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Egg shell quality, with special focus on translucency and product safety

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General Introduction

The unique structure of the avian egg shell enables the egg to maintain its structural integrity and also withstand biological and mechanical onslaught. The eggs produced in Australia are of very high quality. However, it is widely recognized that bacteria such as salmonella are a potential threat to the egg industry so the industry needs to be vigilant in monitoring the presence of these bacteria on or in eggs (Cox *et al*., 2002). It is widely recognized that food-borne pathogens such as Salmonella and *E. coli* are a potential threat to the egg industry. It is generally accepted that horizontal transmission is the most likely source of bacterial contamination of shell eggs. The contents of eggs provide ideal growth media for microorganisms which are hazardous to humans (De Reu *et al*.,2008). Egg contents of the healthy laying hen are usually considered to be sterile. However, all laying hens have a common opening for intestinal, urinary and reproductive tracts which could contribute to external egg shell contamination as the egg passes through this region. The contact between faecal material and the egg shell is often unavoidable and could potentiate the entry of microorganisms into the egg. Also there is a possibility that cracking of egg shells during collection, handling and washing can enhance the entry of microorganisms into the egg. The internal contents and egg shell swabs from eggs collected from various farms across Australia during the AECL Project (UNE/AECL 86) were subjected to bacteriological examination. Our preliminary results after testing internal contents and egg shell surfaces of 500 eggs showed that *Staphylococcus spp* and *E. coli* were the predominant microbes on the egg shell. It is difficult for bacteria to move across an intact good quality egg shell. However, earlier reports indicate that small defects in the egg shell may provide the means for the predominant bacterial spp on the egg shell to penetrate the shell and move into the egg contents. The internal properties of eggs favour survival and growth of contaminating organisms which are Gram-negative (*E coli* and *Salmonella* are gram negative bacteria). Gram negative bacteria have a relatively simple nutritional requirement and have the ability to develop at low temperatures (Board and Tranter, 1995). During egg quality analysis of eggs collected from various farms across Australia, (AECL Project UNE/AECL 86), translucent eggs and microcracks were observed. The translucency in the egg shells appeared to be irrespective of hen age. The role of the phenomenon known as "translucency" is still poorly defined. In addition, it is not clear whether translucency has any role in potentiating the entry of bacteria into the egg contents. The risk that such eggs pose to product safety is uncertain. The proposed project was conducted to define translucency at the ultrastructural level and also to evaluate the extent to which these features increase the likelihood of bacteria penetrating the egg shell. There is little information about the factors responsible for formation of translucent eggs. There is a possibility that nutritional, management or infectious agents could be responsible for the formation of translucent eggs. Earlier, the Dutch strain of *M. synoviae* was found to be one of the factors causing egg shell translucency (Feberwee *et al*., 2009a &b). However there is little information available regarding the positive correlation between Australian strains of *M.synoviae* and egg shell translucency. In the present study, translucent and non translucent eggs were tested for the presence of *M.synoviae*.

Objectives of the present study

The three objectives of this study were

1 To study the ultrastructure of transluscent egg shells

2 To determine the effect of egg shell translucency on the ability of bacteria such as Salmonella and E coli to penetrate the egg shell.

3 To investigate the presence of *Mycoplasma synoviae* in eggs and correlate its presence with egg shell translucency.

Chapter 1

Ultra structure of translucent egg shells

Formation and Structure of Avian egg shell

The oviduct is divided into six distinguishable regions, infundibulum, magnum, isthmus, tubular shell gland, shell gland pouch, and vagina. Each region is morphologically different and involved in some way in the process of egg formation, i.e. in the process of formation of chalazae, thin and thick albumen, inner and outer shell membranes and a calcified shell around the central mass of yolk (Gilbert, 1971). After spending 3-4 hours in the albumen forming regions of the oviduct (Infundibulum and magnum), the egg enters the isthmus and stays there for a short time. According to Draper *et al*. (1972), three parts of the oviduct, isthmus, tubular shell gland and shell gland pouch, are involved in the process of shell formation. The reticular formation of the isthmus starts depositing a continuous deposit of fibrous layers around the rotating egg mass. In the granular isthmus, the substance of the core and mantle of shell membrane fibres is deposited onto the egg. The process of fabrication occurs inside the gland cells of the granular isthmus. The gland cells of the functioning oviduct contain electron dense material as a filament. The epithelial cells of the tubular shell gland produce and secrete the mammillary cores (Stemberger *et al*., 1977). Mammillary cores are seeding sites on which crystallization of the egg shell begins. The integrity of the mammillary region of the egg shell plays a vital part in egg shell quality. Mammillary cores are initial templates for the calcification of the egg shell, hence any change in the morphology or composition of the mammillary cores could affect the egg shell structure and quality. Mammillary caps, type A mammillae and type B mammillae are important components of the mammillary region (Brackpool, 1995). The mammillary caps with extensive contact with egg shell membrane are thought to hold the calcified egg shell more firmly to the membranes which ultimately enhances shell strength (Solomon, 1991). The characteristic appearance of mammillary caps when they join closely to one another is called confluence.

After formation of the mamillary cores, the egg enters the shell gland pouch with the shell membranes still relatively loose and stays there for at least 20 hours. The shell gland pouch starts pouring a watery secretion into the egg and this process is called “plumping” (Simkiss & Taylor, 1971). According to Simkiss and Taylor (1971), formation of egg shell pores may be the effect of this continual secretion. The process of shell formation is initiated in the isthmus with the formation of the shell membranes and continues with the formation of the mammillary cores in the tubular shell gland. The main part of shell mineralization occurs in the shell gland pouch. Most of the calcium used for shell formation is derived from the blood although a small amount may be present in the shell gland pouch (Hohman and Schraer, 1966). The mitochondria of the gland cells in the shell gland pouch accumulate sufficient calcium ions. Epithelial cells play a major role in active transport of calcium from the blood stream. Simkiss and Tyler (1957) suggested that the mammillary core surrounded by sulphated protein is a chelating agent which removes calcium from the blood (chelation is the union between a metallic ion and a chelating molecule). The carbonate ions may then displace the chelating agent from calcium to form a calcium carbonate complex. Calcium

secretion is thought to be under the control of hormones (Eastin and Spaziani, 1978). According to Simkiss & Taylor (1971), in the shell gland pouch during egg shell formation, calcium moves across the epithelial cells of the shell gland. The mitochondrial cells can store a small quantity of calcium ions in the absence of calcification (Hohman and Schraer, 1966). Calcium-binding protein, which is known to mediate the transport of calcium across the intestinal wall, has been found in the avian shell gland pouch (Rabon *et al*., 1991). Diets containing 3.6 % calcium are the chief source of calcium during egg shell calcification. Moreover, the transport of calcium ions across the shell gland is also dependent on the healthy reproductive status of the hen. Calcium ions migrate from the serosal layer to the mucosal layer during the presence of an egg in the shell gland (Simkiss and Taylor, 1971). There is still controversy about the site of synthesis of shell pigment. Tamura *et al*. (1965) reported that epithelial cells are responsible for pigment secretion. However, according to Baird *et al*. (1975), the shell pigment protoporphyrin is derived from the blood. The compounds responsible for pigmentation are protoporphyrin, uroporphyrin and coproporphyrin (Solomon, 1987).

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

Materials and methods

Scanning electron microscopy

Eggs were candled and areas of translucency on egg shells were selected and marked with a pencil. The structure of the eggshell was studied in four eggs with egg shell apex abnormality, egg shell translucency and unaffected eggs using SEM. The egg shell pieces of approximately one square centimeter were cut from the selected translucent areas. After soaking the eggs shells in water, the egg shell membranes were manually removed. The outer membrane was removed from the dry shell by ashing, using a Bio Rad RF plasma Barrel Etcher PT 7150, as per described by Brackpool (1995). The mammillary region of the egg shell was examined for ultra structural characteristics as described by Brackpool (1995). Egg shell pieces from non translucent areas were also cut and processed for comparative observations. Observations regarding changes in mammilary cap arrangements, size, early fusion, late fusion depression, erosions, type A and B bodies were made and recorded.

Results

The ultra structural appearance of the egg shells from the non translucent egg shells were in agreement with Brackpool (1995).

The results regarding the appearance of translucent egg shells during candling are presented in Plates (1.1 to 1.3).

In the non translucent egg shells, there were no detrimental changes in the mammillary caps. Mammillary caps were of good quality with extensive attachment with the shell membrane. However, there was alignment of the mammille, where the mammillae appear to “line up” resulting in long continuous grooves between the cones (Roberts and Brackpool, 1994). The ultrastructure of alignment of mammillae is shown in plate 1.4 and 1.5. There was little cuffing (additional calcium around the mammillary cones which appears to contribute to

shell strength) in the translucent egg shells (Plate 1.6). Late fusion of the mammillary layer was not recorded. Depression and erosion of mammillae were also observed.

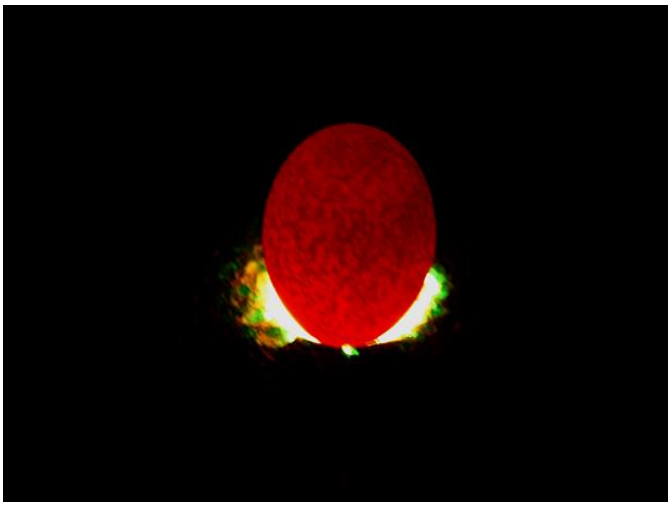

Plate 1.1: Egg shell with extensive translucency

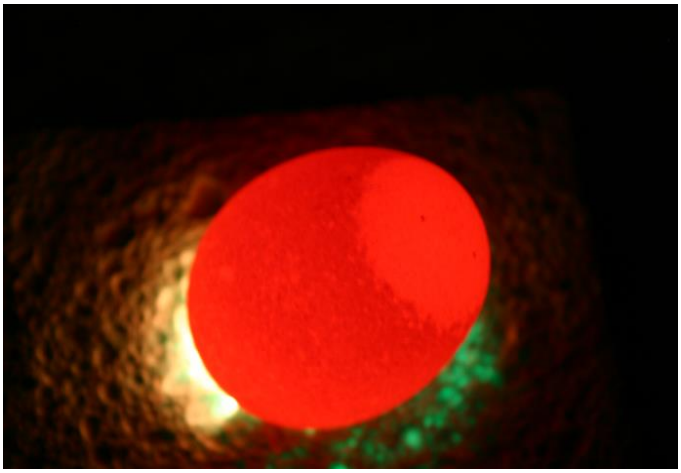

Plate 1.2: Egg shell with translucent patch at the pointy end of the egg

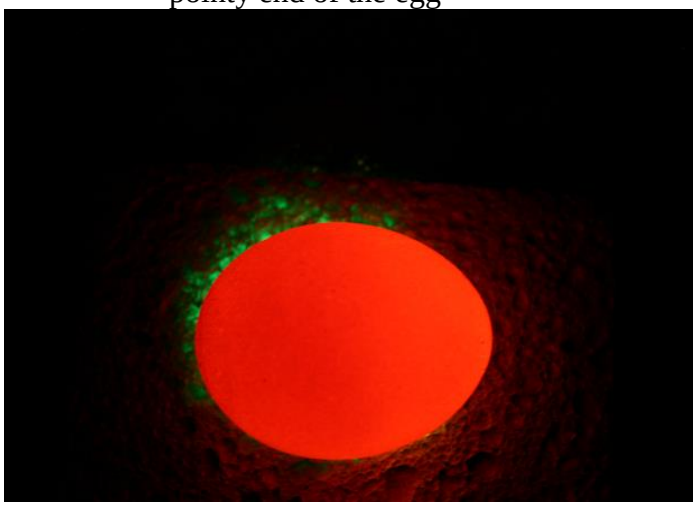

Plate 1.3: Egg shell without translucency.

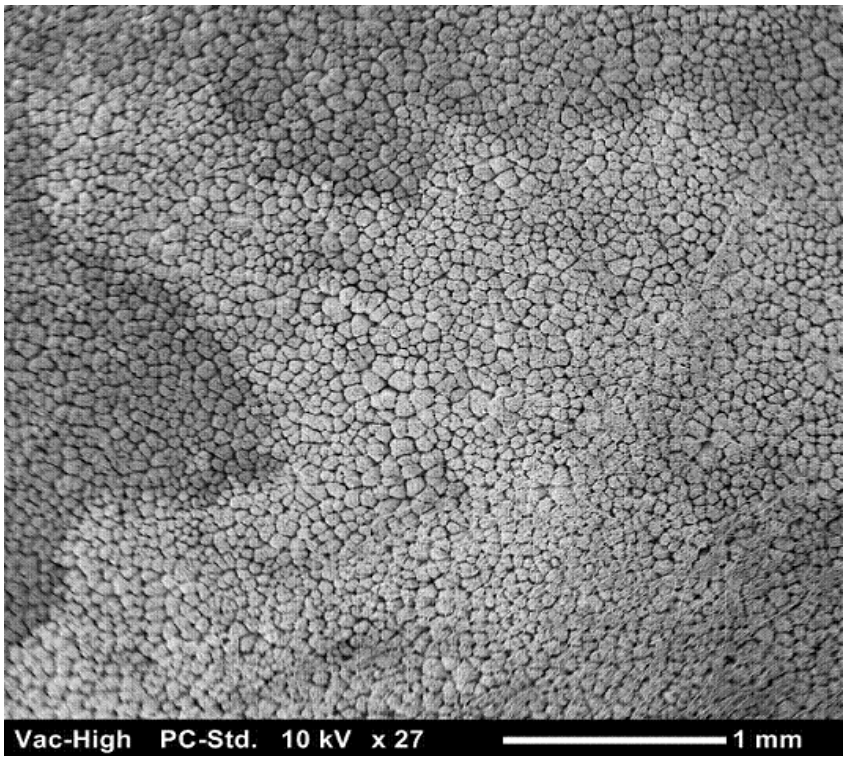

Plate 1.4. The ultrastructure of a translucent egg shell with alignment of mammillae

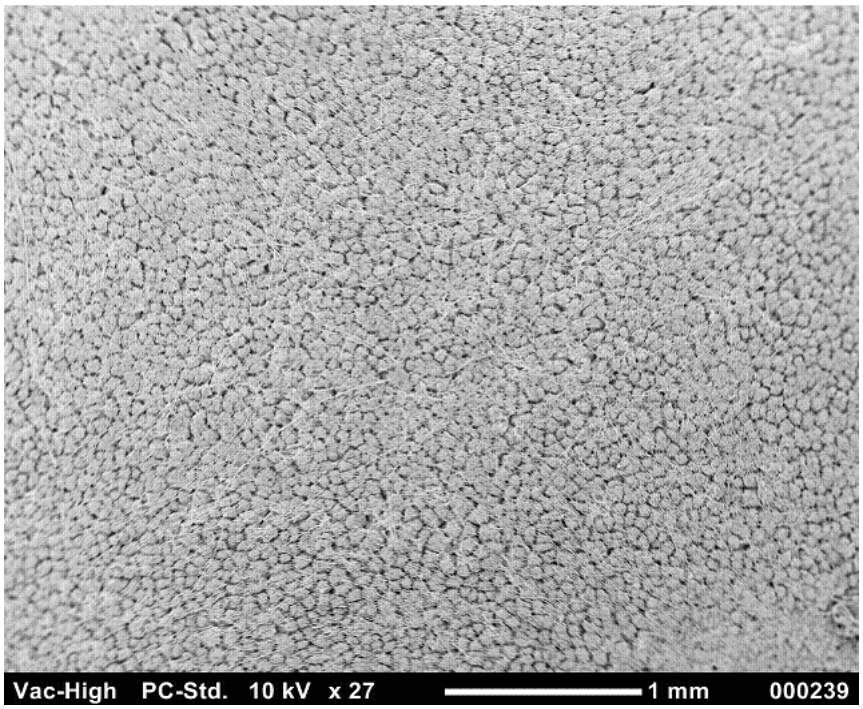

Plate 1.5. The ultrastructure of a normal egg shell.

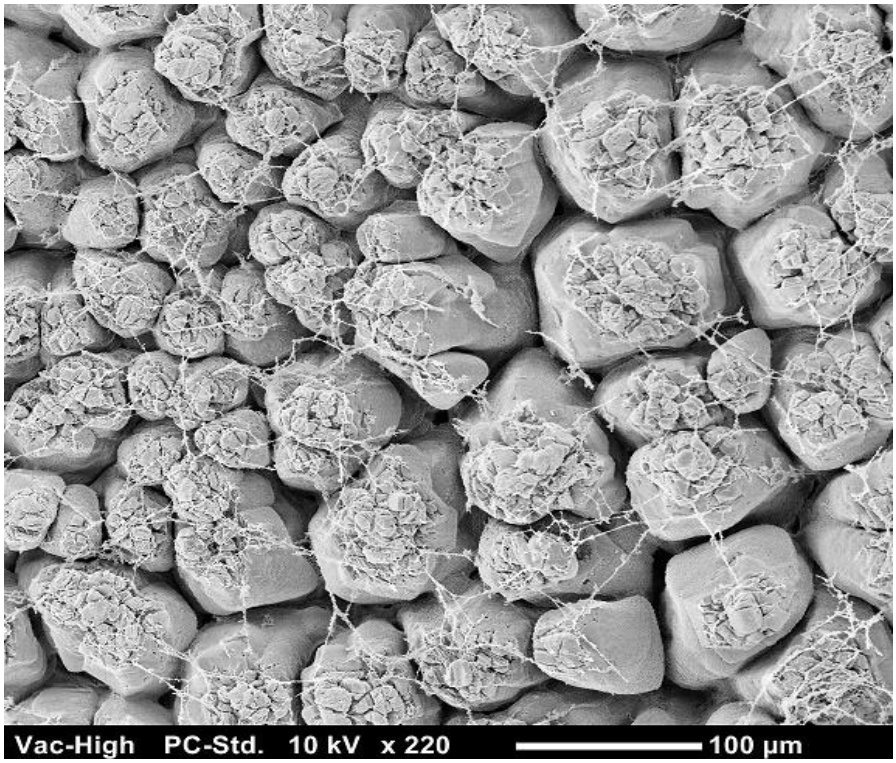

Plate 1.6. Mammillary caps of translucent eggs shells with poor cuffing

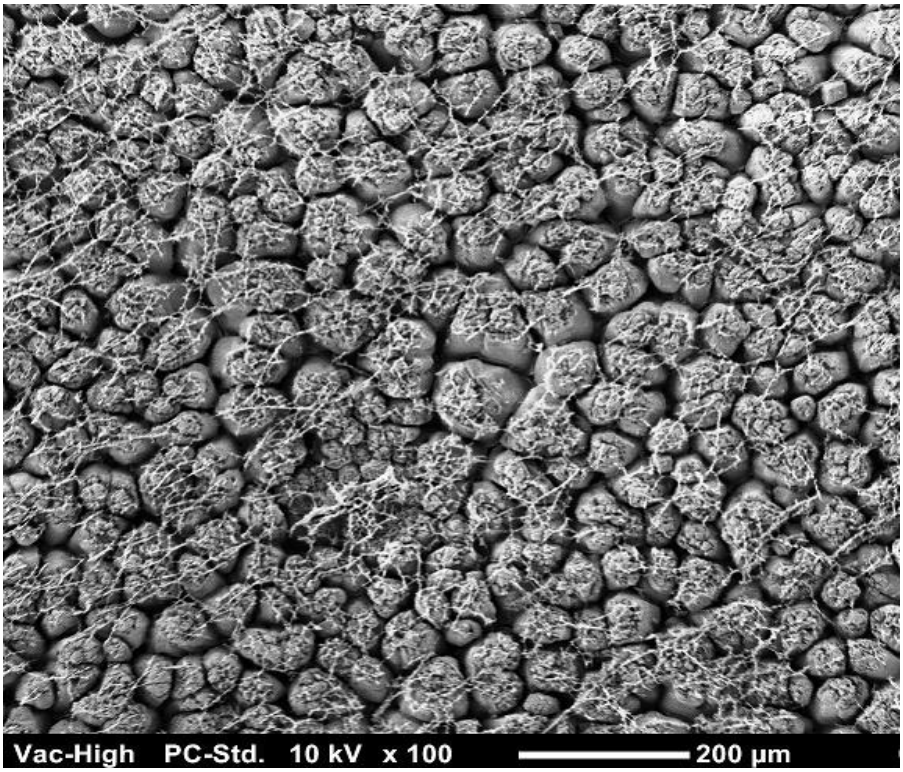

Plate 1.7.Depression in the mammillary caps of translucent egg shell.

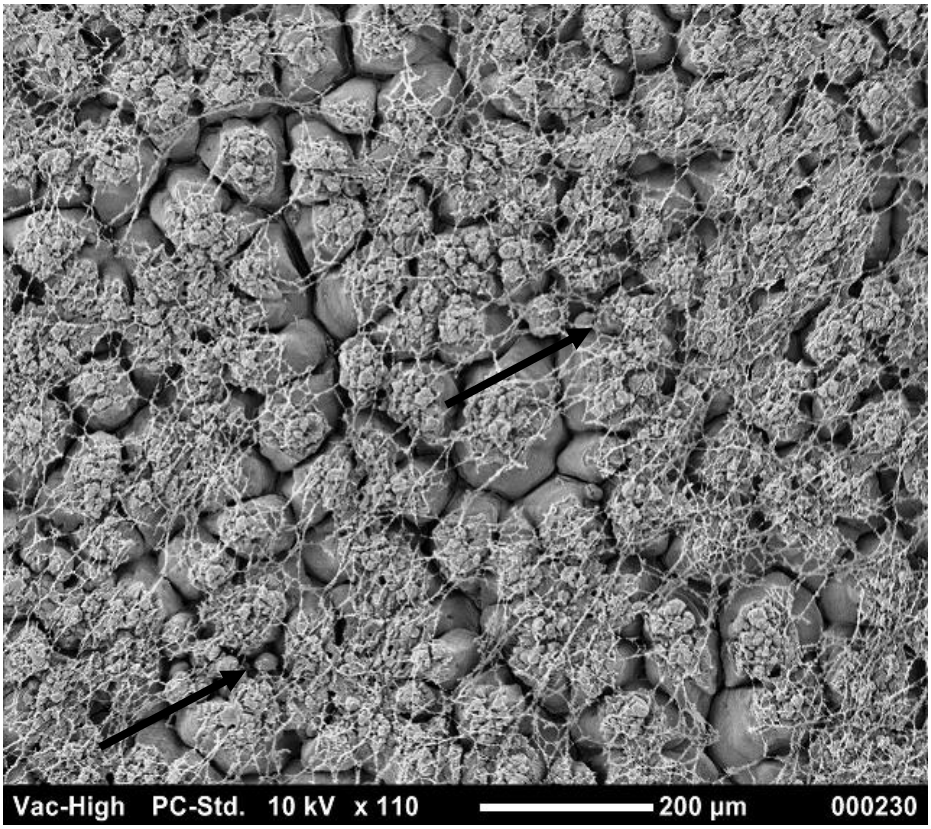

Plate 1.8. Type B bodies in the translucent patch of egg shell (arrows)

(Pitting) of the mammillae of the translucent egg shells was also recorded (Plate 1.7). Type B bodies which are small spherical calcified bodies which have variable contact with membrane fibres were also found in the translucent egg shell (Plate 1.8). Translucency could be one of the egg shell pathology which results in changes in mammillary layer probably due to the changes in the mamillary cores during the early phases of eggshell formation. Translucency ultimately has the potential to increase the incidence of microcracks in egg shells.

Chapter 2

Effect of egg shell translucency on the ability of bacteria such as *Salmonella infantis* and E coli to penetrate the egg shell

Introduction

Eggs produced in Australia are considered a medium to low risk food. The medium risk ranking is mainly because of pathogens like *Salmonella*, *E. coli*, and also some other enteropathogens. Although egg producers are diligent in fulfilling the legal obligations for the production of safe food, the egg industry in Australia is often implicated in public health risks due to the frequent cases of food poisoning. Cage laying systems are the major source of whole shelled eggs for the supermarkets in Australia. Cage eggs are 54.3 per cent of retail grocery sales followed by free range (38.6%) and barn eggs (7.1%). The operation and profitability of barn and free range systems is dependent on the price differential paid for cage and alternate system eggs (Scott *et al*., 2005). The egg contents are an ideal growth media for microorganisms which are hazardous to human. Cooking can destroy or reduce the contaminants/ bacteria in eggs, although it is common practice to eat raw or lightly cooked egg in most parts of the world including Australia. The Australian poultry industry is considered to free from *Salmonella enteritidis* which is of major concern for the food industry all over the world. However, *Salmonella typhimurium* has been isolated from layer farms in Australia (Arzey, 2008). Cox *et al* (2002) reported that *Salmonella infantis* was the predominant pathogen in the Australian egg industry. It is recommended that eggs should be properly cooked or pasteurized as cooking can destroy or reduce the contaminants/ bacteria in eggs (Davis and Breslin, 2003). However, even after cooking of an egg the persistence of heat-stable toxin-producing microorganisms can pose a major threat to human health (Harbrecht and Bergdoll, 2006). The heat stable enterotoxins (STs) are small monomeric toxins that contain multiple cysteine residues whose disulfide bonds account for the heat stability of these toxins. ST-encoding genes (*sta* and *stb*) have been found on transposons and plasmids. It has been reported that enterotoxigenic E coli (ETEC) express STb (Savarina *et al.* 1996; Yamamato and Echeverria 1996). Colicin V plasmids have been reported in the avian pathogenic E coli and are associated with virulent properties like increase in serum survival and resistance to phagocytosis (Waters and Crosa, 1991, Vidotto *et al*., 1991). It is difficult for bacteria to move across an intact good quality egg shell. However, earlier reports indicate that small defects in the egg shell may provide the means for the predominant bacterial spp on the egg shell to penetrate the egg shell and move into the egg contents (De Reu *et al*., 2006). Translucency is one of the egg shell pathology which results in irregular mammillary knobs probably due to the fusion of several mammillary

cores during the early phases of eggshell formation. It is not clear whether translucency has any role in potentiating the entry of bacteria into the egg contents. The risk that such eggs pose to product safety is uncertain. In this study the unwashed eggs collected from the cage front were tested for *Salmonella* and *E. coli spp.* All the E coli isolates were tested for enterotoxin and Colicin V gene. In this study the ultrastructure of translucent egg shells was studied and the influence of egg shell translucency on egg shell penetration and egg content contamination at different temperatures was also investigated.

Materials and Methods

An agar method described earlier by De Reu *et al*. (2006) was adapted to study the bacterial penetration across the egg shell. Briefly, 180 fresh eggs were obtained from commercial Isa brown laying hens. Eggs were divided into two groups (n=90) in a 3 x 3 factorial arrangements. Internal contents of eggs obtained from this flock were tested. Each egg was dipped into 70 % ethanol for 1 min to kill any bacteria on the outside of the shell and was allowed to air dry in a biosafety cabinet. The egg contents were sucked out after making a hole and using a sterile syringe. Eggs were then washed with sterile phosphate buffered saline (PBS; pH 7.2) to remove all the albumen adhering to the membrane. Each egg was then filled with McConkeys agar. After hardening of the agar, the eggs were sealed with paraffin wax. An *Sta* positive *E coli* isolate which was isolated from the egg shell surface in our laboratory was selected for this study. The S. *infantis* strain was obtained from The Institute of Medical and Veterinary Science, Adelaide, Australia). Bacteria stored at -80^{0}C in 50% glycerol were plated on blood and incubated overnight at 37°C. A single colony was then inoculated and grown overnight at 37°C in Brian heart infusion broth (BHI; Oxoid). The broth was then diluted in PBS until 10^{-6} dilution. Enumeration of viable bacteria was performed by serial dilution and plating 100 µl each solution out on McConkeys agar plates and incubation overnight at 37°C. Ten agar filled eggs were immersed for 1 minute in one of three serial dilutions for *E coli* and *Salmonella infantis* (approximately 10^7; $10^{5;}$ 10^3 cells per ml). After inoculation, agar filled eggs were kept at 4°C, room temperature and at 37°C. The eggs were aseptically opened in a biosafety cabinet after 72 hrs to inspect for growth of colonies. Colonies seen nearby the hole were not recorded as penetration. The shell membranes and agar with colony growth were reinoculated onto Triple Sugar Iron agar (TSI; Oxoid) and incubated at 37°C for confirmation. All eggs were candled before filling them with agar and translucency was scored as 0 (no translucency), 1(mild translucency), 2 (moderate translucency) and 3 (severe translucency).

Results

Table 1 and 2 shows the mean values with standard errors for all translucent egg shells (T), penetrated egg shells (Y) and non penetrated egg shells (N). The data are available for individually selected *E coli* and *S. infantis* strains.

Effects of egg shell translucency on E coli penetration

There were no significant differences between the translucency score of total number of inoculated egg shells used in different treatment groups (Table 1). At 37ºC, 6 out of 10 eggs inoculated in 10^7 cfu / ml were positive for egg shell penetration (Plate 2.1). 4 out of 10 eggs inoculated in 10^5 and 10^3 cfu / ml, were positive for egg shell penetration. Six out of 10 eggs were positive for bacterial penetration inoculated at the dose rate of 10^7 cfu and incubated at room temperature (RT). 5 and 3 out of 10 eggs each were found positive for bacterial penetration inoculated in 10^5 and 10^3 cfu respectively.

Bacterial penetration was recorded in two out 10 eggs inoculated at 10^7 cfu and incubated at 37ºC. For the eggs incubated at 37ºC, there were significant differences between egg shell translucencies of penetrated (Y) and non penetrated (N) egg shells inoculated in 10^7 and 10^3 cfu. For the eggs incubated at RT, there were significant differences between egg shell translucencies of penetrated (Y) and non penetrated (N) egg shells inoculated in 10^3 cfu. The bacterial penetration was recorded along the translucent patches of the egg shell. (Plate 2.2)

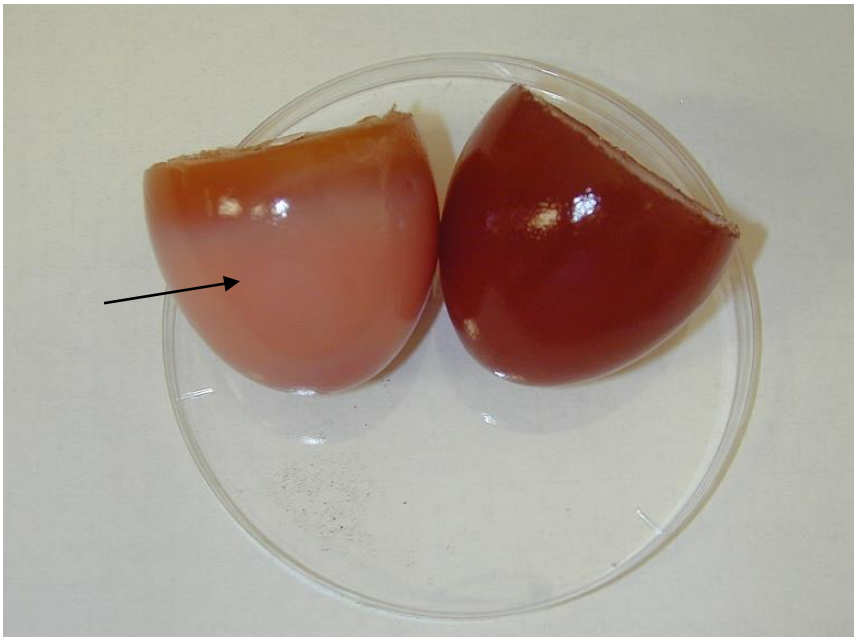

Plate 2.1. Agar filed egg after bacterial penetration (arrow) and egg with no penetration

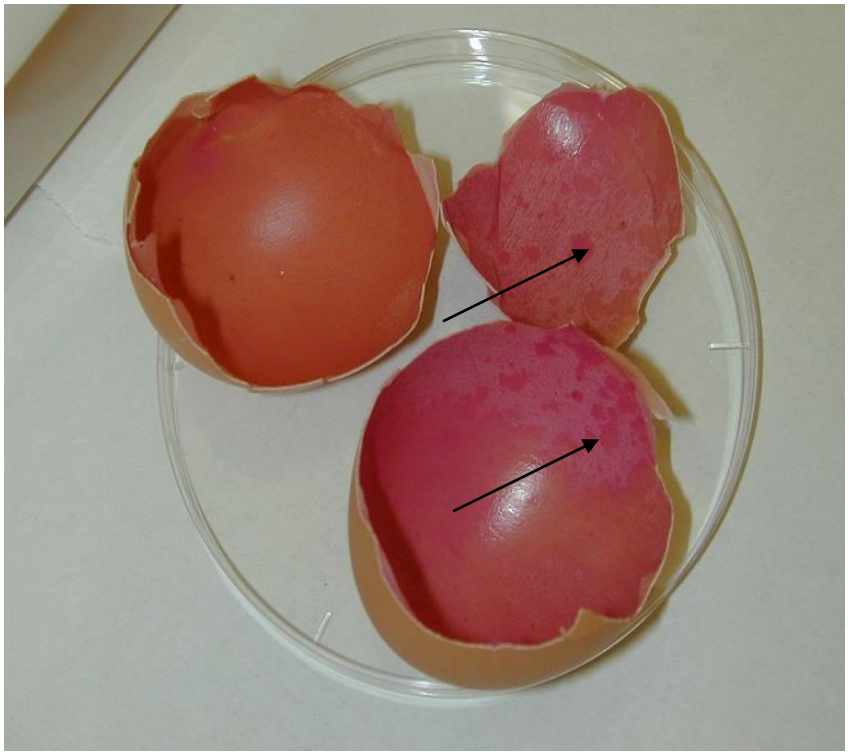

Plate 2.2. Penetration of E coli across the translucent patches of the egg shell (arrow).

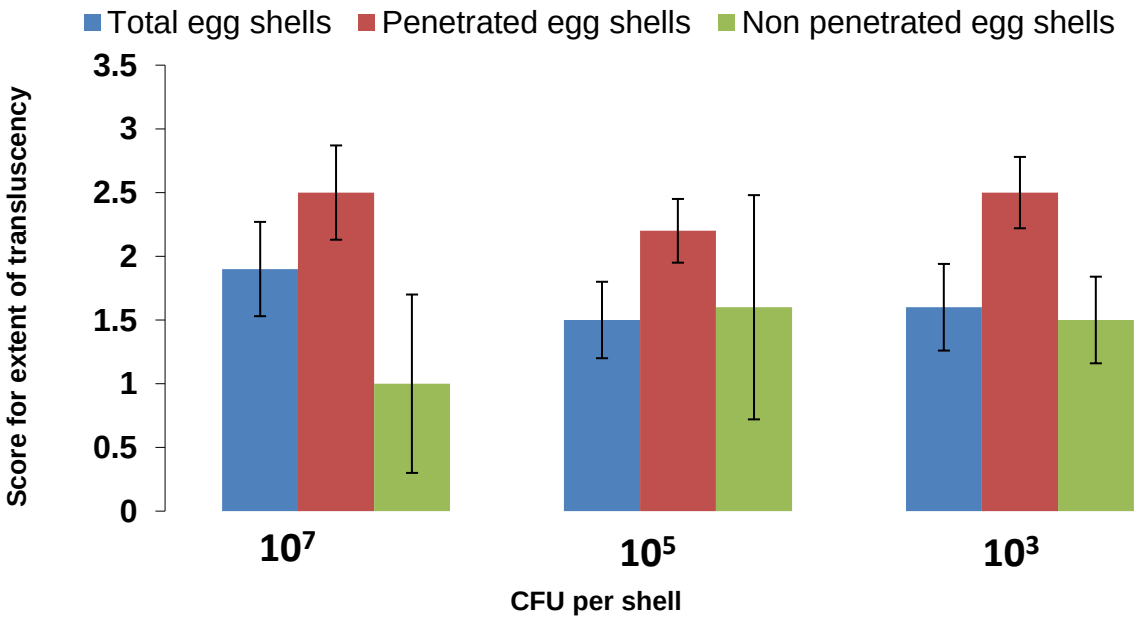

Figure 2.1. Penetration pattern of E coli across translucent and non translucent egg shells at 37ºC.

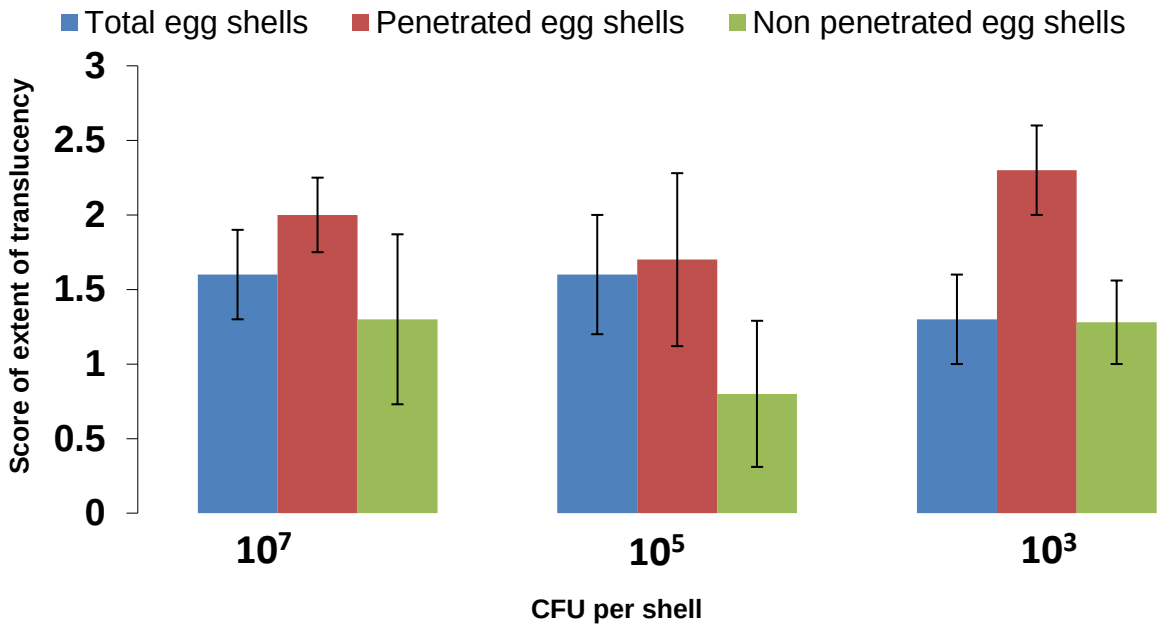

Figure 2.2. Penetration pattern of E coli across translucent and non translucent egg shells at room temperature.

Table 1: Egg shell translucency and egg shell penetration of E coli at 72 hrs

CFU/ml	Temperature	Penetrated egg shells		Mean translucency
10^7	37ºC	T^{es}	10	1.9 ± 0.37
		Y^p	6	2.5 ± 0.37
		N^p	4	1.0 ± 0.70
10^5	37ºC	T	10	1.5 ± 0.30
		Y	4	2.2 ± 0.25
		N	6	1.6 ± 0.88
10^3	37ºC	T	10	1.6 ± 0.34
		Y	4	2.5 ± 0.28
		N	6	1.5 ± 0.34
10^7	RT	T	10	1.6 ± 0.30
		Y	6	2.0 ± 0.25
		N	4	1.3 ± 0.57
10^5	RT	T	10	1.6 ± 0.40
		Y	5	1.7 ± 0.58
		N	5	0.8 ± 0.49
10^3	RT	T	10	1.3 ± 0.30
		Y	3	2.3 ± 0.30
		N	7	1.28 ± 0.28
10^7	4ºC	T	10	1.7 ± 0.36
		Y	2	2.0 ± 0.36
		N	8	1.75 ± 0.45
10^5	4ºC	T	10	1.7 ± 0.36
		Y	0	-
		N	10	-
10^3	4ºC	T	10	2.0 ± 0.36
		Y	0	-
		N	10	-

T^{es-} Total egg inoculated

Y^p- Penetrated egg shells

N^p- Non-penetrated egg shells

CFU- Colony forming unit

Effects of egg shell translucency on *S. infantis* penetration

There were no significant differences between the translucency score of total egg shells (T) inoculated at different dilutions and incubated at various temperatures (Table 2). At 37ºC, 7 out of 10 eggs inoculated in 10^7 cfu were penetrated by *S. infantis* and 4 out of 10 eggs inoculated in 10^5 and 10^3 cfu / ml, were penetrated by *S. infantis*. Seven out of 10 eggs were positive for bacterial penetration which were inoculated at the dose rate of 10^7 cfu and incubated at room temperature. Four and three out of ten eggs each were found positive for bacterial penetration inoculated in 10^5 and 10^3 cfu respectively.

Bacterial penetration was recorded in two out 10 eggs inoculated with 10^7 cfu and incubated at 4ºC. *S. infantis* penetration was not recorded in any of the egg shells from the remainder of the treatments. For the eggs incubated at 37ºC, there were significant differences between egg shell translucencies of penetrated (Y) and non penetrated (N) egg shells inoculated in 10^7 and 10^3 cfu. The bacterial penetration was recorded along the translucent patches of the egg shell (Plate 2.3).

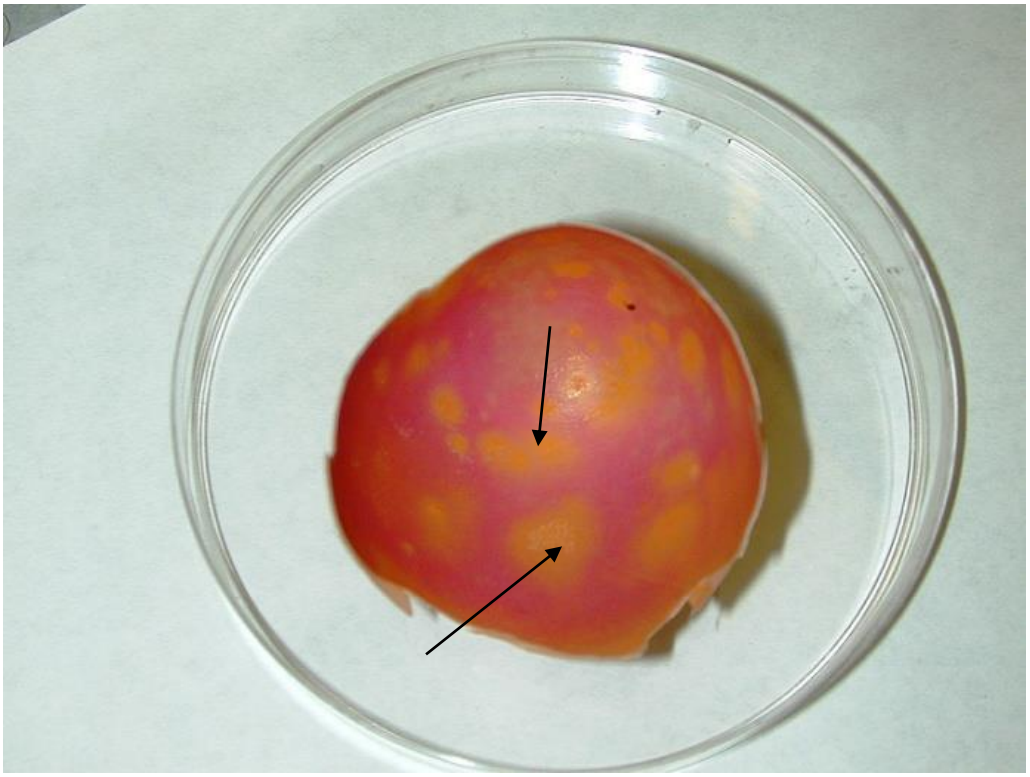

Plate 2.3. Penetration of *S. infantis* across the translucent patches of the egg shell (arrow)

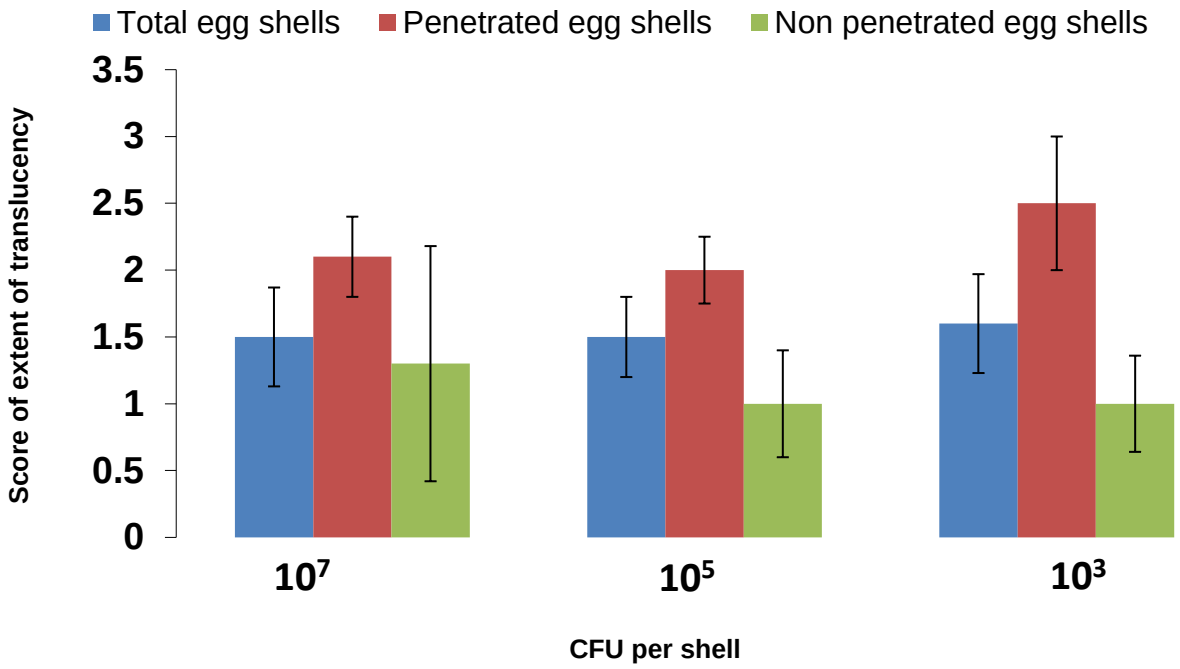

Figure 2.3. Penetration pattern of *S. infantis* across translucent and non translucent egg shells at 37ºC.

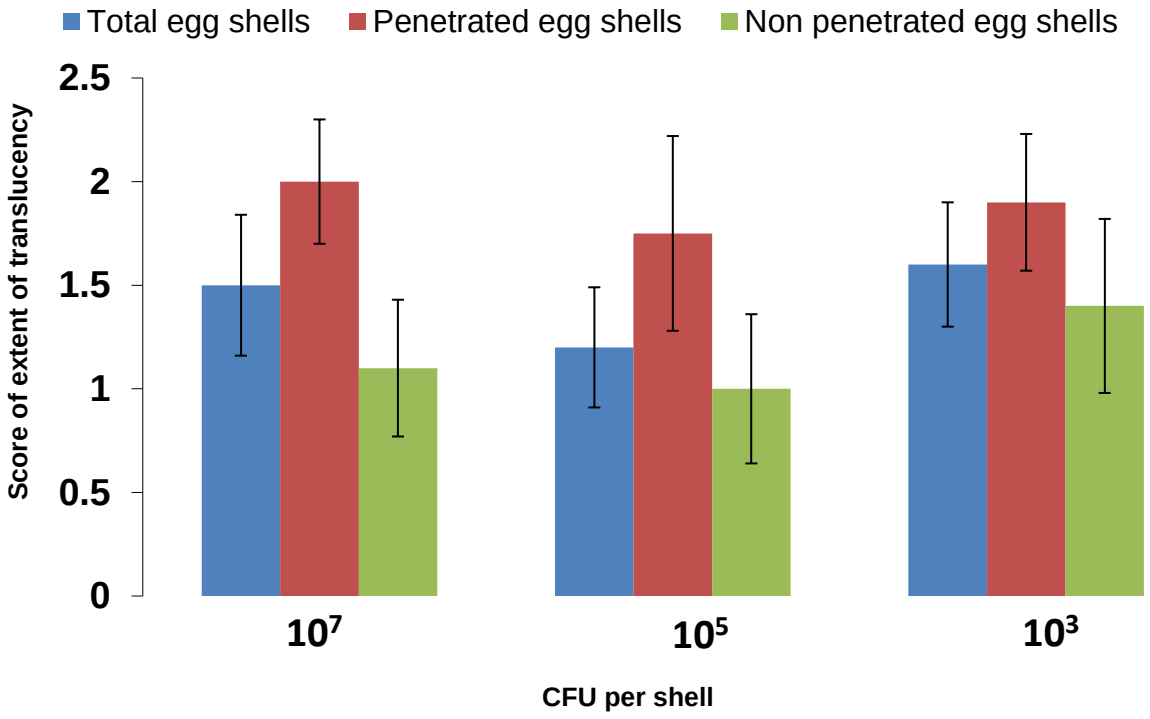

Figure 2.4. Penetration pattern of *S. infantis* across translucent and non translucent egg shells at room temperature.

Table 2: Egg shell translucency and egg shell penetration of *Salmonella infantis* at 72 hrs

CFU/ml	Temperature	Penetrated egg shells		Mean translucency
10^7	37ºC	T^{es}	10	1.5 ± 0.37
		Y^p	7	2.1 ± 0.30
		N^p	3	1.3 ± 0.88
10^5	37ºC	T	10	1.5 ± 0.30
		Y	6	2.0 ± 0.25
		N	4	1.0 ± 0.40
10^3	37ºC	T	10	1.6 ± 0.37
		Y	4	2.5 ± 0.50
		N	6	1.0 ± 0.36
10^7	RT	T	10	1.5 ± 0.34
		Y	7	2.0± 0.30
		N	3	1.1± 0.33
10^5	RT	T	10	1.2 ± 0.29
		Y	4	1.75 ± 0.47
		N	6	1.0 ± 0.36
10^3	RT	T	10	1.6 ± 0.30
		Y	3	1.6 ± 0.33
		N	7	1.4 ± 0.42
10^7	4ºC	T	10	1.6 ± 0.37
		Y	2	2.5 ± 0.50
		N	8	1.2 ± 0.36
10^5	4ºC	T	10	1.6 ± 0.34
		Y	0	-
		N	10	-
10^3	4ºC	T	10	1.9 ± 0.37
		Y	0	-
		N	10	-

T^{es-} Total egg inoculated

Y^p- Penetrated egg shells

N^p- Non-penetrated egg shells

CFU- Colony forming unit

Chapter 3

Detection of *Mycoplasma synoviae* from translucent and non translucent eggs

Introduction

Mycoplasmas are commensals and pathogens of various avian species, especially poultry. *Mycoplasma synoviae* (*M. synoviae*) may cause respiratory disease, synovitis, or may result in a silent infection. *M. synoviae* are known to be transmitted vertically through eggs (Jordan 1979). *M. synoviae* strains vary in infectivity and virulence and infections may sometimes be unapparent. *M. synoviae* is considered to be the second most important mycoplasma affecting chickens (Stipkovits & Kempf, 1996; Kleven, 2003). It causes respiratory disease and subsequent condemnations due to airsacculitis in broilers and peritonitis and mortality in layers.. Earlier, the prevalence of egg *M. synoviae* antibody in egg yolk has been found to be the suitable approach for assessing the flock prevalence of *M.* synoviae infection in layer hens (Hagan *et al*., 2004). A British respiratory isolate of *M. synoviae* has been found to be vertically transmitted in broiler breeders (Macowan *et al*., 1984). Novel egg shell abnormalities characterised by altered shell surface, shell thinning and cracks were correlated with the Dutch strain of *M. synoviae* (Feberwee, 2009a & b). Polymerase chain reaction (PCR) has become a valuable diagnostic test to aid in the diagnosis of Mycoplasma infection. The primary advantage of PCR is that it is a rapid and sensitive method of direct detection of the organism's nucleic acid from the clinical samples. PCR has been developed for detection of Mycoplasma from tracheal and choanal-cleft swabs (Gracia *et al*., 2005). In the present study the attempts were made to set up a PCR reaction for the detection of *M. synoviae* from the egg vitelline membrane to study its vertical transmission and also to study whether there is any correlation between translucent egg shells and the presence of *M. synoviae* in such eggs.

Materials and Methods

DNA extraction from egg vitelline membrane

Approximately 355 egg (230 eggs from laying hens and 125 turkey eggs) were tested for the detection of *M. synoviae*. For DNA extraction from the vitelline membrane samples, an egg was first cracked and drained of as much egg white as possible. The yolk was then placed in a sterile petridish and phosphate buffered saline (PBS) was added. The vitelline membrane was then rinsed with sterile PBS to remove the adhering egg yolk and stored in 1.5 ml microcentrifuge tubes at -20ºC. DNA extraction of the vitelline membranes or extraction from Mycoplasma cultures (*Mycoplasma synoviae* vaccine strain, Bioproperties) was performed using the Qiagen DNeasy Blood & Tissue Kit ® according to manufacturer's instructions. To each sample (about 0.2 ml), 180 µl of buffer ATL was added followed by 20 µl of proteinase K. The samples were then incubated for up to three hours (usually

required about 1 ½ hours) at 56ºC in a waterbath. Samples were then vortexed and 200 µl of Buffer AL was added. The samples were vortexed again, then 20 µl of ethanol was added and samples again vortexed, The resulting solution was passed through a DNeasy Mini spin column and centrifuged at 13,000 rpm in a Clements 100 microcentrifuge for 1 minute. The flow–through was discarded and the samples were then washed first with 500 µl of buffer AW1 and then 500 µl of buffer AW2 and again spun at 13,000 rpm for 1 minute. Following a final centrifugation to remove residual buffer, 30 µl of Buffer AE was added and left for 10 minutes. Samples were then spun at 13,000 rpm for 1 minute and the flow through collected and stored at -29ºC.

Yolk interference test

It was found earlier that egg yolk had a greater negative effect on PCR amplification than the egg white (Xiaohua *et al*., 2007). Egg yolk is in close contact with the vitelline membrane of the egg. Experiments were also conducted to test for the interference of egg yolk on the PCR reaction for the detection of *M. synoviae*. DNA was extracted from 1 ml Aliquots of Mycoplasma vaccine as mentioned above. *M. synoviae* DNA was serially diluted up to 1x 10^{-3}. One µl of serially diluted mycoplasma DNA was added to the swabs taken from the egg vitelline membrane which had yolk adhered to it. All the samples were then tested for PCR. In another experiment, the egg vitelline membrane was washed in sterile phosphate buffered saline (PBS) to remove as much yolk as possible. One µl of serially diluted *M. synoviae* DNA was then added to the washed vitelline membrane. The PCR was then carried out. Undiluted *M. synoviae* DNA extracted from a vaccine strain was used as a positive control for PCR.

Standard PCR reaction

The DNA extracted from the vitelline membrane was subjected to the PCR reaction using the primer sequences (Table 3.1) to confirm the presence of the desired fragment cloned earlier. Each reaction mixture contained 1 X reaction buffer (Fisher), 1.8 mM $MgCl_2$, 200 µM dNTPs, 1 uM of each primer, 1 U Taq polymerase, and 100 pg DNA template made up to 25 µl with MilliQ water. Samples were amplified using an Eppendorf® Mastercycler. DNA extracted from *M.synoviae* vaccine strain was used in every reaction as a positive control. The details of primers used in the PCR reactions and size of amplified product are described in Table 3.1. The PCR was performed in a thermal cycler using the following temperatures and times for 40 cycles: 94ºC for 30 sec, 55ºC for 120 sec, and 72ºC for 120 sec. The final extension was at 72ºC for 300 sec. PCR products were purified using a PCR purification kit (Promega Wizard® PCR Preps DNA purification system) as per manufacturer's recommendation. Twelve µl of PCR product at a concentration of 200 ng/µl was sent to the DNA Sequencing Facility (Macquarie University), where reactions were performed and sequencing data were generated. The data were then forwarded by e-mail back to the sender for analysis.

Scanning electron microscopy

The structure of the eggshell was studied in two eggs with egg shell apex abnormalities and in two unaffected eggs using scanning electron microscopy. The egg shells were processed as described before in materials and methods section of chapter 1.

Results

A 486 bp fragment of the 16S rRNA gene of *M. Synoviae* vaccine was amplified using non quantitative PCR (standard PCR). The sequence of the amplicon matched the sequence published earlier by Volokhov *et al*. (2006), indicating that no errors were introduced during PCR. The *M. synoviae* could not be detected by PCR in vitelline membrane which had egg yolk adhered to it, however *M. synoviae* was detected in vitelline membranes which were washed in sterile phosphate buffered saline to remove the yolk contents. The egg vitelline membrane of all 230 hen eggs tested negative for the presence of *M. synoviae*. Mycoplasma was detected in the vitelline membrane of thirteen of the turkey eggs.

Scanning electron microscopy of the egg shell with the egg shell apex abnormality revealed poor mamillary caps with erosions and type B bodies (Plate 3.1). These abnormalities were not recorded in the normal egg shells (Plate 3.2).

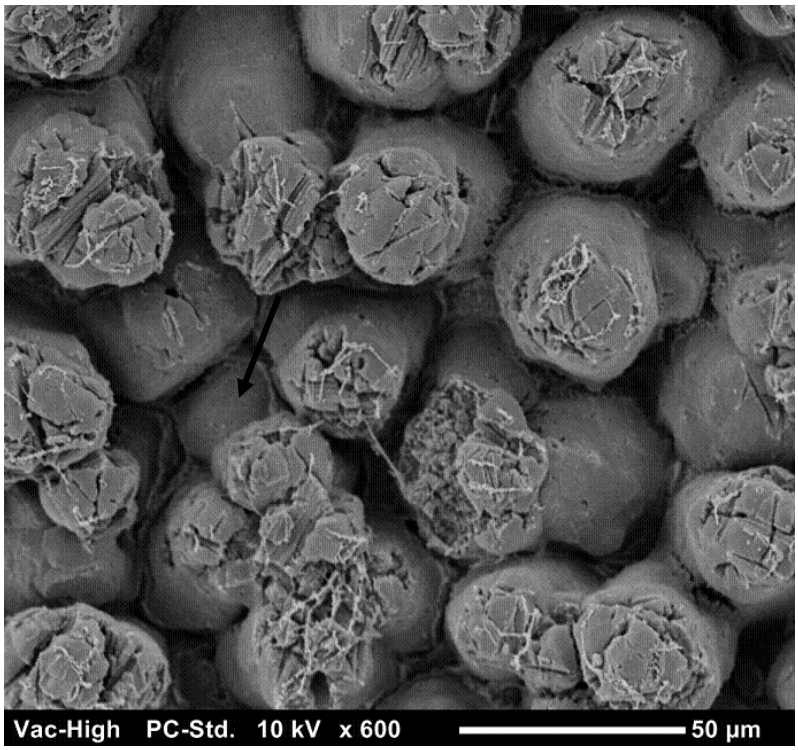

Plate 3.1. Poor mammillary caps with type B bodies (arrow) in the egg shell with abnormalities on the apex.

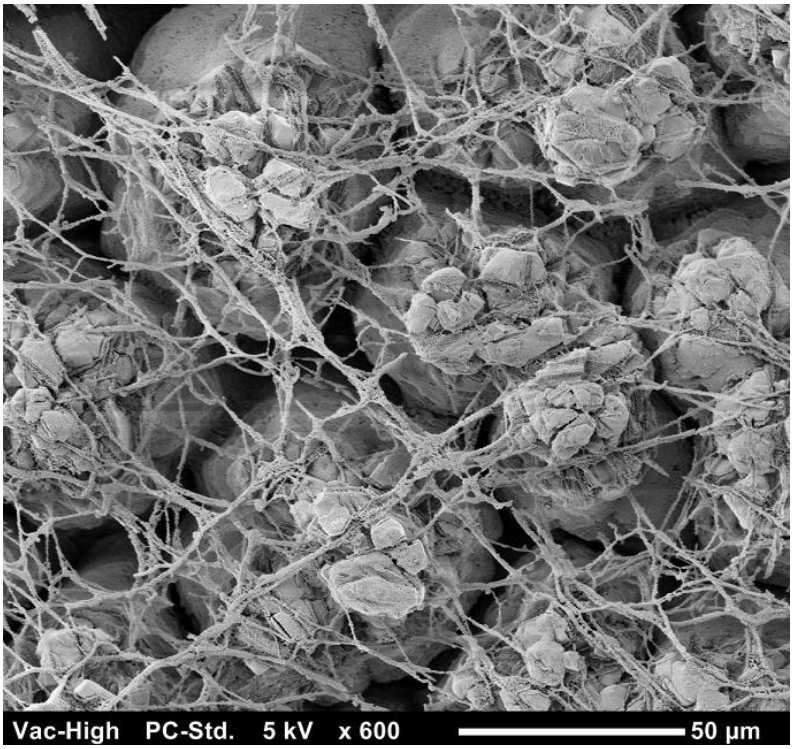

Plate 3.2. Good quality mammilary caps in the egg shell with no abnormalities.

Discussion

Egg shell quality has different meaning for various researchers. To the egg industry, a good quality egg means the provision of an acceptable egg to the consumer. Egg shell quality can be influenced by very many factors like age, strain of hen, temperature, disease (Roberts, 2004). The mammillary region of the egg shell plays a very important role in egg shell quality and it has been recognised by various workers (Solomon, 1991, Brackpool, 1995). Mammillary cores are the initial templates of the calcified egg shell and hence any changes in the morphology of the egg shell quality can lead damage to the structure of the egg shell (Brackpool, 1995). In the present study, translucent egg shells had good quality mammillary caps with extensive attachment with the shell membrane; however there was alignment of the mammillae where the mammillae appeared to "line up" resulting in long continuous grooves between the cones. These groves in the egg shells are thought to lower the resistance of egg shells to bacterial penetration (Solomon, 1991). The ultrastructure of aligned mammillae is shown in Plates 1.1 and 1.3. Cuffing is thought to be responsible for increasing bacterial resistance to penetration and, in this study there was poor cuffing in the translucent egg shells. Depression and erosion of mammillary caps was also recorded in the translucent egg shells. Depressions or erosions are caused by accumulation of oviduct debris on the shell membrane prior to the egg shell calcification process. Pitting can reduce the shell's resistance to bacterial penetration (Nascimento *et al*., 1992)

Type B bodies, which are small spherical calcified bodies which have variable contact with membrane fibres, were found in the translucent egg shells. Type B bodies are normally

present in avian egg shells, although a large number of type B bodies can disrupt the mammillary layer of the egg shell thereby potentiating the entry of bacteria (Nacimento *et al*., 1990).

E. coli strains isolated from the egg shells were selected for the egg shell penetration studies. *S. infantis* strain was selected due to its importance to the Australian egg industry (Cox *et al*., 2002). In the present study, there was no significant difference between the translucency score of the total number of eggs shells used for bacterial penetration study which indicates equal distribution of translucent eggs amongst the treatment groups. Cox *et al.* (2002) further observed that *S. infantis* inoculated onto the surface of the egg could penetrate the egg shell and had the potential to grow within the contents of the egg. Our findings regarding the penetration of *S. infantis* strain across the egg shell are in agreement with Cox *et al*., (2002)., although the translucency score was significantly higher in penetrated egg shells compared to non penetrated egg shells. Our finding regarding the penetration of E coli across the translucent egg shell cannot be compared to those of other workers, owing to a dearth of literature in this area. At 4ºC, only two egg shells were penetrated at high dose rate of the bacterial inoculums. The current finding highlights the importance of storing eggs appropriately throughout the supply chain. The regime of temperature variation, along with poor egg shell quality, could facilitate bacterial penetration across the egg shell. Earlier, Oliveira and Silva (2000) and Aydin *et al* (2004) reported the presence of viable bacterial cells on the shells of intact eggs at refrigeration temperatures.

Board and Tranter (1995) reported that the extent of contamination of hatching eggs was in the range from 2 up to 7 log (10^2 up to 10^7) colony forming units (CFU) per eggshell. In egg washing experiments, Knape *et al*. (2002) and Favier et al. (2000), reported an average initial eggshell contamination of 6.33 and 4.55 log CFU/ eggshell respectively.

In the current experiment, although the dose of bacterial inoculation was very high, it was within the normal contamination range described in earlier studies by Board and Tranter (1995) and Knape *et al*. (2002). However during this study, eggs were washed in 70% ethanol prior to external inoculation which may have damaged the cuticle, reducing its protective properties. There is still the possibility that pathological lesions in egg shells like cuffing, type B bodies and depressions, seen in the transluscent egg shells, could have potentiated the entry of bacteria across the egg shell. The translucent egg shell surface can increase the likelihood of internal contamination of eggs. In this study, however, the bacterial contamination of the egg shell was not quantified and further research is needed in this area.

A Dutch strain of *M. synoviae* has been found to be one of the factors responsible for formation of translucent eggs (Feberwee *et al*., 2009 a &b). During earlier studies, although M. *synoviae* was isolated from the oviduct without any histopathological lesions, attempts were not made to detect the *M. synoviae* from the egg vitelline membrane. There is little information about the effects of Australian strains of *M. synoviae* on the oviduct and possibility of the formation of translucent egg shells or egg shells with apical abnormalities. Hagan *et al*. (2004) reported that screening of antibodies against *M. synoviae* in eggs by ELISA is a suitable method for prevalence studies, so such an approach could be used during future investigations.

In the present study, Mycoplasma was not detected either in translucent or non translucent egg shells from poultry, however it would be hard to correlate such findings without doing further detailed investigation regarding the study the effects of Australian strains of *M. synoviae* on the oviduct and egg quality of mature laying hens. This could be performed by

studying the histopathology of the oviduct andisolation/detection of Mycoplasma from the oviduct and eggs.

It was also found that egg yolk interferes in the PCR reaction during the detection of Mycoplasma. This finding is in agreement with earlier report by (Xiahoua *et al*., 2007) who reported the similar findings during detection of Castor Toxin Contamination in eggs.

Implications of this study and further research in this area

1 In this study the ultrastructure of the translucent egg shell was studied. Little information is available regarding the factors responsible for the formation of egg shell translucency and further research is required to study whether egg shell translucency is related to nutritional, managemental or any disease factors. The present study was conducted using small sample size and quantitative studies are essential using large number of samples.

2 In the second experiment, the effects of translucency on the ability of food borne pathogens like *Salmonella infantis* and *E coli* were studied. Egg shell translucency is responsible for bacterial penetration and there was significant correlation between the egg shell translucency and egg shell penetration by *S. infantis* and *E coli*. Both the strains of bacteria were able to penetrate the translucent egg shells, even at very low dose. The penetration, however, was reduced in both translucent and non translucent eggs at 4^{o}C which highlights the importance of storage of eggs at refrigeration temperature.

3 *M. synoviae* was not isolated from either translucent or non translucent eggs from chickens in the present study; however further studies are required regarding the simultaneous detection and isolation of Australian strains of *M. synoviae* from oviduct. As this organism has been associated with eggshell defects, it is essential to establish whether or not this organism can be detected from egg contents up to a particular period after infection. In Australia, *M. synoviae* has been isolated from the chickens affected with infectious synovitis (Morrow *et al*., 1990). *M. synoviae* has also been isolated from the egg yolk of embryonated eggs from ducks (Bencina *et al*., 1988). Considering the negative effects of egg yolk on the PCR reaction, it is essential to carry out a pre-enrichment procedure before carrying out PCR reaction for detection of Mycoplasma from egg vitelline membrane or egg yolk. It is also essential to study the possibility of formation of translucent eggs due to synergistic effects of Australian uterotropic strains of infectious bronchitis virus and *M. synoviae* in the oviduct of mature laying hens. This is particularly important as Australian strains of infectious bronchitis virus can cause negative effects on egg internal quality, shape index and shell colour.

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Plain English Compendium Summary

Project Title:	**Egg shell quality, with special focus on translucency and product safety**
Poultry CRC Project No.:	CRC 01/09
Researcher:	Dr. Kapil Chousalkar[a], and Associate Professor Brian Cheetham[b]
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Project Overview	In this project, egg shell translucency was studied and defined at a microscopic level. The ability of bacteria such as E coli and Salmonella to penetrate translucent egg shells was studied. A polymerase chain reaction was set up and standardised for rapid detection of Mycoplasma synoviae from the eggs to study whether their presence is responsible for formation of translucent eggs.
Background	During egg quality analysis of eggs collected from various farms across Australia AECL Project (UNE/AECL 86), translucent eggs and microcracks were observed. The translucency in the egg shells appeared to be irrespective of hen age. The role of the phenomenon known as "translucency" is still poorly defined. In addition, it is not clear whether translucency has any role in potentiating the entry of bacteria into the egg contents. The risk that such eggs pose to product safety is uncertain. The proposed project was conducted to define translucency at the ultrastructural level and also to evaluate the extent to which these features increase the likelihood of bacteria penetrating the egg shell. There is little information about the factors responsible for formation translucent eggs. There is a possibility that nutritional, managemental or infectious agent could be responsible for the formation of translucent eggs. Earlier, a Dutch strain of *M. synoviae* was found to be one of the factors responsible for egg shell translucency (Feberwee *et al*., 2009a &b), however there is little information available regarding the positive correlation between Australian strains of *M.synoviae* and egg shell translucency. In the present study translucent and non translucent eggs were tested for the presence of *M.synoviae*.
Research	Egg shell translucency was studied at ultrastructural level by scanning electron microscopy. The ability of E coli and Salmonella to penetrate egg shells was studied by the agar moulding technique (filling eggs with agar and the dipping them into bacterial suspension). The translucent and non translucent eggs were tested using a polymerase chain reaction to detect Mycoplasma synoviae.
Project Progress	Completed
Implications	1 In this study, the ultrastructure of translucent egg shells was studied. Little information is available regarding the factors responsible for the formation of egg shell translucency and further research is required to study whether egg shell translucency is related to nutritional, managemental or any disease factors. 2 In the second experiment, the effects of translucency on the ability of food borne pathogens like Salmonella and E coli were studied. The egg shell translucency is responsible for bacterial penetration and there was

		significant correlation between egg shell translucency and egg shell penetration by *S. infantis* and *E coli*. Both the strains of bacteria were able to penetrate the translucent egg shells even at very low dose. The penetration however was hindered in both translucent and non translucent eggs at 4ºC which highlights the importance of storage of eggs at refrigeration temperature.
	3	*M. synoviae* was not isolated from either translucent or non translucent eggs from chickens in the present study; however further studies are required regarding the simultaneous detection and isolation of Australian strains of *M. synoviae* from oviduct. As this organism has been associated with eggshell defects, it is essential to establish whether or not this organism can be detected from egg contents up to particular period after infection. In Australia *M. synoviae* has been isolated from the chickens affected with infectious synovitis (Morrow *et al*., 1990). *M. synoviae* has also been isolated from the egg yolk of embryonated eggs from ducks (Bencina *et al*., 1988). Considering the negative effects of egg yolk on PCR reaction, it is essential to carry out pre enrichment procedure before carrying out PCR reaction for detection of Mycoplasma detection from egg vitelline membrane or egg yolk. It is also essential to study the possibility of formation of translucent eggs due to synergistic effects of Australian uterotropic strains of infectious bronchitis virus and *M. synoviae* in the oviduct of mature laying hens. This is particularly important as Australian strains of infectious bronchitis virus can cause negative effects on egg internal quality, shape index and shell colour.
Publications	In preparation	