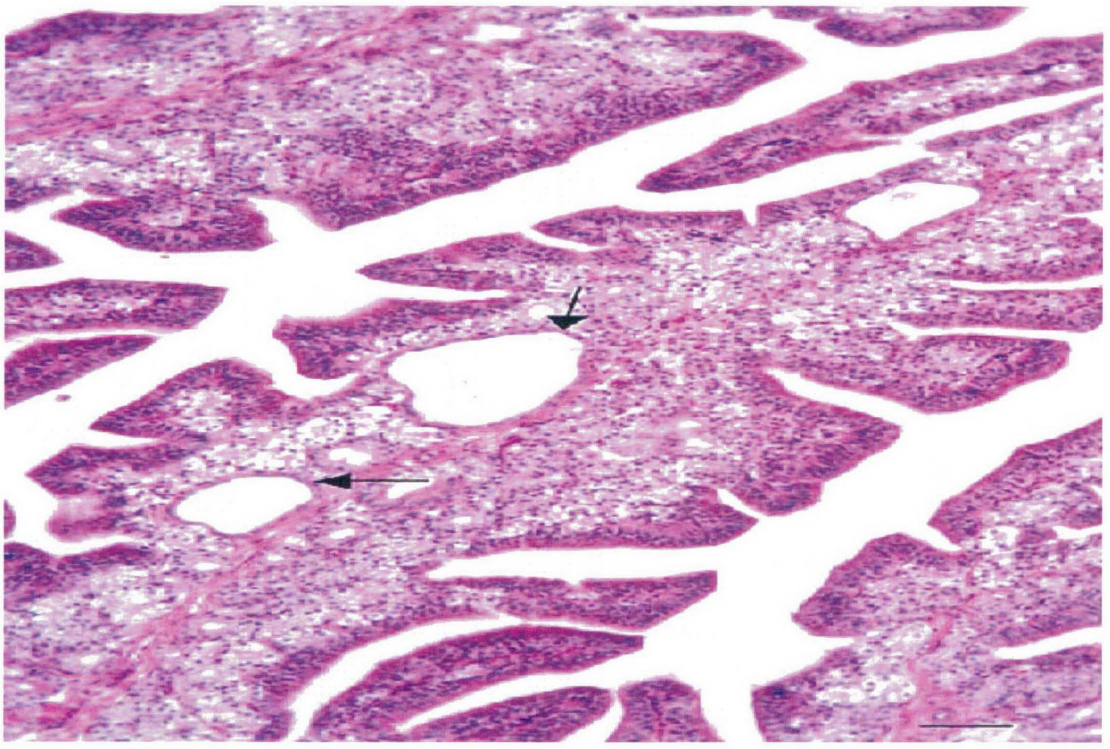


Plate 8.1: Isthmus of T strain infected hen at 12 days p.i. H &E x 200. Note dilated glands with cell debris in the lumen (Arrow) Scale bar represents 50 µm (egg low in magnum)

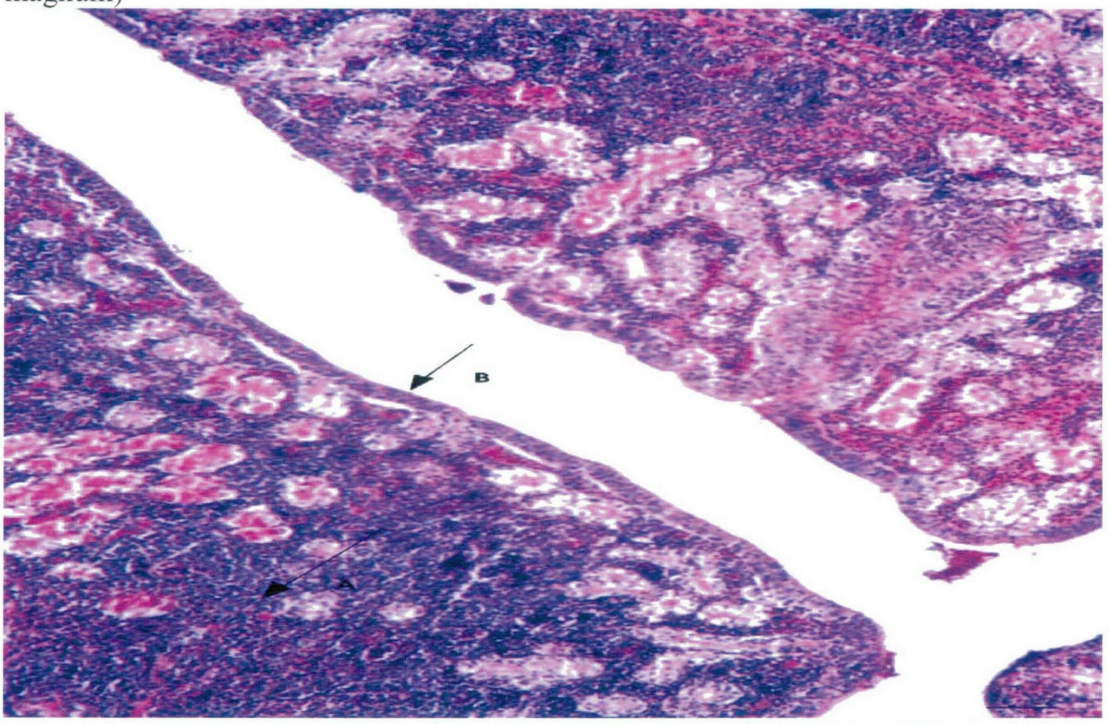


Plate 8.2: Isthmus of T strain infected hen at 30 days p.i. H &E x 200. Scale bar represents 50 µm (no egg in the oviduct). Note the severe lymphocyte infiltration (A) and loss of cilia from surface epithelium (Arrow B)

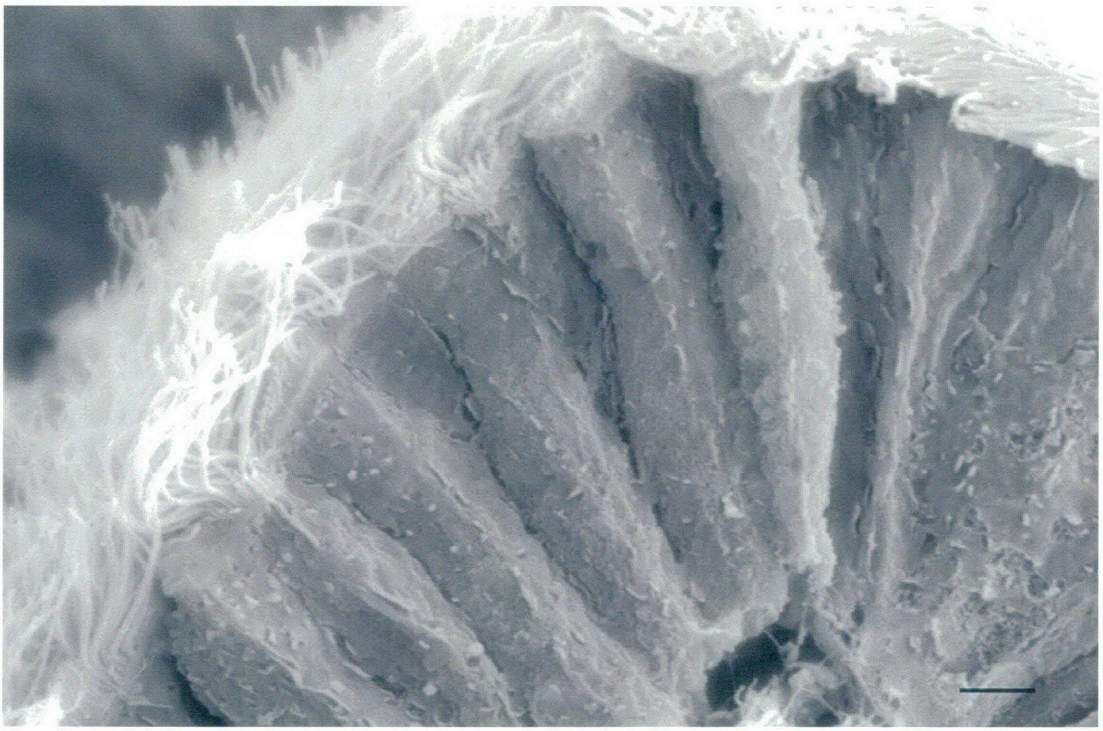


Plate 8.3: Scanning electron micrograph of isthmus of hen from control group x 4000. Scale bar represents 5 µm

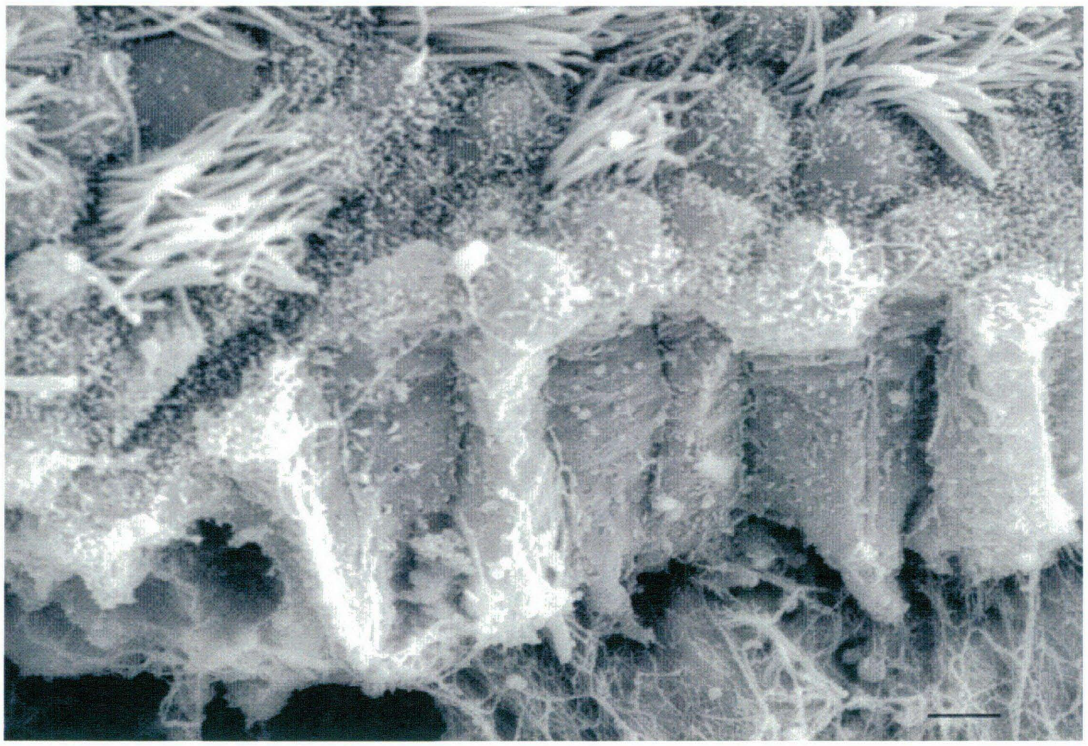


Plate 8.4: Scanning electron micrograph of isthmus of hen from T strain-infected group at 12 days p.i. x 4000. Scale bar represents 5 µm (egg in the mid magnum) Note the loss of cilia from individual cell.

Plate 8.5: Transmission electron micrograph of isthmus of T strain-infected hen at 30 days p.i. Note the virus particles in the cisternae of RER (A). (no egg in the oviduct) X 25000 Scale bar represents 200 nm.

8.3.2 Changes in tubular shell gland (TSG) and shell gland pouch (SGP)

Most of the cytopathologcal changes in the TSG and SGP were similar so the results are presented together. The tubular shell gland, also known as the red region, is the distal part of the isthmus where the process of shell formation is initiated (Solomon, 1975). The histology, scanning and transmission electron microscopic findings regarding TSG and SGP were in accordance with Solomon (1983) (Plates, 8.6; 8.8; 8.10 and 8.12). Detailed information regarding this region has been described in chapter 6

In general, pathological changes were not recorded in the TSG and SGP of hens from the N1/88 group. However, in one hen which had an atrophied oviduct and was killed on day 24 p.i., large lymphoid nodules were recorded in the interglandular space of the TSG and SGP. Virus particles were observed in the gland cell epithelium of the shell gland pouch of this hen.

In T strain-infected hens, there was no cilia loss or glandular dilatation in any of the hens killed. However, in one out of two hens killed on each of 20 and 24 days p.i., lymphoid nodules were recorded in the muscularis area of the TSG and SGP. Severe pathology was observed in the TSG and SGP of hens which were out of production. In hens which had stopped laying, patchy cilia loss (Plate 8.7, 8.9 and 8.11) and increased deposits of RER with virus particles were recorded in ciliated cells. Vacuoloids were not recorded in most of the granular cells of the SGP. although vacuoloids were observed in the SGP of a control hen with no egg in the oviduct. The glands in both the TSG and SGP were occasionally dilated. Mitochondria in non-ciliated cells and glandular epithelial cells appeared swollen and degenerated (Plate 8.13). In some non-ciliated cells, the cristae of the mitochondria were disrupted A few lipid droplets were also recorded in gland cells of the SGP. The apical border of most of the ciliated cells in the SGP and TSG appeared disrupted and all mitochondria had degenerated. Budding of virus particles was observed in dilated RER in both ciliated and non-ciliated cells. The infiltration of plasma cells and lymphocytes into the submucosa was intense. The microvilli of the surface epithelium and glandular epithelium were not affected. All parts of TSG and SGP in control hens appeared normal.

Table 8.2: Lesion score in tubular shell gland (TSG) and shell gland pouch (SGP) of hens infected with T and N1/88 strains of IBV

	Days post inoculation																							
	2		4		6		8		10		12		14		16		18		20		22		24		**P value**	
Lesions	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	G	D
Cilia loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-
Gland dilatation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-
Lymphocyte infiltration	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0.5	0	0	0	0.5	1	NS	NS

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

Mean of lesion score from two hens, G- Group, D- days p.i.

Significance assumed at $p<0.05$. NS - 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

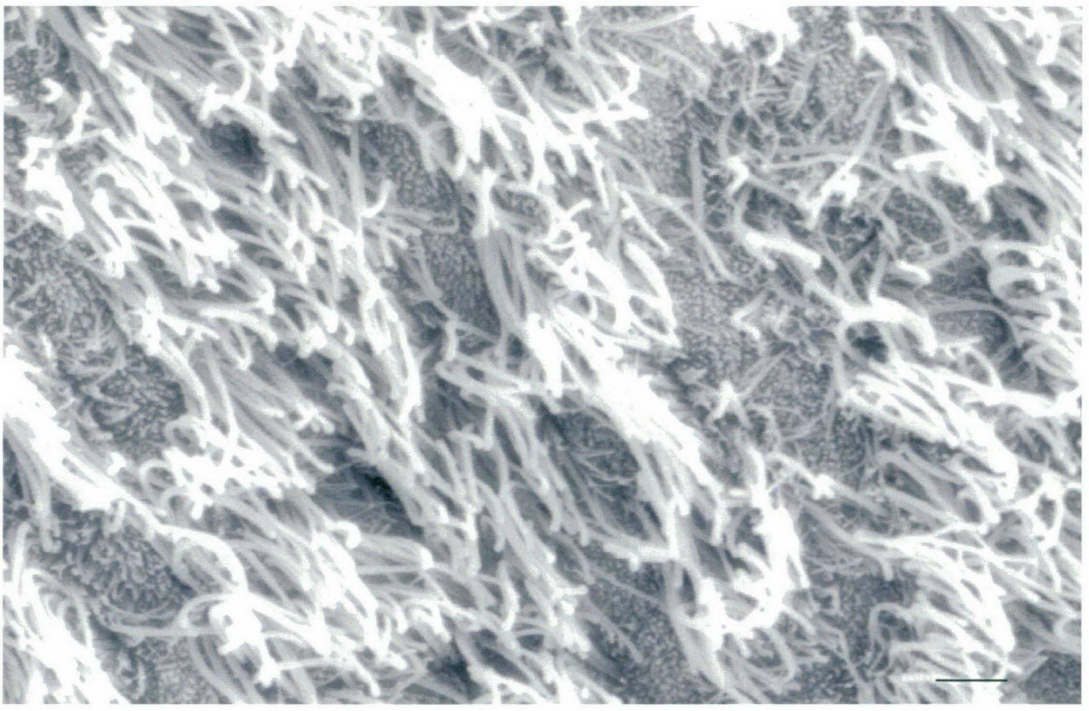


Plate 8.6: Scanning electron micrograph of tubular shell gland of hen from control group x 6000. Scale bar represents 2 μm (egg in the mid-magnum)

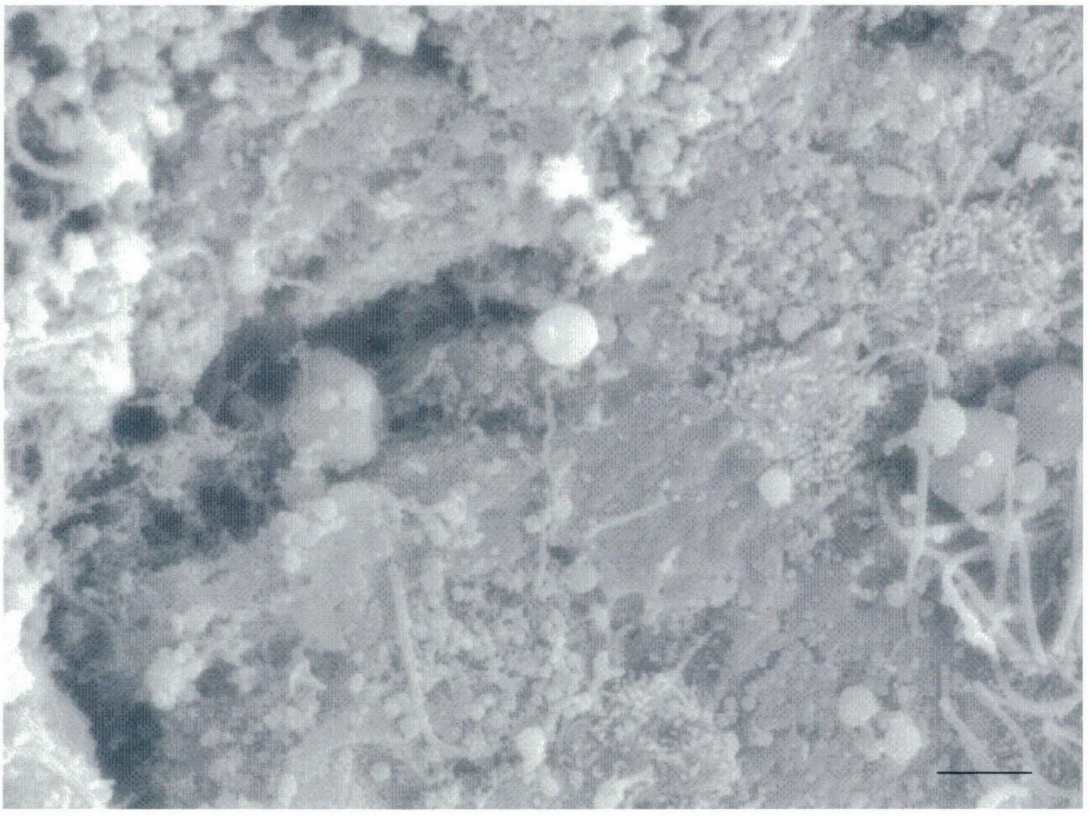


Plate 8.7: Scanning electron micrograph of isthmus of hen from T strain-infected group at 30 days p.i. x 6000. Scale bar represents 2 μm (no egg in the oviduct)

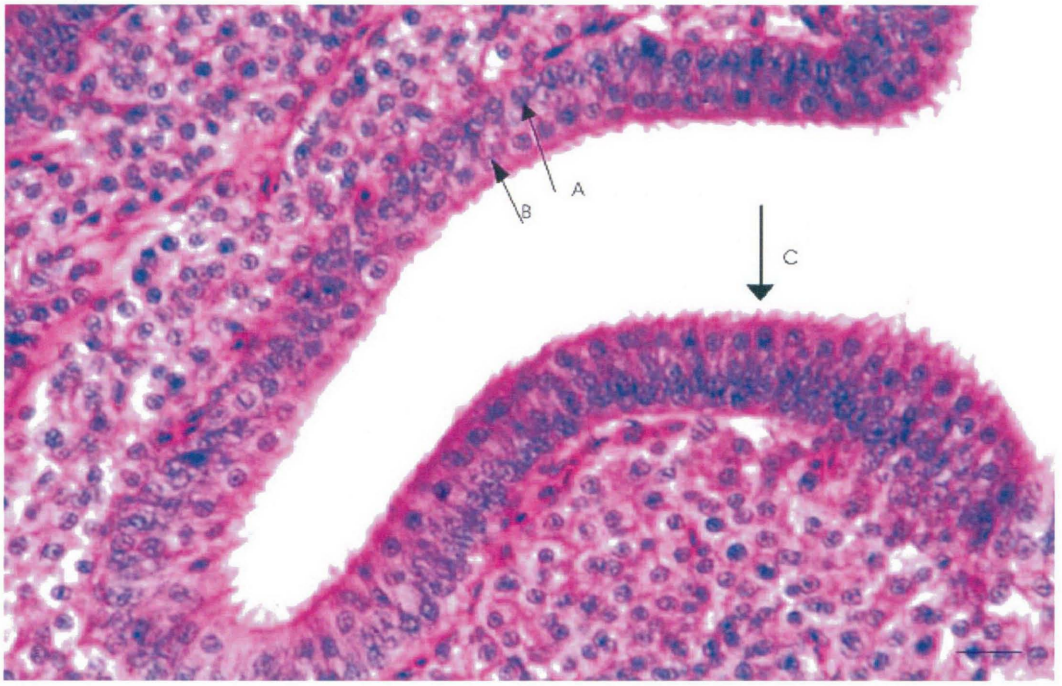


Plate 8.8: Shell gland of hen from control group at 8 days p.i. H &E x 400. Scale bar represents 25 µm (egg low in magnum). Basal cells of surface epithelium (A), Apical cells of surface epithelium (B), cilia (C)

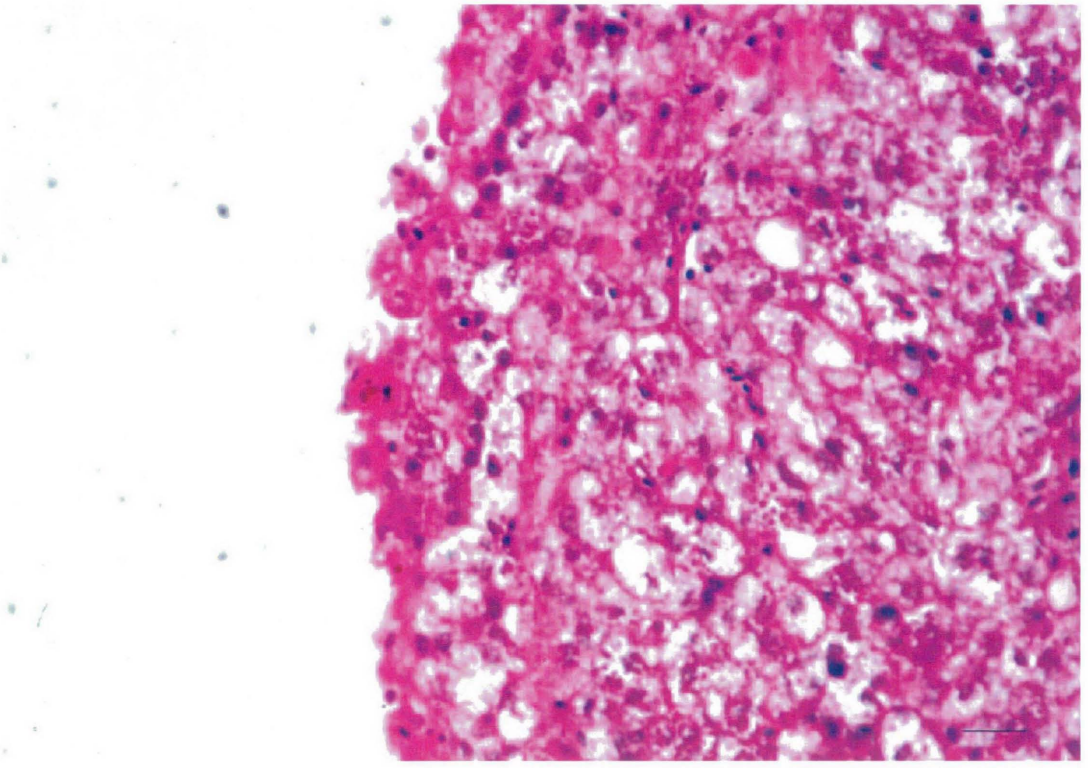


Plate 8.9: Shell gland of T strain-infected hen at 30 days p.i. H &E x 400. Scale bar represents 25 µm (no egg in the oviduct). Note loss of cilia from epithelium and change is architecture of surface epithelium.

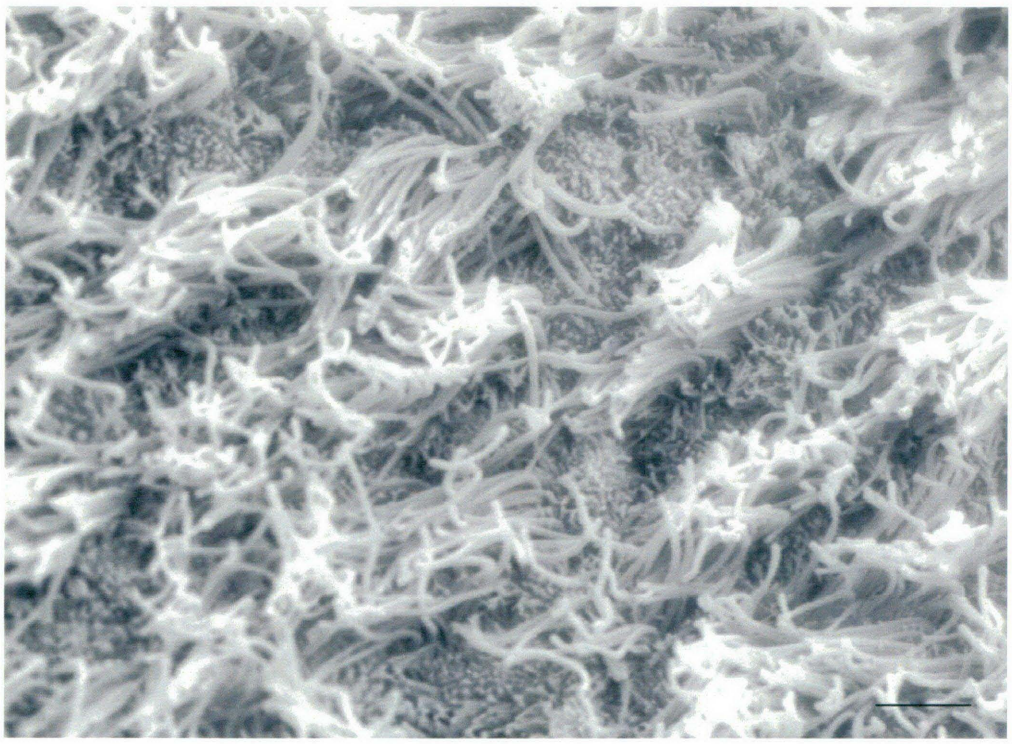


Plate 8.10: Scanning electron micrograph tubular shell gland of hen from control group x 6000. Scale bar represents 2 μm (no egg in the oviduct)

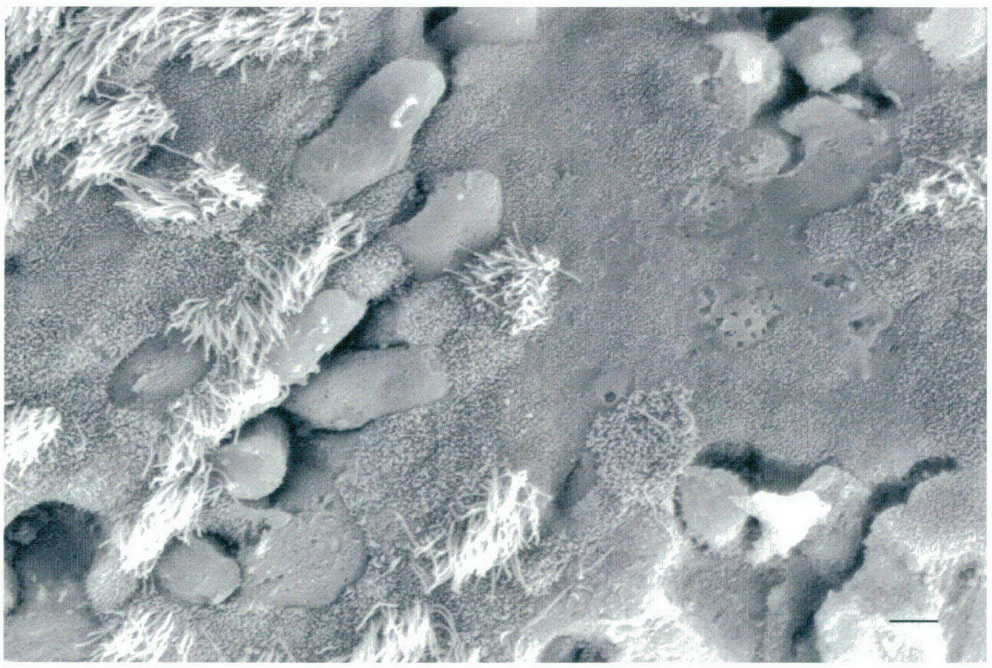


Plate 8.11: Scanning electron micrograph of shell gland of hen from T strain-infected group at 30 days p.i. x 6000. Scale bar represents 2 μm (no egg in the oviduct). Note patchy loss of cilia.

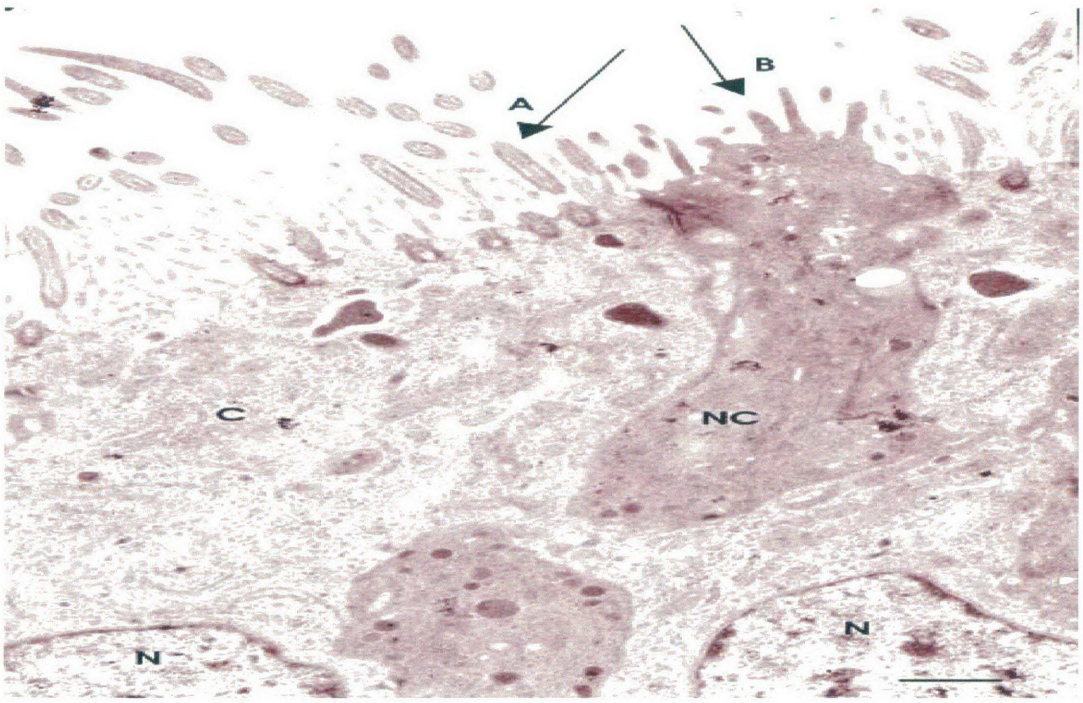


Plate 8.12: Transmission electron micrograph of Shell gland of control hen at 8 days p.i. x 5000. Scale bar represents 1 µm. Cilia (A), Microvillus (B), Ciliated cell (C), Non ciliated cell (NC), Nucleus (N).

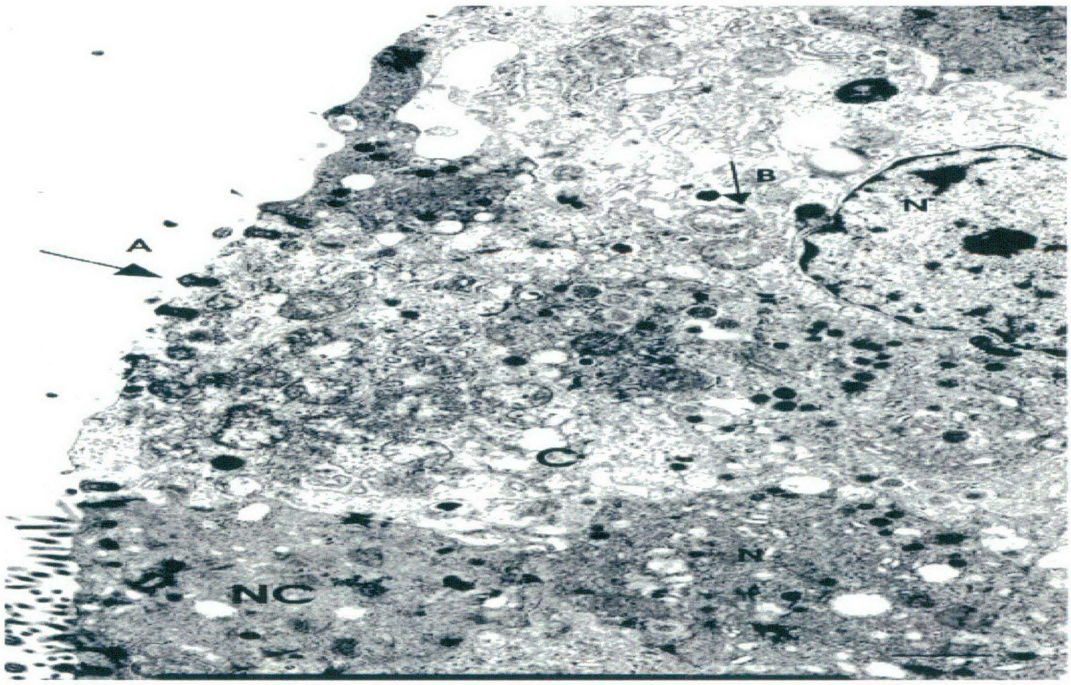


Plate 8.13: Shell gland pouch of hen from T strain-infected group at 30 days p.i. Note the loss of cilia from ciliated cell (A). Also note the degenerated mitochondria (B) (no egg in the oviduct) x 5000. Scale bar represents 1 µm.

8.4 Discussion

In the present study, there was no deterioration of egg shell quality (except for egg shell colour) or production unlike the findings of Sevoian and Levine (1957), Cook (1971) and Munner *et al.* (1986) which recorded the drop in egg production and deterioration of egg shell quality. All these studies were also conducted using limited numbers of birds and reported variation in the individual response to IBV infection. However, it is important to note that, in the above studies, the Massachusetts or Arkansas strain of IBV was used. This indicates that a severe decline in egg shell quality is not necessarily a common feature of Australian strains of IBV. The small number of hens used in the present study may have made production changes difficult to detect. In our preliminary study, we observed pathology in the TSG and SGP of T- and N1/88-infected unvaccinated White Leghorn hens at 65 weeks of age (Chapter 3). The differences between the findings of the previous and present studies could be attributed to age and breed of the hens. However, cytopathology in the albumen-forming and shell-forming regions of the oviduct of hens which were out of production suggests that IBV has the potential to infect all parts of the oviduct. Virus particles were observed in shell-forming regions of the T- strain infected hens up to 30 days p.i., and such hens are a potential source of virus shedding in the flock.

During egg shell formation, the isthmus plays an important role in the formation of egg shell membranes. Cytopathology in the isthmus could cause abnormal shell membrane formation. The intracisternal granules in the RER deposits of the isthmus are a means of regulating protein synthesis (Solomon, 1983), and their absence could be responsible for disturbance in protein synthesis. Mammillary cores in the egg shell are the initial templates for the calcified shell and are secreted by epithelial cells of the TSG (Solomon, 1975). Pathology in the epithelial cells (both surface and glandular) of the tubular shell gland could be responsible for formation of abnormal mammillary cores resulting in formation of a poor quality mammillary layer. The mammillary layer is the crystalline foundation of the egg shell (Brackpool, 1995), hence any alteration in this layer could affect the quality of whole egg shell. However cytochemical studies are essential to clarify this possibility. Moreover, the TSG and SGP play a vital role in the process of egg shell calcification. Solomon (1983) suggested that the columnar epithelium contributes calcium during egg formation.

Mitochondria also store calcium ions (Simkiss and Taylor, 1971). The degeneration of surface epithelial cells and changes in mitochondria may alter the rate of calcification and all this could affect the process of egg shell formation. It is interesting to note the paler shells during days 4 to 8 p.i., without any pathology in the shell gland on those days, but further investigation is essential to explain this finding. Egg shell colour is an important aspect of egg quality for the markets in Europe and Australia where there is a preference for dark brown shelled eggs. Hence, paler egg shells may not be regarded well by consumers. In this trial, severe pathology was observed in the shell gland mucosa of hens which were out of production. Breen and Bruyn (1969) suggested that vacuoloids in the shell gland resynthesise the disintegrated granules which are utilized for the secretory product. The absence of vacuoloids in the shell gland of T-infected hens which have stopped laying indicates the absence of the secretory cycle and hence one of the possible causes of reduced production of secretory product. It is also possible that degenerative changes in the shell gland of IBV infected hens are responsible for insufficient sex steroid stimulation resulting in cessation of egg production. This fact was suggested earlier by Nevalanen (1969) who found degenerative changes in the shell gland mucosa of calcium-deficient hens which were out of lay. However, studies regarding changes in hormonal levels during infection are necessary to prove this. The degenerative changes in the shell-forming regions could be reversible or irreversible in some hens and hens may stop laying either temporarily or permanently. Such changes may not be very common during infection with T and N1/88 strains of IBV in adult Isa Brown hens, as only three hens which were out of lay showed changes in the isthmus, TSG and SGP. All the above findings support the view of McMartin (1968b) and Munner *et al.* (1986) that there could be an individual variation in symptoms amongst the hens during IBV infection varies greatly. The egg shell-forming region of the avian oviduct is highly complex and secretes a range of egg shell matrix proteins, hence it would still be difficult to confirm the cause of thin or soft shells during infection. We did not observe the soft or thin shelled eggs, mentioned by earlier researchers as a typical finding of IBV infection in hens (Sevoian and Levine, 1957). However, it would be interesting to conduct ultrastructural studies with the Massachusetts strain of IBV, the strain known to cause decline in egg production and deterioration of shell quality, on a larger scale. Such studies could not be conducted in Australia due to quarantine restrictions. The Massachusetts strain of IBV has been reported to be more pathogenic for the oviduct

of baby chickens as compared to T strain (Crinion and and Hofstad, 1972a). As there are no published records of direct effects of Australian IBV strains on the mature oviduct and on egg shell quality, the present study addresses earlier speculations about Australian strains of IBV. It would be interesting to study effects of IBV infection on egg shell matrix proteins such as osteopontin (Fernandez *et al.*, 2003) pertaining to its possible role in egg calcification.

Chapter 9

Egg and egg shell quality in Isa Brown laying hens during infection with Australian strains of infectious bronchitis virus

9.1 Introduction

IBV infection in layers is potentially a major threat to the egg industry as egg quality problems currently cost millions of dollars a year. In the past, some studies have been undertaken to study the effects of IBV on egg quality after infecting chickens at an early age (Crinion, 1972). However, little if any research has been done to document the series of changes in different egg quality parameters after IBV infection in adult hens. Sevoian and Levine (1957) observed changes in internal and external egg quality after infecting adult laying hens with the Massachusetts strain of IBV. Surprisingly, since this study, little effort has been made to study the changes in egg internal quality and egg shell quality parameters during IBV infection. Australian strains of IBV are thought by some to be a cause of deterioration of egg internal quality and egg shell quality but documentation to prove or disprove this opinion is lacking. The effects of Australian strains of IBV on the oviduct of laying hens have received little research attention. Vaccination of laying hens in the rearing phase offers some protection of shell quality (Jolly, 2005) when laying hens are challenged with the nephropathogenic T-strain of IBV. The present study was undertaken to study the effects of IBV on egg internal quality and egg shell quality in unvaccinated laying hens challenged with the nephropathogenic T-strain, as compared with the more respiratory N1/88-strain of IBV.

During the experiment, shape index was found to be significantly lower in both infected groups when it was measured during 6-10 weeks post infection. These measurements were made because elongated eggs were noticed in the infected groups during the first half of the experiment. Therefore, a follow-up trial was conducted to study the time frame of the effects of IBV on egg shape index. Very little information

is available about changes in shape index during IBV infection. Decrease in shape index as hens get older has been reported earlier by Leary (1999). It has been suggested that increase in egg size with increased hen age is due to an increase in the size of developing follicles (Williams and Sharp, 1978). However, very little work has been done to find out the exact cause of elongated eggs.

There is still uncertainty regarding the relationship between the shape of an egg and its ability to resist load. It has been postulated that rounder eggs have lower deformation than elongated eggs. Aitken (1971) concluded that egg shape may be determined from the combined effects of the resistance to deformation exerted by the connective tissue of the uterine wall and peristaltic moments of the oviduct. Also, variation in the pattern of peristaltic movements and the architecture of the shell gland connective tissue might influence egg shape. Other factors such as volume of albumen, size of isthmus, breed and flock variation, laying cycle and laying period may influence egg shape (Scanes *et al.*, 2004).

However, the incidence of change in egg shape due to any disease has not been investigated previously.

9.2 Material and Methods

The details of materials and methods from the main trial are described in chapter 2.

The 36 hens used in the follow-up trial were the control birds remaining from the main trial. They were raised and maintained as described earlier in chapter 2. All the hens were fed with commercially available layer mash. *Ad libitum* feed and water were available at all times. Strict biosecurity was maintained throughout the follow-up experiment. Egg production and any visual defects in the laid eggs were recorded daily. All hens received 16 hrs of light per day.

At 40 weeks of age, 30 hens were divided equally into two groups and were exposed to one of two strains of IBV: T or N1/88 strains. Six hens were left unchallenged as a control. The unchallenged group remained in the original isolation shed and hens to be challenged were relocated into two additional sheds. Eggs were collected daily for

11 days post-infection (p.i.) and analysed for all measures of external and internal quality as described in chapter 2. The length and breadth of eggs was measured daily for three weeks and shape index was calculated as described in chapter 2.

Data were analysed by two way factorial ANOVA and Fisher's protected Least Significance Difference test was used to distinguish differences between means. Repeated measures ANOVA was not used owing to the fact that not all hens laid each day and also to allow for the fact that birds were progressively removed from the flocks during the first 24 days post-infection. Significance was assumed at $P<0.05$.

9.3 Results

9.3.1 Clinical findings regarding egg quality

All the hens were negative for IBV antibodies before infection. There were no significant effects of challenge or time in relation to challenge on egg production, also there were no significant interactions (Figure 9.1). However, one hen from the N1/88 group and two hens from the T-infected group stopped laying during the second week post infection (p.i.). Loss of shell colour was noticed visually in both infected groups from 4 to 8 days p.i. Overall, feed intake did not vary significantly over the weeks after challenge or amongst the groups and there were no significant interactions. However, when data for each week were analysed separately, weekly feed intake was significantly lower during the 2^{nd} (769 ± 5.74 g/bird/week) and 4^{th} (772 ± 5.79 g/bird/week) weeks post-infection in the T-infected group (Figure 9.2). There were no visible deformities in egg shells in the N1/88 infected group, but an occasional occurrence of black-spotted shelled egg shells was noted in the T-infected group.

Figure 9.1 Weekly egg production during the main trial before and across the 10 weeks post infection

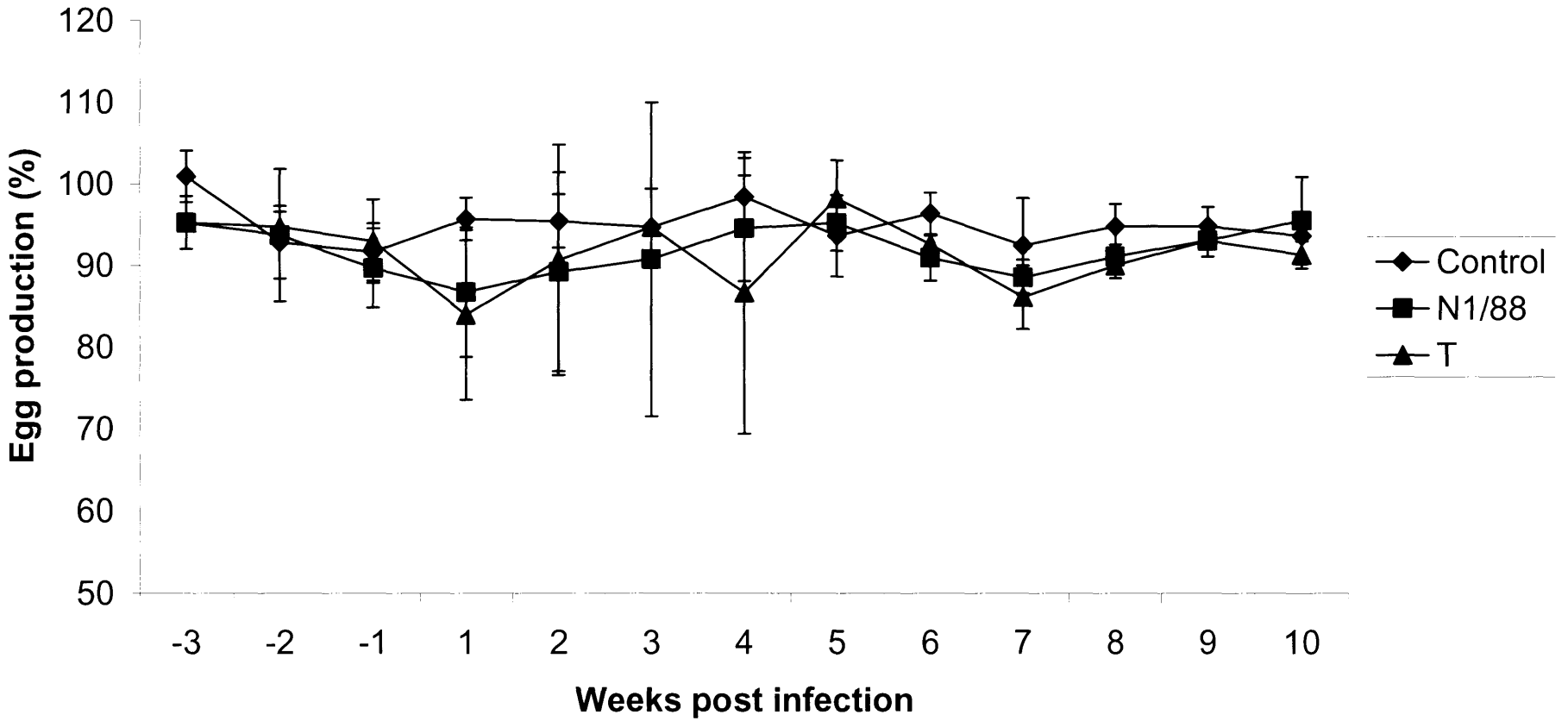


Figure 9.2. Feed intake before and across 4 weeks post infection in relation to treatment group and weeks post-infection

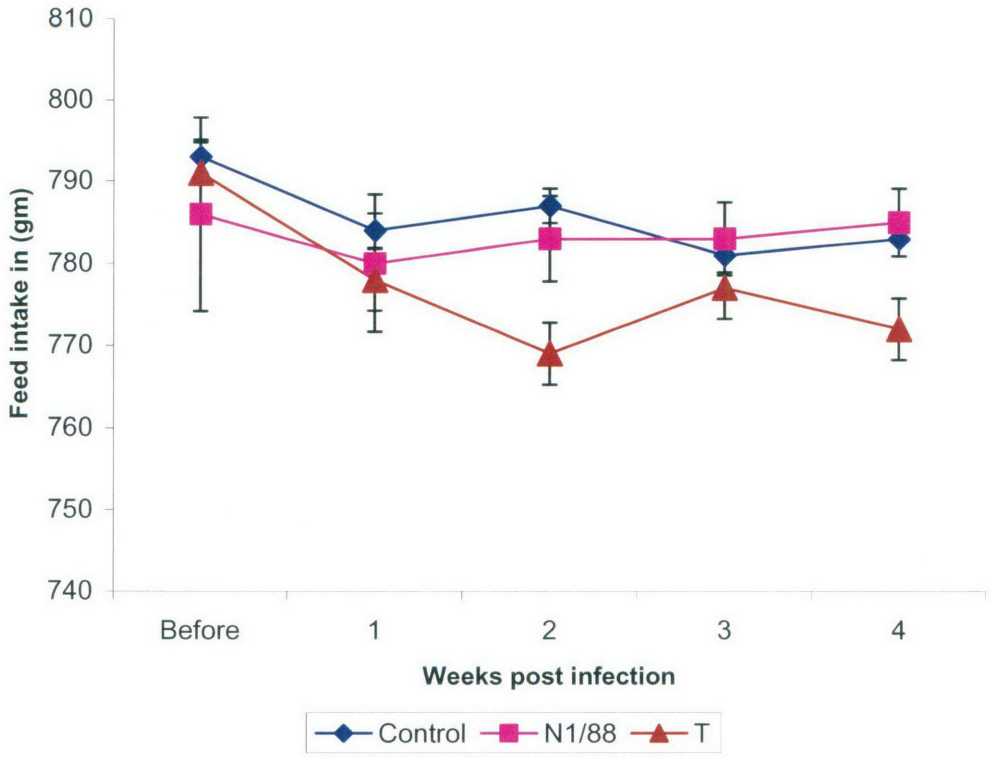


Table 9.1. Significant effects on egg parameters before challenge during the main experiment

Measurements	Groups	3 weeks preinfection	2 weeks preinfection	1 week preinfection	All weeks	P values		
						G	W	G x W
Egg weight	Control	55.3± 0.6	55.7± 0.5	56.1± 0.2	55.7± 0.1			
	N1/88	54.5± 0.6	55.1± 0.5	55.7± 0.2	55.1± 0.2	N	*	N
	T	55.6± 0.6	55.8± 0.5	56.2± 0.2	55.8± 0.1			
	All	55.1± 0.3	55.5± 0.2	56.1± 0.1				
Breaking strength	Control	41.6± 1.2	43.5± 0.5	42.2± 0.3	42.3± 0.3			
	N1/88	44.8± 1.1	44.7± 1.0	42.9± 0.3	44.1± 0.3	*	*	N
	T	42.7± 0.9	44.1± 0.7	42.6± 0.3	43.1± 0.3			
	All	43.0± 0.6	44.1± 0.2	42.6± 0.2				
Deforming unit	Control	255.7± 5.0	266.3± 4.2	257.0± 1.6	259.6± 2.8			
	N1/88	257.5± 5.6	265.0± 4.9	259.3± 1.9	260.6± 1.7	N	*	N
	T	256.5± 4.2	265.7± 4.1	257.8± 1.8	260.0± 1.6			
	All	256.5± 1.5	265.6± 2.5	258.0± 1.0				
Percent shell	Control	10.46± 0.13	10.37± 0.11	10.19± 0.03	10.34± 0.03			
	N1/88	10.50± 0.12	10.41± 0.13	10.25± 0.04	10.38± 0.04	N	*	N
	T	10.44± 0.11	10.31± 0.09	10.25± 005	10.34± 0.04			
	All	10.46± 0.07	10.34± 0.06	10.23± 0.02				
Shell weight	Control	5.77± 0.07	5.77± 0.07	5.71±0.02	5.75± 0.02			
	N1/88	5.72± 0.08	5.72± 0.06	5.70±0.02	5.72± 0.02	N	N	N
	T	5.80± 0.07	5.76± 0.07	5.75±0.03	5.76± 0.03			
	All	5.76± 0.04	5.75± 0.04	5.73± 0.01				
Shell thickness	Control	438.9± 3.5	439.1± 2.8	436.0± 1.1	438.0± 2.2			
	N1/88	438.0± 4.0	437.9± 3.3	435.6± 1.5	437.1± 1.3	N	N	N
	T	441.0 ± 3.9	440.3± 3.9	438.2± 1.5	439.0± 1.3			
	All	439.3± 2.2	439.2± 2.0	436.6± 0.8				

Mean ± Standard Error. G = Treatment Group, W = Week, N = Non significant, * Significant at $P< 0.05$

9.3.2 Egg quality during main trial

9.3.2 (i) Prior to challenge

In eggs laid over the 3 weeks before challenge, egg weight, deformation and percent shell varied significantly over the weeks (Table 9.1). Although not statistically significant, egg weight in the N1/88 infected group before infection tended to be lower than for the other two groups. There was a significant main effect of treatment group and week of experiment on shell breaking strength. Breaking strength of eggs laid by hens in the control group was lower as compared to eggs laid by the other two groups. However, there was no statistically significant interaction between treatment group and time prior to challenge.

9.3.2 (ii) Post-challenge

For the measures of egg shell quality, there was a significant effect of time post-infection as the hens grew older. However, there was a significant effect of treatment group only for egg shell breaking strength and there were no statistically significant interactions between treatment group and time p.i. for any of the measures of egg shell quality (Tables 9.2, 9.3).

Over the ten weeks p.i., there were significant main effects of treatment group (P= 0.0001) and week p.i. (P = 0.0001) for albumen height and Haugh units. Also there were significant interactions between treatment group and weeks p.i. for albumen height (P= 0.0012) and Haugh units (P= 0.0336). Differences were not recorded within these parameters before challenge. When analysed on a weekly basis and compared to control hens, the albumen height in the N1/88 group was significantly lower at 2 (8.73 ± 0.09 mm), 5 (9.43 ± 0.13 mm) and 6 (8.31 ± 0.22 mm) weeks p.i. and Haugh units were significantly lower at 1 (95.5 ± 0.6), 5 (96.5 ± 0.5) and 6 (91.2 ± 1.1) weeks p.i. Except for the 8^{th} week p.i., albumen height and Haugh units were significantly lower from the first week until the end of the experiment in the T-infected group (Figures 9.3 and 9.4).

Overall, there were no significant differences amongst the treatment groups for yolk colour score but there was a significant variation over the weeks in relation to

challenge (P= 0.0001) and a significant interaction between group and week of experiment (P= 0.0001). Yolk colour score was significantly lower in T-infected hens at 2 (10.24 ± 0.05), 3 (10.28 ± 0.05) and 4 (10.31 ± 0.06) weeks p.i. Yolk score did not vary significantly among the treatment groups or over time from 5 to 10 weeks post challenge (Figure 9. 5).

Over the 10 weeks after challenge, there were significant main effects of treatment (P= 0.0001), time in relation to challenge (P= 0.0001) and a significant interaction between treatment group and time p.i. (P= 0.0001) for shell reflectivity. As compared to the control group, shell reflectivity was significantly higher in the N1/88 group in the 1st (31.3 ± 0.4 %) and 2nd (30.5 ± 0.2 %) weeks p.i. whereas, in the T-infected hens, reflectivity was significantly higher from 1 to 5 weeks p.i. From 6 weeks to 10 weeks p.i., no significant effects were recorded for shell reflectivity (Figure 9.6).

When shape index was measured from 6 to 10 weeks p.i., compared to control hens, it was significantly lower in the eggs laid by hens of the T-infected and N1/88 infected group only at 8 weeks p.i.

Table 9.2: Effects of infectious bronchitis virus strains on egg weight, breaking strength and deformation of unvaccinated laying hens during the main experiment.

		Weeks post infection											P values		
Measurements	G	1	2	3	4	5	6	7	8	9	10	All weeks	G	W	G x W
Egg wt (g)	C	57.5± 0.3	58.6± 0.2	58.3± 0.3	58.7± 0.2	58.7± 0.3	59.4± 0.5	59.2± 0.7	59.3± 0.6	59.4± 0.6	59.5± 0.5	58.8± 0.2			
	N1/88	57.2± 0.3	56.7± 0.4	57.4± 0.2	57.9± 0.3	58.4± 0.4	59.2± 0.6	59.3± 0.8	59.5± 0.7	59.8± 0.7	60.0± 0.4	58.5± 0.2	N	*	N
	T	58.1± 0.5	57.6± 0.2	58.2± 0.2	58.7± 0.3	59.4± 0.3	59.4± 0.6	59.2± 0.9	59.7± 0.7	60.0± 1.0	60.8± 0.5	59.1± 0.4			
	All	57.6± 0.2	57.6± 0.1	58.0± 0.1	58.5± 0.1	58.9± 0.2	59.3± 0.3	59.2± 0.4	59.5± 0.4	59.7± 0.4	60.1± 0.3				
Breaking strength (N)	C	40.5± 0.6	39.9± 0.4	38.5± 0.5	38.4± 0.5	42.4± 0.5	42.1± 1.8	42.2± 1.3	39.3± 1.3	43.5± 1.0	43.2± 1.6	41.1± 0.2			
	N1/88	41.7± 0.4	42.0± 0.4	41.8± 0.4	41.0± 0.4	43.5± 0.7	44.7± 1.1	41.6± 1.5	42.3± 1.2	47.7± 1.2	45.3± 1.4	43.1± 0.1	*	*	N
	T	40.3± 0.7	41.1±0.4	39.7± 0.4	40.4± 0.5	42.0± 0.5	43.5± 1.4	40.4± 1.6	42.4± 1.4	44.6±0.9	42.5± 1.2	41.7± 0.2			
	All	40.8± 0.3	41.0± 0.2	40.0± 0.2	39.9± 0.	42.6± 0.3	43.5± 0.8	41.4± 0.8	41.2± 0.8	45.1± 0.6	43.6± 0.8				
Deformation (µm)	C	270.7± 7.9	263.3± 6.1	276.5± 7.4	259.6±7.1	263.2±4.9	238.2±5.6	236.3±5.5	239.6± 13.	252.2±4.5	251.4± 4.8	255.1±1.9			
	N1/88	264.7± 5.4	268.5± 4.0	275.1± 4.3	267.2±4.2	257.7±4.3	248.6±6.8	241.0±7.2	240.8± 8.2	247.7±5.6	256.6± 6.7	256.7±1.3	N	*	N
	T	265.0± 8.5	265.2± 4.2	271.0± 5.1	251.2±4.1	261.8±5.5	249.0±5.9	227.3±5.7	252.7± 9.0	257.6±5.7	246.0± 5.0	254.6±1.5			
	All	267.0± 4.3	265.7± 2.7	273.8± 3.3	58.7±3.0	261.0±2.9	245.9± 3.6	234.8± 3.5	244.4± 6.2	252.7± 3.0	251.2± 3.1				

Mean ± Standard Error, C- Control hens, N- non significant, * Significant at P< 0.05, G- Treatment Group, W- Weeks

Table 9.3: Effects of infectious bronchitis virus strains on egg shell weight, shell thickness and percentage shell of unvaccinated laying hens during the main experiment

		Weeks post infection											P value		
Measurements	G	1	2	3	4	5	6	7	8	9	10	All	G	W	G x W
Shell weight	C	5.80± 0.05	5.87± 0.03	5.81±0.03	5.82± 0.03	5.97± 0.04	6.11± 0.08	6.08± 0.08	6.00± 0.07	6.02± 0.06	6.09± 0.10	5.95± 0.02			
(g)	N	5.74± 0.03	5.67± 0.05	5.74± 0.04	5.80± 0.05	5.90± 0.05	6.01± 0.08	6.03± 0.15	6.06± 0.10	6.02± 0.10	6.24± 0.09	5.92± 0.02	N	*	N
	T	5.73± 0.05	5.79±0.04	5.85± 0.03	5.82± 0.03	5.93± 0.05	6.12± 0.10	6.08± 0.11	6.10± 0.10	6.10± 0.12	6.26± 0.10	5.95± 0.01			
	All	5.76± 0.02	5.77± 0.02	5.80± 0.02	5.81± 0.02	5.93± 0.02	6.08± 0.05	6.06± 0.06	6.05± 0.05	6.05± 0.05	6.19± 0.05				
Shell thickness	C	442.0± 2.2	441.2± 1.7	439.2± 1.6	438.9±1.9	447.0± 1.4	449.6± 2.6	448.4± 3.6	449.0± 2.7	449.0± 2.3	448.9± 5.8	444.4± 0.6			
(µm)	N	440.0± 1.4	438.0± 2.0	438.9± 1.9	438.4± 2.8	444.8± 2.6	449.2± 3.3	447.7± 7.9	448.8± 3.4	449.7± 3.4	452.2± 4.1	443.9± 0.7	N	*	N
	T	437.1± 3.0	438.1± 3.0	440.5± 2.0	438.6± 1.8	445.7± 2.2	449.7± 4.5	448.0± 5.2	449.0± 3.9	450.0± 4.5	452.7± 4.0	444.3± 0.8			
	All	439.8± 1.3	438.9± 1.4	439.7± 1.1	438.7± 1.2	445.8± 1.2	449.5± 2.1	448.1± 3.2	449.0± 1.9	449.6± 2.0	451.1± 2.8				
% shell	C	10.06± 0.07	10.02± 0.05	10.02± 0.06	9.97± 0.05	10.17±0.06	10.29±0.11	10.27±0.11	10.13±0.07	10.14±0.07	10.22±0.15	10.11±0.02			
	N	10.05± 0.07	10.03± 0.08	9.98± 0.07	9.98± 0.07	10.08±0.09	10.18±0.17	10.14±0.17	10.19±0.08	10.05±0.09	10.40±0.14	10.11±0.02	N	*	N
	T	10.04± 0.11	10.07± 0.07	10.08± 0.06	9.92± 0.05	10.06±0.10	10.33±0.20	10.25±0.06	10.20±0.07	10.15±0.07	10.33±0.19	10.13±0.02			
	All	10.05± 0.05	10.03± 0.03	10.03± 0.03	9.95± 0.03	10.10±0.05	10.27±0.10	10.22±0.07	10.17±0.04	10.12±0.04					

Mean ± Standard error, C- Control hens, N- non significant, * Significant at P< 0.05, G- Treatment Group, W- Weeks

Figure 9.3. Albumen height before infection and for 10 weeks post-infection for the three IBV treatment groups. Mean ± S.E.

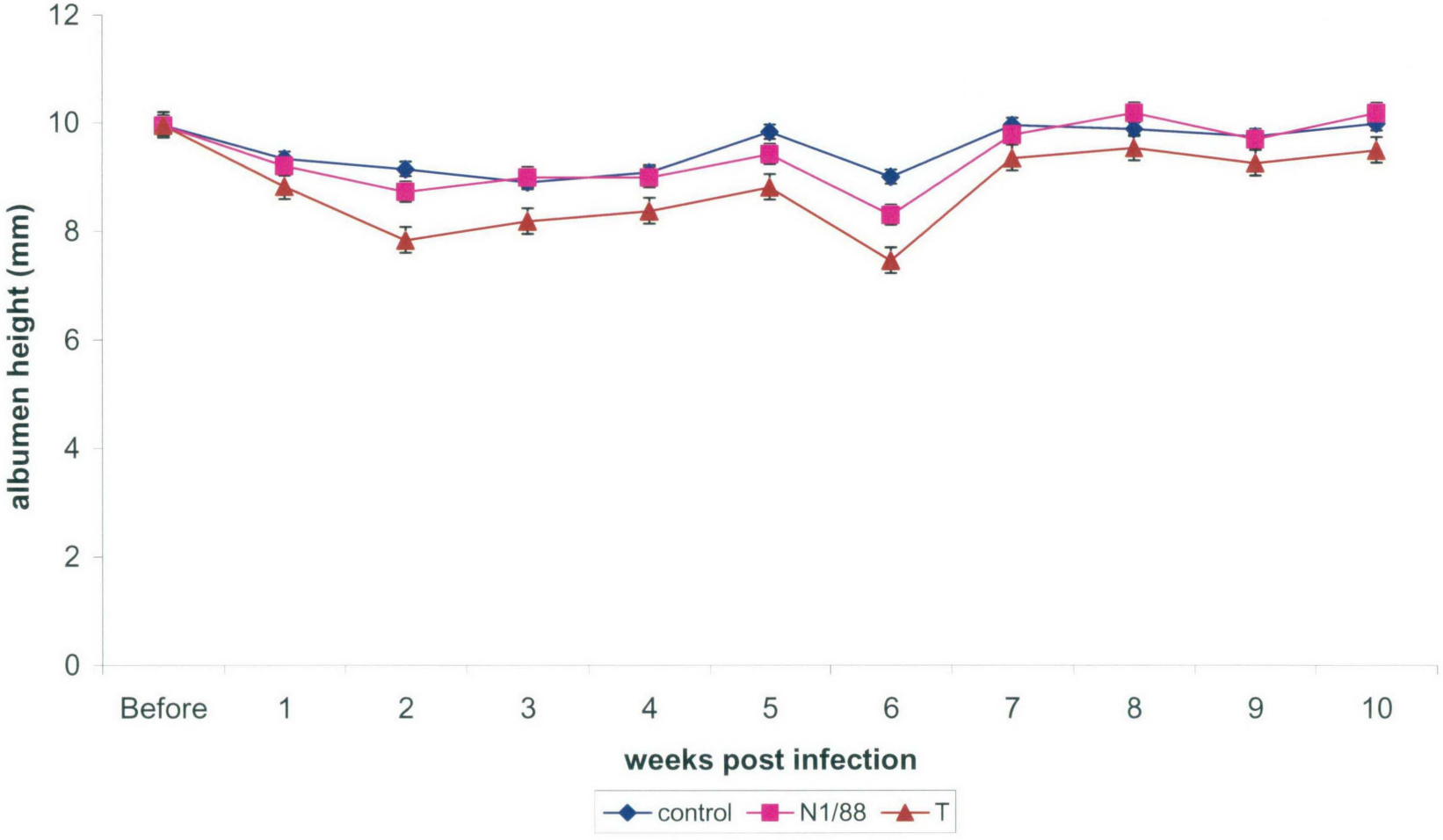


Figure 9.4. Haugh units before infection and for 10 weeks post-infection for the three IBV treatment groups. Mean ± S.E.

Figure 9.5. Yolk score before infection and for 10 weeks post-infection for the three IBV treatment groups. Mean ± S.E.

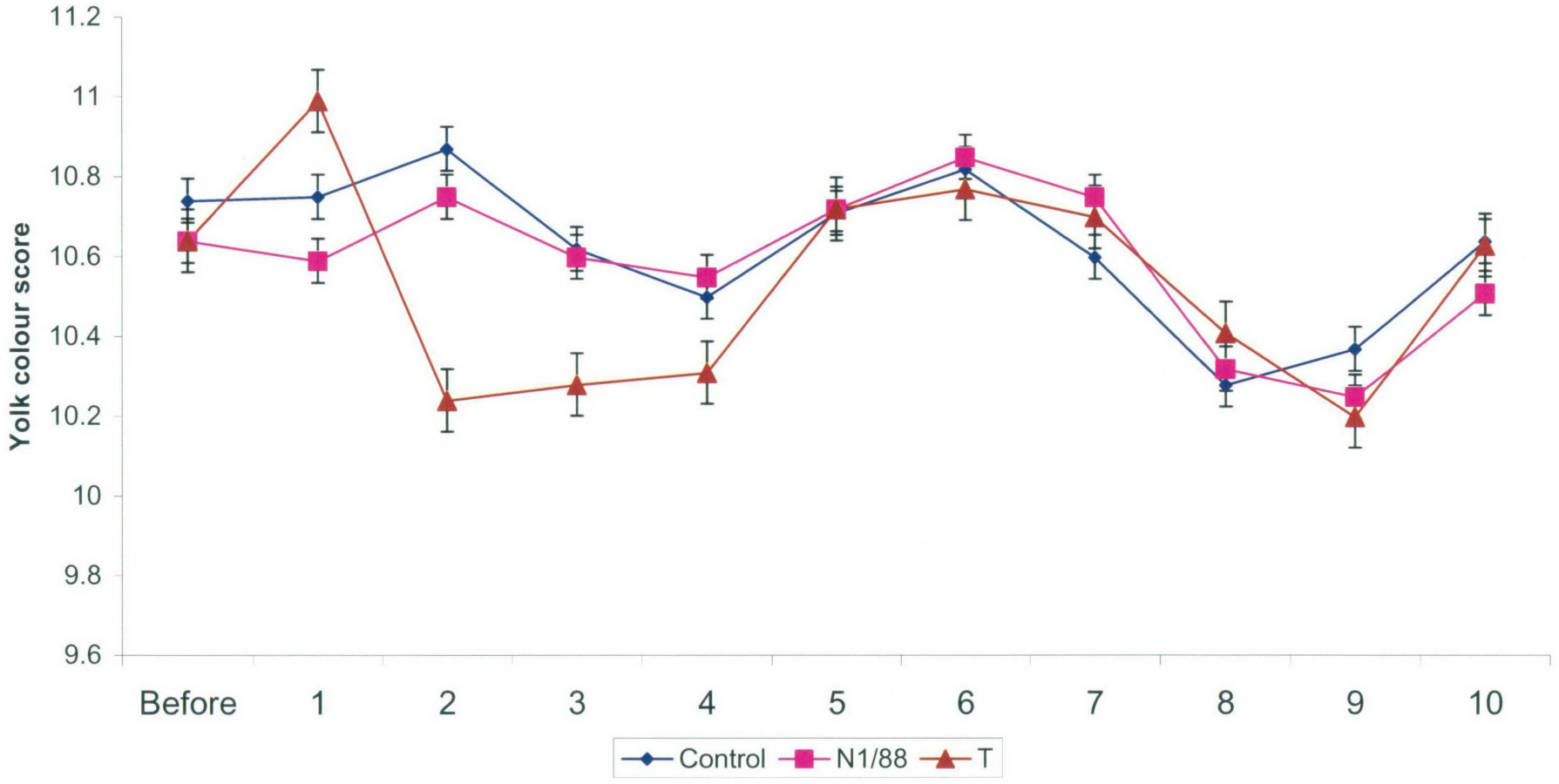


Figure 9.6. Shell reflectivity before infection and for 10 weeks post-infection for the three IBV treatment groups. Mean ± S.E.

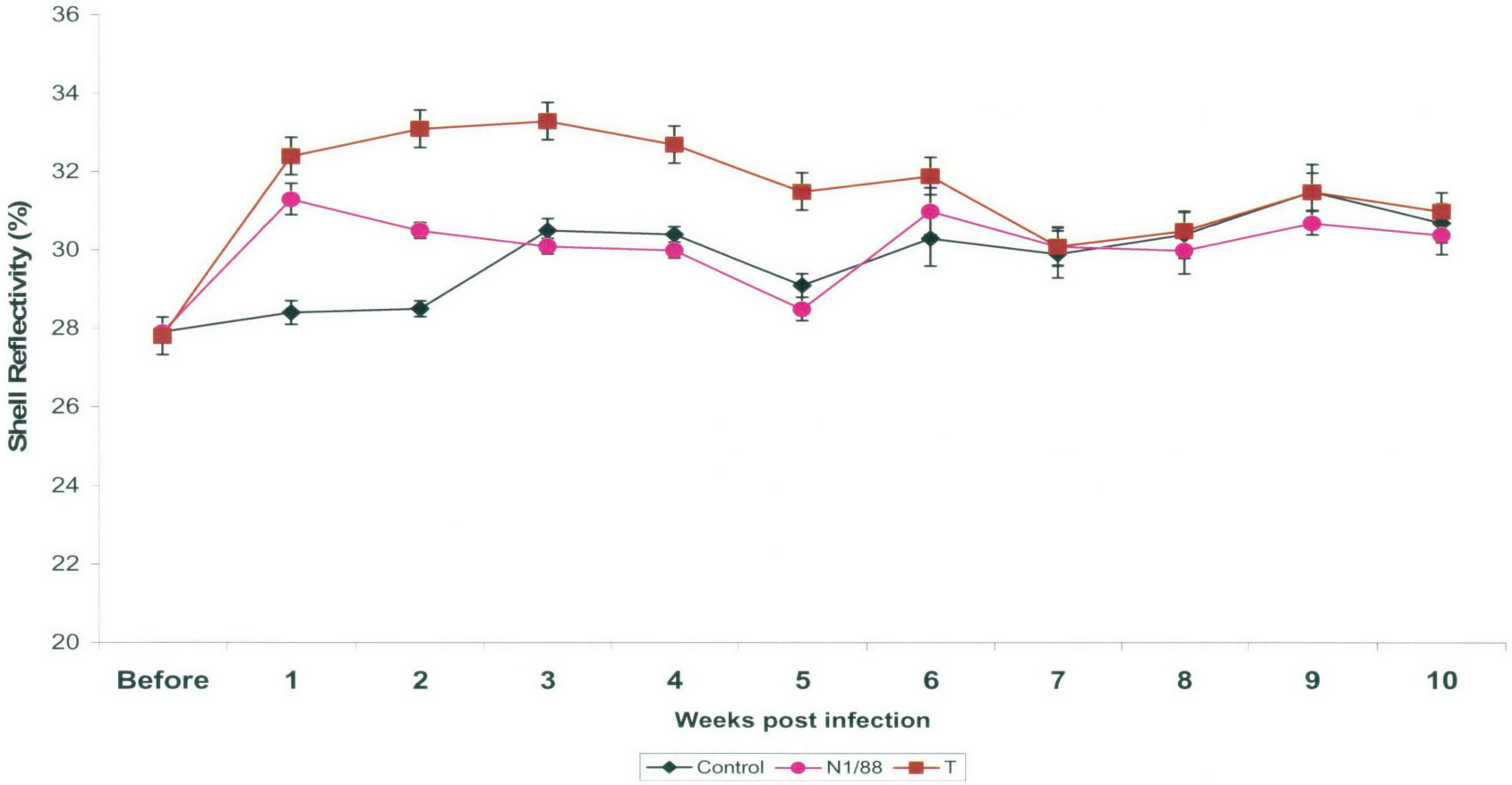
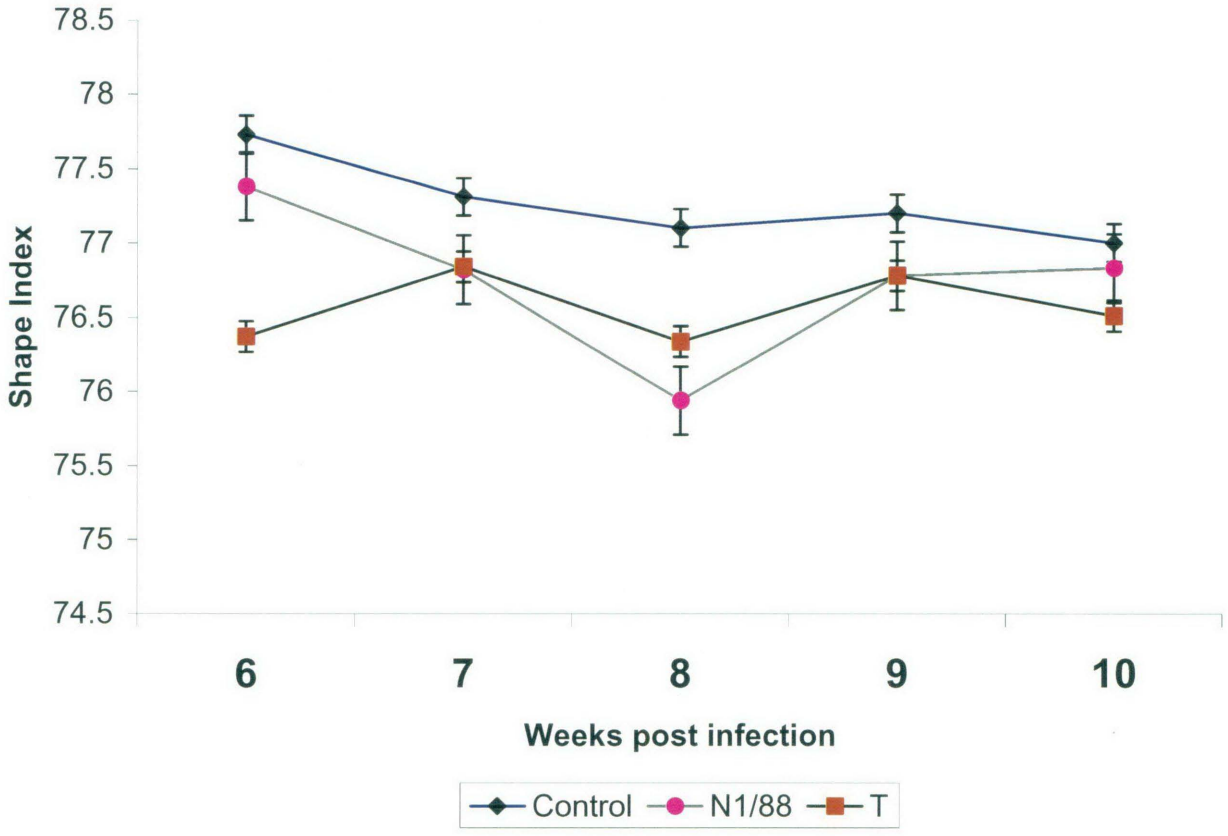


Figure 9.7. **Shape index from 6 to 10 weeks post infection for the three IBV treatment groups during the main experiment. Mean ± S.E.**



9.4 Egg quality during the follow-up trial

There was no decline in egg production during the three weeks of the follow-up trial (Table 9.4). Most of the hens in the T- and N1/88-infected groups had shown respiratory symptoms such as sneezing and rattling between 3 and 10 days p.i. and visual loss of shell colour was recorded between 4 and 10 days p.i. Thin or corrugated or soft shelled eggs were not observed in any of the infected groups.

Overall, reflectivity did not vary significantly over the time post infection (Table 9.5a). However, there was significant variation between treatment groups and significant interactions between group and days p.i. Egg shell reflectivity was significantly higher in the T- and N1/88-infected groups from 4 to 11 days p.i.

Albumen height varied significantly across the days post-infection and also among the treatment groups. However, there was no significant interaction between days p.i. and treatment group. Haugh units were significantly different amongst the groups, but there was no significant variation over time (Table 9.5b). Overall as compared to the control, albumen height and Haugh units were lower in the N1/88 group and even lower in the T-infected group. There were no significant effects on yolk score over time or among treatment groups and there was no interaction between treatment group and time p.i.

Other egg external quality parameters such as egg weight, shell breaking strength, shell deformation, shell weight, shell thickness and percentage shell did not vary significantly over the weeks post infection (Tables 9.5a,c-e). Also, for these parameters, there were no significant differences among the treatment groups and no interaction between treatment group and days p.i..

The eggs collected daily from infected hens for 3 weeks p.i., during the follow-up trial, showed the time course of change in shape index. Eggs laid by infected groups were more elongated (lower shape index) as compared to the control. The shape index varied significantly among the groups (P= 0.0009), but not over the weeks p.i. and there was no interaction between groups and weeks p.i. In N1/88-infected hens, shape index was significantly lower on the 1st (76.1 ± 0.34 %) 2nd (76.2 ± 0.38 %) and 3rd

(75.5 ± 0.3 %) weeks p.i., whereas, in T infected hens, egg shape index was significantly lower during the 2nd (76 ± 0.27 %)and 3rd (76.3 ± 0.43 %) weeks p.i.(Figure 9.8) Overall, egg shape index was lower in the T-strain and even lower in N1/88-infected hens as compared to the control.

Table 9.4: Effects of two strains of IBV on egg production during the follow-up trial

						P values		
Measurements	**G**	**1**	**2**	**3**	**All wks**	**G**	**W**	**G*W**
Egg Production (%)	**K**	97.61 ± 5.66	92.85 ±3.36	100.00 ±0.00	96.82 ±2.18	NS	NS	NS
	N1/88	87.09 ±5.93	94.28 ±3.68	99.04 ±5.71	93.47 ±3.05			
	T	95.23 ± 6.12	88.57 ±8.57	89.52 ±1.34	91.11 ±3.42			
	All	93.31 ±3.39	91.90 ±3.18	96.19 ±2.13				

Table 9.5a: Effects of two strains of IBV on shell reflectivity and egg weight during the follow-up trial

		Days post infection												P values		
Measurements	**G**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**All wks**	**D**	**G**	**G*D**
Reflectivity	**K**	33.16	32.33	33.50	30.83	30.50	29.00	29.00	29.16	27.16	30.66	29.40	30.49			
(%)		±1.30	±1.62	±1.38	±1.70	±1.38	±2.04	±1.42	±1.77	±0.94	±1.78	±1.53	±0.47			
	N1/88	33.06	30.73	35.26	34.73	36.28	33.62	35.92	35.28	36.28	36.00	35.75	34.81			
		±1.11	±0.89	±1.06	±0.99	±1.09	±1.59	±1.42	±1.31	±0.98	±1.02	±1.23	±0.36	NS	<0.0001	0.0146
	T	31.27	32.58	34.58	35.64	39.90	35.09	36.86	35.06	36.16	36.00	35.63	35.13			
		±0.91	±0.86	±0.74	±1.08	±1.34	1.11	±0.99	±1.13	±0.96	±1.10	±1.12	±0.33			
	All	32.46	31.69	34.69	34.42	35.38	33.52	35.14	34.14	34.53	35.00	34.57				
		±0.65	±0.59	±0.60	±0.71	±0.83	±0.93	±0.86	±0.84	±0.85	±0.76	±0.85				
Egg weight	**K**	59.95	59.45	57.61	59.10	59.23	58.12	59.90	59.23	54.83	61.68	60.28	59.04			
		±0.82	±0.51	±0.95	±1.22	±1.34	±0.96	±1.44	±0.93	±4.06	±1.73	±1.57	±0.52			
(gm)	**N**	58.84	60.24	59.86	59.56	58.86	59.12	57.95	59.34	58.35	59.20	57.91	59.05			
		±1.12	±1.00	±1.22	±1.22	±1.11	±1.32	±0.94	±1.21	±0.98	±0.85	±0.99	±0.32	NS	NS	NS
	T	61.82	60.59	59.51	60.11	58.92	60.40	60.48	60.02	55.75	59.20	62.01	59.88			
		±1.22	±1.06	±0.99	±1.13	±1.23	±1.23	±1.04	±1.00	±3.07	±1.09	±1.06	±0.42			
	All	60.07	60.22	59.33	59.70	58.95	59.56	59.36	59.61	56.71	59.66	59.95				
		±0.71	±0.59	±0.68	±0.71	±0.69	±0.77	±0.65	±0.65	±1.41	±0.64	±0.65				

Mean ± Standard Error. K– control, G- Treatment Group, D- day p.i., NS- not statistically significant, N1/88 and T are the strains of IBV

Table 9.5b: Effects of two strains of IBV on albumen height and Haugh units during the follow-up trial

		Days post infection												P values		
Measurements	**G**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**All wks**	**G**	**D**	**G*D**
Albumen height (mm)	**K**	10.13 ±0.55	10.33 ±0.39	9.91 ±0.43	9.36 ±0.19	9.61 ±0.19	9.77 ±0.25	9.38 ±0.55	9.61 ±0.32	9.48 ±0.44	9.70 ±0.37	9.36 ±0.33	9.70 ±0.11			
	N1/88	9.55 ±0.35	9.10 ±0.36	9.05 ±0.28	9.01 ±0.37	9.12 ±0.39	8.73 ±0.35	9.00 ±0.21	8.53 ±0.31	8.67 ±0.57	8.94 ±0.31	8.46 0.45	8.94 ±0.11	<0.0001	0.0201	NS
	T	10.48 ±0.24	8.65 ±0.43	8.85 ±0.32	8.48 ±0.23	8.62 ±0.33	8.83 ±0.50	8.48 ±0.27	8.48 ±0.40	8.44 ±0.42	9.02 ±0.48	8.32 ±0.20	8.76 ±0.11			
	All	9.98 ±0.22	9.16 ±0.25	9.13 ±0.19	8.86 ±0.19	9.04 ±0.22	8.96 ±0.27	8.84 ±0.17	8.69 ±0.23	8.73 ±0.30	9.11 ±0.23	8.57 ±0.22				
Haugh units	**K**	99.50 ±2.22	100.5 ±1.60	99.00 ±1.86	96.50 ±0.80	97.50 ±0.84	98.75 ±1.03	96.66 ±2.26	97.66 ±1.33	96.33 ±1.83	97.33 ±1.49	96.20 ±1.42	97.81 ±0.48			
	N	97.05 ±1.66	94.46 ±1.80	94.26 ±1.67	95.06 ±1.55	94.85 ±1.83	92.37 ±1.67	94.78 ±1.13	91.92 ±1.76	93.35 ±2.83	94.14 ±1.53	91.91 ±2.42	94.14 ±0.55	<0.0001	NS	NS
	T	100.5 ±1.20	91.91 ±2.49	93.00 ±1.65	91.57 ±1.11	92.72 ±1.70	92.81 ±2.89	91.60 ±1.33	91.00 ±2.79	92.00 ±2.30	94.41 ±2.31	90.63 ±0.89	92.80 ±0.62			
	All	98.71 ±0.99	94.63 ±1.33	94.66 ±1.06	93.91 ±0.86	94.61 ±1.06	93.69 ±1.55	93.74 ±0.86	92.51 ±1.43	93.40 ±1.53	94.84 ±1.12	92.17 ±1.15				

Mean ± Standard Error. K– control, G- Treatment Group, D- day p.i., NS- not statistically significant, N1/88 and T are the strains of IBV

Table 9.5c: Effects of two strains of IBV on shell breaking strength and deformation during the follow-up trial

		Days post infection												P values		
Measurements	**G**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**All wks**	**G**	**D**	**G*D**
Deformation	**K**	245.0	236.6	246.6	236.6	246.6	267.5	243.3	243.3	275.0	258.3	286.0	252.2			
μm		±5.6	±13.0	±11.1	±14.5	±18.0	±29.2	±11.7	±13.8	±22.1	±33.8	±18.0	±5.4			
	N1/88	236.0	230.6	239.3	242.6	241.4	265.0	255.0	257.8	259.2	253.5	256.6	247.8			
		±9.6	±7.5	±8.07	±6.5	±11.9	±11.8	±5.5	±24.9	±9.5	±10.5	±11.9	±3.5	NS	NS	NS
	T	228.1	236.6	244.1	245.7	283.6	276.3	247.3	268.0	251.6	280.0	250.9	255.5			
		±14.8	±7.5	±7.8	±7.8	±35.8	±29.7	±9.5	±26.6	±5.3	±37.9	±7.6	±6.1			
	All	235.0	233.9	242.4	242.8	257.4	270.8	249.7	259.7	259.3	264.3	259.6				
		±6.7	±4.8	±4.9	±4.7	±14.2	±15.1	±4.9	±15.0	±6.1	±15.8	±6.9				
Breaking	**K**	41.81	38.75	43.98	43.40	47.30	40.20	37.94	37.09	42.83	35.15	34.32	40.35			
strength		±2.9	±3.4	±2.29	±3.70	±3.92	±1.89	±3.08	±4.68	±2.93	±2.89	±4.36	±1.06			
N	**N**	35.94	38.07	42.43	41.46	38.23	47.00	43.84	42.01	45.33	42.01	44.19	41.56			
		±2.95	±2.14	±4.11	±1.55	±2.97	±1.90	±1.35	±1.56	±1.65	±1.57	±1.58	±0.73	NS	NS	NS
	T	39.39	38.53	41.84	40.23	38.45	42.26	40.84	41.52	41.80	40.69	42.16	40.72			
		±2.54	±1.16	±2.99	±1.62	±2.70	±1.59	±2.08	±1.86	±1.50	±1.73	±1.57	±0.59			
	All	38.23	38.36	42.49	40.30	40.06	43.55	41.54	40.96	43.54	40.23	41.63				
		±1.73	±1.19	±2.15	±1.09	±1.87	±1.16	±1.19	±1.27	±1.07	±1.14	±1.32				

Mean ± S.E. K– control, G- Treatment Group, D- day p.i., NS- not statistically significant, N1/88 and T are the strains of IBV

Table 9.5d. Effects of two strains of IBV on yolk colour score and percentage shell during the follow-up trial

		Days post infection												P values		
Measurements	**G**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**All wks**	**G**	**D**	**G*D**
Yolk colour score	**K**	10.16 ±0.16	10.66 ±0.42	10.33 ±0.21	9.66 ±0.21	9.66 ±0.21	9.75 ±0.25	10.50 ±0.34	10.16 ±0.40	10.50 ±0.34	9.66 ±0.33	10.00 ±0.31	10.11 ±0.09			
	N1/88	10.26 ±0.34	10.06 ±0.31	10.80 ±0.24	9.60 ±0.69	10.21 ±0.40	10.37 ±0.32	9.92 ±0.37	9.50 ±0.32	9.71 ±0.26	10.21 ±0.35	9.75 ±0.37	10.03 ±0.11	NS	NS	NS
	T	10.54 ±0.31	9.25 ±0.47	9.83 ±0.40	9.71 ±0.45	9.45 ±0.39	10.27 ±0.41	10.46 ±0.21	10.26 ±0.22	10.33 0.25	10.16 ±0.47	9.72 ±0.35	10.01 ±0.11			
	All	10.34 ±0.19	9.87 ±0.24	10.36 ±0.19	9.65 ±0.34	9.83 ±0.23	10.21 ±0.22	10.25 ±0.18	9.94 ±0.18	10.09 ±0.17	10.09 ±0.23	9.78 ±0.21				
Percent shell %	**K**	9.96 ±0.35	9.78 ±0.28	10.63 ±0.23	10.28 ±0.33	10.54 ±0.38	9.98 ±0.47	9.90 ±0.30	10.21 ±0.45	11.19 ±1.16	9.91 ±0.39	10.03 ±0.59	10.23 ±0.15			
	N	9.27 ±0.43	9.39 ±0.39	9.43 ±0.47	9.82 ±0.52	10.08 ±0.19	10.55 ±0.21	10.31 ±0.21	10.11 ±0.24	10.27 ±0.20	9.78 ±0.44	10.16 ±0.16	9.88 ±0.11	NS	NS	NS
	T	9.24 ±0.41	9.99 ±0.21	10.30 ±0.31	9.74 ±0.33	9.44 ±0.38	9.97 ±0.22	9.62 ±0.37	9.88 ±0.25	10.74 ±0.73	10.34 ±0.23	10.08 ±0.15	9.94 ±0.11			
	All	9.39 ±0.25	9.68 ±0.20	9.96 ±0.25	9.87 ±0.26	9.94 ±0.18	10.18 ±0.15	9.95 ±0.19	10.03 ±0.16	10.62 ±0.35	10.01 ±0.22	10.11 ±0.13				

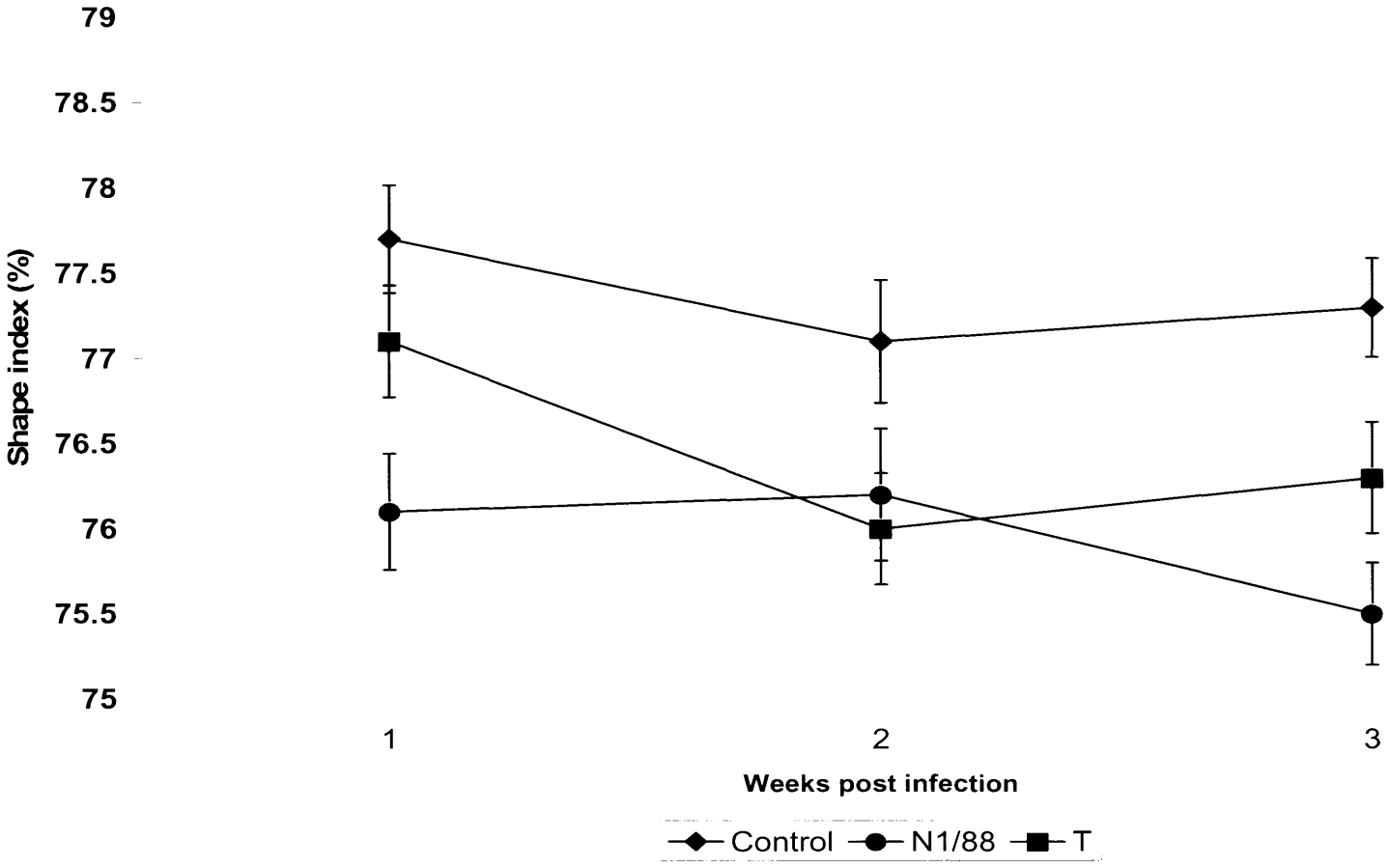
Mean ± S.E. K– control, G- Treatment Group, D- day p.i., NS- not statistically significant, N1/88 and T are the strains of IBV

Table 9.5e. Effects of two strains of IBV on shell weight and shell thickness during the follow-up trial

		Days post infection												P values		
Measurements	**G**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**All wks**	**G**	**D**	**G*D**
Shell weight	**K**	5.96 ±0.17	5.81 ±0.17	6.11 ±0.07	6.06 ±0.15	6.25 ±0.27	5.80 ±0.24	5.91 ±0.13	6.03 ±0.21	5.91 ±0.21	6.08 ±0.18	6.02 ±0.24	6.00 ±0.05			
	N1/88	5.47 ±0.29	5.66 ±0.26	5.66 ±0.32	5.85 ±0.32	5.92 ±0.11	6.23 ±0.16	5.96 ±0.10	6.00 ±0.17	5.97 ±0.10	5.63 ±0.23	5.97 ±0.09	5.83 ±0.06	NS	NS	NS
	T	5.70 ±0.24	6.05 ±0.14	6.11 ±0.16	5.84 ±0.18	5.55 ±0.22	6.02 ±0.19	5.80 ±0.21	5.93 ±0.18	6.00 ±0.11	5.90 ±0.15	6.26 ±0.14	5.92 ±0.05			
	All	5.64 ±0.16	5.83 ±0.13	5.91 ±0.16	5.88 ±0.15	5.85 ±0.11	6.06 ±0.11	5.88 ±0.10	5.97 ±0.10	5.97 ±0.07	5.81 ±0.12	6.09 ±0.08				
Shell Thickness µm	**K**	438.2 ±7.7	432.0 ±9.6	447.6 ±7.8	443.8 ±8.0	461.4 ±13.9	434.9 ±8.5	445.0 ±9.5	447.3 ±14.3	438.4 ±10.4	434.8 ±15.1	440.7 ±14.9	442.5 ±3.3			
	N	414.4 ±16.3	417.0 ±15.3	431.2 ±14.8	432.6 ±19.5	443.0 ±7.6	453.5 ±6.4	446.0 ±5.4	446.0 ±8.0	436.9 ±4.8	421.7 ±14.8	441.3 ±3.9	433.7 ±4.8	NS	NS	NS
	T	415.1 ±13.0	440.3 ±6.6	438.4 ±10.5	429.0 ±6.7	415.5 ±11.8	449.7 ±8.0	433.5 ±12.0	447.6 ±8.2	452.1 ±8.2	438.1 ±9.2	450.3 ±7.5	437.4 ±3.8			
	All	419.1 ±8.9	428.2 ±7.6	436.8 ±7.7	433.1 ±8.7	436.8 ±6.6	448.4 ±4.7	440.5 ±5.7	446.9 ±5.1	442.9 ±4.2	430.3 ±7.7	444.7 ±4.2				

Mean ± S.E. K– control, G- Treatment Group, D- day p.i., NS- not statistically significant, N1/88 and T are the strains of IBV

Figure 9.8: Shape index for three weeks post infection for the three IBV treatment groups during the follow-up trial. Mean ± S.E.



9.5 Discussion

There were no significant effects of IBV infection on egg production although mean production over the 10 weeks p.i. was highest in the control group (95.0%) and lower in the T (91.6%) and N1/88 (92.0%) groups. Also, during the follow-up trial, there was no decline in egg production. It may be that the relatively small number of hens in each treatment group made detection of production effects of IBV more difficult. On the other hand, Cook (1971) observed a drop in egg production in a small number of hens after infecting them with the Massachusetts strain of IBV. Sevoian and Levine (1957) also reported a drop in egg production of 42 hens infected with American strains of IBV. Reduction in egg production may be an effect of the Massachusetts strain but not necessarily of other strains of IBV.

IBV clearly has major effects on albumen height and Haugh units and, except for 8 weeks p.i., these parameters in the T-infected group were significantly lower than controls from the first week p.i. until the end of experiment, indicating prolonged effects of IBV. The fluctuation in the N1/88 group could be due to selective removal of hens showing low albumen height for other studies. This finding is consistent with results from the earlier study of ultrastructural changes in the magnum during T-and N1/88-infection (see chapter 8) and thus indicates that both of these Australian strains of IBV have tropism for the upper reproductive tract. The deterioration in albumen height and Haugh units was also noticed in the follow-up trial. Our findings support the previous suggestion that IBV is associated with thinning of albumen (Sevoian and Levine, 1957; Butler *et al.*, 1972). The significantly lower yolk score in T-infected hens from 2 to 4 weeks p.i. could be attributed to the transient decrease in feed intake observed in the T group. However, overall, feed intake did not vary significantly among the treatment groups. This finding regarding feed intake was recorded earlier by Roberts (2005). In the follow-up experiment, there were no differences in the yolk score of infected compared to control hens.

The significant increase in shell reflectivity in the T-infected group was reported earlier by Jolly *et al*, (2005). However, this result was found in vaccinated HyLine Brown and HyLine Grey hens with no significant effect on vaccinated Isa Brown hens. Temporary loss of shell colour from N1/88 infection has not been reported

previously but reduction in shell colour was also observed during the follow-up experiment. Reduction in shell colour during IBV infection with other strains has been reported in the past (Cook and Huggins, 1986). In the present experiment, shell reflectivity was measured using a sensitive shell reflectivity meter and pale shells were recorded visually for a short period during both the main and follow-up trials. Abnormal shell whitening (increase in shell reflectivity) usually occurs due to the deposition of a superficial calcareous layer on the egg shell cuticle (Hughes, *et al.*, 1986) and is observed in stress-related egg retention in the shell gland (Mills, *et al.*, 1991). The shell whitening in this study could be attributed to IBV infection-induced stress on hens. Also, porphyrin pigment imparts colour to the egg shells of brown egg layer hens (Baird *et al.*, 1975). Alteration in pigment deposition during IBV infection is a possible cause of egg shell whitening, although further studies are essential to prove this. However, paler egg shells may not be regarded well by consumers. Other measures of egg shell quality did not vary significantly. Cook (1971) found deterioration of egg shell quality after infecting laying hens with the Massachusetts strain of IBV. Such findings were not recorded in the present study, which could be due to differences in the breed of hen or strain of virus. The trend towards the hens in the N1/88-infected group having lower egg weight and higher breaking strength may be due to inherent differences among hens that were present before challenge, although there was no significant variation in egg weight among the treatment groups. Deformation was not different among the treatment groups, a finding that is in accordance with the study of Roberts (2005). Increase in shell weight and shell thickness towards end of the experiment could be due to increase in egg weight as hens get older. Such findings were reported earlier in Isa Brown hens by Leary (1999). There was no obvious pattern in shell percentage.

In the follow-up trial, in both the T and N1/88 treatment groups, shape index was lower to the end of the experiment, indicating long term effects of IBV on egg shape index. Little is known about the variation in egg shape index during IBV infection and hence the cause is still speculative. Possible causes are changes in the composition of the albumen and alteration of oviduct movements during infection. However, further research is needed to prove this. The different degree of coordination between inner circular or outer longitudinal muscle layer of the shell gland and/or secretion of albumen from the magnum could be responsible for formation of more elongated eggs

and changes in egg shape index (Scanes *et al.*, 2004). Further research needs to be conducted to explore the facts in this context.

A decrease in egg shape index with progressive increase in hen age was reported earlier by Leary (1999), but in the present study, the hens were in their early lay period. Overall, shape index did not vary significantly among treatment groups during the main experiment but there were differences when treatment groups were compared separately at 6 and 8 weeks p.i. However, it should also be noted that shape index was not measured during the first five weeks post-infection.

In conclusion, in the present study, when the hens were challenged with two strains of IBV (T and N1/88), there was a deterioration in albumen quality which was for a relatively short period in N1/88-infected hens but more prolonged in T-strain infected hens. This finding reflects the uterotropism of these two Australian strains of IBV for the fully-functional oviduct. Yolk score was lower than the controls only in T-infected hens which could be due to the decrease in feed intake in this treatment group. Egg shell quality was affected only by changes in shell colour and shape index.

In the present study, the hens in different treatment groups were housed in the different sheds to avoid cross-infection. This arrangement was essential for the experiment but it is possible that shed effects had some influence on the egg quality measurements.

Chapter 10

Isolation of infectious bronchitis virus from different tissues and faecal samples after experimental infection in laying hens

10.1 Introduction

Infectious bronchitis virus (IBV) is reported to have a wide tissue tropism which affects nearly all the epithelial layers in the body of poultry birds (Dhinakar Raj and Jones, 1997a). The virus has been isolated from the trachea (Ignjatovic *et al.*, 2002), kidney (Cumming, 1962, Hofstad and Yoder, 1966), intestine (Maiti *et al.*, 1986, Jones and Ambali, 1987, Lucio and Fabricant, 1990) and faeces (Alexander and Gough, 1977). Australian strains of infectious bronchitis virus have been isolated mainly from either the kidney in cases of uraemia (Cumming, 1962) or from the trachea (Ignjatovic *et al.*, 2002). The T strain of IBV was isolated originally from the kidney (Cumming, 1962) and N1/88 was isolated from the trachea of a vaccinated broiler flock (Ignjatovic and McWaters, 1991). Under experimental conditions, it has been observed that T strain can multiply and induce pathology in both the trachea and kidney (Ignjatovic *et al.*, 2002). Sapats and coworkers (Sapats *et al.*, 1996c) observed that the N1/88 strain, which is genetically and antigenically different from T, can multiply and induce histopathology only in the trachea. However, in our earlier trial, it was observed that N1/88 can multiply and induce histopathology in the kidney (Roberts, 2006). Both strains have been found to induce pathology in the albumen-producing (Chapter 7) and shell-forming regions of the oviduct (Chapter 8). The present study was undertaken to isolate two strains of IBV, T and N1/88, from the trachea, kidney, lower gut contents and faecal samples at different intervals after IBV infection in commercial laying hens. The detail regarding isolation of virus from the oviduct is described in chapter 11.

10.2 Materials and Methods

The details of the experiment have been described in chapter 5. The details of the procedure for virus isolation have been described in chapter 2 (Section 2.10).

10.3 Results

All samples from the control hens were negative for IBV.

The results of virus isolation for T strain IBV are presented in Table 10.1. In the T-infected group, virus was detected from the trachea from days 4 to 16 p.i. except for day 14 where virus was not detected in the trachea of any of the hens. Also, virus could not be detected in any of the hens from day 18 p.i. onwards. Virus was detected in the kidney from days 6 to 18 p.i. Virus was not detected in any kidney of any hen between 20 and 24 days p.i., although virus was persistent in the kidney of one hen which had stopped laying and was killed on day 30 p.i. Virus was recovered from the lower gut contents of hens except on days 2, 4, 14, 20 and 22. Overall, amongst the T strain-infected group, out of 26 hens killed, virus was isolated from the trachea of 9 (34%), kidney of 14 (53 %) and gut contents of 10 (38%) hens.

The pattern of N1/88 strain IBV isolation is presented in Table 10.2. For the N1/88 infected group, virus was detected from the trachea on days 4 to 16 p.i. except for day 14, p.i. On day 14 p.i., virus was not detected in the trachea of any of the hens. Also, virus could not be detected in any of the hens from day 18 p.i. onwards. Virus was detected in the kidney of all hens sampled from days 6 to 18 p.i. and was detected in kidney of one out of two hens killed on each of days 4, 8, 10, 16 and 22 p.i. Virus was recovered from the gut contents of one of two hens killed on days 6, 8, 10 and 24 p.i. Overall, for the N1/88 infected group, out of 24 hens killed, virus was isolated from the trachea of 12 (50%), kidney of 5 (20%) and gut contents of 4 (16%) hens.

In the T strain-infected group, virus was consistently isolated from the faecal samples of two out of five hens from one to nine weeks p.i. In the N1/88-infected group, virus was isolated from the faeces of three hens in the first week, and two hens during the second and third weeks. Virus was also isolated from the faeces of one hen during the

8th week p.i. The faecal samples of the hens from control group were negative throughout the experiment.

Table 10.1: Isolation of T strain infectious bronchitis virus at different time intervals post-infection. Number of positive samples out of a total number of samples are presented

Strain of virus	Days P I	Trachea	Kidney	Gut contents
T	2	0/2	0/2	0/2
	4	2/2	0/2	0/2
	6	2/2	2/2	1/2
	8	2/2	1/2	1/2
	10	1/2	2/2	1/2
	12	1/2	2/2	2/2
	14	0/2	2/2	0/2
	16	1/2	2/2	1/2
	18	0/2	2/2	2/2
	20	0/2	0/2	0/2
	22	0/2	0/2	0/2
	24	0/2	0/2	1/2
	30	0/2	1/2	1/2
Total Samples Positive		9/26 (34%)	14/26 (53%)	10/26 (38%)

Table 10.2: Isolation of N1/88 strain of infectious bronchitis virus at different time intervals post-infection. Number of positive samples out of a total number of samples are presented.

Strain of virus	Days P I	Trachea	Kidney	Gut contents
N1/88	**2**	**1/2**	**0/2**	**0/2**
	4	**2/2**	**1/2**	**0/2**
	6	**2/2**	**0/2**	**1/2**
	8	**2/2**	**1/2**	**1/2**
	10	**2/2**	**1/2**	**1/2**
	12	**2/2**	**0/2**	**0/2**
	14	**0/2**	**0/2**	**0/2**
	16	**1/2**	**1/2**	**0/2**
	18	**0/2**	**0/2**	**0/2**
	20	**0/2**	**0/2**	**0/2**
	22	**0/2**	**1/2**	**0/2**
	24	**0/2**	**0/2**	**1/2**
Total Samples Positive		**12/24 (50%)**	**5/24 (20%)**	**4/24 (16%)**

Table 10.3: Comparative pattern of isolation of infectious bronchitis virus from faeces of infected laying hens. Number of positive samples out of a total number of samples are presented

Weeks post infection	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
IBV strain T	2/5	2/5	2/5	2/5	2/5	2/5	2/5	2/5	2/5
IBV strain N1/88	3/5	2/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5

10.4 Discussion

All the samples from the control hens were negative for virus which indicates that all the hens were kept free from IBV infection throughout the experiment.

Virus was detected to a greater extent from the trachea of the N1/88-infected group (50%) as compared to the T strain-infected group (34%) which is not surprising as N1/88 is known to have a predilection for the respiratory tract (Ignjatovic *et al.*, 1991). T strain IBV has been also isolated previously from the respiratory tract (Ignjatovic *et al.*, 2002). This observation supports our earlier report regarding histopathology of T strain IBV in the trachea (see Chapters 3 and 5).

T strain IBV is known to be nephropathogenic (Cumming, 1962,) and has been isolated from the kidney of T strain-infected chickens at regular intervals between 3 to 10 days of post infection (Ignjatovic *et al.*, 2003). However, in the present study, N1/88 virus was also isolated from the kidney, which is in accordance with Roberts (2005), and also correlates with the finding regarding the histopathology of N1/88 in the kidney of White Leghorn hens and cockerels (Chapter 3) and also in the kidneys of Isa Brown hens (Chapter 5). Earlier, Sapats and coworkers (Sapats *et al.*, 1996b) reported that N1/88 has the capacity to multiply in the respiratory tract only but our

finding proves that N1/88 also has the capacity to multiply and induce pathology in the kidneys of adult laying hens.

Our finding regarding the isolation of N1/88 strain from gut samples has not been reported previously. In the past, Ambali and Jones (1990) reported the pathogenesis of an enterotropic strain of virus in chickens and Alexander and Gough (1977) isolated T strain from caecal tonsils of three week old Rhode Island Red chickens challenged with IBV. Alexander and Gough (1977) further reported haemorrhages in the caecal tonsils. However, in the preliminary experiment regarding histopathological observations during infection of N1/88 and T strains of IBV in White Leghorns in full lay, pathology was not observed in the caecal tonsils. Past and present observations suggest that breed and age of chickens are important factors in the induction of enteric infection. Also, it is possible that, when mature birds are challenged, both T and N1/88 strains of IBV can persist in the gut contents without causing any pathology. In such cases, the intestine may be involved as a major provider of virus for other target organs and for excretion in the faeces. It would be interesting to study the histopathology of other enteric organs as isolation of IBV has also been reported from various parts of the enteric tract by Lucio and Fabricant (1990). Escorcia *et al.* (2002) studied ultramicroscopic changes, along with the presence of virus particles, in the proventriculus and gizzard of challenged pathogen-free chicken embryos.

Isolation of N1/88 strain IBV from the faeces has not been reported earlier. However, the T strain of IBV was isolated from faecal samples of T strain-infected hens up to 225 days post infection (Alexander and Gough, 1977). Excretion of virus and its subsequent isolation from faeces could be due to the urates present in the faeces. Hence, in this study, besides the faecal samples, gut samples were also processed for virus isolation.

Virus was consistently isolated from the faecal samples of two hens in the T infected group from 1 to 9 weeks post infection. Such hens can be persistent virus shedders and these hens appeared healthy throughout the experiment. Shedding of virus in such fashion by apparently healthy birds could be an important means of introducing virus into new premises (Jones and Ambali, 1987).

All these findings regarding virus isolation from various tissues of hens indicate that IBV has a wide tissue tropism and has a capacity to multiply in many tissues of chickens (Cavanagh, 2003).

The tropism of Australian strains for the kidney and trachea has been studied and it was reported that there has been a drift in pathogenicity of Australian strains of IBV towards more respiratory strains (Ignjatovic *et al.*, 2002). However, our findings suggest a need to study the tropism of Australian IBV strains for other tissues.

Chapter 11

Detection of infectious bronchitis virus from the oviduct of laying hens by virus isolation and RT-PCR

11.1 Introduction

As described earlier, IBV virus particles have been detected in different parts of the oviduct by electron microscopy. IBV has been isolated from the reproductive tract of hens in clinical cases (Pradhan *et al.*, 1982; Maiti *et al.*, 1986) and has also been isolated from the oviduct (Jones and Jordan, 1971, 1972), egg yolk (Fabricant and Levine, 1951) and egg vitelline membrane (Cook, 1971) during challenge experiments. There is no record of isolation of Australian strains of IBV from the fully-functional oviduct of laying hens from field or experimental conditions, although this virus is considered a potential threat to the layer industry in Australia. Also, little work has been done on rapid detection of this virus directly from the fully-functional oviduct of IBV-infected hens.

A reverse-transcriptase polymerase chain reaction (RT-PCR) test has been developed for rapid detection of IBV from the trachea and kidney (Mardani *et al.*, 2006 a & b). However, in the past, no efforts were made to detect the virus from oviduct tissue by RT-PCR. The present study was undertaken to detect two strains of IBV, T and N1/88, from the oviduct at different intervals post-infection (p.i.). T-strain IBV was originally isolated from the kidney of laying hens (Cumming, 1962) and N1/88 strain IBV was isolated from the trachea of vaccinated broilers (Ignjatovic *et al.*, 1991).

11.2 Materials and Methods

Details of the experimental procedure have been described in chapter 5. The tissue processing for virus isolation and viral RNA isolation for RT-PCR is described in detail in chapter 2 (Section 2.10). All oviduct segments (entire oviduct) were scraped for the virus isolation. As different parts of the oviduct were also collected to study

the histopathology and cytopathology, it was difficult to avoid cross-contamination among the different regions of the oviduct.

11.3. Results and Discussion

11.3.1 Detection of IBV by virus isolation

All samples from the control group were negative for IBV. The virus was not detected in hens from either of the infected groups until day 10 p.i., except for one hen from the T strain-infected group (Table 11.1). The virus was detected in the oviduct of all hens from both the IBV challenge groups on days 10 and 12 p.i. On day 14 p.i., one hen from each of the T and N1/88 groups was positive for IBV. Virus was detected in the oviduct of one hen on each of days 16 and 18 p.i. in the T-infected group. However, in the N1/88-group, virus could only be detected in the oviduct of the hen that was out of lay and sacrificed on day 24 p.i. Virus was also detected in the oviducts of both the hens from the T-infected group which were out of lay and sacrificed on day 30 p.i. Earlier, Sevoian and Levine (1957) reported pathology in the mature oviduct on day 10 p.i. Jones and Jordan (1972) isolated the Massachusetts strain of IBV from the oviduct of hens between 5 and 11 days post-infection. Virus has been isolated also from the oviduct of young chickens (Crinion and Hofstad, 1971b) and from the pooled ovaries and oviduct of samples from field cases by Maiti and coworkers (Maiti *et al.*, 1982).

11.3.2 Comparison of virus isolation and RT-PCR

Using RT-PCR, IBV was detected in all samples which were positive by egg embryo inoculation (virus isolation). Also, samples which were negative by chicken egg embryo inoculation were negative when tested with RT-PCR. (Table11.1). There was no cross contamination between the two IBV challenge groups. Hence, we observed a similar pattern of virus detection through virus isolation in embryonated eggs and RT-PCR (Plate 11.1). Although RT-PCR is the most rapid method of diagnosis for the presence of IBV, it provides only a limited amount of information about the extent of virulence or pathogenicity of the strain of IBV.

Table 11.1 .Detection of T and N1/88 strain of IBV from oviduct samples at different days post infection by chicken embryo inoculation and RT-PCR

Days post inoculation	Hen	Detection by virus isolation in embryonated eggs		Detection by RT-PCR	
		T	N1/88	T	N1/88
2	1	-	-	-	-
	2	-	-	-	-
4	1	-	-	-	-
	2	-	-	-	-
6	1	-	-	-	-
	2	-	-	-	-
8	1	+	-	+	-
	2	-	-	-	-
10	1	+	+	+	+
	2	+	+	+	+
12	1	+	-	+	-
	2	+	+	+	+
14	1	+	-	+	-
	2	-	+	-	+
16	1	-	-	-	-
	2	+	-	+	-
18	1	-	-	-	-
	2	+	-	+	-
20	1	-	-	-	-
	2	-	-	-	-
22	1	-	-	-	-
	2	-	-	-	-
24	1	-	-	-	-
	2	-	+	-	+

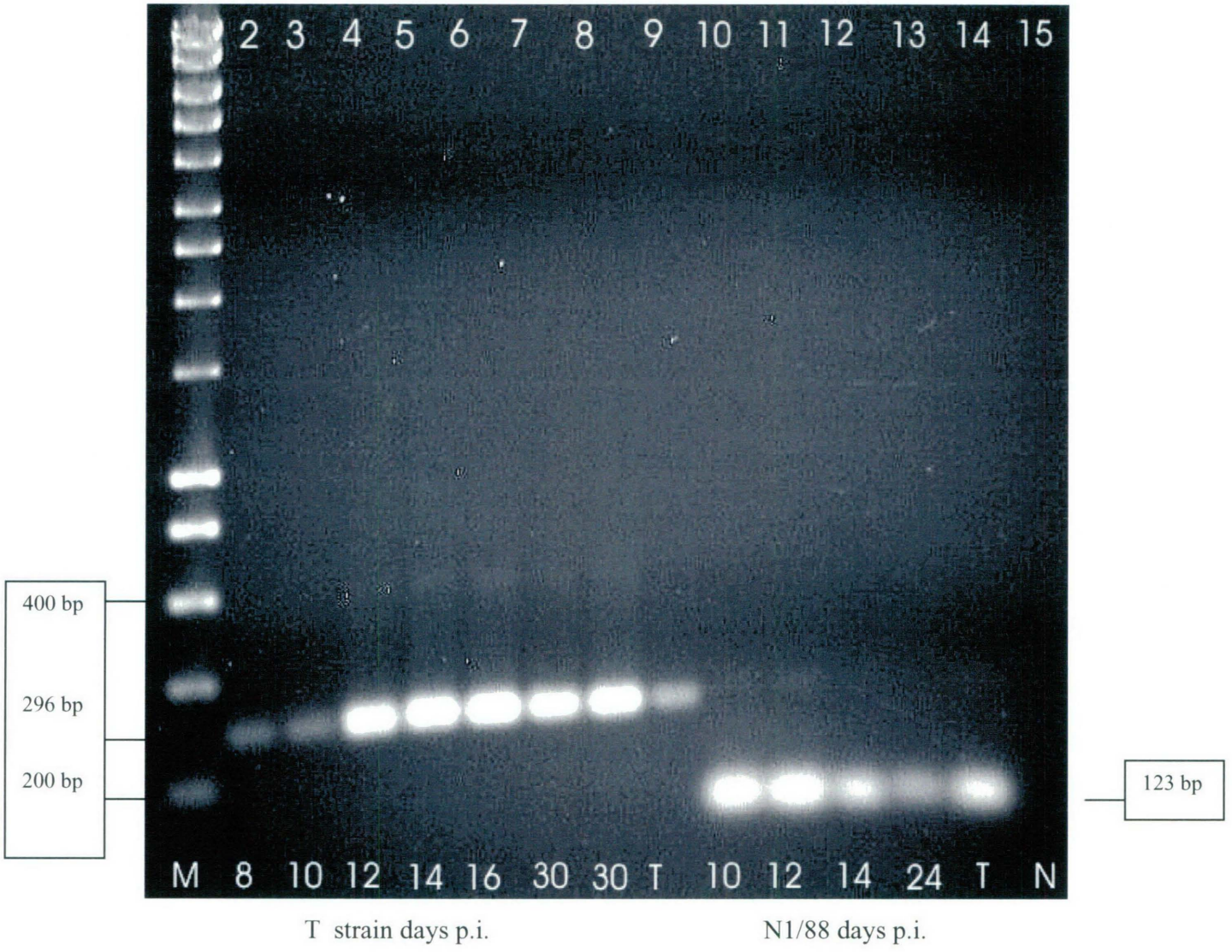


Plate 11.1: Detection of IBV by RT-PCR from oviduct samples at different days p.i. Hens were inoculated with T and N1/88 strains of IBV by the intraocular route. Lane 1, marker; lanes 2 to 6, T strain of IBV detected on 8, 10, 12, 14 and 16 days p.i., respectively; lanes 7 and 8, T strain of IBV detected on day 30 p.i.; lane 9, positive control for T; lane 10 to 13, N1/88 strain of IBV detected on 10, 12, 14 and 24 days p.i., respectively; lane 14, positive control for N1/88, lane 15, negative control

Based on the present results, it could be concluded that both techniques are equally sensitive for the detection of virus from the oviduct. In the present study, virus could not be detected in the oviduct of all the challenged hens which is indicative of the variation of susceptibility for the infection amongst the birds of the infected flock

11.3.3 Comparison of ultra structural findings and RT-PCR

IBV was isolated mostly between 10 and 14 days post infection. This correlated with the ultrastructural findings (Chapter7). However, in the T-infected group, virus particles were not observed by electron microscopy in any part of the oviduct of hens on days 8, 16 and 18 p.i. However, lymphocyte infiltration was noticed by light microscopy in those hens. This could be due to the size of the tissue sample processed for electron microscopy. Although electron microscopy is an excellent tool to study the extent of cytopathology during virus infection, RT-PCR would be the preferred technique over electron microscopy for rapid and sensitive detection of IBV from the oviduct.

11.3.4 Comparison of Australian and overseas strains with respect to uterotropism

Australian strains of IBV have been classified mainly on the basis of their tropism for the kidney and respiratory tract (Ignjatovic *et al.*, 2002). The present finding suggests that Australian strains of IBV are also uterotropic. The T-strain of IBV has been reported to be pathogenic for the oviduct of young chickens (Crinion and Hofstad, 1972a). Our findings suggest that T-strain IBV is potentially pathogenic for the fully-functional oviduct and can persist in the oviduct as long as 30 days after infection. We also observed virus particles in the oviduct of these hens at 30 days p.i. by electron microscopy (Chapter 7). T and N1/88 strains of IBV are genetically different and have been placed in subgroup 1 and subgroup 2, respectively, based on variation in their nucleocapsid (N) and S1 glycoprotein (S) gene sequences (Sapats *et al.*, 1996a; Sapats *et al.*, 1996b). Both the S and N proteins of the T (subgroup 1) strain of IBV share a high level of identity with most of the European and American strains of IBV. However, both of these proteins in the N1/88 strain share only a low level of identity with European and American strains (Ignjatovic *et al.*, 2006). Both of the IBV strains,

T and N1/88, despite of their antigenic and genetic disparity, have the capacity to replicate in the oviduct of hens of different ages and breeds. It is, however, interesting that very few efforts have been made to establish a direct correlation between IBV and the Haugh unit problems or shell quality problems reported in the literature. IBV has been isolated from the trachea or kidney of layers having a history of low egg production. In almost all instances, the presence of virus in the trachea or kidney was correlated with the decreased egg production. However, it is equally important to isolate or detect the virus from the oviduct to ascertain the cause of egg quality and production problems amongst the layer flock. In the present study, IBV was isolated mostly from the oviduct of hens showing low albumen heights but no egg shell quality problems. Hence it would not be prudent to suggest a direct relation between infection with IBV and poor egg shell quality. It is necessary to isolate or detect the virus from the shell-forming regions of the oviduct to confirm the exact cause of deterioration in egg shell quality even in an IBV susceptible flock.

In the past, a drop in egg production has been recorded in the hens challenged with the Massachusetts strain of IBV (Cook, 1971) but this was not recorded in the present trial. However, it would be interesting to study the persistence of the Massachusetts strain of IBV in the oviduct, as the strain has been reported to be more pathogenic as compared to T (Crinion and Hofstad, 1972a).

11.4. Conclusion

In the T-strain infected group, out of 26 hens killed at different days p.i., oviducts of 10 hens were positive for IBV. However, in the N1/88 group, out of 24 hens killed, IBV was detected in the oviduct of 6 hens. Both these Australian strains of IBV have varying degrees of uterotropism and it is possible that all the Australian strains of IBV have pathogenicity for the fully-functional oviduct. However, further research needs to be done to confirm this fact. Given the large number of existing IBV strains, it would not be possible to screen all the strains for uterotropism *in-vivo*. However, it may be possible to study this aspect by combining *in-vivo* and *in-vitro* experiments.

Chapter 12

General discussion and conclusions

Even after 60 years of on-going virus isolation and development of a number of vaccines, there is continuous emergence of new strains due to the ability of infectious bronchitis virus (IBV) to mutate which makes the study of this virus very complicated. Infectious bronchitis virus continues to be a major concern to the poultry industry all around the world. Most of the strains of IBV have been classified as nephropathogenic or respiratory although the virus has the potential to multiply in other organs as well. Australia has an interesting history of infectious bronchitis virus which starts from the isolation of a highly nephropathogenic strain, T strain, in the 1960s (Cumming, 1962). Recently, most of the IBV strains isolated from the field have been respiratory (Ignjatovic *et al.*, 2002). However, it should be mentioned that all recent strains of IBV have been isolated from broiler flocks.

The interaction of IBV with the fully-functional oviduct has been a matter of conjecture. Broadfoot (1956) reported abnormal oviducts in chickens that were infected at a young age. The first extensive study was then carried out by Sevoian and Levine (1957) who reported deterioration of egg production and shell quality following exposure to IBV. Cook (1971) also conducted an IBV challenge trial in laying hens confirming the deterioration of egg shell quality and production. Even after using a variety of vaccine strains, the extent of protection offered to the oviduct is still speculative.

In Australia, there is still much debate regarding IBV and egg quality. Many IBV strains have been isolated over the past few years, apparently the result of independent mutation (Details are described in chapter 1). Most frequently, virus has been isolated from either the kidney or trachea. The tropism of most of the Australian IBV strains has been studied by Ignjatovic and coworkers (Ignjatovic *et al.*, 2002). There has been a drift in pathogenicity of the virus and most of the virus strains recently isolated came from the respiratory tract. However, there is no record regarding the extent of

tropism for the reproductive tract although IBV is known to have detrimental effects on egg production and quality (see reviews by Dhinakar Raj and Jones, 1997a; Ignjatovic and Sapats, 2000; Cavanagh, 2003).

Chapter three describes the comparative pathogenicity of IBV for laying hens and cockerels. The severity of microscopic lesions in hens was high as compared to the cockerels which contradicts the earlier finding by Cumming (1967) that cockerels are more susceptible than hens to IBV infection. The oviduct of laying hens even at 65 weeks of age was susceptible to IBV infection. However, to avoid the confounding effects of age of the hens, a further trial was conducted using laying hens with fully functional oviducts at 30 weeks of age.

It is concluded from chapter four that rearing phase vaccination does not offer full protection to the oviduct. However, the Hyline Grey hens used in that experiment were 110 weeks old, so these results might have been influenced by the hens' age as changes occurring in the oviduct as hens get older and loss of immunity cannot be excluded. Although it has been shown that regular revaccination can affect egg shell quality (Sulaiman *et al.*, 2004) and rearing phase vaccination can protect egg internal quality (Jolly, 2005), comprehensive microscopic studies at regular intervals post infection are essential to study the cellular changes occurring in the oviduct during vaccination/challenge experiments pertaining to IBV effects on internal egg quality. The results reported by Sulaiman *et al.* (2004) and Jolly (2005) regarding the effects of IBV on the oviduct of vaccinated hens cannot be compared with those of other authors, owing to a scarcity of literature regarding the response of the normal and fully-functional oviduct during IBV infection.

Chapter five describes the overall gross observations and histopathology of the Harderian gland, trachea and kidney of unvaccinated Isa Brown laying hens, following challenge with two strains of IBV. There was a rise in plasma IBV antibody titres after the infection in both challenge groups which indicates that both the treatment groups experienced a significant virus infection. Also, the control group remained negative for IBV antibodies throughout the experiment. The antibody titres were at highest at 10 weeks post infection in both the groups, a finding similar to that

reported earlier by Animas and coworkers (Animas *et al.*, 1994). The antibody titre of T strain-infected hens was higher than the N1/88 infected hens.

Both the strains of IBV were equally pathogenic for the Harderian gland which indicates that both the strains have a similar predilection for the Harderian gland. The avian Harderian gland plays a vital role in local production of antibodies for protection of the upper respiratory tract against air borne viruses such as IBV (Toro *et al.*, 1996). Association of local immunoglobulins with resistance against IBV has been reported earlier (Baba *et al.*, 1990; Gallego *et al.*, 1992). Plasma cell infiltration and lymphocyte infiltration intensified in the Harderian gland during the IBV infection and this has been reported earlier (Dalevaar and Kouwenhoven, 1976; Toro *et al.*, 1996). The increase in plasma cell number and lymphocyte infiltration strongly suggests the participation of these cells in the immune response. The plasma cell is a major immunoglobulin-secreting cell. The IBV specific response of IgA in lachrymal fluid has been described earlier by Toro and coworkers (Toro *et al.*, 1997). IgA antibody can agglutinate particulate antigens and plays an important role in virus neutralization. IgA antibody also has a unique capacity of acting inside the cell, although the mechanism by which this neutralization occurs is not still clear. Also, under experimental conditions, increased plasma cell and lymphocyte infiltration has been observed during the attack of inert antigens such as sheep RBCs. Under field conditions, the increase in plasma cell and lymphocyte infiltration could be due to environmental pollutants. It is important to isolate IBV from the Harderían gland to confirm its presence and to rule out the possibility of environmentally-induced histopathological changes in the Harderian gland. Such studies have been conducted by Toro and coworkers (Toro *et al.*, 1996) using the indirect immunofluroscence test and chicken embryo inoculation to detect a vaccine strain of IBV in the Harderian gland. It would be interesting to study the persistence of virulent strains of virus in the Harderian gland. The occurrence of globular leucocytes in the Harderian gland at regular intervals, observed in the present study, has not been reported earlier.

As the name indicates, IBV is known to have a primary predilection for the respiratory tract. The virus initially replicates in the upper respiratory tract and then disseminates to the other tissues. Being epitheliotropic in nature, the virus replicates in ciliated and mucus-producing cells (Nakamura, *et al.*, 1991). In the present

experiment, there was loss of cilia and hypertrophy of mucus glands as has been reported earlier (Nakamura *et al.*, 1991; Arshad *et al.*, 2003). The cilia loss and degeneration of mucus glands appears to be an important feature as the mucociliary system helps to remove foreign antigens like dust or bacteria from the respiratory tract (Ross and Corrsin, 1974). The damage caused by IBV to the respiratory tract could make it easier for bacteria to invade the tissue which may complicate the respiratory infection. Earlier, Williams and coworkers (Williams *et al.*, 1985) reported increased mortality in 8 day old specific pathogen free chickens during a mixed infection of *E. coli* and IBV in the respiratory tract. The IBV strain N1/88 induced severe pathology in the trachea as compared to the T strain. N1/88 strain has been reported to have a predilection for the respiratory tract of chickens (Sapats, *et al.*, 1996b). Out of 50 hens from both the challenge groups, thirteen hens from the T group and 15 hens from the N1/88 group showed respiratory symptoms. Such variation in respiratory symptoms amongst the infected group was also observed by Munner and coworkers (Munner *et al.*, 1986) during infection of layers with the Arkansas strain of IBV.

The T strain of IBV induced pronounced histopathology in the kidneys of unvaccinated Isa Brown laying hens, as compared to the N1/88 strain. Pathology was also observed in the kidneys of N1/88-infected White Leghorns, Isa Brown hens and also in White Leghorn cockerels, which indicates that the N1/88 strain of IBV has the potential to induce lesions in the kidneys of adult birds. However, in the present experiment, there was no swelling of the kidneys in any of the hens killed from either the T- or N1/88-infected group. Although lesions such as necrotic foci and collecting duct dilatation were observed in the kidneys of White Leghorn hens in both the infected groups in our preliminary trial, such microscopic lesions were not observed in the trial with Isa Brown hens. Also, Roberts (2005) reported enlarged kidneys in T-infected White Leghorn hens which were not observed in the Isa Brown hens. This could be attributed to differences in breed or age of the hens. There were no significant differences in right or left kidney weights or right and left kidney weights as a percentage of body weight amongst the virus challenged and control groups. Also, plasma sodium and potassium values did not differ amongst the groups. Afanador and Roberts (1994) found increased kidney weights and kidney weights as a percentage of body weight after challenging 4 weeks old male broilers with T strain IBV. The differences between these two studies were both the ages and sex of the

chickens. Difference in observations in the above studies underlines the role of intrinsic influences such as age and sex in the pathogenesis of IBV nephritis. However, other extrinsic factors such as cold stress may also exacerbate IB nephritis. Chubb and coworkers (Chubb *et al.*, 1976) reported more than 50% mortality in 6 week old chickens subjected to IBV combined with cold stress. The effect of cold stress on IB-induced nephritis in chickens at 30 weeks of age remains to be determined and needs further investigation. There were no significant differences among the body weights of the treatment groups.

Almost all parts of the oviduct show cellular changes during the transit of the egg through the oviduct. The main aim of killing control hens at different egg positions in the oviduct during the present study was to differentiate between the normal physiological changes occurring during egg formation and the virus-induced pathological changes in the oviduct. To best of our knowledge, this is the first study to compare the IBV-infected oviduct with the normal oviduct at various egg positions.

Understanding of the interaction of IBV with the oviduct has been somewhat limited. IBV effects on egg production and quality have been frequently quoted by many researchers (see reviews by Dhinakar Raj and Jones, 1997a; Ignjatovic and Sapats, 2000; Cavanagh, 2003; Cavanagh, 2007). With respect to the effects of IBV on the oviduct and egg shell quality of laying hens, most of the reviews refer to the study Sevoian and Levine (1957). However, in Sevoian and Levine's study, no attempts were made to isolate virus from the oviduct to provide confirmation of direct effects of IBV on the fully-functional oviduct. Also, a mixture of three strains was used and the serotypes were not mentioned. Sevoian and Levine (1957) observed pathology in the oviduct (although in different regions of the oviduct from those in which pathology was observed in the present study) of infected and stressed hens. This raises the question as to whether thin or soft-shelled eggs observed during Sevoian and Levine's trial were a direct IBV effect or a disease-induced stress effect? Stress is one of the major factors responsible for deterioration in egg shell quality (Brackpool, 1995, Roberts, 2004). Nevalainen (1964) observed pathology in the oviduct of calcium-deprived hens which were out of lay. IBV infection reduces the feed intake in hens (Sevoian and Levine, 1957), so considering Nevalainen's (1964) conclusion, reduced feed intake and subsequent reduction in calcium supply via the feed to

individual hens could also induce pathology in the shell gland. Hence it is important to conduct a differential diagnosis between stress and IBV-induced pathology in the shell gland which can be done only by demonstration of the presence of virus in the oviduct. In the present study, feed intake was significantly lower in the T-infected group during 2 and 4 weeks post infection and pathology was not recorded in the shell gland of many hens except for those which were out of lay. Virus particles were observed in the shell gland of hens which were out of lay. This was further confirmed by electron microscopy, virus isolation and RT-PCR experiments. Hence it is established that pathological lesions in those hens were caused by IBV.

Egg shell quality defects such as thin, soft or corrugated eggs reported in the literature during IBV outbreaks could be the indirect effect of general illness amongst the hens. It is also possible from the observations of the present study that Australian strains of IBV do not cause deterioration of egg shell quality in the fully-functional oviduct of Isa Brown hens. As the American Massachusetts strain of IBV is considered pathogenic for the oviduct of young chickens, it is important to study whether this strain of virus has the same effects on the mature, fully-functional oviduct. It is very surprising that so few efforts have been made between 1957 and 2007 to investigate IBV-induced pathology in the fully-functional oviduct and simultaneous effects on egg shell quality. Under commercial field conditions, IBV has been isolated from the oviduct of layer flocks with a history of decreased egg production (Maiti, *et al.*, 1984, Wahi *et al.*, 1990; Christopher *et al.*, 1996). However, none of the above reports mentioned abnormal egg shell quality of those flock from which IBV was isolated. All the above reports are from India and it is important to note that Indian strains of IBV, isolated from the oviduct of birds from flocks with a history of decreased egg production but without deterioration in egg shell quality, are antigenically similar to the Massachusetts strain M41 of IBV (Pradhan *et al.*, 1982; Christopher *et al.*, 1996). In another study, Jones and Jordan (1971) demonstrated the presence of IBV in the uterus of experimentally-infected hens by immunofluorescence and virus was detected from various parts of the oviduct, although nothing was mentioned about its effect on egg shell quality. The assumption that thin albumen provides a poor template for egg shell deposition, resulting in wrinkled eggs needs further investigation as, in present study, wrinkled eggs were not observed in any of the infected groups despite thin albumen being produced during IBV infection.

The results of the present study suggest that IBV replicates in the upper parts (albumen-forming regions) of the oviduct and thereby affects egg internal quality. However, whether IBV has direct effects on the formation of thin, corrugated or rough shelled eggs (which is considered to be a typical sign of IB) remains uncertain. The deterioration of albumen quality and Haugh units in infected hens could be due to disturbance in the physiology of cell organelles, as described in chapter 7. Individual body performance and functioning of such cell organelles can vary from hen to hen. Hence, some hens recover normal function despite severe damage to the cells whereas others cannot. It remains difficult to determine the exact reason for cessation of egg production in some hens, but non-functional Golgi bodies, dilated RER and hence possible alteration in protein synthesis of affected cells, could be contributory factors. Hormonal studies are required to prove or disapprove the suggestion that insufficient sex steroid stimulation results in cessation of egg production during IBV infection. The role of cilia in the oviduct is also very important for sperm motility (Johnson, 2000) and movement of the ovum away from the ovary (Fujii, *et al.*, 1981). However, the role of cilia during egg passage through the magnum and the shell-forming regions of the oviduct is still speculative and needs further investigation. Such knowledge may explain the cause of the stagnated yolk material observed in the oviduct during this experiment. Also, irreversible atrophy of the oviduct during IBV infection could be the cause of cessation of egg production.

It has been assumed that release of any secretory product in the oviduct is stimulated by the local stimulus of the presence of the yolk (Gilbert, 1969) in a manner similar to the control of secretion in digestive tract which is related to the presence of food. However, in the present study, yolk material was found in the oviduct of hens which were out of lay, which underlines the need for further studies to confirm the earlier assumption regarding a local stimulus.

Chapter 9 suggests that IBV has effects on egg shell quality but these effects were only in terms of egg shell colour and shape index. However, long-lasting effects were observed on egg internal quality of the T strain-infected group. Hence, based on the microscopic findings and results of egg internal quality, both T and N1/88 strains of IBV can cause significant effects on egg internal quality in terms of albumen height

and Haugh units. It was, however, interesting to note the loss of shell colour during IBV infection without any sign of pathology in the shell gland (the shell gland is the major site for deposition of egg shell pigment). This would not be of any significance for the egg markets in Asia or North America as white eggs are preferred in such countries. However, in Australia and Europe, brown shelled eggs are the norm. Thin and corrugated egg shells were not observed in either of the infected groups and such observations were also recorded earlier by Berry (1964).

Burmester and Card (1939) concluded that the production of chalazae depends upon the mechanical separation of strands of mucus derived from cells of the magnum, although Scott and Huang (1941) were of the opinion that the infundibulum has a role in the formation of the chalazae. The separation of yolks from albumen during egg breakout, observed during this trial, could be due to the cytopathology in the infundibulum and magnum, which supports the latter finding that both the infundibulum and magnum have roles in the formation of chalazae.

Age of hens at the time of IBV infections would be expected to have an influence on the pathogenesis of IBV for the reproductive tract. However, Crinion *et al.* (1971b), after infecting baby chickens, found 26 % non-layers with 83 % non-patent and 17% patent oviducts, which supports the view of Broadfoot *et al.* (1956) that the oviduct of younger chickens is more likely to be affected by IBV than that of older chickens. Jones and Jordan (1972) found abnormal or poorly-formed oviducts in hens challenged with the M41 strain of Massachusetts at day old. Crinion and Hofstad (1972a) reported that the Australian T strain of IBV induced pathological lesions in the immature oviduct. However, it is still unclear if any of the Australian strains of IBV has long term effects like those reported for the Massachusetts strain and further study needs to be done in this context. Jones and Ambali (1987) also reported variation in pathogenicity of IBV strains in day-old chicks. It would be interesting to study the extent of pathogenicity caused by the N1/88 strain in the immature and undifferentiated oviduct.

Overall, T-strain was more pathogenic for the fully-functional oviduct as compared to N1/88. In the present study, out of 50 infected hens in each challenge group, only three hens, one from the N1/88-infected group with a non-patent oviduct and two

from the T-infected group with patent oviducts, were out of lay. The T and N1/88 strains of virus represent two different subgroups; strain T belongs to subgroup 1 and N1/88 belongs to subgroup 2 (Sapats, 1996 a& c). Both the strains of IBV induced moderate to severe cytopathology in the albumen-forming regions of the oviduct. This indicates that most of the strains belonging to subgroup 1 and subgroup 2 could have tropism towards the reproductive tract. Besides T and N1/88, there are 61 strains of IBV in Australia and it would be a difficult task to assess their uterotropism in the fully-functional oviduct owing to the labour and time required. It could be investigated, however, by assessing the pathogenicity of other IBV strains in oviduct organ culture. Oviduct organ culture has been used by some authors to study the virulence of IBV (Pradhan *et al.*, 1986; Dhinakar Raj and Jones, 1996a). Different parts of the oviduct have also been used in organ culture experiments to demonstrate the replication of IBV (Peters *et al.*, 1979). Field studies could then be conducted following selection of IBV strains on the basis of oviduct organ culture results.

The microscopic lesions in the oviduct of the White Leghorns were more severe than those observed in Isa Brown hens. However, from these results, it would not be prudent to conclude that White Leghorns are more susceptible to IBV infection owing to the age difference between the birds used in the two studies (65 weeks for White Leghorns and 30 weeks for the Isa Brown hens at the commencement of the experiment). The pathology which was consistent in the shell-forming regions of the White Leghorn hens, tubular shell gland and shell gland pouch, was not observed in the Isa Brown hens. Even though the shell-forming regions showed microscopic lesions in the White Leghorns, there were no significant effects on their egg shell quality (Roberts, 2005)

Reproductive and respiratory tropism of T and N1/88 strains of IBV were not directly related in the present study. Despite severe respiratory signs, the reproductive performance of some hens remained unaffected. Similar observations were reported by McMartin (1968b) during the infection of a British isolate of the Massachusetts serotype of IBV. Also, it was interesting that the hens which were out of lay and had virus particles in their oviduct did not show any respiratory symptoms during 3 to 9 days following virus infection. Both T and N1/88 strains of IBV varied in their effect on the respiratory tract. Many hens from both the infected groups showed no clinical

respiratory signs following virus infection. Similar observations were reported by McMartin (1968b). The lack of uniformity in the respiratory signs in infected hens was recorded also by Munner *et al.* (1986). Hens which had not shown any external clinical respiratory symptoms showed pathological lesions and virus was also isolated from their respiratory tract. Also, there was no direct relationship between presence of virus in the kidney and in the oviduct. It appears from the present study that reproductive performance of the Isa Brown layer flock to the IBV infection was irregular as some hens were completely unaffected and did not even show the inferior albumen quality. This could be attributed to variation in the natural resistance against IBV infection. Cumming (1963) described 50% loss of egg production during natural T strain IBV infection in White Leghorns. However, in the present experiment, there was no deterioration of egg production. This could be either because of the small number of hens used in the experiment or the result of breed differences. Earlier, Cumming (1963) noted that Australorps were less severely affected compared to White Leghorns during a natural outbreak of avian nephrosis.

The present study partially supports the *in-vivo* findings of Peters *et al.* (1979) who found that, unlike kidney organ cultures, magnum and uterus organ cultures were susceptible to IBV infection regardless of the age of chicken. The kidneys of Isa Brown hens after IBV infection showed a considerable amount of resistance compared to the infundibulum and magnum regions of the oviduct. The uterus (shell gland pouch) was the least affected.

In Australia, all layer flocks are currently vaccinated for IBV. However, the level of protection offered to the oviduct by current vaccines is still speculative. In earlier studies by Box *et al.* (1988) and Sulaiman *et al.* (2004), regular revaccination (which is practised in most parts of the world amongst layer flocks) can negatively affect shell quality. If this is the case, it would be interesting to study whether the deterioration of egg shell quality in frequently-vaccinated hens is stress or virus related. In another study, Jolly (2005) observed that rearing phase vaccination can offer protection for IBV effects on internal egg quality but not for egg production throughout the laying cycle. It is important to investigate the breadth of protection offered by current vaccines by histopathology and virus isolation studies. Also it would be interesting to study the *in- vivo* effects of vaccine strain on the oviduct. The

effects of IBV on egg shape index need further investigation. The watery albumen observed during the infection amongst the infected groups could be the cause of change in egg shape. The shell gland of the oviduct is a very complex structure which secretes a range of matrix proteins. Extensive immunohistochemical and immunocytochemical studies are essential to confirm whether or not IBV has any effect on the egg shell matrix proteins.

Persistence of IBV in the faeces of laying hens has been reported previously by Cook (1968) and Alexander and Gough (1977). The virus was isolated from cloacal swabs for as long as 18 and 30 weeks post-infection. In the present experiment, faecal samples were collected for 9 weeks post-infection and two hens out of five in the T group were persistently positive. Such hens, even if they appear healthy, can act as persistent shedders of the virus. Under field conditions, introduction of IBV to a layer farm could be due to introduction of infected hens or via movement of people (Cumming, 1969). The isolation of virus from faecal samples would be an efficient way to check the status of a flock for virus shedding. At the same time, pathogenic strains of virus should be differentiated from vaccine strains. Shedding of vaccine strains of IBV has been reported earlier by Naqui *et al.* (2003). Detection of virus in faeces could be done rapidly by molecular tests such as restriction length fragment polymorphism or sequencing of genes (Mardani *et al.*, 2006a & b). Both strains of the virus were also isolated from lower gut contents in the present study. The recovery of virus from other tissues besides respiratory tract and kidney could be due to viraemia resulting in spread of virus to other tissues.

Chapter 11 describes a comparative analysis between two diagnostic tests, RT-PCR and virus isolation. Both techniques were equally sensitive for virus detection from the oviduct. All oviduct samples from infected groups which were positive by chicken embryo inoculation were also positive for RT-PCR at different days post-infection. Molecular techniques such as RT-PCR are more rapid and less laborious than chicken embryo inoculation. However, the results of RT-PCR do not necessarily correlate with the infectivity of the virus. Both techniques are less time consuming than electron microscopy. However, electron microscopy is an excellent tool for studying the extent of cytopathology in the tissues and to confirm the presence of virus particles. RT-PCR would be a convenient tool to check for any cross-contamination

between flocks during experimental trials. Although egg embryo inoculation and RT-PCR were found to be equally sensitive for virus detection in the oviduct, it is important to conduct such comparative studies with other organs such as the trachea and kidney. It has been suggested that RT-PCR is very sensitive and could detect a smaller quantity of virus present in tissue than could egg embryo inoculation. In the present trial it is possible that scraping from the relatively large surface area of the oviduct yielded sufficient inoculums to produce similar positive results from chicken embryo inoculation and RT-PCR.

Sevoian and Levine (1957) found that IBV induces pathology in the oviduct at approximately the 10^{th} day p.i. The present study supports this earlier finding as virus particles, along with cytopathological and histopathological changes were recorded at 10 days p.i. However, virus was isolated from the oviduct from the 8^{th} day p.i. onwards. This could be the result of the eclipse phase of virus replication.

The Australian strains of infectious bronchitis virus used in the present study appear to have a predilection towards the albumen-forming regions of the mature reproductive tract of Isa Brown laying hens despite the genetic or antigenic variation between the T and N1/88 strains. T strain of IBV, in particular, can produce long term or even permanent damage to this region of the oviduct. The two IBV strains also have the capacity to induce pathology in the shell-forming regions of some hens. Both strains were found to affect shell quality, but only in terms of shell colour and shape index, not in terms of soft-shelled, thin or corrugated eggs. Effects such as soft-shelled or corrugated eggs, reported earlier in the literature during IBV infection, could be due to the indirect effects of IBV (such as general illness). The T strain of IBV was found to persist in faecal samples up to 9 weeks post infection.

References

Adzhar, A., Shaw, K., Britton, P. & Cavanagh, D. (1996). Universal oligonucleotides for the detection of infectious bronchitis virus by the polymerase chain reaction. *Avian Pathology* **25**, 817-836.

Australian Bureau of Statistics (2006). Agricultural Commodities, Australia, 2004-05. Cat no. 7121.0

Afanador, G. & Roberts, J. R. (1994). Effect of nephropathogenic infectious bronchitis viruses on renal function in young male broilers chickens. *British Poultry Science* **35**, 445-456.

Aitken, R. N. C. & Johnston, H. S. (1963). Observations on the fine structure of infundibulum of the avian oviduct. *Journal of Anatomy* **97**, 87-99.

Aitken, R. N. C. (1971). The oviduct in *Physiology and biochemistry of domestic fowl.* Vol 3, Eds D.J. Bell and B.M. Freeman. Acedemic press London. pp 1237-1290

Albassam, M. A., Winterfield, R. W. & Thacker, H. L. (1986). Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. *Avian Diseases* **30**, 468-476.

Alexander, D. J. & Gough, R. E. (1977). Isolation of avian infectious bronchitis virus from experimentally infected chickens. *Research in Veterinary Science* **23**. 344-347

Alexander, D. J., Gough, R. E. & Pattison, M. (1978). A long-term study of the pathogenesis of infection of fowls with three strains of avian infectious bronchitis virus. *Research in Veterinary Science* **24**, 228-233.

Alvarado, I. R., Villegas, P., El-Attrache, J. & W., J. M. (2006). Detection of Massachusetts and Arkansas Serotypes of Infectious Bronchitis Virus in Broilers.

Avian Diseases **50**, 292-297.

Ambali, A. G. & Jones, R. C. (1990). Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. *Avian Diseases* **34**, 804-881.

Animas, S. B., Otsuki, K., Hanayama, M., Sanekata, T. & Tsubokura, M. (1994). Experimental infection with avian infectious bronchitis virus (Kagoshima-34 strain) in chicks at different ages. *Journal of Veterinary Medical Science* **56**, 443-447.

Arshad, S. S., Al-Salihi, K. A. & Noordin, M. M. (2003). Ultrastructural pathology of trachea in chicken experimentally infected with infectious bronchitis Virus-MH-5365/95. *Annals of Microscopy* **3**, 43-47.

Baba, T., Kawata T., Masumoto K. & T., K. (1990). Role of the harderian gland in immunoglobulin A production in chicken lacrimal fluid. *Research in Veterinary Science* **19**, 20-24.

Bain, J. M. & Hall, J. M. (1969). Histological changes in oviduct during chalaza formation. *Australian Journal of Biological sciences* **22**, 653-656.

Baird, T., Solomon, S. E. & Tedstone, D. R. (1975). Localisation and characterization of egg shell porphyrins in several avian species. *British Poultry Science* **16**, 201-208

Bakst, M. R. & Howarth (1975). SEM preparation and observations of the hen's oviduct. *The anatomical record* **181**, 211-225.

Bakst, M. R. (1978). Scanning electron microscopy of the oviduct mucosa apposing the hens ovum. *Poultry Science* **57**, 1065-1069.

Barr, D. A., Reece, R. L., O' Rurke, D., Button, C. & Faragher, J. T. (1988). Isolation of Infectious bronchitis virus from the flock of racing pigeons. *Australian Veterinary Journal* **65**, 228.

Bayry, J., Goudar, M. S., Nighot, P. K., Kshirsagar, S. G., Ladma, B. S., Gleb, J., Ghalsasi, G. R. a. & Kolte, G. N. (2005). Emergence of a nephropathogenic avian infectious bronchitis virus with a novel genotype in India. *Journal of clinical Microbiology* **43**, 916-918.

Beach, J. R. & Schalm, O. W. (1936). A filtrable virus distinct from that of laryngeotracheitis virus. *Poultry Science* **15**, 199-206.

Beaudette, F. R. & Hudson, C. B. (1937). Cultivation of the virus of Infectious bronchitis. *Journal of American Veterinary and Medical association* **90**, 51-58.

Berry, D. M. (1965). Inactivated infectious bronchitis vaccine. *Journal of Comparative Pathology* **75**, 409-415

Biswal, G. (1954). Additional Histological Findings in the chicken reproductive tract. *Poultry Science* **33**, 843-851.

Boltz, D. A., Nakai, M. & Bahr, J. M. (2004). Avian Infectious Bronchitis Virus: A Possible Cause of Reduced Fertility in the Rooster. *avian Diseases* **48**, 909-915.

Bourne, F. J., Davison, T. F., Morris, T. R. & Payne, L. N. (1996). Research priorities in future in, *Poultry immunology*, Poultry Science Symposium series, Vol 4 Eds T.F. Davison, T.R. Morris& L.N.Payne pp. 419-432. Abington:Carfex.

Boursnell, M. E. G., Binns, M. M., Brown, T. D. K., Canavag, D. & Tomley, F. M. (1989). Moleculor biology of avian infectioius bronchitis virus. *Veterinay Microbiology and Immunology* **5**, 65-82.

Box, P. G., Beresford, A. V. & Roberts, B. (1988). Protection of laying hens against infectious bronchitis with inactivated vaccines. *The Veterinary Record* **106**, 264-268.

Brackpool, C. E. (1995). Egg shell ultrstructure as an indicator of egg shell quality in laying hens. In *Animal Physiology*. Armidale: University of New England.

Breen, P. C. & De Bruyn, P. P. H. (1969). The Fine Structure of the secretory cells of the Uterus (Shell Gland) of the Chicken. *Journal of Marphology* **128**, 35-66.

Broadfoot, D. I., Pomeroy, B.S., Smith, W.M. (1956). Effects of infectious bronchitis in baby chicks. *Poultry Science* **35**, 757-762.

Bronzoni, R. V. M., Pinto, A. A. & Montassier, H. J. (2001). Detection of infectious bronchitis virus in experimentally infected chickens by an antigen-competitive ELISA. *Avian Pathology* **30**, 67-71.

Burmester, B.R. & Card, L.E. (1939). The effect of resecting the so-called chalaziferous region of the hen's oviduct on the formation of the subsequent eggs. *Poultry science* **18**, 138-145.

Butcher, G. D., Winterfield, R. W. & Shapiro, D. P. (1990). Pathogenesis of H13 nephropathogenic infectious bronchitis virus. *Avian Diseases* **34**, 916-921.

Butler, E. J., Curtis, M. J., Pearson, A. W. & McDougall, J. S. (1972). Effect of infectious bronchitis on the structure and composition of egg albumen. *Journal of science food and Agriculture* **23**, 359-369.

Callison, S. A., Jackwood, M. W. & Hilt, D. A. (2001). Molecular characterization of infectious bronchitis virus isolates foreign to the United States and comparison with United States isolates. *Avian Diseases* **45**, 492-499.

Callison, S. A., Hilt, D. A., Boynton, T. O., Sample, B. F., Robison, R., Swayne, D. E. & Jackwood, M. W. (2007). Develpoment and evaluation of real time taqman RT-PCR assay for the detection of Infectious bronchitis virus from infected chickens. *Journal of Virological Methods* **138,** 60-65.

Canavag, D., Darbyshire, J. H., davis, P. & Peters, R. W. (1984). Induction of humoral neutralizing and heamagglitunation inhibitating antibodies by spike proteins of avain infectious bronchitis viurs. *Avian Pathology* **13**, 573-583.

Canavag, D., davis, P. J., Darbyshire, J. H. & Peters, R. W. (1986). Coronavirus IBV : Virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus nutrilalizing or heamaglutination inhibiting antibody or induce chicken tracheal protection. *Journal of general virology* **67**, 1435-1442.

Canavag, D. & Naqui, S. (1997). Infectious bronchitis, in *Diseases of poultry*, 10 edn. Eds B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif, Iowa State University Press, Ames, Iowa. pp 511-526

Cavanagh, D. & Davis, P. J. (1988). Evolution of avian coronavirus IBV: sequence of the matrix glycoprotein gene and intergenic region of several serotypes. *Journal of General Virology* **69**, 621-629.

Cavanagh, D. (2003). Severe acute respiratory syndrome vaccine develpoment: experiences of vaccination against avian infectious bronchitis coronavirus. *Avian Pathology* **32**, 567-582.

Cavanagh, D. (2005). Coronaviruses in poultry and other birds. *Avian Pathology* **34**, 439-448.

Chandra, M. (1988). Comparative nephropathogenecity of infectious bronchitis virus in bursectomized and nonbursectomized chickens. *American Journal of Veterinary Research* **49**, 831-834.

Chen, B. Y., Hosi, S., Nunoya, T. & Itakura, C. (1996). Histopathology and immunohistochemistry of renal lesions due to infectious bronchitis virus in chicks. *Avian Pathology* **25**, 269-283.

Chen, B. Y. & Itakura, C. (1996). Cytopathology of chick renal epithelial cells experimentally infected with avian infectious bronchitis virus. *Avian Pathology* **25**, 675-690.

Chen, B. Y. & Itakura, C. (1997). Histopathology and immunochemistry of renal lesions due to avian infectious bronchitis virus in chicks uninoculated and previously

inoculated with highly virulent infectious bursal disease. *Avian Pathology* **26**, 607-324.

Chew, P. H., Wakenell, P. S. & Farver, T. B. (1997). Pathogenicity of attenuated infectious bronchitis viruses for oviducts of chickens exposed in ovo. *Avian Diseases* **41**, 598-603.

Chong, K. T. & Apostolov, K. (1982). The pathogenesis of nephritis in chicken induced by infectious bronchitis virus. *Journal of Comparative Pathology* **92**, 199-211.

Christopher, S. D., Sheela, S., Veni, S., Rajashree, B. R., Murugan, M., Rajendran, M. P., Anbumani, S. P. & Doraiswami, J. (1996). An outbreak of infectious bronchitis in poultry: Epidemiology, clinicopathology and isolation and identification of virus. *Indian Journal of Animal sciences* **66**, 755-759.

Chubb, R. C. & Ma, V. (1974). Some observations on the propogation of avian infectious bronchitis virus in tissue culture. *Australian Veterinary Journal* **50**, 63-68.

Chubb, R. C., Wells, B. A. & Cumming, R. B. (1976). Some immunological aspects of a recent Australian isolate of infectious bronchitis virus. *Australian Veterinary Journal* **52**, 378-381.

Condron, R. J. & Marshall, A. T. (1985). Pathogenesis of Infectious bronchitis nephritis 2. Studies of water and electrolyte balance in colostomised chickens. *Avian Pathology* **14**, 509-520.

Condron, R. J. & Marshall, A. T. (1986). Patogenesis of Infectious bronchitis nephritis: Morphometric analysis of kidney proximal tubular epithelium in chickens. *Journal of Comparative Pathology* **96,** 47-61.

Conrad, R. M. & Scott, H. M. (1938). The Formation of the Egg of the Domestic Fowl. *Physiological Reviews* **4**, 481-493.

Cook, J. K. A. & Garside, J. S. (1967). A study of the infectious bronchitis status of a group of chicks hatched from infectious bronchitis infected hens. *Research in Veterinary Science* **8**, 74-82.

Cook, J. K. A. (1968). Duration of experimental infectious bronchitis in chickens. *Research in Veterinary Science* **9**, 506-514.

Cook, J. K. A. (1971). Recovery of Infectious bronchitis virus from eggs and chicks produced by experimentally inoculated hens. *Journal of Comparative Pathology* **81**, 203-211.

Cook, J. K. A., Darbyshire, J. H. & Peters, R. W. (1976). The use of chicken tracheal organ cultures for isolation and essay and assay of avain infectious bronchitis virus. *Archives of Virology* **50**, 109-118.

Cook, J. K. A. (1984). The classification of new serotypes of infectious bronchitis virus isolated from poultry flocks in Britain between 1981 and 1983. *Avian Pathology* **13**, 733-741.

Cook, J. K. A. & Huggins, M. B. (1986). Newly isolated serotypes of infectious bronchitis virus: their role in disease. *Avian Pathology* **15**, 129-138.

Cook J.K.A., Brown, A. J. & Bracewell C.D. (1987). Comparision of haemagglutination inhibition test and serum neutralization test in tracheal organ cultures for typing infectious bronchitis virus strains. *Avian pathology* **16**, 505-511.

Cook, J. K. A., Orbell, S. J., Woods, M. A. & Huggins, M. B. (1999). Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. *Avian Pathology* **28**, 477-485.

Cook, J. K. A., Chesher, J., Baxendale, W., Greenwood, N., Huggins, M. B. & Orbell, S. J. (2001). Protection of chickens against renal damage caused by a nephropathogenic bronchitis virus. *Avian Pathology* **30**, 423-426.

Cowen, B. S. & Hitchner, S. B. (1975). Characterization of a new infectious bronchitis virus isolate. III. Cell-culture adaptation of Clark 333. *Avian Diseases* **19**, 6-11.

Creger, C. R., Phillips, H. & Scott, J. T. (1976). Formation of egg shell. *Poultry Science* **55**, 1717-1723.

Crinion, R. A. P., Ball, R. A. & Hofstad, M. S. (1971a). Pathogenesis of oviduct lesions in immature chickens following exposure to infectious bronchitis virus at one day old. *Avian Diseases* **15**, 32-41.

Crinion, R. A. P., Ball, R. A. & Hofstad, M. S. (1971b). Abnormalities in laying chickens following exposure to infectious bronchitis virus at one day old. *Avian Diseases* **15**, 42-48.

Crinion, R. A. P. (1972). Egg quality and production following infectious bronchitis virus exposure at one day old. *Poultry Science* **51**, 582-585.

Crinion, R. A. P. & Hofstad, M. S. (1972a). Pathogenicity of four serotypes of avian infectious bronchitis virus for the oviduct of young chickens of various ages. *Avian Diseases* **16**, 351-363.

Crinion, R. A. P. & Hofstad, M. S. (1972b). Pathogenicity of two embryo-passage levels of avian infectious bronchitjis virus for the oviduct of young chickens of various ages. *Avian Diseases* **16**, 967-973.

Crossley, J., Ferrando, G., & Eiler, H. (1980). Distribution of adrenergic receptors in domestic fowl oviduct. Poultry science, 59, 2331-2335

Cubillos, A., Ulloa, J., Cubillos, V. & Cook, J. K. A. (1991). Characterization of strains of infectious bronchitis virus isolated in Chile. *Avian Pathology* **20**, 85-99.

Cumming, R. B. (1962). The etiology of "uraemia" of chickens. *Australian*

Veterinary Journal **38**, 554.

Cumming, R. B. (1963). Infectious avian nephrosis (uraemia) in Australia. *Australian Veterinary Journal* **39**, 145-147.

Cumming, R. B. (1967). Studies on Infectious bronchitis Nephrosis/ nephritis (Uremia). Armidale: University of New England.

Cumming, R. B. (1969a). The control of avian infectious bronchitis/nephrosis in Australia. *Australian Veterinary Journal* **45**, 200-203.

Cumming, R. B. (1969b). Studies on avian infectious bronchitis virus. 3. Attempts to infect other avian species with the virus. *Australian Veterinary Journal* **45**, 312-314.

Cumming, R. B. (1970a). Studies on Australian infectious bronchitis virus. IV. Apparent farm-to-farm airborne transmission of infectious bronchitis virus. *Avian Diseases* **14**, 191-195.

Cumming, R. B. (1970b). The value of inactivated vaccines in chickens challanged with Australian nephrosis (Uremia) outbreaks. In *Proceedings of the 14th World Poultry Congress*, pp. 189-195. Madrid, Spain.

Cumming, R. B. & Chubb, R. C. (1988). The pathogenesis of nephritis evoked by Australian IB viruses In *Proceedings of the 1st International Symposium on Infectious Bronchitis*, pp. 129-132. Rauischholzhausen, Germany.

Cunningham, C. H. (1970). Avian Infectious bronchitis. *Advances in veterinary science and comparative medicine* **14**, 105-148.

Darbyshire, J. H. & Peters, R. W. (1984). Sequential develpoment of humoral immunity and accessment of protection in chickens following vaccinations and challange with avian infectious bronchitis virus. *Research in Veterinary Science* **37**, 77-86.

Davelaar, F. G. & Kouwenhoven, B. (1976). Changes in the Harderian gland of the chicken following conjunctival and intranasal infection with infectious bronchitis virus in one- and 20-day-old chickens. *Avian Pathology* **5**, 39-50.

Davidson, M. (1986). Histological studies of changes in the magnum of domestic hen associated with the production of "watery white" eggs. *British Poultry Science* **27**, 353-354.

De Witt, J. J., Mekkes, D. R., Kouwenhoven, B. & Verheijden, J. H. M. (1997). Sensitivity and specificity of serological tests for infectious bronchitis virus antibodies in broilers. *Avian Pathology* **26**, 105-118.

De Witt, J. J., de Jong, M. C. M., Pijpers, A. & Verheijden, J. H. M. (1998). Transmission of infectious bronchitis virus within vaccinated and unvaccinated groups of birds. *Avian Pathology* **27**, 464-471.

De Witt, J. J. (2000). Detection of infectious bronchitis virus. *Avian Pathology* **29**, 71-93.

Dhinakar Raj, G. & Jones, R. C. (1996a). An *in vitro* comparison of the virulence of seven strains of infectious bronchitis virus using tracheal and oviduct cultures. *Avian Pathology* **25**, 649-662.

Dhinakar Raj, G. & Jones, R.C. (1996b). Protectotypic differentiation of avian infectious bronchitis viruses using an in vitro challenge model. *Veterinary Microbiology* **53**, 239-252

Dhinakar Raj, G. & Jones, R. C. (1996c). Local antibody production in the oviduct and gut of hens infected with enterotropic strain of Infectious bronchitis virus. *Veterinary Immunology and Immunopathology* **53**, 147-161.

Dhinakar Raj, G. & Jones, R. C. (1997a). Infectious bronchitis virus: immunopathogenesis of infection in the chicken. *Avian Pathology* **26**, 677-706.

Dhinakar Raj, G. & Jones, R. C. (1997b). Growth of infectious bronchitis virus vaccines in oviducts derived from oestrogen-treated chicks and embryos. *Vacccine* **15**, 163-168.

Di Matteo, A. M., Sonez, M. C., Plano, C. M. & Lawzewitsch, v. (2000). Morphological observations on respiratory tracts of chickens after hatchery infectious bronchitis vaccination and formaldehyde fumigation. *Avian Diseases* **44**, 507-518.

Draper, M. H., Davidson, M. F., Davidson, G. & Johnston, H. S. (1972). The fine structure of the fibrous membrane forming region of the oviduct of domestic fowl. *Quarterly Journal of Experimental Physiology* **57**, 297-309.

Duff, R. H., Macdonald, J. W. & McMartin, D. A. (1971). Infection od day old chicks with Infectious bronchitis virus and subsequent anatomical abnormalities. *The Veterinary Record* **88**, 315.

Eastin, J. W. C. & Spaziani, E. (1978). On control of calcium secretion in the avian shell gland (Uterus). *Biology of Reproduction* **19**, 493-504.

Eastin, J. W. C. & Spaziani, E. (1978). On mechanism of calcium secretion in the avian shell gland (Uterus). *Biology of Reproduction* **19**, 505-518.

El Houafdi, M. D., Jones, R. C., Cook, J. K. A. & Ambali, A. G. (1986). The isolation and characterization of six avain infectious bronchitis virus isolated from Morocco. *Avian Pathology* **15**, 93-105.

Escorcia, M., Jackwood, M. W., Lucio, B., Petrone, V. M., López, C., Fehervari, T. & Téllez, G. (2000). Characterization of Mexican strains of avian infectious bronchitis isolated during 1997. *Avian Diseases* **44**, 944-947.

Escorcia, M., Fortoul, T. I., Petrone, V. M., Galindo, F., López, C. & Téllez, G. (2002). Gastric gross and microscopic lesions caused by the UNAM-97 variant strain of infectious bronchitis virus after the eighth passage in specific pathogen-free chicken embryos. *Poultry Science* **81**, 1647-1652.

Fabricant, J. & Levine, P. P. (1951). The persistence of infectious bronchitis virus in eggs and tracheal exudates of infected chickens. *The Cornell veterinarian* **41**, 240-246.

Farnendez, M. S., Escobar, C., Lavelin, I., Pines, M. & Arias, J. L. (2003). Localization of osteopontin in oviduct tissue and egg shell during different stages of avian egg laying cycle. *Journal of Structural Biology* **143**, 171-180.

Ferreira, H. L., Pilz , D., Mesquita, L. G. & Cardoso, T. (2003). Infectious bronchitis virus replication in the chicken embryo related cell line. *Avian Pathology* **32**, 413-417.

Fertuck, H.C & Newstead, J. D. (1970). Fine and structural observation on magnum mucosa in quail and hen oviducts. *Cell and Tissue Research* **103**, 447-459.

Fujii S., Yoshimura, Y., Okamoto, T. & Tamura, T. (1981). Regional morphology of hens oviduct with special reference to mechanism of engulfing of the ovulated ovum. *Journal of Faculty of applied biological sciences, Hiroshima University* **10**, 87-94.

Fulton, R. M., Reed, W. M. & Thacker, H. L. (1993). Cellular response of the respiratory tract of chickens to infection with Massachusetts 41 and Australian T infectious bronchitis viruses. *Avian Diseases* **37**, 951-960.

Gallego, M., Del Cacho, E., Arnal, C. & Bascuas, J. A. (1992). Local immune response in the chicken Harderian gland to antigen given by different ocular routes. *Research in Veterinary Science* **52**, 38-43.

Ganapathy, K. & Bradbury, J. M. (1999). Pathogenicity of Mycoplasma imitans in mixed infection with infectious bronchitis virus in chickens. *Avian Pathology* **28**, 229-237.

Ganapathy, K., Cargill, P. W. & Jones, R. C. (2005). A comparison of methods of

inducing lachrymation and tear collection in chickens for detection of virus-specific immuoglobulins after infection with infectious bronchitis virus. *Avian Pathology* **34**, 248-251.

Gilbert, A. B. (1969). Effect of foreign object in the shell gland on egg production of hen fed with calcium deficient diet. *British Poultry Science* **10**, 83-88.

Gilbert (1971). The egg in reproduction in, *Physiology and biochemistry of domestic fowl.* Vol 3, Ed D.J. Bell and B.M. Freeman. Acedemic press London. Pages 1401-1410

Goldstein, D. L. & Skadhauge, E. (2000). Renal and extrarenal regulation of body fluid composition in, *Strukie's Physiology*, 5 edn, Eds G.C. Whittow, Academic press,London. Pp 265-297

Gough, R. E. & alexander, D. J. (1978). Comparison of serological tests for the measurment of the primary immune response to avian infectious bronchitis virus vaccine. *Veterinary Microbiology* **2**, 289-301.

Gough, R. E. & Alexander, D. J. (1979). Comparison of duration of immunity in chickens infected with a live infectious bronchitis vaccine by three different routes. *Research in Veterinary Science* **26**, 329-332.

Gough, R. E., Randall, C. J., Dagless, M. D., Alexander, D. J., Cox , W. J. & Pearson, D. (1992). A new strain of Infectious bronchitis virus infecting domestic fowl in Great Britain. *Veterinary Record* **130**, 493-494.

Gough, R. E., Cox , W. J., Winkler, C. E., Sharp, M. W. & Spackman, D. (1996). Isolation and identification of infectious bronchitis virus from pheasants. *Veterinary Record* **138**, 208-209.

Guatelli, J. C., Gingeras, T. R. & Richman, D. D. (1989). Nucleic acid amplification in vitro: detection of sequences with low copy numbers and application to diagnosis of human immunodeficiency virus type 1 infection. *Journal of Microbiological reviews* **2**, 217-226.

Guittet, M., Picault, J.P., Le Coq, H., Lamande, J. & Bennejean, G. (1988). Strategy of protection of the layers against infectious bronchitis virus. In *Proceedings of the IInd International Symposium on Infectious Bronchitis*. Rauischholzhausen, West Germany. pp 298-307

Guy, J. S., Smith, L. G., Breslin, J. J., Vaillancourt, J. P. & Barnes, H. J. (2000). High mortality and growth depression experimentally produced in young turkeys by dual infection with enteropathogenic Escherichia coli and turkey coronavirus. *Avian Diseases* **44**, 105-113.

Handberg, K. J., Nielson, O. L., Pedersen, M. W. & Jorgensen, P. H. (1999). Detection and strain differentiation of infectious bronchitis virus from tracheal tissues from experimentally infected chickens by reverse transcriptase polymerase chain reaction. Comparision with immunohistochemical techniques. *Avian Pathology* **28**, 327-335.

Hara, K. T., Sakuma, Y., Sakai, S., Nagata, M. & Aoki, F. (2003). Dynamic Changes in the Expression of Protein Tyrosine Phosphatases During Preimplantation Mouse Development: Semi-Quantification by Real-Time PCR. *Journal of Reproduction and Development* **49**, 323-328.

Heath, B. H. (1969). The effect of unilateral ureteral ligation on mortality in chickens experimentally infected nephrosis inducing infectious bronchitis. *Australian Veterinary Journal* **45**, 225-227.

Heath, B. H. (1970). Chemical pathology of nephrosis induced by an infectious bronchitis virus. *Avian Diseases* **14**, 95-106.

Hill, R. W., Lorenz, F.W. (1956). Studies on egg changes following avian respiratory diseases. *Poultry Science* **35**, 409-417.

Hodges, R. D. (1974). The Female reproductive system in, *The Histology of the Fowl.* Academic press Inc. London, pp 33-417

Hofstad, M. S. & Yoder, H. W., Jr. (1966). Avian infectious bronchitis - virus distribution in tissues of chicks. *Avian Diseases* **10**, 230-239.

Hofstad, M. S. (1967). Immunity following aerosol exposure to high-embryo-passage avian infectious bronchitis virus. *Avian Diseases* **11**, 452-458.

Hofstad, M. S. (1978). Avian Infectious bronchitis virus in, *Diseases of Poultry*, 7th edn. Eds M.S. Hofstad, B.W. Calnek, C.F. Helmboldt, W.M. Reid, H.W. Yoder Jr. Iowa: Iowa State University press, Iowa. pp 487-504.

Hofstad, M. S. (1981). Cross immunity in chickens using seven isolates of avain infectious bronchitis virus. *Avian Diseases* **25**, 650-654.

Hohman, W. & Schraer, H. (1966). The intarcellular distribution of calcium in the mucosa of avian shell gland. *Journal of Cell Biology* **30**, 317-331.

Holland, P. M., Abramson, R. D., Watson, R. & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of Thermus aquaticus. In *proceedings of National Academy of Science*, pp. 7276-7280. USA.

Hopkins, S.R. & Yoder, H.W. (1984). Increased incidence of airsacculitis in broilers infected with mycoplasma synoviae and chicken-passaged infectious bronchitis vaccine virus. *Avian Diseases*, **28**, 386-396.

Hopkins, S. R. & Beard, C. W. (1985). Studies on methods of determining the efficacy of oil emulsion vaccines against infectious bronchitis virus. *Journal of American Veterinary and Medical association* **187**, 305.

Hughes, B. O., A.B., G. & Brown, M. F. (1986). Categorisation and causes of abnormal egg shells. *British Poultry Science* **27**, 325-327.

Husbands, D. H. & Brown, W. O. (1965). Sex differences in composition and

acetate incorporation in to liver lipids of the adult fowl. *Comparative biochemistry and physiology* **14**, 445-451.

Ignjatovic, J. & Mcwaters, P. G. (1991). Monoclonal antibodies to three structural proteins of avian infectious bronchitis virus:Characterization of apitopes and antigenic differentiation of Australian strains. *Journal of general virology* **72**, 2915-2922.

Ignjatovic, J. & Galli, L. (1994). The S1 glycoprotein but not the Nor M proteins of Infectious bronchitis virus induces protection in vaccinated chickens. *Archives of Virology* **138**, 117-134.

Ignjatovic, J. & Galli, L. (1995). Immune responses to structural proteins of avian infectious bronchitis virus. *Avian Pathology* **24**, 313-332.

Ignjatovic, J., Sapats, S. I. & Ashton, F. (1997). A long-term study of Australian infectious bronchitis viruses indicates a major antigenic change in recently isolated strains. *Avian Pathology* **26**, 535-552.

Ignjatovic, J. (1998). Epdemiology of Infectious Bronchitis Virus in Australia. In *International symposium on infectious bronchitis*, pp. 84-88. Rauischholzhausen,West Germany.

Ignjatovic, J. & Sapats, S. (2000). Avian infectious bronchitis virus. *Rev sci tech Off int Epiz* **19**, 493-508.

Ignjatovic, J., Ashton, D. F., Reece, R., Scott, P. & Hooper, P. (2002). Pathogenicity of Australian strains of avian infectious bronchitis virus. *Journal of Comparative Pathology* **123**, 115-123.

Ignjatovic, J., Reece, R. & Ashton, D. F. (2003). Susceptibility of three genetic lines of chicks to infection with a nephropathogenic T strain of Avian infectious bronchitis virus. *Journal of Comparative Pathology* **128**, 92-98.

Ignjatovic, J., Gould, G. & Sapats, S. (2006). Isolation of variant infectious

bronchitis virus in Australia that further illustrates diversity among emerging strains. *Archives of Virology* **151**, 1567-1585.

Ishiguro, T., Saitoh, J., Yawata, H., Yamagishi, H., Iwasaki, S. & Mitoma, Y. (1995). Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. *Annals of Biochemistry* **229**, 207-213.

Ito, N. M. K., Miyaji, C. I. & Capellaro, C. E. M. P. D. M. (1991). Studies on broilers IBV and IB-like virus from guinea fowl. In *2nd International symposium on Infectious bronchitis* pp. 302-307. Edited by E. F. H.-R. Kaleta, U. Giessen: Justus Leibig University.

Jackwood, M. W., Yousef, N.M.H., Hilt, D.A. (1997). Further development and use of a molecular serotype identification test for infectious bronchitis virus. *Avian Diseases* **41**, 105-110.

Jackwood, M. W., Hilt, D.A. & Callison, S.A. (2003). Detection of infectious bronchitis virus by real-time reverse transcriptase-polymerase chain reaction and identification of a quasispecies in the Beaudette strain. *Avian Diseases* **47**, 718-724.

Johnson, A. L. (2000). Reproduction in the female in Strukie's Avian Physiology. 5th edn. Eds G.C. Whittow, Academic press, London. pp 569-596.

Johnston, H. S., Aitken, R. N. C. & Wyburn, G. M. (1963). The Fine Structure of Uterus of the domestic fowl. *Journal of Anatomy* **97**, 333-444.

Jolly, M. (2001). Histological examination of kidneys of vaccinated and unvaccinated Isa Brown cockerels, exposed to T strain infectious bronchitis. In *Animal Physiology*. Armidale: University of New England.

Jolly, M. (2005). Protection of egg quality in laying hens by rearing phaseinfectious bronchitis vaccination. In *Animal science*. Armidale: University of New England.

Jolly, M., Roberts, J. R. & ball, W. (2005). Effect of IB vaccination strain during rearing on egg production and quality following challange with IB virus during laying cycle. *In Proceedings of Australian Poultry science Symposium*, pp. 1-8. Sydney.

Jones, R. C., Jordan, F.T.W. (1970). The exposure of day-old chicks to infectious bronchitis and the subsequent development of the oviduct. *The Veterinary Record* **87**, 504-505.

Jones, R. C. & Jordan, F. T. W. (1971). The site of replication of infectious bronchitis virus in the oviduct of experimentally infected hens. *Veterinary Record* **89**, 317-318.

Jones, R. C. & Jordan, F. T. W. (1972). Persistence of virus in the tissues and development of the oviduct in the fowl following infection at day old with infectious bronchitis virus. *Research in Veterinary Science* **13**, 52-60.

Jones, R. C. (1974). Nephrosis in laying chickens caused by Messachusetts-type of infectious bronchitis virus. *Veterinary Record* **95**, 319.

Jones, R. C. & Ambali, A. G. (1987). Re-excretion of an enterotropic infectious bronchitis virus by hens at point of lay after experimental infection at day old. *The Veterinary Record* **120**, 617-618.

Jordan, F. T. W. & Nassar, T. J. (1973). The survival of infectious bronchitis (IB) virus in water. *Avian Pathology* **2**, 91-101.

Kami, K. & Yasuda, Y. (1984). Immunoelectron microscopical demonstration of endogenous avidin in secretory granules of the hen oviduct mucosa: a preliminary study. *The Histochemical Journal* **16**, 835-842.

Keeler, C. L., Reed, K. L., Nix, W. A. & GElb, J. J. (1998). Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. *Avian Diseases* **42**, 275-284.

Kidd, I. M., Clark, D. A. & Emery, V. C. (2000). A non-radioisotopic quantitative competitive polymerase chain reaction method: application in measurement of human herpesvirus 7 load. *Journal ofVirological Methods* **87**, 177-181.

King, D. & Hopkins, S. R. (1984). Rapid serotyping of Infectious bronchitis virus haemagglutination inhibition test. *Avian Diseases* **28**, 727-733.

Klieve, A. V. & Cumming, R. B. (1988). Infectious bronchitis: safety and protection in chickens with maternal antibody. *Australian Veterinary Journal* **65**, 396-397.

Kusters, J. G., Niesters, H. G., Bleumink-Pluym, N. M., Davelaar, F. G., Horzinek, M. C. & Van der Zeijst, B. A. (1987). Molecular epidemiology of infectious bronchitis virus in The Netherlands. *Journal of general virology* **68**, 343-352.

Landman, W. J. & Feberwee, A. (2004). Aerosol-induced Mycoplasma synoviae arthritis: the synergistic effect of infectious bronchitis virus infection. *Avian Pathology* **33**, 591-598.

Leary, A. (1999). Factors affecting egg and egg shell quality in several strains of laying hen. In *Animal Physiology*. Armidale: University of New England.

Liu, S., Chen, J., Chen, J., Kong, X., Shao,Y., Han,Z., Feng, L., Cai, X.,Gu, S., & Liu, M. **(2005).** Isolation of avian infectious bronchitis coronavirus from domestic peafowl (Pavo cristatus) and teal (Anas). *Journal of General Virology* **86**, 719-725.

Locatelli, G., Santoro, F., Veglia, F., Gobbi, A., Lusso, P. & Malnati, M. S. (2000). Real-time quantitative PCR for human herpesvirus 6 DNA. *Journal of clinical Microbiology* **38**, 4042-4048.

Lougovskaia , N. N., Lougovskoi , A. A., Bochkov , A. A., Batchenko , G. V., Mudrak , S. V., Drygin , V. V., Borisov , A. V., Borisov , V. V. & Gusev, A. A. (2002). Detection and estimation of avian infectious bronchitis virus antigen by a novel indirect liquid-phase blocking enzyme-linked immunosorbent assay using

chicken and rabbit affinity purified immunoglobulins. *Avian Pathology* **31**, 549-557.

Lucio, B. & Hitchner, S. B. (1970). Differentiation and detection of Infectious bronchitis virus subtypes by immunofluroscence. *Avian Diseases* **14**, 9-24.

Lucio, B. & Fabricant, J. (1990). Tissue tropism of three cloacal isolates and Massachusetts strain of infectious bronchitis virus. *Avian Diseases* **34**, 865-870.

Lukert, P. D. (1965). Immunofluorescence of avian infectious bronchitis virus in primary chicken embryo kidney, liver, lung, and fibroblast cell cultures. *Archives fur die gesamte virusforschung* **19**, 265-272.

Mahecha, G.A.B. Oliveria, C.A., Balzuweit, K. & Hess, R.A. (2002). Epididymal lithiasis in roosters and efferent ductule in testicular damage. *Reproduction* **124**, 824-831.

Maiti, N. K., Sharma, S. N. & Sambyal, D. S. (1984). Isolation of Infectious bronchitis virus from intestine and reproductive organs of laying hens with dropped egg production. *Avian Diseases* **29**, 509-513.

Makita, T. & Kiwaki, S. (1968). The fine structure of indundibulum of quails oviduct. *Journal of Zootechnical science.* **39,** 246-254

Makita, T. & Sandborn, E. B. (1970). Scanning electron microscopy of secretory granules in albumen gland cells of laying hen oviduct. *Experiemtal Cell Research.* **60**.

Makita, T., Kiwaki, S. & Sandborn, E. B. (1973). Secretion granules in the ciliated cells of avian oviduct. *Archives of Histology, Japan* **35**, 209-224.

Mao, K. M., Sultana, F., Howlider, M. A. R., Iwasawi, A. & Yoshizaki, N. (2006). The magnum isthmus junction of the fowl oviduct participates in the formation of avian type shell membrane. *Zoological science* **23**, 41-47.

Mardani, K., Browning, G. F., Ignjatovic, J. & Noormohammadi, A. H. (2006a).

Rapid differentiation of current infectious bronchitis vaccine strains and field isolates in Australia. *Australian veterinary journal* **84**, 59-62.

Mardani, K., Noormohammadi, A. H., Ignjatovic, J. & Browning, G. F. (2006b). Typing infectious bronchitis virus strains using reverse transcription-polymerase chain reaction and restriction fragment length polymorphism analysis to compare the 3' 7.5 kb of their genomes. *Avian Pathology* **35**, 63-69.

Matthews, J. A. & Kricka, L. J. (1988). Analytical strategies for the use of DNA probes. *Annals of Biochemistry* **169**, 1-25.

Matthijs, M. G. R., Van Eck, J. H. H., Landman, W. J. M. & Stegeman, J. A. (2003). Ability of Massachusetts-type infectious bronchitis virus to increase colibacillosis susceptibility in commercial broilers: a comparison between vaccine and virulent field virus. *Avian Pathology* **32**, 473-481.

Mccallion, D. J. (1953). A cytological and cytochemical study of the shell gland of domestic hen. *Canadian Journal of Zoology* **31**, 577-589.

Macdonald, J.W., Randall, C.J. & McMartin, D.A. (1980). An inverse age resistance of chicken kidneys to infectious bronchitis virus. *Avian Pathology* **9,** 245-259.

McDonald, J. W., Randall, C. J., McMartin, D. A. & Dagless, M. D. (1983). Immunity following inoculation of H120 and H52 vaccine strains of infectious bronchitis virus in to the crop of the domestic fowl. *Avian Pathology* **12**, 379-383.

McDougall, J. S. (1968). Infectious bronchitis in laying fowls. *The Veterinary Record* **83**, 84-86.

McFerran, J., Cahill HT, Young JA & CL, W. (1971). Isolation of infectious bronchitis virus from newborn chicks and dead-in-shell embryos. *Veterinary Record* **89**, 560-561.

Mcintosh, K. (1974). Coronavirus : a comparative review. *Current topics in Microbiology and Immunology* **63**, 85-129.

McMartin, D. A. (1968a). Preliminary investigation of methods for evaluating inactivated vaccines for infectious bronchitis virus. *British Veterinary Journal* **124**, 36-41.

McMartin, D. A. (1968b). The pathogenicity of an infectious bronchitis virus for laying hens, with observations on pathogenesis. *British Veterinary Journal* **124**, 576-581.

McMartin, D. A. & Macleod, H. (1972). Abnormal oviducts in laying hens following natural infections with infectious bronchitis at an early age. *British Veterinary Journal* **128,** XIX-XXI.

McMartin, D. A. (1993). *Infectious Bronchitis, in "Virus Infections of Birds"*:Eds, M.S. McNulty, Elsevier Science Publishers B.V. pp 249-275

Meulemans, G., Carlier, M. C., Gonze, M., Petit, P. & Vandenbroeck, M. (1987). Incidence, chcracterization and prophylaxis of nephropathogenic avian infectious bronchitis virus. *Veterinary Record* **120**, 205-206.

Mills, A. D., Nys, Y., Gautron, J. & Zawadski, J. (1991). Whitening of brown shelled eggs: Individual variation and relationships with age fearlessness, oviposition interval and stress. *British Poultry Science* **32**, 117-129.

Mongin, P. & Carter, N. W. (1977). Studies on The Avian Shell Gland During Egg Formation:Aqueous and Electrolytic Composition of the Mucosa. *British Poultry Science* **18**, 339-351.

Moody, A., Sellers, S. & Bumstead, N. (2000). Measuring infectious bursal disease virus RNA in blood by multiplex real-time quantitative RT–PCR. *Journal of Virological Methods* **85**, 55-64.

Muneer, M. A., Halvorson, D. A., Sivanandan, V., Newman, J. A. & Coon, C. N. (1986). Effect of infectious bronchitis (Arkansas strain) on laying chickens. *Avian Diseases* **30**, 644-647.

Muneer, M. A., Newman, J. A., Halvorson, D. A., Sivanandan, V. & Coon, C. N. (1987). Effects of avian infectious bronchitis virus (Arkansas strain) on vaccinated laying chickens. *Avian Diseases* **31**, 820-828.

Nagano, H., Tsuchimoto, M., Hohdatsu, T., Yamagami, T., Ide, S., Eiguchi, Y., Tanaka, Y. & Yamagishi, H. (1990). Enzyme-linked immunosorbent assay for the detection of infectious bronchitis virus (IBV) antigen with monoclonal antibody. *The Japanese Journal of Veterinary Science* **52**, 657-659.

Nakamura, K., Cook, J. K. A., Otsuki, K., Huggins, M. B. & Frazier, J. A. (1991). Comparative study of respiratory lesions in two chicken lines of different susceptibility infected with infectious bronchitis virus: histology, ultrastructure and immunohistochemistry. *Avian Pathology* **20**, 241-257.

Naqui, S. A. (1990). Monoclonal antibody based immunoperoxidase procedure for rapid detection of Infectious bronchitis virus in infected tissues. *Avian Diseases* **34**, 893-898.

Naqui, S., Gay, K., Patalla, P., Mondal, S. & Liu, R. (2003). Establishment of Persistent Avian Infectious Bronchitis Virus Infection in Antibody-Free and Antibody-Positive Chickens. *avian Diseases* **47**, 594-601.

Nevalainen, T. J. (1969). Electron Microscope observations on the Shell gland Mucosa of Calcium-deficient Hens (Gallus domesticus). *Anatomical Record* **164**, 127-140.

Niubo, J., Perez, J. L., Carvajal, A., Ardanuy, C. & Martin, R. (1994). Effect of delayed processing of blood samples on performance of cytomegalovirus antigenemia assay. *Journal of clinical Microbiology* **32**, 1119-1120.

O'Malley, B. W. (1967). *In vitro* hormonal induction of a specific protein (avidin) in

chicken oviduct. *Biochemistry* **6**, 2546- 2551.

Otsuki, K., Noro, K., Yamamoto, H. & Tsubokura, M. (1979). Studies on avian infectious bronchitis virus (IBV). II. Propagation of IBV in several cultured cells. *Archieves of virology* **60**, 115-122.

Otsuki, K., Huggins, M. B. & Cook, J. K. A. (1990). Comparison of the susceptibility to avian infectious bronchitis virus infection of two inbred lines of white leghorn chickens. *Avian Pathology* **19**, 467-475.

Owen, R. L., Cowen, B. S., Hattel, A. L., Naqui, S. A. & Wilson, R. A. (1991). Detection of viral antigen following exposure of one day old chicken of the Holland 52 strain of Infectious bronchitis virus. *Avian Pathology* **20**, 663-673.

Palmiter, R. D. & Gutman, G. A. (1970). Fluorescent antibody localization of ovalbumin, conalbumin, ovomucoid and lysozyme in chick oviduct magnum. *Journal of Biological Chemistry*, 6459-6461.

Palmiter, R. D. (1972). Regulation of protein synthesis in chick oviduct. I. Independent regulation of ovalbumin, conalbumin, ovomucoid, and lysozyme induction. *Journal of Biological Chemistry* **25**, 6450-6461.

Parsons, D., Ellis, M. M., Cavanagh, D. & Cook, J. K. A. (1992). Characterization of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. *Veterinary Record* **131**, 408-411.

Patterson, S. & Bingham, R. W. (1976). Electron microscope observations on entry of Avian Infectious bronchitis virus in to susceptible cells. *Archives of Virology* **52**, 191-200.

Pensaert, M. & Lamberechts, C. (1994). Vaccination of chickens against a belgian nephropathogenic strain of infectious bronchitis virus B1648 using attenuated homologous and heterologous strains. *Avian Pathology* **23**, 631-641.

Peters, R. W., Darbyshire, J. H. & Cook, J. K. A. (1979). The susceptibility of chicken kidney and oviduct organ cultures to a vaccine strain of infectious bronchitis virus. *Research in Veterinary Science* **26**, 38-40.

Picault, J. P., Drouin, P., Toux, J. Y., Guittet, M. & Bennjean, G. (1988). Infectious bronchitis in France. In *Proceedings of the IInd International Symposium on Infectious Bronchitis*, pp. 3-22. Rauischholzhausen, Germany.

Pohl, R. (1974). The histopathogenesis of the nephrosis-nephritis syndrome. *Avian Pathology* **3**, 1-13.

Pradhan, H. K., Mohanty, G. C. & Verma, K. C. (1982). Isolation and Characterization of viral agents from reproductive tract of young chickens. *Indian Journal of Poultry Science* **17**, 143-148.

Pradhan, H. K., Mohanty, G. C. & Rajya, B. S. (1984). Comparative sensitivities of oviduct and tracheal organ cultures and chicken embryo kidney cell cultures to Infectious bronchitis virus. *Avian Diseases* **27**, 594-601.

Purcell, D. A. & McFerran, J. B. (1972). The histopathology of infectious bronchitis in the domestic fowl. *Research in Veterinary Science* **13**, 116-122.

Rabon, H. W., Roland, D. A. S. & Clark, A. J. (1991). Uterine calcium binding protein activity of non laying hens and hens laying hard shelled or shell less eggs. *Poultry Science* **70**, 2280-2283.

Ramneek,.M., Mitchell, N.L. & McFarlaneR.G. (2005). Rapid detection and characterisation of infectious bronchitis virus (IBV) from New Zealand using RT-PCR and sequence analysis. *New Zealand Veterinary Journal* **53**, 457-461.

Randall, C. J. & Reece., R. L. (1996). Respiratory system in, Colour *Atlas of Avian Histopathology*. Academic press, Mosby- Wolfe publishing company, London. pp 145-170

Ratanasethakul, C. & Cumming, R. B. (1981). Comparison of Australian infectious bronchitis vaccine viruses. In *Proceedings of 4th Australasian Poultry and Stock feed Convention*, pp. 134-140. Perth.

Ratnasethakul, C. & Cumming, R. B. (1983a). The effect of route of infection and strain of virus on the pathology of Infectious bronchitis *Australian Veterinary Journal* **60**, 209-213.

Ratanasethakul, C. & Cumming, R. B. (1983b). Immune response of chickens to various routes of administration of Australian infectious bronchitis vaccine. *Australian Veterinary Journal* **60**, 214-216.

Roberts, J. R. (2004). Factors affecting egg internal quality and egg shell quality in laying hens. *Journal of Poultry science* **41**, 161-177.

Roberts, J. R. & Ball, M. (2004). Egg quality guidelines for the Australian egg industry. Australian egg Corporation Ltd. Sydney.

Roberts, J. R., Ball, W., Chubb, R. C., Sulaiman, A. & Jolly, M. (2004). Serological methods for Infectious bronchitis virus in laying hens. In *Australian Poultry Science Symposia*. Sydney.

Roberts, J. R. (2005). Preliminary studies on the effects of Infectious bronchitis on unvaccinated laying hens. In *Queensland Poultry Science Symposium*, pp. 169-177. Gatton.

Roland, D. A., Farmer, M. & Marple, D. (1985). Calcium and its relationship to excess feed consumption, body weight, egg size, fat deposition, shell quality, and fatty liver hemorrhagic syndrome. *Poultry Science* **64**, 2341-2350.

Ross, S. M. a. & Corrsin, S. (1974). Results of an analytical model of mucociliary pumping. *Journal of applied Physiology* **37**, 333-340.

Sapats, S., Ashton, D. F., Wright, P. J. & Ignjatovic, J. (1996a). Novel variation in

N protein of Avian Infectious Bronchitis virus. *virology* **226**, 412-417.

Sapats, S., Ashton, D. F., Wright, P. J. & Ignjatovic, J. (1996b). Sequence analysis of S1 glycoprotein of infectious bronchitis viruses:Identification of novel genotypic group in Australia. *Journal of general virology* **77**, 413-418.

Sapats, S. I., Wright, P. J. & Ignjatovic, J. (1996c). Sequence analysis of the S1 glycoprotein of Australian strains of infectious bronchitis virus. *Proceedings of the Australian Poultry Science Symposium* **8**, 33-38.

Scanes, C.G., Brant, G. & Ensminger, M.E. (2004) Poultry Biology in, *Poultry science*, 4th Edn, Pearson Education Ltd, New Jersey. pp 22-45.

Schalk, A. F. & Hawn, M. C. (1931). An appearently new respiratory disease of chick. *Journal of American Veterinary and Medical association* **78**, 143-422.

Scott, H. M. & Huang, W. (1941). Histological Observations on the Formation of the Chalaza in the Hen's egg. *Poultry Science* **20**, 402-405.

Scott, T. R., Savage, M. L. & Olah, I. (1993). Plasma cells of chicken harderian gland. *Poultry Science* **72**, 1273-1279.

Sevoian, M. & Levine, P. P. (1957). Effects of infectious bronchitis on the reproductive tracts, egg production, and egg quality of laying chickens. *Avian Diseases* **1**, 136-164.

Sharma, S. N. & Adhalakha, S. C. (1996). Cultivation of virus, *In: Text book of Veterinary microbiology*. Vikas publishing house Pvt. LTD, New Delhi. pp 69-73.

Shimada, K. & Asai, I. (1978). Uterine contraction during the ovulatory cycle of the hen. *Biology of Reproduction* **21**, 1057.

Shimakura, S. & Hirai, K. (1971). Incidence of avian nephrosis in Japan with special reference to isolation and transmission test of infective agents. *Japanese*

Journal of veterinary science **33**, 163-173.

Simkiss, K. & Tyler, C. (1957). A histochemical study of the organic matrix of hen egg shells. *Quarterly Journal of Microscopical Science* **98**, 19-28.

Simkiss, K. & Taylor, T. G. (1971). Shell formation. In *Physiology and biochemistry of domestic fowl*, pp. 1331-1342. Edited by B. M. Freemman & D. J. Bell. London: Academic press.

Smith, H. W., Cook, J. K. A. & Parsell, Z. E. (1985). The experimental infection of chickens with mixtures of infectious bronchitis virus and E. coli. *Journal of general virology* **66**, 777-786.

Solomon, S. E. (1975). Studies of **isthmus** region of domestic fowl. *British Poultry Science* **16**, 255-258.

Solomon, S. E. (1983). The Oviduct. *Physiology and biochemistry of Domestic Fowl.* Vol 4. Eds D.J. Bell and B.M. Freeman. Acedemic press London. pp 379-419.

Solomon, S. E. (1987). Egg shell pigmentation In : Egg quality- Current problems and recent advances. In *Poultry science Symposium*. Eds R. G. Wells & C. G. Belyavin. Butterworth & Co Ltd, London. pp 147-157.

Solomon, S. E. (1991). The ovary and oviduct in, *Egg and egg shell quality*. London: Wolfe Publishing Limited. London. pp 11-32

Song, C. S., Lee, Y. J., Lee, C. W., Sung, S. W., Kim, J. H., Mo, I. P., Izumya, Y., Jang, H. K. & Mikami, T. (1998). Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by recombinant baculovirus. *Journal of General Virology* **79**, 719-723.

Spackman, D. & Cameron, I. R. (1983). Isolation of infectious bronchitis virus from pheasants. *Veterinary Record* **113**, 354-355.

Stemberger, B. H., Mueller, W. J. & Leach, R. M. (1977). Microscopic study of the Initial stages of egg shell calcification. *Poultry Science* **56**, 537-543.

Stevens, A. (1990). *The haematoxylins, in "Theory and Practice of Histological Techniques".* Edinburgh, UK.

Sturkie, P. D. (1955). Absorption of egg yolk in body cavity of hen. *Poultry Science* **34**, 736

Sulaiman, A., Roberts, J. R. & ball, W. (2004). Regular revaccinations for infectious bronchitis virus in laying hens: advantages and disadvantages. In *Australian Poultry Science Symposia*. Sydney.

Sultana, F., Yokoe, A., Mao, K. M. & Yoshizaki, N. (2003). The perialbumen layer: a novel structure in the envelopes of an avian egg. *Journal of Anatomy* **203**, 115-122.

Survashe, B. D., Itken, I. D. a. & Powell, J. R. (1979). The response of harderian gland of the fowl to antigen given by the ocular route I. Histologicical changes. *Avian Pathology* **8**, 77-93.

Tamura, T., S., F., Kunisaki, H. & Yamane, M. (1965). Histological observations on the quail oviduct with reference to pigment (porphyrin) in uterus. *Journal of Faculty of Fisheries and Animal Husbandry , Hiroshima University* **6**, 37-57.

Tomley, F. M., Mockett, M. E. G., Boursnell, M. E. G., Binns, M. M., Cook, J. K. A., Brown, T. D. K. & Smith, G. L. (1987). Expression of the infectious bronchitis virus spike protein by recombinant vaccinia virus and induction of neutralizing antibodies in vaccinated mice. *Journal of General Virology* **68**, 2291-2298.

Toro, H., Godoy, V., Larenas, J., Reyes, E. a. & Kaleta, E. F. (1996). Avian infectious bronchitis virus: Viral persistence in the Harderian gland and histological changes after eye drop vaccination. *Avian Diseases* **40**, 114-120.

Toro, H., Espinoza C., Ponce V., Rojas V., Morales MA. & EF., a. K. (1997). Infectious bronchitis: effect of viral doses and routes on specific lacrimal and serum

antibody responses in chickens. *Avian Diseases* **41**, 379-387.

Tsukamoto, Y., Kotani, T., Shiraishi, Y., Kawamura, H. & Sakuma, S. (1996). Epithelial cell proliferation of collecting ducts and ureters in the regenerating process of interstitial nephritis caused by infectious bronchitis virus. *Avian Pathology* **25**, 95-102.

Tsukamoto, Y., Matsumoto, T., Kotani, T., Taira, E., Takaha, N., Miki, N., Yamate, J. & & Sakuma, S. (1999). The expression of gicerin, a cell adhesion molecule, in regenerating process of collecting ducts and ureters of the chicken kidney following infection with a nephrotropic strain of infectious bronchitis virus. *Avian Pathology* **26**, 245-255.

Tyrrel, D. A., Alexander, D. J., Alemida, J. D. & other authors (1978). Corinoviridae: Second report. *Intervirology* **10**, 321-328.

Uenaka, T., Kishimoto, I., Sato, S., Animal, S. B., Ito, T., Otsuki, K. & Cook, J. K. A. (1998). Intracloacal infection with avian infectious bronchitis virus. *Avian Pathology* **27**, 309-312.

Vandekerchove, D., De Herdt, P., Laevens, H., Butaye, P., Meulemans, G. & Pasmans, F. (2004). Significance of interactions between Escherichia coli and respiratory pathogens in layer hen flocks suffering from colibacillosis-associated mortality. *Avian Pathology* **33**, 289-302.

Wadey, C. N. & Faragher, J. T. (1981). Australian infectious bronchitis viruses: identification of nine subtypes by a neutralisation test. *Research in Veterinary Science* **30**, 70-74.

Wahi, S. K., Dwivedi, P. N., Oberoi, M. S., Sharma, S. N. & Singh, B. (1990). Epdemiology and Microbiology of egg production drops in Poultry. *Indian Journal of Comparative Microbiology Immunology and Infectious diseases* **11**, 141-144.

Warren, D. C. & Scott, H. M. (1935). The time factor in egg production. *Poultry Science* **14**, 195-207.

Watzinger, F., Horth, E. & Lion, T. (2001). Quantitation of mRNA expression by competititive PCR using non-homologous competitors containing a shifted restriction site. *Nucleic Acid Research* **29**, E52.

Wideman, R. F. & Cowen, B. S. (1987). Effect of dietary acidification on kidney damage induced in immature chickens by excess calcium and infectious bronchitis virus.*Poultry science* **66**, 626-633.

Williams, J. B. & Sharp, P. J. (1978). Ovarian morphology and rates of ovarian follicular development in laying broiler breeders and commercial egg producing hens. *British Poultry Science* **19**, 387-395.

Williams, S., Cook, J. K. A. & Parsell, Z. E. (1985). The experimental infection of chickens with mixtures of Infectious bronchitis virus and Escherichia coli. *Journal of general virology* **66**, 777-786.

Williams, A. K., Wang, L., Sneed, L. W. & Collisson, E. W. (1993). Analysis of hypervariable region in the 3'non coding end of infectious bronchitis virus genome. *Virus Research* **28**, 19-27.

Wilson, S. J. & Sharp, P. J. (1976). Induction of luteinizing hormone release by gonadal steroids in the ovariectomized domestic hen. *Journal of Endocrinology* **71**, 87-98.

Winterfield, R. W., Fadly, A. M. & Bickford, A. A. (1972). The immune response to infectious bronchitis virus determined by respiratory signs, virus infection, and histopathological lesions. *Avian Diseases* **16**, 260-269.

Wright, P. A. L., Burns, R. B., Rothwell, B. & Mackenzie, G. M. (1971). The harderian gland of domestic fowl.I histology with reference to genesis of plasma cells and Russel bodies. *Journal of Anatomy* **120**, 307-315.

Wyburn, G. M., Johnston, H. S., Draper, M. H. & Davidson, M. F. (1970). The

Fine structure of the Infundibulum and magnum of the oviduct of *Gallus domesticus*. *Quarterly Journal of Experimental Physiology* **55**, 213-232.

Wyburn, G. M., Johnston, H. S., Draper, M. H. & Davidson, M. F. (1973). The Ultrastructure of the Shell Forming Region of the Oviduct and the Development of the Shell of Gallus Domesticus. *Quarterly Journal of Experimental Physiology* **58**, 143-151.

Yachida, S., Aoyama, S., Takahashi, N., Iritani, Y. & Katagiri, K. (1981). Influence of temperature of incubation on chicken embryo tracheal organ cultures and chick embryos infected with strains of avian infectious bronchitis virus. *Research in Veterinary Science* **31**, 14-18.

Yu, J. Y. & Marquadart, R. R. (1973). Develpoment, cellular growth and function of Avian oviduct. *Biology of Reproduction* **8**, 283-298.

Zaghini A., Martelli G., Roncada P., Simioli M., Rizzi L. (2005). Mannanoligosaccharides and aflatoxin B1 in feed for laying hens: effects on egg quality, aflatoxins B1 and M1 residues in eggs, and aflatoxin B1 levels in liver. *Poultry Science* **84**, 825-832

Zanella, A. (1988). Avian infectious bronchitis: properties and application of attenuated vaccine prepared with nephropathogenic strain A2-23/74. In *Proceedings of the IInd International Symposium on Infectious Bronchitis*. Rauischholzhausen, West Germany.

Zeigel, R.F. & Dalton A.J. (1962). Speculations based on the morphology of Golgi systems in several types of protein secreting cells. *Journal of Cell Biology* 15, 45-54.

Zicca, A., Cadoni, A., Leprini, A., Millo, R., Lydyard, P. M. & Grossi, C. E. (1982). Immunofluorescent and ultrastructural analysis of plasma cell degeneration in chickens harder's gland. *Develpoment and comparative immunology* **6**, 131-139.

Appendix

Appendix 1

Daily egg internal quality analysis amongst three IBV treatment groups

Measurements	Group	Day -21	Day -14	Day -7	Day -6	Day -5	Day -4	Day -3	Day -2	Day -1	Day 0	All groups	P values For days	P values For group	P value G*D
Albumen height (mm)	Control	9.96 ±0.18	9.79 ±0.22	10.06 ±0.20	10.12 ±0.23	9.77 ±0.12	10.04 ±0.12	10.03 ±0.16	10.10 ±0.20	10.08 ±0.15	9.69 ±0.16	9.96 ±0.05	NS	NS	NS
	N1/88	9.95 ±0.18	9.85 ±0.17	9.97 ±0.15	10.02 ±0.17	9.71 ±0.18	10.10 ±0.13	9.98 ±0.17	9.99 ±0.18	9.92 ±0.17	10.03 ±0.12	9.95 ±0.05			
	T	9.86 ±0.13	9.84 ±0.14	10.06 ±0.15	10.14 ±0.16	9.97 ±0.21	10.03 ±0.08	10.02 ±0.13	10.08 ±0.17	10.01 ±0.13	9.62 ±0.16	9.96 ±0.04			
	All	9.92 ±0.09	9.83 ±0.10	10.03 ±0.09	10.09 ±0.10	9.81 ±0.10	10.06 ±0.06	10.01 ±0.08	10.06 ±0.10	10.00 ±0.08	9.77 ±0.09				
Haugh units	Control	99.46 ±0.87	98.77 ±1.23	99.97 ±0.96	100.23 ±1.06	99.15 ±0.57	100.28 ±0.54	100.0 ±0.63	100.0 ±0.95	100.1 ±0.65	98.27 ±0.84	99.62 ±0.27	NS	NS	NS
	N1/88	99.49 ±0.80	99.15 ±0.74	99.39 ±0.80	100.0 ±0.73	98.65 ±0.92	100.2 ±0.58	99.50 ±0.76	99.62 ±0.85	99.29 ±0.84	100.1 ±0.58	99.55 ±0.24			
	T	99.05 ±0.63	99.05 ±0.63	99.67 ±0.71	100.3 ±0.73	99.46 ±0.95	99.43 ±0.46	99.86 ±0.55	99.87 ±0.78	99.17 ±0.57	97.9 ±0.77	99.42 ±0.21			
	All	99.33 ±0.44	99.00 ±0.49	99.66 ±0.47	100.21 ±0.47	99.06 ±0.47	99.96 ±0.30	99.80 ±0.37	99.84 ±0.49	99.70 ±0.40	98.71 ±0.43				
Yolk score	Control	10.70 ±0.11	10.65 ±0.14	10.68 ±0.19	10.69 ±0.16	10.56 ±0.14	10.59 ±0.14	10.70 ±0.11	10.54 ±0.26	10.45 ±0.11	10.54 ±0.14	10.61 0.05±	NS	NS	NS
	N1/88	10.81 ±0.10	10.60 ±0.12	10.51 ±0.16	10.66 ±0.13	10.73 ±0.17	10.68 ±0.11	10.54 ±0.10	10.64 ±0.12	10.60 ±0.14	10.63 ±0.10	10.64 ±0.04			
	T	10.70 ±0.14	10.58 ±0.11	10.51 ±0.18	10.57 ±0.10	10.56 ±0.11	10.74 ±0.10	10.68 ±0.11	10.67 ±0.10	10.52 ±0.20	10.82 ±0.13	10.64 ±0.04			
	All	10.74 ±0.07	10.61 ±0.07	10.56 ±0.10	10.64 ±0.07	10.62 ±0.08	10.67 ±0.07	10.64 ±0.06	10.62 ±0.09	10.53 ±0.09	10.67 ±0.07				

Measurements	Group	Day 1	Day 3	Day 5	Day -7	Day -9	Day -11	Day -13	Day -15	Day -17	Day -19
Albumen height (mm)	**Control**	9.68 ±0.10	9.22 ±0.16	9.05 ±0.20	9.17 ±0.14	9.16 ±0.13	9.13 ±0.12	8.89 ±0.16	8.93 ±0.17	8.91 ±0.15	9.24 ±0.13
	N1/88	9.76 ±0.13	8.69 ±0.25	9.10 ±0.27	8.73 ±0.19	8.53 ±0.13	8.90 ±0.13	8.79 ±0.15	9.01 ±0.18	9.21 ±0.17	9.10 ±0.13
	T	9.58 ±0.11	8.39 ±0.19	8.19 ±0.22	7.82 ±0.13	7.48 ±0.18	8.10 ±0.11	7.96 ±0.14	8.17 ±0.14	8.42 ±0.16	8.34 ±0.17
	All	9.67 ±0.06	8.77 ±0.12	8.82 ±0.13	8.48 ±0.10	8.41 ±	8.65 ±0.07	8.48 ±0.09	8.65 ±0.10	8.80 ±0.10	8.84 ±0.09
Haugh units	**Control**	98.09 ±0.49	96.04 ±0.72	97.84 ±0.79	94.33 ±1.23	93.95 ±1.31	95.09 ±0.59	93.34 ±1.32	94.19 ±0.83	94.28 ±0.76	95.94 ±0.64
	N1/88	98.46 ±0.68	92.77 ±1.48	95.03 ±1.22	93.25 ±0.88	92.59 ±0.67	94.58 ±0.58	93.70 ±0.79	94.14 ±0.86	95.91 ±0.77	95.57 ±0.55
	T	96.45 ±1.10	91.47 ±1.04	92.22 ±1.18	88.49 ±0.77	87.40 ±0.79	90.17 ±0.63	89.17 ±0.87	90.00 ±0.81	91.71 ±0.88	91.14 ±1.03
	All	97.66 ±0.46	93.48 ±0.65	95.38 ±	91.61 ±0.57	91.40 ±0.60	92.97 ±0.39	91.95 ±0.59	92.48 0.51	93.68 ±0.50	93.92 ±
Yolk score	**Control**	10.56 ±0.09	10.95 ±0.11	10.71 ±0.28	11.00 ±0.10	10.62 ±0.08	10.99 ±0.08	10.71 ±0.22	10.46 ±0.12	10.68 ±0.09	10.64 ±0.10
	N1/88	10.72 ±0.07	10.67 ±0.14	10.30 ±0.35	11.02 ±0.83	10.34 ±0.09	10.79 ±0.10	10.92 ±0.08	10.30 ±0.11	10.67 ±0.11	10.59 ±0.12
	T	11.12 ±0.07	10.95 ±0.14	10.81 ±0.17	10.45 ±0.08	10.04 ±0.08	10.14 ±0.09	10.37 ±0.10	10.47 ±0.10	10.00 ±0.08	10.27 ±0.10
	All	10.80 ±0.05	10.86 ±0.07	10.62 ±0.16	10.79 ±0.05	10.34 ±0.05	10.59 ±0.06	10.63 ±0.08	10.42 ±0.06	10.38 ±0.06	10.48 ±0.06

Measurements	Group	Day 21	Day - 23	Day 25	Day 27	Day - 34	Day - 41	Day - 48	Day - 55	Day - 62	All groups	P values For days	P values For group	P value G*D
Albumen height (mm)	**Control**	9.14 ±0.15	8.88 ±0.12	9.52 ±0.17	10.21 ±0.19	9.01 ±0.17	9.97 ±0.18	9.90 ±0.19	9.39 ±0.19	10.00 ±0.17	9.31 ±0.03	<0.0001	<0.0001	0.0038
	N1/88	8.92 ±0.10	8.98 ±0.18	9.30 ±0.19	9.57 ±0.17	8.31 ±0.22	9.79 ±0.19	10.24 ±0.21	9.10 ±0.24	10.21 ±0.19	9.11 ±0.04			
	T	8.38 ±0.11	8.44 ±0.12	8.55 ±0.14	9.16 ±0.18	7.47 ±0.14	9.36 ±0.17	9.55 ±0.28	8.67 ±0.19	9.51 ±0.13	8.42 ±0.04			
	All	8.82 ±0.07	8.73 ±0.08	9.05 ±0.10	9.60 ±0.11	8.19 ±0.12	9.71 ±0.10	9.88 ±0.13	9.07 ±0.12	9.90 ±0.10				
Haugh units	**Control**	95.10 ±0.79	94.03 ±0.58	97.01 ±0.79	99.67 ±0.58	95.00 ±0.94	99.12 ±0.81	98.59 ±0.82	96.37 ±0.88	98.82 ±0.81	95.99 ±0.21	<0.0001	<0.0001	NS
	N1/88	94.46 ±0.48	94.20 ±0.86	96.16 ±0.87	96.87 ±0.64	91.20 ±1.11	98.32 ±0.79	99.88 ±0.88	94.37 ±1.12	99.66 ±0.85	95.08 ±0.21			
	T	91.55 ±0.61	91.53 ±0.60	91.69 ±0.77	94.11 ±0.89	86.25 ±0.86	95.60 ±0.82	95.96 ±1.47	92.06 ±1.03	96.33 ±0.67	91.43 ±0.22			
	All	93.72 ±0.39	93.15 ±0.40	94.50 ±0.51	96.64 ±0.49	90.41 ±0.68	97.71 ±0.49	98.08 ±0.65	94.38 ±0.60	98.25 ±0.47				
Yolk score	**Control**	10.38 ±0.08	10.48 ±0.09	10.78 ±0.08	10.62 ±0.09	10.82 ±0.13	10.60 ±0.12	10.28 ±0.15	10.37 ±0.31	10.64 ±0.15	10.66 ±0.03	<0.0001	0.0022	<0.0001
	N1/88	10.35 ±0.11	10.77 ±0.15	10.89 ±0.19	10.56 ±0.18	10.55 ±0.10	10.75 ±0.28	10.32 ±0.28	10.25 ±0.13	10.51 ±0.13	10.61 ±0.03			
	T	10.51 ±0.10	10.19 ±0.10	10.73 ±0.12	10.72 ±0.13	10.77 ±0.13	10.70 ±0.10	10.41 ±0.17	10.20 ±0.08	10.63 ±0.13	10.46 ±0.02			
	All	10.41 ±0.05	10.44 ±0.06	10.79 ±0.07	10.64 ±0.08	10.71 ±0.07	10.68 ±0.10	10.33 ±0.11	10.28 ±0.12	10.60 ±0.08				

Measurements	Group	Day -21	Day -14	Day -7	Day -6	Day -5	Day -4	Day -3	Day -2	Day -1	Day 0	All groups	P values For days	P values For group	P value G*D
Reflectivity %	**Control**	27.46 ±0.54	28.11 ±0.51	24.00 ±0.34	28.09 ±0.51	28.34 ±0.64	28.81 ±0.53	28.61 ±0.57	28.45 ±0.57	28.13 ±0.55	28.11 ±0.58	27.90 ±0.18	<0.0001	NS	NS
	N1/88	27.52 ±0.47	28.11 ±0.56	24.80 ±0.38	28.16 ±0.62	28.42 ±0.58	28.60 ±0.47	28.36 ±0.65	28.75 ±0.55	28.35 ±0.60	28.18 ±0.65	27.97 ±0.18			
	T	27.20 ±0.45	28.05 ±0.47	24.48 ±0.32	28.24 ±0.47	28.36 ±0.48	28.80 ±0.51	28.17 ±0.49	28.73 ±0.44	27.96 ±0.48	27.96 ±0.54	27.88 ±0.15			
	All	27.39 ±0.28	28.09 ±0.29	24.45 ±0.20	28.17 ±0.31	28.41 ±0.33	28.74 ±0.29	28.37 ±0.33	28.68 ±0.30	28.14 ±0.31	28.07 ±0.33				
Egg weight g	**Control**	55.33 ±0.63	55.72 ±0.53	56.00 ±0.75	55.99 ±0.58	55.75 ±0.46	56.36 ±0.57	56.25 ±0.63	56.29 ±0.59	56.21 ±0.63	56.54 ±0.54	56.03 ±0.18	0.0428	0.0584	NS
	N1/88	54.59 ±0.69	55.14 ±0.57	55.36 ±0.64	55.43 ±0.89	55.30 ±0.52	55.57 ±0.57	55.80 ±0.71	56.15 ±0.60	55.97 ±0.61	56.19 ±0.52	55.53 ±0.20			
	T	55.68 ±0.63	55.86 ±0.52	56.05 ±0.62	56.22 ±0.42	55.85 ±0.70	56.04 ±0.55	56.61 ±0.64	56.50 ±0.73	56.25 ±0.54	56.60 ±0.57	56.18 ±0.18			
	All	55.21 ±0.37	55.58 ±0.31	55.78 ±0.38	55.89 ±0.37	55.62 ±0.32	55.99 ±56.22	56.22 ±0.38	56.32 ±0.37	56.14 ±0.34	56.45 ±0.31				
Egg shell weight g	**Control**	5.77 ±0.07	5.77 ±0.07	5.74 ±0.06	5.71 ±0.06	5.68 ±0.06	5.75 ±0.05	5.76 ±0.09	5.68 ±0.06	5.67 ±0.06	5.73 ±0.05	5.73 ±0.02	NS	NS	NS
	N1/88	5.72 ±0.08	5.72 ±0.06	5.65 ±0.09	5.70 ±0.08	5.71 ±0.07	5.70 ±0.06	5.68 ±0.07	5.70 ±0.08	5.70 ±0.07	5.71 ±0.06	5.71 ±0.02			
	T	5.80 ±0.07	5.76 ±0.07	5.79 ±0.10	5.77 ±0.06	5.69 ±0.10	5.76 ±0.10	5.83 ±0.08	5.72 ±0.09	5.77 ±0.10	5.78 ±0.07	5.74 ±0.02			
	All	5.76 ±0.04	5.75 ±0.04	5.72 ±0.05	5.73 ±0.04	5.69 ±0.04	5.74 ±0.04	5.76 ±0.05	5.70 ±0.04	5.72 ±0.04	5.74 ±0.04				

Measurements	Group	Day 1	Day 3	Day 5	Day -7	Day -9	Day -11	Day -13	Day -15	Day -17	Day -19	Day 21	Day -23
Reflectivity %	**Control**	27.69 ±0.42	29.02 ±0.65	28.69 ±0.62	27.66 ±0.52	28.93 ±0.42	29.12 ±0.41	29.23 ±0.60	30.88 ±0.50	31.44 ±0.40	30.42 ±0.54	30.47 ±0.49	30.56 ±0.49
	N1/88	29.18 ±0.48	32.35 ±0.72	33.16 ±0.88	30.29 ±0.42	31.33 ±0.48	30.06 ±0.41	29.17 ±0.45	30.45 ±0.49	30.73 ±0.51	30.30 ±0.45	30.42 ±0.40	29.27 ±0.45
	T	29.11 ±0.33	34.71 ±0.63	34.66 ±1.08	33.47 ±0.45	34.17 ±0.57	32.24 ±0.43	32.24 ±0.51	33.12 ±0.51	34.52 ±0.52	33.39 ±0.45	32.68 ±0.53	32.24 ±0.49
	All	28.65 ±0.24	31.97 ±0.43	31.77 ±0.54	30.88 ±0.32	31.43 ±0.33	30.66 ±0.26	30.47 ±0.33	31.66 ±0.31	32.51 ±0.32	31.57 ±0.30	31.18 ±0.28	30.88 ±0.30
Egg weight g	**Control**	57.38 ±0.40	57.64 ±0.62	57.53 ±0.92	58.49 ±0.43	58.76 ±0.40	58.82 ±0.56	58.88 ±0.61	58.73 ±0.49	57.60 ±0.51	58.49 ±0.50	59.06 ±0.56	58.66 ±0.48
	N1/88	56.34 ±0.43	57.67 ±0.78	58.13 ±0.80	56.59 ±0.76	56.58 ±0.60	57.09 ±0.78	58.04 ±0.47	57.47 ±0.49	56.69 ±0.55	57.44 ±0.62	58.11 ±0.52	58.41 ±0.57
	T	58.32 ±0.59	58.08 ±0.71	58.13 ±1.58	57.49 ±0.38	58.02 ±0.48	57.51 ±0.48	58.62 ±0.40	58.55 ±0.39	57.42 ±0.44	57.63 ±0.52	59.09 ±0.57	59.59 ±0.47
	All	57.36 ±0.28	57.80 ±0.40	57.89 ±0.62	57.46 ±0.32	57.76 ±0.30	57.76 ±0.35	58.53 ±0.28	58.27 ±0.26	57.27 ±0.28	57.85 ±0.31	58.75 ±0.32	58.96 ±0.29
Egg shell weight g	**Control**	5.79 ±0.06	5.82 ±0.09	5.80 ±0.10	5.84 ±0.05	5.90 ±0.07	5.87 ±0.04	5.88 ±0.07	5.77 ±0.05	5.78 ±0.04	5.81 ±0.05	5.83 ±0.07	5.82 ±0.05
	N1/88	5.67 ±0.05	5.76 ±0.06	5.80 ±0.07	5.65 ±0.08	5.68 ±0.06	5.70 ±0.09	5.78 ±0.09	5.74 ±0.05	5.71 ±0.06	5.72 ±0.10	5.85 ±0.07	5.82 ±0.07
	T	5.77 ±0.08	5.67 ±0.09	5.78 ±0.14	5.75 ±0.05	5.82 ±0.11	5.80 ±0.07	5.90 ±0.06	5.81 ±0.04	5.83 ±0.04	5.76 ±0.06	5.81 ±0.05	5.88 ±0.06
	All	5.75 ±0.03	5.75 ±0.50	5.79 ±0.06	5.74 ±0.04	5.80 ±0.04	5.78 ±0.04	5.86 ±0.04	5.78 ±0.03	5.78 ±0.03	5.77 ±0.04	5.83 ±0.03	5.85 ±0.03

Measurements	Group	Day 25	Day 27	Day -34	Day -41	Day -48	Day -55	Day -62	All groups	P values For days	P values For group	P value G*D
Reflectivity %	**Control**	29.56 ±0.48	29.59 ±0.49	30.39 ±0.74	29.93 ±0.62	30.43 ±0.62	31.57 ±0.72	30.79 ±0.56	29.74 ±0.12	<0.0001	<0.0001	<0.0001
	N1/88	28.78 ±0.48	28.35 ±0.54	31.03 ±0.69	30.10 ±0.52	30.00 ±0.62	30.77 ±0.37	30.40 ±0.57	30.32 ±0.12			
	T	32.07 ±0.49	30.90 ±0.52	31.96 ±0.65	30.10 ±0.60	30.51 ±0.63	31.53 ±0.75	31.03 ±0.71	32.50 ±0.13			
	All	30.44 ±0.31	29.79 ±0.31	31.20 ±0.40	30.04 ±0.33	30.33 ±0.36	31.32 ±0.38	30.75 ±0.35				
Egg weight g	**Control**	58.33 ±0.48	59.17 ±0.41	59.45 ±0.55	59.25 ±0.74	59.30 ±0.61	59.47 ±0.64	59.56 ±0.50	58.59 ±0.13	<0.0001	0.0015	NS
	N1/88	58.08 ±0.60	58.85 ±0.60	59.21 ±0.67	59.34 ±0.88	59.51 ±0.79	59.86 ±0.77	60.04 ±0.46	57.84 ±0.15			
	T	59.56 ±0.41	59.20 ±0.53	59.48 ±0.65	59.28 ±0.90	59.73 ±0.71	60.05 ±1.06	60.80 ±0.59	58.58 ±0.13			
	All	58.79 ±0.28	59.08 ±0.30	59.38 ±0.36	59.29 ±0.48	59.51 ±0.40	59.77 ±0.47	60.11 ±0.30				
Egg shell weight g	**Control**	5.95 ±0.06	5.99 ±0.04	6.11 ±0.08	6.08 ±0.08	6.00 ±0.07	6.02 ±0.06	6.09 ±0.10	5.88 ±0.01	<0.0001	NS	NS
	N1/88	5.77 ±0.07	6.02 ±0.07	6.01 ±0.08	6.03 ±0.15	6.06 ±0.10	6.02 ±0.10	6.24 ±0.09	5.85 ±0.02			
	T	5.85 ±0.06	6.04 ±0.07	6.12 ±0.01	6.08 ±0.11	6.10 ±0.10	6.10 ±0.12	6.26 ±0.10	5.87 ±0.01			
	All	5.86 ±0.04	6.02 ±0.03	6.08 ±0.05	6.05 ±0.05	6.05 ±0.05	6.05 ±0.05	6.19 ±0.05				

Measurements	Group	Day -21	Day -14	Day -7	Day -6	Day -5	Day -4	Day -3	Day -2	Day -1	Day 0	All groups	P values For days	P values For group	P value G*D
Deformation µm	**Control**	255.74 ±5.27	266.36 ±4.24	259.42 ±6.37	254.88 ±4.97	260.43 ±5.09	261.83 ±4.29	255.85 ±4.71	257.95 ±4.18	250.00 ±4.83	255.90 ±4.06	259.6 ± 2.8			
	N1/88	257.54 ±5.61	265.09 ±4.98	263.65 ±8.27	264.40 ±7.52	263.26 ±4.24	257.00 ±5.52	250.00 ±4.64	264.22 ±4.38	258.43 ±4.10	253.83 ±4.30	260.6 ± 1.7	NS	0.0337	NS
	T	256.54 ±4.28	265.71 ±4.11	255.13 ±5.29	260.52 ±3.93	265.12 ±7.00	257.42 ±3.99	254.22 ±5.82	256.12 ±3.96	260.18 ±5.05	253.65 ±7.14	260.0 ± 1.6			
	All	256.52 ± 1.5	265.60 ± 2.5	259.55 ±3.97	260.20 ±3.24	262.85 ±3.12	258.70 ±2.65	253.30 ±2.94	259.34 ±2.41	256.55 ±2.71	254.42 ±3.22				
Breaking Strength N	**Control**	41.6 ± 1.2	43.5 ± 0.5	42.75 ±1.18	42.89 ±0.94	42.41 ±0.85	42.30 ±0.85	42.61 ±1.21	42.48 ±1.26	42.39 ±0.97	40.05 ±1.15	42.3 ± 0.3			
	N1/88	44.8 ± 1.1	44.7 ± 1.0	43.48 ±1.27	43.31 ±1.04	42.50 ±0.81	43.05 ±0.96	43.00 ±1.18	43.38 ±0.84	42.96 ±0.87	42.09 ±0.81	44.1 ± 0.3	0.0470	0.0398	NS
	T	42.7 ± 0.9	44.1 ± 0.7	43.10 ±1.20	43.05 ±1.16	42.79 ±1.02	42.51 ±0.91	43.18 ±0.85	41.94 ±1.21	42.49 ±1.07	42.20 ±1.30	43.1 ± 0.3			
	All	43.06 ± 0.6	44.1 ± 0.2	43.13 ±0.70	43.09 ±0.62	42.56 ±0.51	42.62 ±0.52	42.94 ±0.62	42.58 ±0.65	42.62 ±0.56	41.49 ±0.65				
Shell thickness µm	**Control**	438.9 ± 3.5	439.19 ±2.81	437.98 ±3.06	432.38 ±3.86	432.19 ±2.62	439.17 ±2.41	436.80 ±4.19	435.32 ±2.81	433.82 ±3.37	439.24 ±2.59	436.67 ±1.00			
	N1/88	438.0 ± 4.0	437.95 ±3.39	433.33 ±5.22	432.75 ±5.23	434.71 ±3.54	438.55 ±2.89	434.54 ±3.23	434.64 ±5.99	437.65 ±3.92	438.93 ±3.34	436.16 ±1.31	NS	0.0295	NS
	T	441.0 ± 3.9	440.39 ±3.95	440.63 ±4.19	436.64 ±4.19	432.99 ±4.13	438.89 ±5.16	441.65 ±3.10	436.40 ±5.08	440.08 ±3.84	438.51 ±3.81	439.26 ±1.33			
	All	439.3 ± 2.2	439.22 ±2.02	437.04 ±2.52	434.29 ±2.60	433.55 ±1.97	438.87 ±2.19	437.72 ±2.02	435.48 ±2.78	437.38 ±2.17	438.87 ±1.93				

Measurements	Group	Day 1	Day 3	Day 5	Day -7	Day -9	Day -11	Day -13	Day -15	Day -17	Day -19	Day 21	Day -23
Deformation μm	**Control**	287.44 ±15.63	251.36 ±8.25	272.56 ±15.65	252.39 ±6.54	269.43 ±16.22	268.85 ±7.71	275.83 ±8.82	271.02 ±8.29	282.55 ±18.51	253.52 ±5.99	273.88 ±18.56	250.83 ±7.70
	N1/88	257.44 ±5.14	276.75 ±13.60	259.66 ±6.61	252.41 ±5.38	278.40 ±7.58	278.11 ±8.05	279.52 ±7.16	267.12 ±7.08	279.88 ±8.37	271.92 ±7.36	257.11 ±5.84	275.14 ±9.08
	T	241.91 ±5.16	275.23 ±14.98	289.63 ±26.32	259.56 ±6.77	270.83 ±7.01	268.85 ±7.71	273.30 ±7.56	283.95 ±11.65	255.82 ±6.37	248.36 ±6.73	252.50 ±9.14	253.26 ±6.30
	All	262.33 ±5.99	267.38 ±7.22	273.33 ±9.93	254.89 ±3.68	273.04 ±6.35	271.81 ±4.54	275.87 ±4.54	275.06 ±5.71	270.77 ±6.68	256.66 ±3.96	261.63 ±7.18	258.41 ±4.39
Breaking Strength N	**Control**	40.15 ±0.78	39.52 ±1.22	42.27 ±1.08	40.13 ±0.73	40.25 ±0.77	39.38 ±0.64	39.56 ±0.98	38.90 ±0.78	37.19 ±0.90	39.57 ±0.75	37.83 ±1.14	38.03 ±0.67
	N1/88	41.13 ±0.74	41.49 ±0.91	42.86 ±0.93	42.49 ±0.88	41.31 ±0.76	42.14	43.00 ±0.91	42.18 ±0.62	40.33 ±0.69	40.87 ±0.80	41.22 ±0.67	41.04 ±1.00
	T	40.51 ±0.85	39.85 ±1.06	40.73 ±2.44	40.65 ±0.81	42.21 ±0.87	41.09 ±0.70	41.62 ±0.77	40.61 ±0.78	37.15 ±0.77	38.09 ±0.88	41.77 ±0.89	41.81 ±0.72
	All	40.65 ±0.45	40.26 ±0.62	42.02 ±0.86	41.21 ±0.48	41.24 ±0.46	40.93 ±0.43	41.42 ±0.51	40.60 ±0.44	38.07 ±0.47	39.35 ±0.49	40.26 ±0.55	40.37 ±0.47
Shell thickness μm	**Control**	440.45 ±2.91	440.28 ±4.63	445.86 ±4.00	441.92 ±3.10	439.70 ±3.47	441.88 ±2.57	444.00 ±3.57	438.17 ±2.23	435.89 ±2.36	439.99 ±2.74	438.05 ±3.99	438.92 ±3.48
	N1/88	437.44 ±2.05	439.71 ±2.78	444.31 ±2.77	437.40 ±3.88	437.45 ±3.04	439.22 ±3.50	442.42 ±4.34	438.16 ±2.77	436.42 ±2.76	435.18 ±7.05	441.39 ±3.25	438.39 ±3.83
	T	437.80 ±3.84	436.15 ±5.25	437.56 ±7.89	437.53 ±3.05	438.66 ±5.76	438.43 ±6.66	443.89 ±4.61	440.59 ±2.33	437.30 ±3.39	435.80 ±3.41	438.48 ±3.38	441.87 ±2.58
	All	438.58 ±1.75	438.73 ±2.52	443.04 ±2.87	438.65 ±1.95	438.58 ±2.39	439.66 ±2.95	443.49 ±2.50	439.15 ±1.41	436.62 ±1.74	436.96 ±2.55	239.32 ±2.04	439.96 ±1.85

Measurements	Group	Day 25	Day 27	Day -34	Day -41	Day -48	Day -55	Day -62	All groups	P values For days	P values For group	P value G*D
Deformation µm	**Control**	263.81 ±5.90	262.50 ±8.40	238.26 ±5.68	236.36 ±5.55	239.68 ±13.10	252.28 ±4.53	251.47 ±4.87	262.27 ±2.66			
	N1/88	262.50 ±7.34	252.96 ±4.63	248.62 ±6.87	241.07 ±7.28	240.80 ±8.26	247.77 ±5.63	256.66 ±6.75	263.95 ±1.80	< 0.0001	NS	NS
	T	264.43 ±7.24	258.41 ±8.52	249.03 ±5.98	227.33 ±5.75	252.75 ±9.09	257.66 ±5.72	246.00 ±5.08	260.44 ±2.10			
	All	263.74 ±4.05	258.00 ±4.40	245.90 ±3.63	234.83 ±3.56	244.41 ±6.20	252.71 ±3.02	251.20 ±3.17				
Breaking Strength N	**Control**	41.52 ±0.66	43.44 ±0.79	42.14 ±1.84	42.24 ±1.32	39.36 ±1.36	43.53 ±1.05	43.26 ±1.65	40.23 ±0.23			
	N1/88	42.89 ±1.00	45.04 ±1.07	44.71 ±1.14	41.63 ±1.56	42.33 ±1.26	47.75 ±1.26	45.33 ±1.44	42.37 ±0.22	< 0.0001	< 0.0001	N
	T	40.81 ±0.73	43.75 ±0.91	43.59 ±1.49	40.44 ±1.65	42.49 ±1.47	44.64 ±0.90	42.51 ±1.21	41.01 ±0.22			
	All	41.36 ±0.45	44.05 ±0.53	43.58 ±0.85	41.46 ±0.86	41.28 ±0.80	45.13 ±0.64	43.63 ±0.85				
Shell thickness µm	**Control**	446.46 ±2.23	447.73 ±1.84	449.64 ±2.66	448.44 ±3.68	449.07 ±2.74	449.02 ±2.34	448.93 ±5.83	442.77 ±0.77			
	N1/88	441.20 ±2.97	448.50 ±4.33	449.29 ±3.32	447.76 ±7.90	448.86 ±3.46	449.79 ±3.43	452.21 ±4.18	441.38 ±0.91	< 0.0001	NS	NS
	T	442.75 ±2.97	449.56 ±3.42	449.79 ±4.56	448.05 ±5.24	449.08 ±3.90	450.08 ±4.68	452.74 ±4.00	441.44 ±1.01			
	All	443.49 ±1.64	448.68 ±1.94	449.57 ±2.16	448.10 ±3.23	449.01 ±1.92	449.60 ±2.01	451.16 ±2.80				

G- Group, D- Days, NS- Non significant

Appendix 2

10 % Neutral Buffered Formalin (Fixative for Histology)

Concentrated formalin	10 ml
Distilled water	90 ml
Sodium dihydrogen phosphate (anhydrous)	0.35 gm
Disodium hydrogen phosphate (anhydrous)	0.65 gm

Tissue processing for histopathology

50% alcohol	1 hour
70% alcohol	1 hour
90% alcohol	1 hour
90% alcohol	1 hour
Absolute alcohol	1 hour
Absolute alcohol	1 hour
Absolute alcohol	2 hours
Xylene	1 hour
Xylene	1.5 hours
Xylene	1.5 hours
Paraffin wax	2 hours
Paraffin wax (with vacuum)	1 hour

Staining solutions

Harris Haematoxylin

Haematoxylin	2.5 gm
Absolute alcohol	25 ml
Potassium alum	50 gm
Distilled water	500 ml
Mercuric oxide	1.25 gm
Glacial acetic acid	15 ml

Steps for making Haematoxylin stain

Dissolve the Haematoxylin in absolute alcohol
Add potassium alum in fairly hot distilled water in 2 litre flask
Remove from heat and add Haematoxylin solution in alcohol to potassium alum solution in water.
Boil the mixture for maximum one minute with constant stirring
Remove from heat and slowly add mercuric oxide, reheat to a simmer until solution turns dark purple
Cool the stain rapidly by plunging it in to sink containing ice
Add glacial acetic acid, when solution is cold (addition of 2-4 ml of acetic acid per 100 ml solution increases precision of nuclear stain)
Stain can be stored and used maximum for three months

Eosin

Eosin yellow	1.0 gm
Distilled water	20 ml
95% alcohol	80 ml

Acid alcohol

70% alcohol	99 ml
Hydrochloric acid (HCl)	1 ml

Alcian blue

Alcian blue	0.5 gm
Distilled water	100 ml
Glacial acetic acid	3 ml

Haematoxylin and Eosin staining protocol

Xylol I	**5 minutes**
Xylol II	**5 minutes**
Absolute alcohol I	**3 minutes**
Absolute alcohol II	**3 minutes**
80% alcohol	**2 minutes**
50% alcohol	**2 minutes**
Distilled water	**1 minute**
Harris haematoxylin	**15 minutes**
Rinse in tap water	**5 minutes**
Acid-alcohol rinse	**4 dips**
Running tap water	**30 minutes**
70% alcohol	**2 minutes**
90% alcohol	**2 minutes**
Absolute alcohol II	**2 minutes**
Eosin	**3 minutes**
Absolute alcohol IV	**3 minutes**
Absolute alcohol V	**3 minutes**
Xylol III	**2 minutes**
Xylol IV	**3 minutes**
Mount in Eukitt or Depex	

Alcian blue and Haematoxylin staining protocol

Xylol I	**5 minutes**
Xylol II	**5 minutes**
Absolute alcohol I	**3 minutes**
Absolute alcohol II	**3 minutes**
80% alcohol	**2 minutes**
50% alcohol	**2 minutes**
3% acetic acid	**3 minutes**
Alcian blue	**15 minutes**
Distilled water	**1 minute**
Harris haematoxylin	**10 minutes**
70% alcohol	**2 minutes**
90% alcohol	**2 minutes**
Absolute alcohol II	**2 minutes**
Absolute alcohol IV	**3 minutes**
Absolute alcohol V	**3 minutes**
Xylol III	**2 minutes**
Xylol IV	**3 minutes**

Mount in Eukitt or Depex

Nutrient broth/Antibiotic mixture preparation

Prepare nutrient broth (Oxoid Nutrient broth, code CM1) as directed and autoclave.

Add

Penicillin	10,000 IU/ml
Streptomycin	10 mg/ml
Gentamycin	250 µg/ml
Oxytetracyclin	50 µg/ml

Appendix 3

Phosphate buffer saline for scanning and transmission electron microscopy

1. Prepare a 0.2 M solution of dibasic sodium phosphate with

$Na_2HPO_4 . 2H_2O$	35.6g	
or $Na_2HPO_4 . 7H_2O$	53.65g	**(X)**
or $Na_2HPO_4 . 12H_2O$	71.64g	
Distilled water to make	1000 ml.	

2. Prepare a 0.2 M solution of monobasic sodium phosphate with

$NaH_2PO4 . H_2O$	27.6g	
or $NaH_2PO4. 2H_2O$	31.21g	**(Y)**
Distilled water to make	1000 ml.	

3. Prepare the **0.1 M** phosphate buffer by mixing 36 ml. of 0.2 M dibasic sodium phosphate with 14 ml. of 0.2 M monobasic sodium phosphate and diluting to 1000 ml. with distilled water. (pH 7.2)

Fixative (Karnovski's method)

0.2M buffer	50ml
10% Para formaldehyde in water	20ml
25% glutaraldehyde in water	10ml
Distilled water to make	100ml

Osmium tetraoxide

Tissue processing for transmission electron microscopy (TEM)

Fix the tissue in fixative for three hours

Samples dissected out parallel to the fibre axis, approximately 3-4 mm long and 1mm wide.

Wash tissue pieces for two times for 10 minutes in 0.1 M washing buffer

Add osmium tetraoxide and keep tissues in it for 2 hors rotating continuously.

Wash the samples with 0.1 M phosphate buffer for 15 minutes.

Dehydration in alcohol series

50%	10 mins
70%	10 mins
80%	10 mins
90%	10 mins
95%	10 mins
100%	10 mins
100%	10 mins

Pipette the osmium out for the first stage, from the first sample and pour about 10ml of 50% ethanol into the tube, start the timer (10 mins) and then continue on with the rest of the samples. At 10 mins change the first sample, and restart the timer, continue this way through the samples to the last 100% stage. Here the resin may need to be prepared.

Infiltration with alcohol/Spurrs resin

2:1	2 - 6 hours ROOM TEMP
1:1	Overnight
1:2	2 – 6 hours
Full strength	overnight

Orientate the sample as desired

Keep the samples for polymerisation for 24 hrs at 65 ^{0}C.

Tissue processing for scanning electron microscopy (SEM)

Fix the tissue in fixative for three hours

Samples dissected out parallel to the fibre axis, approximately 3-4 mm long and 1mm wide.

Wash tissue pieces for four times for 10 minutes in 0.1 M washing buffer

Add osmium tetraoxide and keep tissues in it for 2 hours rotating continuously.

Wash the samples with 0.1 M phosphate buffer for 15 minutes.

Dehydration in alcohol series

50%	10 mins
70%	10 mins
80%	10 mins
90%	10 mins
95%	10 mins
100%	10 mins
100%	10 mins

Transfer the tissues in to the boat along

Critical point drying to remove all the alcohol from tissues using liquid Co_2

Mount the sample on aluminium stub

Gold coat the sample

View under scanning electron micrograph

Spurr's Resin for TEM

ERL 4206	10g	(vinyl cyclohexene dioxide)	resin
DER 732	6g	(diglycidyl ether of polypropylene)	flexibiliser
NSA	26g	(nonenyl succinic anhydride)	hardener
DAME	0.36g	(dimethylaminoethanol)	accelerator

(prepare fresh resin at all times for the embedding)

Stains for electron microscopy

Uranyl acetate (Stock solution)

Uranyl acetate	4 gm
Distilled water	100 ml

Centrifuge at 1000 rpm for 5 min.

Uranyl acetate (working solution)

Uranyl acetate (Stock solution)	2 ml
Absolute ethanol	98 ml

Lead citrate

Lead citrate anhydrous	1.33 g
Sodium citrate dehydrate	1.76 g
Distilled water	30 ml

Shake vigorously for 1 minute and intermittently for 30 mins

1N Sodium hydroxide	8 ml

Make up to 50 ml with distilled water mix by inversion

Leave on the bench at room temperature at least for 12 hrs before use.

Appendix 4

Isolation of viral RNA from allantoic fluid using RNAeasy kit (Quiagen)

400 µl of allantoic fluid

↓

Add 300 µl of RTL buffer (add 5 µl of B-Mercaptoethanol)

↓

Vortex and keep at RT for 10 min

↓

Add 700 µl of 70 % ethanol and mix it properly with the pipette

(Make 70% ethanol by using mol bio grade ethanol and miliQ water)

↓

Transfer 700 µl of above mixture to spin column placed in the collection tube

(provided with the kits)

↓

Centrifuge it at 10000 rpm for 20 sec.

↓

Discard the flow through

↓

Add 700 µl of RW1 buffer

↓

Centrifuge it at 10000 rpm for 20 sec.

↓

Discard the flow through

↓

Add 500 µl of RPE buffer

↓

Centrifuge it at 10000 rpm for 20 sec.

↓

Discard the flow through

↓

Add 500 µl of RPE buffer

↓

Centrifuge it at 10000 rpm for 1 min

↓

Transfer spin columns in to new collection tube and Centrifuge it at 10000 rpm for 1 min (this is to remove the traces of RPE from RNA)

↓

Transfer spin column in to new microcentrifuge tube and add 30 µl of Rnase free water to spin column. Centrifuge it at 10000 rpm for 1 min to elute RNA from spin column. Transfer RNA to -70 ASAP

Important notes

Don't throw the spin columns before quantifying RNA. If yield of RNA is low, you can always re elute RNA by following last step of this protocol. Use gloves all the time and maintain cool conditions to avoid RNA degradation.

Be quick and alert while doing RNA isolation. Don't perform RNA isolation for more than 8-9 samples at a time. You can end up with degradation.

Appendix 4

Refereed publications arising from Ph.D. project

Chousalkar, K.K. and Roberts, J.R. (2006). Histopathology of two serotypes (T & N1/88) of avian infectious bronchitis virus (IBV) in vaccinated and unvaccinated birds. ***Proceedings of the Australian Poultry Science Symposium***, **18**: 135-138

Chousalkar, K.K., Roberts, J.R. and Reece, R. (2007) Histopathology of Two Australian serotypes of Infectious Bronchitis Virus in hens vaccinated in rearing phase. ***Poultry Science***. 86: 59-62

Chousalkar, K.K., Roberts, J.R. and Reece, R. (2007). Comparative Histopathology of Two Serotypes of Infectious bronchitis virus (T & N1/88) in laying hens and Cockerels. ***Poultry Science.*** 86: 50-58

Chousalkar, K.K. and Roberts, J.R. (2007). Ultra structural study of infectious bronchitis virus infection in infundibulum and magnum of commercial laying hens. ***Veterinary Microbiology.*** 122: 223-236

Chousalkar, K.K. and Roberts, J.R. (2007). Pathogenesis of two strains of Infectious bronchitis virus for the oviduct of unvaccinated laying hens. ***Proceedings of the Australian Poultry Science Symposium***, 19, 211-214

Chousalkar K.K. and Roberts, J.R. (2007). Ultra structural observations on effects of infectious bronchitis virus in egg shell-forming regions of the oviduct of the commercial laying hen. ***Poultry Science,*** 86, 1915-1919

Chousalkar, K.K. and Roberts, J.R. (2007).Egg and egg shell quality during experimental Infectious bronchitis virus infection in unvaccinated laying hen. ***Proceedings of the Australian Poultry Science Symposium***, 19, 215-218

Chousalkar, K.K. and Roberts, J.R. (2007) Persistence of infectious bronchitis virus in tissues and faeces of experimentally infected laying hens. ***Proceedings of Queensland poultry science symposium***, 13, 99-106

Other conference papers arising from Ph.D. project

Roberts, J.R. and **Chousalkar, K.K.** (2006) Effects of two strains of infectious bronchitis virus on vaccinated laying hens. ***Proceedings of the European Poultry Congress, Verona, Italy***

Chousalkar, K.K. and Roberts, J.R. (2007) Effects of infectious bronchitis challenge on egg quality in unvaccinated laying hens. ***Proceedings of Queensland poultry science symposium***, 13, 107-114

Roberts, J.R. and **Chousalkar, K.K.** (2007) Egg and egg shell quality during experimental infectious bronchitis virus infection in laying hens. ***Proceedings of the European Symposium on the Quality of Eggs and Egg Products, Prague, September 2-5.***