CHAPTER 2 LITERATURE REVIEW

2.1 Chicken coccidiosis

Chicken coccidiosis is a parasitic disease that causes severe losses in poultry meat and egg production. This disease is an intestinal infection caused by obligate intracellular protozoan parasites belonging to various species of genus *Eimeria* (Lillehoj and Lillehoj, 2000). Seven *Eimeria* species have been recognized to be capable of infectivity in chickens: *Eimeria acervulina, E. maxima, E. tenella, E. brunette, E. necatrix, E. mitis,* and *E. praecox*. Each species has its own characteristic, prevalence, site of infection, pathogenicity, and immunogenicity as shown in Table 2.1 (Rose and Long, 1980). *Eimeria* enters the host by penetration of epithelial cells of the intestinal mucosa (Trout and Lillehoj, 1996). The parasite subsequently multiplies in the intestinal tract and causes tissue damage. Extensive damage leads to diarrhea, dehydration, blood loss, diminished feed intake and nutrient absorption, reduced body-weight gain, increased susceptibility to other diseases, and mortality (Davies *et al.,* 1963; Turk, 1978; Cook, 1988; McDougald, 2003).

2.1.1 Characteristics of chicken coccidia

Coccidia are microscopic, spore-forming, single-celled protozoan parasites of the phylum Apicomplexan and Sporozoasida class (Levine, 1973; Taylor *et al.,* 2007). These protozoa parasites are capable of infectivity the intestinal tracts of animals. Sexually mature coccidia living in the host animal's intestine fertilize and produce environmentally-resistant spores called oocysts which undergo fecal-oral transmission between hosts. These unsporulated oocysts are covered by a hard shell and can survive many weeks in the soil outdoors as long as 600 days (Farr and Wehr, 1949). They must undergo further development (sporulation) to become infectious (sporulated oocyst) to other chickens. The unsporulated oocyst and sporulated oocyst are shown in Figure 2.1.

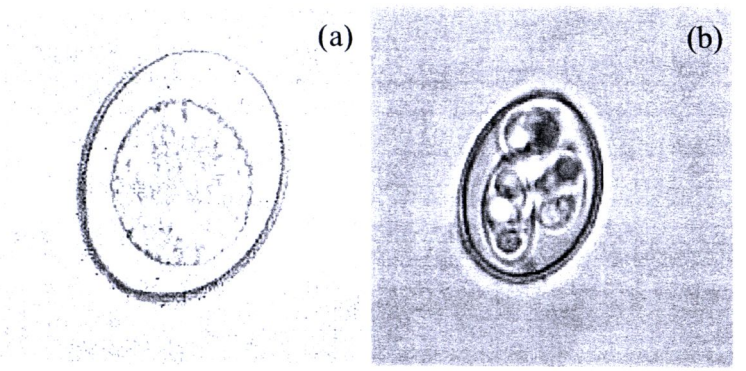


Figure 2.1 Unsporulated (a) and sporulated (b) coccidia oocyst
http://www.damascusroad.ca/Article-Coccidia.htm

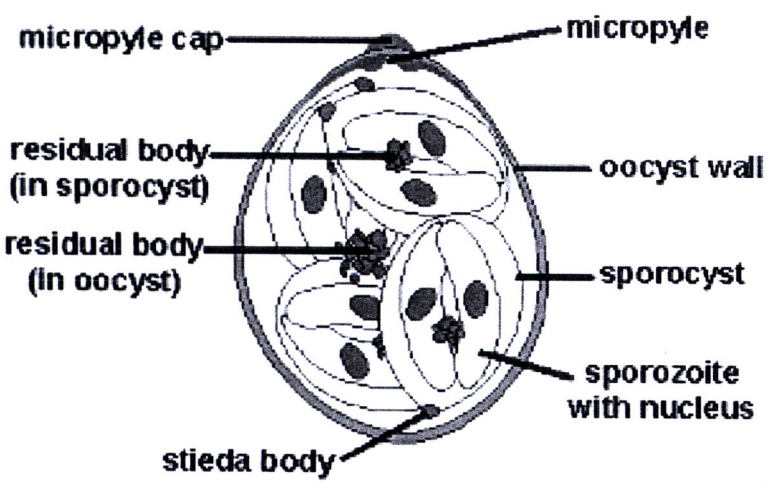


Figure 2.2 Morphology of *Eimeria* oocyst.
http://www.saxonet.de/coccidia/oocyst.htm

Most coccidia in poultry belong to the genus *Eimeria*. Sporulated oocysts of *Eimeria* exhibit a characteristic 1:4:2 configuration, that is, each oocyst contains 4 sporocysts in which each containing 2 sporozoites as shown in Figure 2.2 (Jordan and Pattison, 1996). The oocysts are generally ovoid in shape and vary in size, ranging from 10-40 μm in length by 10-30 μm in width as shown in Table 2.1. *E. maxima* is of the largest size, about 20 x 30 microns while *E. mitis* is the smallest approximately 15 x 16 microns.

Eimeria oocysts contain storage materials in the form of granules, amylopectin and lipid as well as mitochondria and ribosomes. They may contain specialized structures such as polar caps, micropyles, residual and crystalline bodies (Figure 2.2). Micropyle and its cap do not appear in every *Eimeria* species. Bilayer oocyst wall provides an effective protective barrier against the extremes of pH, the action of the detergents (i.e., sodium hypochlorite and dichromate) and enzymes (e.g., proteolytic, glycolytic and lipolytic) (Ryley, 1973).

Table 2.1 Characteristics of chicken *Eimeria spp.*

***Eimeria* species**	**Oocyst size** (μm) (Length x Width)	**Oocyst shape**	**Site of infection**	**Pathogenicity**
E. brunetti	24.6 x 18.8	Oviod	Small and large intestines	High
E. necatrix	20.4 x 17.2	Oblong ovoid	Small intestine, caecum	High
E. tenella	22.0 x 19.0	Ovoid	Caecum	High
E. acervulina	18.3 x 14.6	Ovoid	Anterior small intestine	Moderate
E. maxima	30.5 x 20.7	Ovoid	Mid small intestine	Moderate
E. mitis	16.2 x 16.0	Subspherical	Small and large intestines	Low
E. praecox	21.3 x 17.1	Ovoidal	Small intestine	Low

Source: Jordan and Pattison (1996)

2.1.2 General life cycle of coccidia

Coccidia are obligate, intracellular parasites, which means that they must live and reproduce within an animal cell. However, stages of chicken coccidia appear both within the host as well as outside (Kennedy, 2001). The life cycles of typical *Eimeria* spp. can be divided into three phases of development: sporogony, merogony and gametogony (Hammond, 1973) as shown in Figure 2.3. The developmental stages in the chicken give rise to microscopic eggs (called oocysts) that are shed into the feces of a definitive host. Sporogony taking place outside of the host, is the process (sporulation) by which a one celled sporont (zygote) within the oocyst wall undergoes a series of divisions to form sporozoites (Current *et al.,* 1990). This entails subdivision into four sporocysts each of which contains two sporozoites. At this stage, the sporulated oocyst contains eight sporozoites, each of which is capable of entering a cell in the chicken intestine after the oocyst is eaten. Sporulation requires three conditions: warmth, moisture and oxygen. Under optimal conditions, around 25-30 °C and at least 20 percent moisture in the litter, the oocyst develops within 1-2 days to form a sporulated oocyst which is capable of infecting other chickens. Sporulated oocysts, protected by the thick oocyst wall, are resistant to a fairly wide range of normal environmental conditions and the ability of, at least some, to survive for months or years is a key factor in the epidemiology of coccidial infection. Temperatures above 56 °C and below freezing are lethal, as is desiccation, but oocysts are able to tolerate most disinfectants. Only small molecular weight compounds, such as ammonia and methyl bromide, effectively kill oocysts (Jordan and Pattison, 1996).

The route of infection is through oral ingestion by the chicken. Once ingested, the permeability of the oocyst's environmentally resistant wall is altered by mechanical grinding in the gizzard (Figure 2.5) allowing sporozoites to be released from the sporocysts by the action of pancreatic enzymes, predominantly trypsin, and bile salts. Trypsin acts to degrade the Stidea body (Figure 2.2) which allows the release of sporozoites into the intestine of the definitive host, while bile salts promote sporozoite activity and motility (Current *et al.,* 1990). After excystation, motile infective sporozoites actively enter cells in the epithelium of intestine.

For Merogony stage, sporozoites entering the cells then divide many times producing either a few or many offspring (merozoites) (Kennedy, 2001). The number of asexual generations produced varies from two to four depend on the species of coccidia involved (Current *et al,* 1990). Each merozoite, in turn, may enter another intestinal cell. This cycle may be repeated several times. Because of this cyclic multiplication, large numbers of intestinal cells are destroyed. The Merogony stage then turns to Gametogony stage by which the asexual cycles stop and sex cells are produced (Kennedy, 2001). The gametocytes differentiate into macro and microgametocytes. The microgametocytes produce and release many microgametetes (male); each containing flagellas then migrate to the macrogametocytes. The macrogametocytes develop into macrogemetes (female) which are fertilized by the microgametes resulting in the formation of zygotes. After fertilization, the environmentally resistant oocyst's wall is formed. Oocysts are then released by the host cells and passed into the feces entering the environment (Jordan and Pattison, 1996; Chapman, 1993). The whole process between oocyst ingestion and release may take between 4-6 days to complete (Kennedy, 2001).

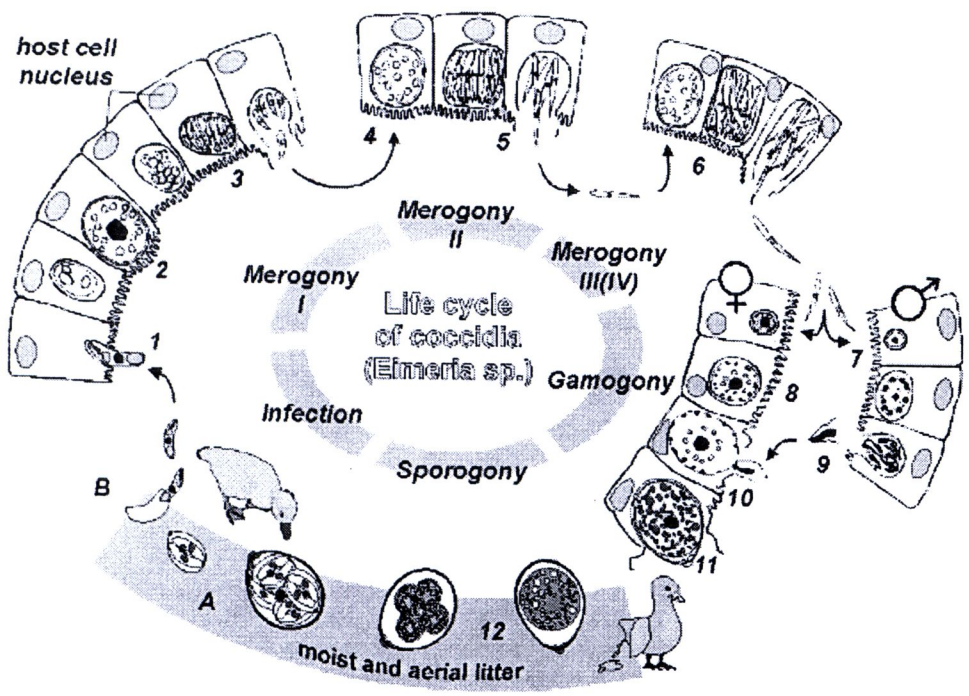


Figure 2.3 General life cycle of coccidia (*Eimeria* spp.)
www.saxonet.de/coccidia/coccid02.htm

2.1.3 Infectious feature and clinical sign of chicken coccidiosis

Coccidiosis in chickens is generally classified as either intestinal or cecal based on the location of the lesions caused by different *Eimeria* species in the gastrointestinal tract (Kennedy, 2001). These locations and the lesions produced are characteristics that help in diagnosing the species present during a post-mortem examination.

Seven *Eimeria* species have been recognized to be capable of infecting chickens and appear to be distributed throughout the world (Rose and Long, 1980; Jordan and Pattison, 1996). They are specific exclusively to chickens therefore; unable to infect other types of fowl or birds or mammals (Badran and Lukesova, 2006). Clearly, each species colonizes in demarcated parts of the host intestine as shown in Figure 2.4. The site and feature of lesions vary between species as described below:

E. brunetti is localized preferentially in the rectum and cloaca causing haemorrhagic lesions in the lower of intestine (Jordan and Pattison, 1996; Witlock and Ruff, 1977). It may ascend to the terminal part of the ileum and the proximal part of the caeca in case of severe infection.

E. necatrix causes both white and red focal lesions with ballooning of the intestinal wall in the mid-intestine of chickens. It develops its schizogony in the jejunum-ileum appearing as white lesions while its gamogony and oocyst formation take place in the caeca but without lesions (Jordan and Pattison, 1996).

E. tenella is localized almost exclusively in the caeca initially with blotchy haemarrhagic lesions, accompanied by haemorrhage into the caecal lumen. In the second stage, at day 1 or 2 of infection, the caeca become pale and shrunken with a thickened wall caused by schizonts in the deep subepithelial position. Host cell debris and oocysts are formed in the caecal lumen (Jordan and Pattison, 1996).

E. acervulina penetrates and completes its entire cycle in the duodenum of chickens (Doran, 1966), but may spread to the ileum during severe infection. Oocysts of *E. praecox* and *E. mitis* can also be found in the duodenum. The lesions caused by this species are characteristically white and rough linear associated with gamonts and oocysts (Jordan and Pattison, 1996).

E. maxima infects the middle intestine (jejunum and beginning of the ileum) but most often ascends into the duodenum. It causes discrete focal, haemorrhagic lesions (Joyner, 1982). The meronts occur in the epithelial cells of the villi of the small intestine while its gamonts are displaced towards the center of the villi and come to lie in their interior (Levine, 1982).

E. mitis colonizes mainly in the ileum with no lesions (Jordan and Pattison, 1996).

E. praecox colonizes in the duodenum and the jejunum with no lesions (Jordan and Pattison, 1996).

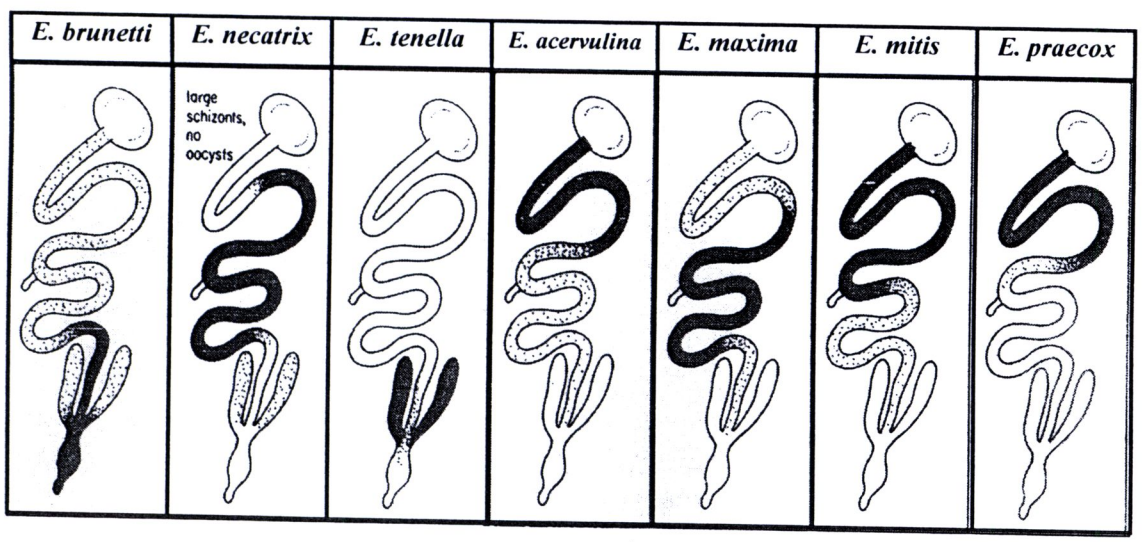


Figure 2.4 Location of the lesions caused by different *Eimeria* species in the gastrointestinal tract.

Although each species has specific development sites in the intestine, sometimes it is difficult to identify them during multiple infections. The most easily recognized clinical sign of severe cecal coccidiosis is the presence of bloody droppings. Dehydration may accompany cecal coccidiosis. Coccidiosis caused by *E. tenella* becomes noticeable at about three days post infection. Chickens start to droop, stop feeding, huddle together and blood begins to appear in the droppings by the fourth day. The greatest amount of blood appears by day five or six, and by the eighth or ninth day, the chicken is either dead or on the way to recovery. Mortality is highest between the fourth and sixth days of infection. Death may occur unexpectedly, owing to excessive blood loss. Chickens that recover may develop a chronic illness (Kennedy, 2001).

Most serious cases of intestinal coccidiosis is caused by *E. necatrix* in comparison with *E. tenella* given that *E. necatrix* does not produce as many oocysts. Therefore, a longer time is usually required for high levels of environmental contamination. Chickens heavily infected with *E. necatrix* may die before any marked change becomes noticeable such as weight loss or the presence of blood in the feces (Kennedy, 2001).

E. acervulina is less pathogenic than *E. tenella* and *E. necatrix*. *E. acervulina* is responsible for sub acute or chronic intestinal coccidiosis in broilers, older chickens and chickens at the point of lay. The clinical signs consist of weight loss and a watery, whitish diarrhea. At postmortem, greyish-white, pinpoint foci or transversely elongated areas are appeared on the outer (or serous) surface of the upper intestine. The pinpoint foci are the areas that consist of dense oocysts and gametes (Kennedy, 2001).

E. maxima produces few marked changes in the small intestine until the fifth day post infection. After that, in severe infections, numerous small hemorrhages occur in the intestine along with a marked production of thick mucus. The intestine then loses its hue becoming flaccid and swollen. The inner surface of the infected intestine is inflamed with the pinkish mucoid intestinal content (Kennedy, 2001).

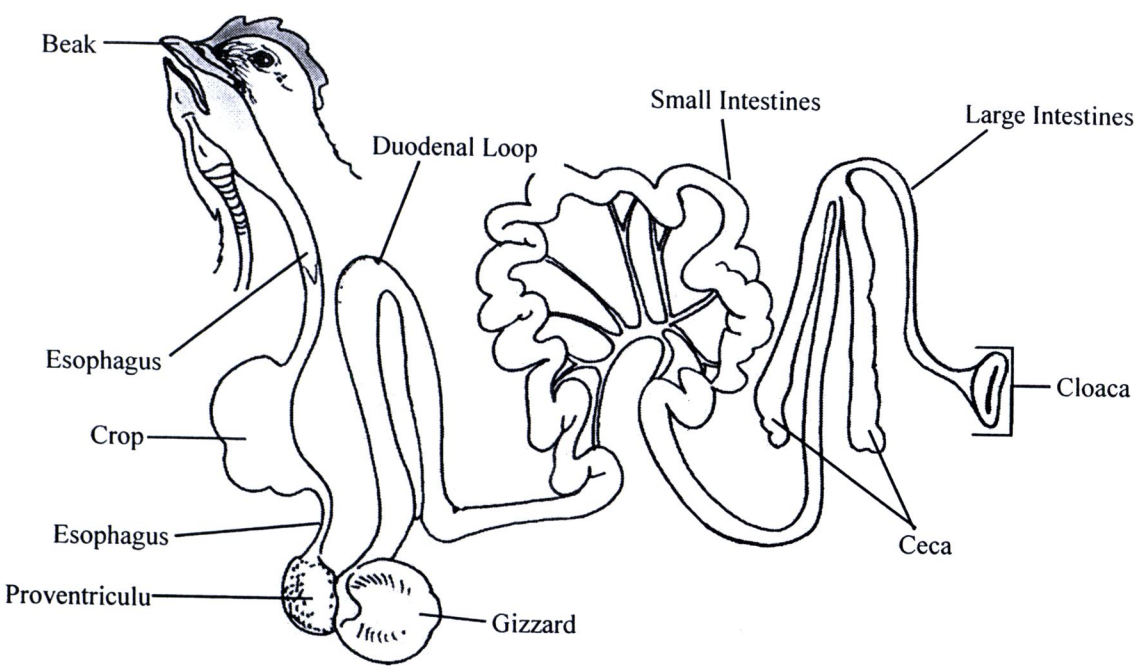


Figure 2.5 The digestive system of poultry (Wilson, 2007).

2.1.4 Control of chicken coccidiosis

Current methods for coccidiosis control are based on the incorporation of anticoccidial agents into feed or water, vaccination (Li *et al.*, 2005).

2.1.4.1 Control with anticoccidial drugs

More than 50 years, anticoccidial feed additives have been used to prevent or treat coccidiosis in poultry. Anticoccidia agents can be classified as follows (Jeffers, 1997; Chapman, 1997; Allen and Fetterer, 2002):

Chemicals: These compounds such as amprolium, nicarbazin and diclazuril are produced by chemical synthesis and have a specific mode of action against parasite metabolism.

Polyether ionophores: They are produced by fermentation of *Streptomyces* or *Actinomadura* and are the most commonly used agents, such as salinomycin, monensin, lasalocid and narasin. They act through a general mechanism of altering ion transport and disrupting osmotic balance in the parasite.

Anticoccidia often have more than one biochemical effect. However, each class of compounds is unique in its type of action exerted on the parasite and its development stage. Diverse modes of action have been described and can be divided into several broad categories as follows (McDougald, 1982; 2003; Chapman, 1997).

Drugs affecting cofactor synthesis: Several drugs affect biochemical pathways that are dependent upon an important cofactor. For instance, amprolium competitively inhibits the uptake of thiamine by the parasite.

Drugs affecting mitochondrial function: These drugs inhibit energy metabolism in the cytochrome system of the *Eimeria*. For instance, quinolones and clopidol inhibit electron transport in the parasite mitochondrion, albeit by different pathways.

Drugs affecting membrane function: Ionophores in common possess the abilities to form lipophylic complexes with alkaline metal cations (Na^+, K^+, and Ca^{++}), and transport these cations through the cell membrane and then affect a wide range of biochemical

processes that are ion transport dependent, such as influx of sodium ions, thus, causing severe osmotic damage. These drugs act against the extracellular stages of the life cycle of the *Eimeria*.

Supplementation of anticoccidial drugs to the feed represents a good preventive measure and is well adapted to large-scale use. However, prolonged utilization of these drugs leads unavoidably to the emergence of *Eimeria* strains that are resistant to all anticoccidial drugs, including ionophores (Ruff and Danforth, 1966; Chapman, 1994; 1997; 1998; Allen and Fetterer, 2002). Resistance may be developed quickly, as in the case of quinolones and clopidol, or it may take several years for the coccidia to become tolerant, as in the case of polyether ionophores (Chapman, 1997; McDougald, 2003; Li *et al.*, 2004). Due to high development cost as well as possibility of drug residues remaining in the product causing market unacceptability, the pharmaceutical industry is disinclined to develop new anticoccidial products (Chapman, 1997).

2.1.4.2 Vaccination

The development coccidia resistant strains to anticoccidial drugs (Chapman, 1997, Williams, 2002), the concern regarding drug residues in poultry products (McEvoy, 2001; Young and Craig, 2001), the pressure forced by consumers to avoid chemotherapeutics and the recent ban several anticoccidial drugs used in broilers by EU (Farrant, 2001) have led to an interesting in the vaccination of poultry against coccidiosis. Currently, vaccine produced from live oocysts is the only viable alternative to the use of anticoccidial drugs. This control strategy has been used by the poultry industry for over 50 years, primarily by broiler breeder followed by layer (Chapman *et al.*, 2002).

Live oocyst vaccines comprise various species of *Eimerria* oocysts, either attenuated or non-attenuated *Eimeria* strains (Shirley, 1992 and Williams, 2002). There are four major brands of vaccines commercially available, whose vaccines are manufactured from either wild type (Coccivac® D/B and Immucox®) and attenuated (Paracox® and livacox®) *Eimeria* species (Williams, 1998; 2002; Chapman *et al.*, 2002). The non-attenuated vaccines contain a mixture of oocysts of wild-type-strain *Eimeria* that will not produce pathogenic effect, but induce immunity. Both non-attenuated and attenuated vaccines provide strong immunity to coccidial infection when applied carefully under

good rearing conditions (Shirley and Long, 1990). Live oocyst vaccination has been shown to be an effective tool for the generation of immunity and protection against subsequent *Eimeria* challenge evidenced by increased body weight gain (Danforth, 1998; Crouch *et al.*, 2003; Williams, 2003), reduced feed conversion (Crouch *et al.*, 2003), and reduced intestinal lesion development (Danforth, 1998; Crouch *et al.*, 2003; Williams, 2003). These vaccines are usually delivered orally in drinking water and feed. Additionally, the non-attenuated vaccines comprise of the most frequently occurring virulent species such as *E. tenella, E. acervulina and E. maxima* rely on the administration of low doses of the vaccines in early life of chickens (Shirley, 1992). Because the parasite could cause lesions to chickens if the vaccines are employed exceeded the recommended dose (Chapman, 2000). However, this has to be carefully administered as chickens that do not ingest the vaccine are susceptible to coccidia infection (Shirley, 1992). The attenuated vaccines consist of *Eimeria* strains incapable of inducing pathogenicity in the host but still confer protective immunity and are also an effective option for live vaccination of chickens. However, drawbacks of live vaccines include safety concern, short shelf-life and difficulties of large-scale production. Since the live vaccines against coccidia are costly to produce given further that these vaccines are strain- and species-specific, a cocktail of antigens may be required in order to raise protective immunity effectively. Therefore, there is continued interest in devising new vaccines using defined recombinant antigens (Innes *et al.*, 2006).

Due to the complex life cycle of *Eimeria* is associated with complicated host immune system, vaccine development has been difficult (Yun *et al.*, 2000). *Eimeria* exhibit a complex life cycle comprising stages both inside and outside of the host. During the stage inside host, there are both intracellular and extracellular stages and both asexual and sexual reproduction. This complexity provides the immune system with only three moments to inhibit *Eimeria* development. The first is when the sporozoites search for a site of penetration and actually bind with the epithelium of chicken intestine. The second is when the sporozoites are in the villus epithelium, inside and between intraepithelial leucocytes. The third moment of possible attack by the immune system is during the passage of the lamina propria into the crypt epithelium (Jeurissen and Veldman, 2002).

Recombinant vaccines may be the long-term sustainable solution while the major challenge ahead is to devise effective ways to deliver these antigens to the immune system in order to stimulate appropriate protective immunity (Innes *et al.*, 2006). For these reasons, development of other control methods for coccidiosis are still needed to reduce economic losses due to coccidiosis.

2.1.4.3 Alternative anticoccidia treatments

Probiotics

In 1989, Fuller has defined probiotics as "a live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance". Probiotic preparations may consist of any number of microorganisms, e.g., single strain or up to eight strains (Fuller, 1989; Timmerman *et al.*, 2004). The use of probiotics aims to fasten the development of a stable and beneficial intestinal microflora, which will lead to improvement of intestinal health and modulate the immune system; therefore, enhancing host resistance to enteric pathogens (Jin *et al.*, 1996; 1998; 2000; Abdulrahim *et al.*, 1999; Zulkifli *et al.*, 2000). Tortuero (1973) showed that *lactobacilli* were capable reducing the severity of clinical signs in *E. tenella* infection. Dalloul *et al.* (2003) reported that a *Lactobacillus* containing diet fed to broilers infected with *E. acervulina* resulted in an immunoregulatory effect on the local immune system and improved the broilers' resistance to *E. acervulina* infection. Furthermore, it has been reported that lactobacillus species inhibit the invasion of *E. tenella in vitro* (Tierney *et al.*, 2004). Recently, Lee *et al.* (2007) showed that *Pediococcus acidilactici* could effectively enhance the resistance of chickens and partially protect against the negative growth effects associated with coccidiosis.

Prebiotics

A prebiotic has been defined by Gibson and Roberfroid (1995) as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or limited number of bacteria in the colon that can improve the host health". Prebiotics are more advantageous when compared with probiotics in that they are target the bacteria already present; e.g., those that are adapted to the gastrointestinal tract environment. Many studies have demonstrated that the non-digestible polysaccharides such as inulin, oligofructose, and oligomannose could enhance growth of the beneficial bacteria (*Bifidobacteria and Lactobacillus*), reduce growth of the

pathogenic bacteria (*E.coli* and *Salmonella*) and also stimulate the immune system of the host (Hidaka *et al.*, 1986; Wang and Gibson, 1993; Gibson and Roberfroid, 1995; Gibson *et al.*, 1995; Gibson, 1999; Gibson and Fuller, 2000; Cummings and MacFarlane, 2002).

Mannanoligosaccharides (MOS), a non-digestable polysaccharide, derived from the cell wall of the yeast saccharomyces cerevisiae, is considered as prebiotics which could be efficiently utilized by lactic acid producing bacteria (Delzenne, 2003). MOS also competes with pathogenic gram-negative bacteria such as *E.coli* and *Salmonella* to bind host cells, resulting in a reduction of colonization of those pathogenic bacteria in host intestine (Ofek *et al.*, 1977; Spring *et al.*, 2000). Fernandez *et al.* (2002) have reported that feeding young chickens supplemented with MOS could increase in a probiotic bacteria *Bifidobacteria* and reduce in susceptibility to *Salmonella enteritidis* colonization. Addition of MOS to the diet of broilers reduced the severity of the infection caused by either *E. tenella* alone (Elmusharaf *et al.*, 2006) or a mixture of *E. acervulina*, *E. maxima* and *E. tenella* (Sun *et al.*, 2005; Elmusharaf *et al.*, 2007).

Chicken egg yolk antibody (IgY)

Antibodies are immunological molecules that play an important role in the defense against invading pathogens. They specifically recognize and bind to foreign antigens, resulting in the activation of a number of immune effector functions capable of selectively eliminating foreign microorganisms, viruses and molecules. Immunotherapy using antibodies are promising as an important therapeutic approach for the treatment of infectious diseases (Newcombe and Newcombe, 2006). Recently, laying chickens have attracted considerable attention as an alternative source of antibodies for the prevention and treatment of infectious gastrointestinal diseases (Hau and Hendriksen, 2005). The major serum antibodies produced by chicken which are transferred into the egg yolk called Immunoglobulin Y (IgY) (Rose and Orlans, 1981). Oral administration of IgY has been proven successful treatment for a variety of gastrointestinal infections such as bovine and human rotaviruses, enterotoxigenic *Escherichia coli* and *Salmonella* ssp. (Karlsson *et al.*, 2004). Lee *et al.* (2009) has demonstrated that supplementation of anti-coccidia IgY antibodies into chicken feed provided protective immunity against coccidia infection.

2.2 Chicken egg

The eggs of avian species have long been recognized as an excellent source of nutrients for humans. In recent years, innovative research revealing the diversity of structure and function of components in eggs has incentive increasing demand to more fully utilize this bioresource. Nowadays, applications of eggs are being developed to take advantage not only of their nutritional contributions in food products but also of their bioactive components that may be used as nutraceutical food ingradients with potential to reduce risk of disease and enhance human health (Mine, 2008). Isolation of valuable components (particularly proteins) from the egg is receiving greater attention (Juneja, 1991). Most of research effort has been focused on the immune proteins found in the egg yolk (Davis and Reeves, 2002).

2.2.1 Structure and chemical composition of hen egg

An understanding of the egg's structural and chemical characteristics provides the fundamental basis for investigation of its bioscience and biotechnology (Mine, 2008). Eggs are composed of three main parts; the eggshell with eggshell membrane, the albumen or egg white, and the yolk (Figure 2.6). The first part of the egg to be developed is the yolk. The yolk is surrounded by albumen layers, which in turn is enveloped by eggshell membrane and finally a hard eggshell (USDA, 2000). The process is complete in 20-24 hours. The yolk comprises about 31% of the total weight of the egg (i.e., an average size 55 grams egg contains 17 grams of yolk). The yolk comprises 51% water, 16% protein, 30.5% fat and some minerals (Table 2.2), and is contained within a fine elastic membrane called the vitelline membrane. Around the yolk is the egg white, a clear jelly-like substance which comprises about 58% of the weight of the egg (i.e., an average 55 grams egg contains 32 grams of egg white). The egg white consists of 88% water, 9% protein and some minerals. Egg white exists in two states: (i) the thick and gelatinous egg white which immediately surrounds the yolk and acts to cushion the yolk and (ii) the thinner egg white which surrounds the thick egg white and is contained by two membranes just inside the shell. These membranes enclose the whole egg and prevent bacterial contamination. The shell of the egg is rigid and brittle, providing protection for its contents. The shell make up 11% of the weight of an egg and approximately 98% of the shell consists of calcium (Davis and Reeves, 2002).

Table 2.2 Chemical composition of hen egg (gram/egg)

Egg component	Water	Protein	Free sugar	Oligo saccharides	Lipid	Mineral	Total
Egg yolk (31%)	9.1 (31%)	3.1 (16%)	0.1	0.1	5.8 (30.5%)	0.3	18.5
Egg white (58%)	28.9 (88%)	3.5 (9%)	0.1	0.2	0.0	0.2	32.9
Egg shell and membrane (11%)	0.1	0.4	-	-	-	5.9	6.4
Whole egg (100%)	38.1	7.0	0.2	0.3	5.8	6.4	57.8

Sources: Adapted from Sugino *et al.* (1997)

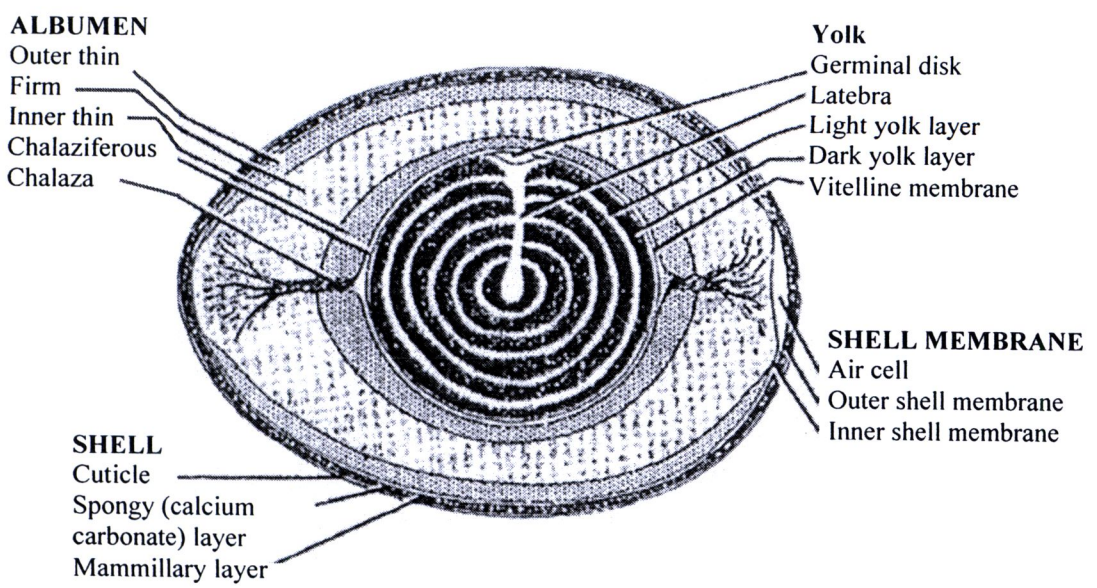


Figure 2.6 Structural components of the eggshell, eggshell membrane, albumen, and yolk (USDA, 2000).

2.2.2 Egg yolk

Egg yolk is homogeneously emulsified fluid. The major portion of egg yolk consists of lipoproteins which can be separated into plasma fraction (supernatant) and granular fraction (precipitate) in 4:1 proportion, respectively, using ultracentrifugation (Davis and Reeves, 2002; Sugino *et al.*, 1997). The major components of plasma fraction are low-density lipoproteins and livetins, a protein fraction in ether-treated egg yolk that remains in solution after the precipitation of the rest of the yolk solids with water (Plimmer, 1908) which normally occupies nearly 62% and 10% of overall egg yolk solid contents, respectively. Livetins, lipid-free, globular glycoprotein, are major components of water-soluble egg yolk protein (Davis and Reeves, 2002; Williams, 1962). Livetins could be classified into three type: α-livetin (MW 80 kDa), β-livetin (MW 40-42 kDa), and γ-livetin (MW 180kDa). Livetins transferred from blood serum, are identified as serum albumin (α-livetin) and immunoglobulin (Ig) (γ-livetin) which is immunologically analogous to the plasma protein fraction in the blood of mammals (Williams, 1962). In particular, γ-livetin is denoted as IgY because it is structurally different from the IgG of a mammal (MW 150kDa). Recently, the application of IgY as the specific antibody obtained from egg yolk in preventing infectious disease has received much attention (Yamamura *et al.*, 1995).

2.3 Chicken egg yolk immunoglobulin (IgY)

2.3.1 Antibody

Antibodies are soluble glycoproteins produced by one of white blood cells called a plasma cells, of vertebrates that are secreted into blood serum and other body fluids such as saliva, milk and etc. (Male *et al.*, 2006; Elgert, 1996). These proteins belong to a family of globular proteins (globulins) and involved in immunological reactions. They are also called immunoglobulins (Igs) (Elgert, 1996; Rao, 2006). Antibodies are produced in specific response to stimulation by foreign substances called antigens that enter the organism (Male *et al.*, 2006; Hau and Hendriksen, 2005). Their function is to eliminate their specific antigens or microorganisms carrying those antigens previously recognized (Male *et al.*, 2006). Antibodies specifically recognize and bind to foreign antigens, resulting in the activation of a number of immune effector functions capable of selectively eliminating foreign microorganisms, viruses and molecules (Newcombe and Newcombe, 2006).

2.3.2 Antibody structure and class

The basic structural unit of an antibody consists of four polypeptides. Two identical copies of heavy (~55kD) and light (~25kD) chains are held together by disulfide bonds such that the resulting molecule is often represented by a schematic Y-shaped molecule of 150 kD (Figure 2.7). Each of these chains composes of constant (C) and variable (V) regions. Light chains consist of a variable amino terminal portion of 110 amino acids with a constant region of equivalent length while heavy chain contains one variable and at least three constant regions, each approximately 110 amino acids long. Amino termini of the light and heavy chains associate to form an antigen-binding domain (Fab) whereas the carboxy terminal region of the two heavy chains fold together to form the Fc domain or crystallizable fraction. The antigen binding domains reside at the tip of the arms while their effector domains reside in the tail. The two arms (Fab) of the antibody molecule are connected to the tail (Fc) by a region rich in proline, threonine, and serine, known as the hinge. This region imparts lateral and rotational movement to the antigen-binding domains, providing the antibody the ability to interact with a variety of antigen presentations (Lipman *et al.*, 2005).

Antibodies are typically made of one or more basic structural unit of Y shape, such as monomer with one unit, dimers with two units or pentamers with five units. Both light and both heavy chains of antibodies are folded into distinct domains, given that the type of heavy chain determines the class and subclass of the antibody (Male *et al.*, 2006). There are five classes of Igs found in mammals which are IgG, IgM, IgA, IgD and IgE (Lipman *et al.*, 2005). whose chains of those Igs are denoted as gamma (γ), mu (μ), alpha (α), delta (δ), and epsilon (ε), respectively. They differ in size, charge, amino acid sequence and carbohydrate content. The IgG and IgA classes can be subdivided into four and two subclasses as (IgG1, IgG2, IgG3, IgG4) and (IgA1, IgA2), respectively. The classes and subclasses together represent nine isotypes each of which is defined by the sequence of the constant region of its heavy chain (Male *et al.*, 2006). The isotypes differ in their biological properties, functional locations and abilities to deal with different antigens, as described in the Table 2.3.

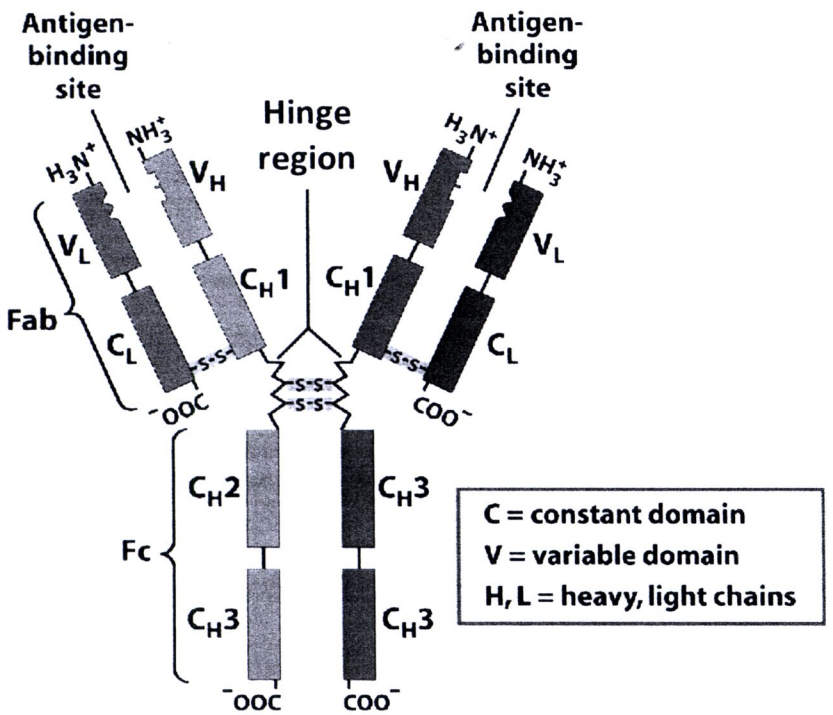


Figure 2.7 The basic structural molecule of an antibody.

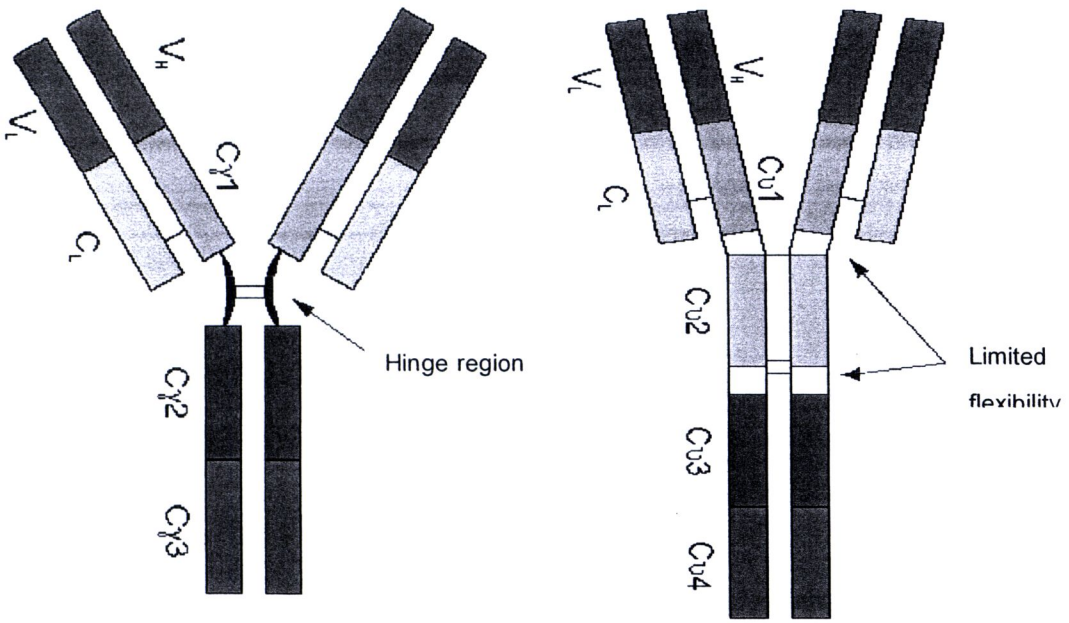
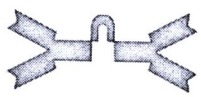
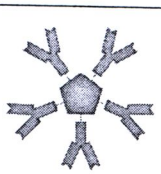


Figure 2.8 The structural molecule of IgG and IgY.

Table 2.3 Antibody isotypes and its function

Classes (Isotypes)	Heavy Chain	Functions	Structure
IgA (IgA1, IgA2)	α1, α2 Alpha	Found in mucosal areas, such as the gut, respiratory tract and urogenital tract, and prevents colonization by pathogens (Underdown and Schiff, 1986). Also found in saliva, tears, and breast milk.	 Dimer
IgD	δ Delta	Functions mainly as an antigen receptor on B cells that have not been exposed to antigens (Geisberger *et al.*, 2006). It has been shown to activate basophils and mast cells to produce antimicrobial factors (Chen *et al.*, 2009)	 Monomer
IgE	ε Epsilon	Binds to allergens and triggers histamine release from mast cells and basophils, and is involved in allergy. Also protects against parasitic worms (Pier *et al.*, 2004)	 Monomer
IgG (IgG1, IgG2, IgG3, IgG4)	γ1, γ2, γ3, γ4 Gamma	Provides the majority of antibody-based immunity against invading pathogens (Pier *et al.*, 2004). The only antibody capable of crossing the placenta to give passive immunity to fetus.	 Monomer
IgM	μ Mu	Expressed both on the surface of B cells and in a secreted form. Eliminates pathogens in the early stages of B cell mediated (humoral) immunity before there is sufficient IgG (Pier *et al.*, 2004; Geisberger *et al.*, 2006)	 Pentamer

2.3.3 Immunoglubolin Y (IgY)

Three serum immunoglobulin classes have been shown to exist in chickens as IgA, IgM and IgY. IgY is the major immunoglobulin which makes up about 75% of the total serum immunoglobulin. The concentrations of IgY, IgA, and IgM in serum have been reported to be 5.0, 1.25, and 0.61 mg/ml, respectively (Leslie *et al.*, 1973). IgA and IgM are homologues of mammalian IgA and IgM while IgY is the functional equivalent to mammalian IgG but different in structural molecule (Warr *et al.*, 1995; Leslie and Clem,

1970; Chalghoumi, 2009). In female avian, IgY is transferred from the serum into the egg yolk to give passive immunity to embryos and neonates, a process that corresponds to the placental IgG transfer in mammals, which confer passive immunity to the fetus (Zhang, 2003). IgA and IgM are transferred to the egg white in limited amounts (Davis and Reeves, 2002). General structure of IgY molecule is similar to the mammalian IgG containing two heavy (Hυ) chains with the molecular mass of 67-70 kDa each and two light (L) chains with the molecular mass of 25 kDa each (Narat, 2003). Parvari *et al.* (1988) suggested that H chain of IgY should be called "υ" and constant region Cυ, as "υ" is the Greek transliteration of the latter Y. The major difference between IgG and IgY is the number of constant regions (C) in H chains of IgG has 3 C regions (Cγ1-Cγ3), while IgY has 4 C regions (Cυ1-Cυ4) (Figure 2.8). One additional C region with the two corresponding carbohydrate chains has a logical consequence in a greater molecular mass of IgY compared to IgG, i.e., 180 and 150 kDa, respectively. IgY is much less flexible than IgG due to the absence of the hinge between Cγ1 and Cγ2, which is a unique mammalian feature. There are some regions in IgY (near the boundaries of Cυ1-Cυ2 and Cυ2-Cυ3) containing proline and glycine residue enabling only limited flexibility (Figure 2.8) (Warr *et al.*, 1995). Recently, it has also been suggested that IgY is a more hydrophobic molecule than IgG (Davalos-Pantoja *et al.*, 2000).

2.3.4 Chicken IgY production

Antibodies are produced by the immune system of an animal in a specific response to exposure to an antigen. They are commonly produced by injection of the antigen of interest into an animal, often in combination with an adjuvant to increase the immune response. The antibody response can be enhanced by subsequent booster injections of the antigen with or without adjuvant. Blood specimens are sampling from the animal to assess the level of antibodies produced, and once a sufficiently high titre has been reached, the antibody is prepared by blood collection followed by serum preparation, with subsequent purification of antibodies from the serum if required (CCAC, 2002). The most frequently used animal species for polyclonal antibody (PAb) induction in the laboratory setting are the rabbit, mouse, rat, hamster, guinea pig, goat, sheep, and chicken (Hanly *et al.*, 1995). The rabbit is the most commonly used animal for the production of PAbs because of their convenient size, ease of handling and bleeding, relatively long life span, high titer and high affinity of antiserum produced (Stills,

1994). Rodents may be suitable when small volumes of PAbs are required (Garvey *et al.*, 1977) whereas farm animals such as sheep, goats, and horses are usually used when larger amounts of PAbs are need. In some cases, chickens may be used for producing large amount of PAbs (Erhard *et al.*, 2000; Schade *et al.*, 1996).

Drawbacks of polyclonal antibody production using mammals are that a process to obtain a vast amount of blood from immunized animals is complicated and purification of antibodies from mammalian blood resulted in low yielding and, at the same time, laborious. In addition, this method also associated with multiple injections of antigens and repeated blood sampling procedures which cause distress to the animals. Disadvantages of the available techniques and concern for animal welfare enhance the interest in developing alternative methods for the production of antibodies (Hua and Hendriksen, 2005; Kim *et al.*, 2000; Narat, 2003).

During the past 20 years, the use of chickens instead of mammals for antibody production has increased. Utilizing chickens as the immunization host yields a number of advantages (Hua and Hendriksen, 2005). Because antibodies are continuously transferred from blood to egg yolk (Bollen and Hau, 1997), it is convenient to harvest and purify the antibodies from the egg yolk of the immunized chickens. From a practical as well as an animal welfare point of view, the use of laying hens for antibody production represents reduction in animal hurt and number of animal use because the painful and invasive blood sampling or animal scarifies are replaced by eggs collection (Chalghoumi, 2009) whilst the antibody productivity of an egg-laying hen is much greater than that of a similar sized mammal. Schade *et al.* (1996) reported the antibody productivity in laying hens is nearly 18 times greater than that in rabbits.

In addition, due to the phylogenetic distance between chickens and mammals, there is greater potential of producing a higher percentage of specific antibody against mammalian antigens when using chickens (Gassmann *et al.*, 1990). It is also possible to obtain a strong immune response with small amounts of antigen in hens thus reducing the amount of antigen required for producing antibodies (Larsson *et al.*, 1998). Highly conserved mammalian proteins sometimes fail to illicit a humoral immune response in animals, such as rabbits, that are traditionally used for generating polyclonal antibody. Since chicken IgY does not cross-react with mammalian IgG and does not bind bacterial

or mammalian Fc receptors, non-specific binding and the risk for false-positive results are reduced, given that the need for cross-species immunoabsorptions is also eliminated (Hadge and Ambrosius, 1984; Larsson *et al.*, 1991; Carlander *et al.*, 2000). These antibodies are directed against more antigenic epitopes and recognize the same proteins in several species, making them more widely useful (Tini *et al.*, 2002; Zhang, 2003).

In order to produce egg yolk antibody (IgY), hens are exposed (usually through intramuscular or subcutaneous injection) to an antigen capable of inducing immune responses leading to the production of specific antibodies to those antigens. The antibodies produced are then naturally transferred to the egg yolk (Loeken and Roth, 1983; Sunwoo *et al.*, 1996). Booster immunizations are usually given at later time on a regular basis to ensure continued transfer of antibodies from hen to the egg yolk (Sunwoo *et al.*, 1996). These antibodies are subsequently extracted from the egg yolk. The isolation process consists of separation of the yolk from the white and then antibodies (present in the yolk) are purified by removing all other materials. (Akita and Nakai, 1992; Ko and Ahn, 2007; DeMeulenaer and Huyghebaert, 2001).

Several critical steps involved are greatly affected the production of polyclonal IgY including (1) animal selection and care, (2) preparation of the antigen, (3) selection of the adjuvant, (4) immunization protocol and (5) collection and extraction of the antibodies. Each step is described below.

2.3.4.1 Animal selection and care

IgY can be produced using chickens bred for commercial egg production as well as those which have been bred free from specific pathogens (SPF chickens). Commercial laying chickens are not only cheaper for purchasing, but they can also be obtained just before they come into production, thereby further reducing the costs associated with antibody production. It is preferable to use chickens for breeding purposes than those used for egg production, because the health status of breeding animal is often better controlled. When immunizing chickens, two facts have to be considered. Firstly, 10-15% of outbred chickens may be non responders or low-responders to certain antigens such as *salmonella pullorum* bacterin, human serum albumin, and synthetic peptides. Secondly, several viral diseases may cause transient immunomodulatory effects which can interfere with antibody production. Thus, if the antibody produced is to be used for

therapeutic purposes, the use of SPF chickens is required. SPF chicken can be obtained from some commercial suppliers in Europe (for example, F.E. Lohmann, Cuxhaven, Germany) and in the United States (for example, Spafas, Preston CT, USA) (Schade *et al.*, 1996). The advantage of using SPF chickens over egg laying chickens is that the former generally give higher antibody titres although conflicting findings have been reported (Hlinak, *et al.*, 1996) in literature.

A basic requirement for the use of chickens is the availability of housing and racing conditions which favour species-specific behavior. A suitable cage for chicken-specific housing must, at the very least, conform to the minimum standards outlined in Table 13 of Directive 86/609/EEC and in the European Convention for the protection of Vertebrate animals used for Experimental or other scientific purposes (Anon, 1986). A specific commercially available cage for housing two chickens (128 x 65 x 80cm) is recommended by Swiss Veterinary administration (Schweizer Bundesamt fur venturinarwesen). Housing chicken in cages under laboratory conditions is advantageous, in that the chicken can be readily located and their health can be easily monitored. Although the housing of chickens in social groups on the floor is desirable from the perspective of animal welfare and social behavior, it is coupled with the problem of egg identification, and with an increased risk of infection (especially for chickens housed outside) (Schade *et al.*, 1996).

The age of the animals is another important consideration because this factor can influence the outcome of the immunization. It is important to use young adults, for whom the immune response is fairly robust and unaffected by previous immune challenges. The robustness of the immune response decreases with age after the period of young adulthood. When chickens are immunized, they should be of egg-laying age by the time antibody is to be harvested (Hanly *et al.*, 1995; Hendriksen and Hau, 2003). It is usual to vaccinate chickens that are of at least 7 weeks old of age (Schade *et al.*, 1996). Another recommended age of chicken for polyclonal antibody production is 18-20 weeks (Hanly *et al.*, 1995; Hendriksen and Hau, 2003). However, Schade *et al.* (1996) also recommended that for antibody production the whole laying period chickens could be used as production machinery depending on the antibody titers induced. It is advisable to start with a group of chicken, and select high responding animals which can then be kept for a longer period of time for antibody production.

Finally, It is important to consider the health status of animals used for the production of antibodies. Since some infectious agents may suppress, modulate, or stimulate the immune system, utilization of disease-free animals minimizes the likelihood of cross-reactivity to other antigens with which the animal's immune system may have encountered previously (Leenaars and Hendriksen, 2005).

2.3.4.2 Preparation of the antigen

Antigen features such as the quality, quantity, and procedure used for preparing antigens are of significant importance on antibody production. The specificity of the immune response obtained depends on the purity of the antigen applied. Tiny impurities (<1%) may prove to be immunodominant (e.g., with many bacterial antigens) and may result in antibodies expressing higher activity against the impurity than against the antigen of interest (Leenaars and Hendriksen, 2005). Purification results in an increased number of specific antibodies associated with extensive absorption procedures to remove many unwanted antibodies (Leenaars *et al.,* 1997). Before immunization, the investigator should consider the toxicity of chemical used to inactivate the microorganism remained in the antigen to be employed including contamination of endotoxins such as lipopolysaccharide produced by those microorganisms (Hendriksen and Hau 2003). The diluents should be endotoxin free, and the pH should be adjusted within physiological limits. Upon administration, these factors are important because they may have a negative effect on the welfare of the animal as well as on the immunological results. Other clearly important factors include the working conditions during antigen preparation, which must be sterile, and also the injection instruments (Leenaars and Hendriksen, 2005).

2.3.4.3 Selection of the adjuvant

Adjuvants are used to stimulate the immune response of experimental animals. The desired antigen is applied in combination with various adjuvant compounds. (Michael *et al.,* 2010). There are more than 100 known adjuvants, which differ in their chemical characteristics, their efficacy in stimulating the immune system, and their secondary side-effects (Chalghoumi *et al.,* 2009). Only a few adjuvants are routinely utilized for polyclonal antibody production including Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), aluminum salts (e.g. $Al(OH)_3$, $AlPO_4$), Quil A, Iscoms, Montanide, TiterMax™, and RIBI™, etc. (Stills, 2005).

FCA is frequently used for the production of polyclonal antibodies because high antibody titers are induced to almost all types of antigens. However, many investigators have reported severe side effects after injection of FCA (Leenaars and Hendriksen, 2005). In mammals, the use of this adjuvant leads systematically to severe inflammation at the injection site. In birds, the use of FCA does not seem to result in the same severe lesions as in mammals (Chalghoumi, 2009). Study by Gassmann *et al.* (1990) suggested that chickens showed higher resistance to tissue damaging potency of FCA than rabbits which are in good agreement with that reported by Svendsen *et al.,* (1996). However, Wanke *et al.* (1996) and Olbrich *et al.* (2002) reported otherwise. To avoid an eventual local tissue reaction, the Freund's incomplete adjuvant (FIA), which is the most effective substitute found to date, becomes the most commonly used adjuvant in immunizing hens for antibody production. Since FIA is less efficient than FCA, some investigators preferred the use of a combination of the two adjuvants: FCA for the first immunization and FIA for the booster immunizations (Kapoor *et al.,* 2000; Li *et al.,* 2006; Chalghoumi *et al.,* 2008). In these studies, good results were achieved with no adverse side-effects reported.

Other types of adjuvant can also be used, such as Specol (Boersma, 1992) and the lipopeptide, Pam_3-Cys-ser-$(Lys)_4$. The adjuvant $ALPO_4$, $AL(OH)_3$ and saponin has been found to induce only very low antibody responses. Thus, it is important to first test the efficacy and quality of emulsion type adjuvants according to standardized procedures (Herbert, 1967).

2.3.4.4 Chicken immunization protocol

Basically, different immunizing protocols for each antigen and for each animal species have to be tested to establish out which method induces the highest serum and egg yolk antibody titer. Different immunization protocols, using different adjuvants, antigen dose and volume, route of injection, vaccination frequency and interval were described below (Michael *et al.,* 2010):

Antigen dose

The dose of antigen influences significantly the immune response and the antibody titre induced. Too much or too little antigen may induce suppression, sensitization, tolerance or other unwanted immunomodulation (Hanly *et al.,* 1995). Generally, for each antigen, various concentrations have to be tested. Behn *et al.* (1996) achieved better results when injecting hens with 0.1 mg of mouse IgG instead of 1.0 mg. Schwarzkopf *et al.* (2000) found that the injection of antigen ranging between 10 μg and 1 mg elicited good antibody responses which are in good keeping with that reported by other studies (Mahn, 1998). The types of antigen employed should also be taken into consideration. Antigen can be presented to the immune system as complex multiantigens (e.g., bacteria, viruses and parasites) or as single antigens (e.g., proteins or polysaccharides) (Leenars *et al.,* 1996). Proteins are recognized to be the most efficient immunogens because of the polymorphism of their structure and the differences existing between species and individuals (Goldsby *et al.,* 2003). 10-100 μg of protein antigen was recommended as preferable dose (Camenisch *et al.,* 1999; Matsuda *et al.,* 1999; Tini *et al.,* 2002). Peptides (molecular weight below 10 kDa) can also be used as antigen, but they should be coupled to carriers (e.g., bovine serum albumin or keyhole limpet haemocyanin) (Schade *et al.,* 2005). Polysaccharides antigens are found to be highly efficient.

Volume and Route of Injection

The most common route for antigen injection in hens for IgY production is the intramuscular route (Schade *et al.,* 2005). Usually 10–100 μg (Tini *et al.,* 2002; Camenisch *et al.,* 1999; Matsuda *et al.,* 1999) of protein antigen in final volume of 0.5-1 mL is employed and injected intramuscularly into the breast muscle at two or three injection sites of 7 to 8-week-old chicken. Some authors prefer antigen injection in the leg. However, the general recommendation states that intramuscular injection in the leg should be avoided, since it can lead to lameness. Chicken can also be injected subcutaneously in the neck. With very young animals, it may be preferable to inject intramuscularly into the breast muscle, because subcutaneous injection is more difficult to perform and can therefore cause more distress (Schade *et al.,* 1996).

Immunization frequency and interval between immunizations

The total number of immunization required depends on the type and dose of the antigen as well as the adjuvant employed. At least two immunizations have to be given. Yolk antibody titers should be checked 14 days after the last immunization. If antibodies titres begin to decrease, booster immunizations can be given during the laying period to maintain production of high levels of specific antibodies up to year (Schade *et al.*, 1996). The success of an immunization protocol depends also on the interval between the first and second and subsequent immunizations. Often reported interval is two to four weeks (Camenisch *et al.*, 1999; Matsuda *et al.*, 1999; Tini *et al.*, 2002).

2.3.4.5 Collection and extraction of IgY

Because of the continuous transfer of antibodies from blood to egg yolk in chickens (Bollen and Hau, 1997), it is convenient to harvest and purify antibodies from the egg yolk of the chicken. Eggs (usually one egg is laid per day) are collected daily and marked for identification. They can be stored for up to one year at 4°C prior to IgY purification. To purify IgY from egg yolk, it is important to understand the composition of the egg yolk first. The information about egg composition was described in 2.2.

Several methods were described in the 1950s for purifying IgY based on the strategy of separation of proteins (livetins) from lipoproteins (lipovitellins) and the rest of the yolk lipids using organic solvent extraction resulting in a rather low yield of antibody. However, purification methods based on organic solvents, e.g., chloroform, remain in use (Kovacs *et al.*, 2004). Other methods are based on affinity chromatography or on dilution of the yolk followed by a freezing-thawing process after which the process consists of ion exchange chromatography and salt precipitations often combining a number of salts like for e.g. polyethylene glycol (PEG), dextran sulfate, dextran blue, sodium sulfate, ammonium sulfate, caprylic acid and sodium citrate may be adapted.

More recently, methods combining chloroform with ammonium sulfate precipitation for removal of lipid have been shown to result in a good yield of antibodies of high purity. λ-carrageenan yield high extraction efficiency given that the lipid content in the supernatant in subsequent to removal of the resulting precipitate was less than 0.4% of that of original egg yolk. Hatta (1990) reported that the IgY remaining in the supernatant was further isolated using DEAE-Sephacel column followed by salting-out

with sodium sulfate resulting in almost pure IgY (98%) whereas the yield was 70-100 mg per egg.

Water dilution method was found to be more superior in terms of ease of use and large scale production of IgY. It is concluded to be simple, rapid and efficient means of purifying IgY with high activity (Akita and Nakai, 1993). The IgY recovery from thiophilic interaction chromatography is close to 100%, providing a simple and efficient means for purifying IgY from egg yolk.

Another efficient method consists of two successive precipitations in polyethylene glycol (PEG), using 3.5% PEG to remove fatty substances, and then 12% PEG to precipitate the IgY (Hansen *et al.,* 1998). Purification of IgY by PEG and ammonium sulphate yielded very pure IgY, which was also capable of neutralizing toxic and lethal components of the *E. carinatus* venom (Meenatchisundaram *et al.,* 2010). An improvement of this method could be achieved by incorporating an emulsification step by adding one volume of chloroform rather than using 3.5% PEG. It is generally expected that about 100 mg of IgY can be recovered per egg yolk (Kovacs *et al.,* 2004).

2.3.5 Large yield and scaleable production of IgY

An industrial scale production of IgY is possible because of the availability of large chicken farms and automation of egg breaking and processing (Kim *et al.,* 2000). A laying hen can produce approximately 300 eggs annually while each egg yolk volume is approximately 15 mL (Wilkie, 2006). The amounts of IgY in yolk are 20-25 mg/ml (Rose and Orlans, 1981), which would supply over 100 grams of antibody per hen per year.

Several studies reported that IgY levels in the egg yolk are not always consistent and may vary within and between chicken populations. Carlander *et al.* (2001) investigated day to day variation of IgY concentration in eggs produced by ten Single Comb White Leghorn (SCWL) hens over a time period of 28 days. They found that there was variability in individual hens but this variability was lower than what was observed among hens. Further, the variation of IgY concentration among chickens of different genetic lines was also studied. Carlander *et al.* (2003) investigated the variation of IgY in eggs of SCWL hens, RIR hens, and a cross between the two genetic lines reported

that highest IgY content was found in eggs from SCWL, although there was high variability between individual hens.

Li *et al.* (1998) compared egg yolk weights, percentage of hen-day production, and content of yolk antibody (IgY) produced from Single-Comb White Leghorn (SCWL) and Rhode Island Red (RIR) hens immunized with bovine serum albumin (BSA). It was found that similar percentages of total IgY as well as BSA-specific antibodies were detected in the eggs of the two lineages of immunized laying hens while there was no significant difference in the activity of the IgY. However, the SCWL hens had greater yolk weights, percentage hen-day production. Therefore, the egg yolk weight and the percentage hen-day production, both of which are greater in the SCWL hens, are considered to be important factors for the efficient production of IgY.

The above-mentioned information indicates that it is possible to increase IgY production by genetic selection within high-producing lines (Carlander *et al.*, 2003) which may be an important step for large-scale production of egg yolk antibodies.

2.3.6 Stability of IgY

2.3.6.1 pH stability

The stability of IgY to acid and alkali has been studied under various conditions. It was found that the activity of IgY was decreased at pH 3.5 or lower and almost completely lost with irreversible change at pH 3 (Shimizu *et al.*, 1988; 1992; 1993). Similar results were reported by Losch *et al.* (1986), Hatta *et al.* (1993) and Lee *et al.* (2002). Rapid decrease of the IgY activity at low pH(s) indicated conformational antigen-binding site. Under alkaline conditions, the activity of IgY remain intact until the pH increased to 11. However, it was markedly diminished at pH 12 or higher (Shimizu *et al.*, 1988; 1992; 1993). Similar results on pH effect were presented by Losch *et al.* (1986), Hatta *et al.* (1993) and Lee *et al.* (2002).

2.3.6.2 Proteolysis stability

IgY is relatively resistant to trypsin or chymotrypsin digestion while, on the contrary, it is fairly sensitive to pepsin digestion. Hatta *et al.* (1993) demonstrated that almost all of the IgY activity was lost following digestion with pepsin given that 39% and 41% of the activity remained even after 8 h of incubation with trypsin and chymotrypsin, respectively. The stability of IgY against pepsin appears to be highly dependent on pH and the enzyme/substrate ratio. At pH 5 or higher, IgY was fairly resistant to pepsin and retained its antigen-binding and cell-agglutinating activities. However, at pH 4.5 or below, both activities were lost (Shimizu *et al.*, 1988).

The results obtained by Hatta *et al.* (1993) who also investigated the IgY behavior with pepsin under different incubation times and pH confirmed the susceptibility of IgY to pepsin at low pH. Digestion of IgY with pepsin at pH 2 resulted in complete hydrolysis of the antibody molecule, leaving only small peptides. However, IgY digested with pepsin at pH 4 retained 91% and 63% of its activity after 1 hour and 4 hours incubation, respectively. After tryptic digestion, IgY retained its antigen binding and cell-agglutinating activities in spite of a definite breakdown of the polypeptides. Unlike the trypsin digestion, no definite cleavage of the IgY chains was observed for chymotryptic digestion and the activities of IgY remained high for these digests (Shimizu *et al.*, 1988; Otani *et al.*, 1991).

2.3.6.3 Temperature and pressure stability

Stability of IgY has been investigated by thermally treated IgY at various temperatures for different periods of time. The binding activity of IgY to antigen decreased with increasing temperature and heating time. According to Shimizu *et al.* (1992) and Hatta *et al.* (1993), IgY is stable at temperature up to 65°C in aqueous condition. The activity of IgY decreased when subjected to heating for 15 min at 70°C or higher (Shimizu *et al.*, 1988; 1992). Further, IgY was denatured when thermally treated at temperatures higher than 75°C (Chang *et al.*, 1999). IgY is relatively stable to pressure variation as no detectable inactivation of IgY was reported when subjected to pressure up to 4,000 kg per cm^2 (Shimizu *et al.*, 1994).

2.3.6.4 Freezing and spray-drying stability

Freezing and freeze-drying are low temperature processes that are usually considered to be less destructive to biomolecule during treatment. However, proteins may easily suffer from a loss of activity as a result of conformational changes, aggregation or adsorption (Skrabanja *et al.,* 1994). There have been some reports on the stability of IgY with regard to the above mentioned methods. Freezing and freeze-drying did not affect the activity of IgY unless repeated several times (Shimizu *et al.,* 1988). However, Chansarkar (1998) showed that frozen or freeze-dried IgY loss some antigen-binding activity with a significant drop in the solubility under the conditions of high salt and protein concentrations. The same findings were observed by Sunwoo *et al.* (2002). Recently, Fu *et al.* (2006) examined the thermal stability of IgY at various temperatures ranging between 25 and 90°C for 15 min treatment, before and after freeze-drying process and found that freeze-dried IgY had shown a good thermal stability with no significant reduction in reactive activity except at 90°C. Additionally, the same conclusion could also be made for the non freeze-dried IgY.

Yokoyama *et al.* (1992) studied properties of IgY powders obtained by spray-drying or freeze drying the water-soluble fraction of egg yolks from *Escherichia coli* immunized hens. As compared to the freeze-dried powder, the spray-dried powder did not show a significant alteration in antibody titres and yields, even when several spray-drying temperatures were attempted (140 to 170°C). However, the moisture content of powder of the powder prepared by spray-drying was higher than that of obtained with freeze-drying.

2.3.7 Storability of IgY

Regarding the relatively high core body temperature of chickens, which is 41°C, it is not surprising that half-life time of IgY is in months and that they retain their activity after 6 month storage at room temperature or four months at 37°C (Larson *et al.,* 1993). However, high salt conditions or addition of stabilizing regents (*e.g.,* sugar) increases the resistance of IgY to heat, acid extreme pH and high pressure (Shimizu *et al.*, 1994; Zhang, 2003). IgY fractions could be stored in 0.9% NaCl, 0.02% NaN_3 at 4°C for 20 years without any significant loss of antibody titer whereas affinity-purified and

biotinylated IgY stored in the same conditions have retained their activities after 5 years (Olovsson and Larsson, 1993).

2.4 IgY used for passive immunization

Passive immunity is the transfer of active immunity in the form of ready-made antibodies from one individual to another. It can also be induced artificially, when high levels of antibodies specific to a pathogen or a toxin are recovered from immunized individual or from patient recovering from the infection and administered to non-immune individual. The antibodies transfer may be carried out via systemic, intravenous or oral route (Chalghoumi *et al.*, 2009). Antigen-specific IgY can be produced on a large scale from eggs laid by chickens immunized with selected antigens (Hatta *et al.*, 1997). Therefore, the use of IgY for passive immunization has been studied extensively, demonstrating its effectiveness in preventing or treating infectious diseases caused by various pathogens in animal models, especially those of the intestinal tract (Table 2.4). In this case, antibodies are usually administered by mixing with the feed in several forms: whole eggs powder, whole yolks powder, water soluble fraction powder or purified IgY material.

From Table 2.4, although positive results have been obtained against some pathogens in mice, pigs and calves, very little research has been done to assess the ability of hen-egg antibodies in reducing enteric pathogen shedding and gastrointestinal infections in poultry.

Table 2.4 Effect of passive immunization by enteric pathogen-specific IgY

Pathogen	Animal species	Effects	Reference
Salmonella	Mice	Preventing mice challenged with *S. Enteriditis* from experimental salmonellosis	Peralta *et al.*, 1994
		Protecting mice challenged with *S. Enteritidis* or *S. Typhimurium* from experimental salmonellosis	Yokoyama *et al.*, 1998a
	Calves	Preventing fatal salmonellosis in neonatal calves exposed to *S. Typhimurium* or *S. Dublin*	Yokoyama *et al.*, 1998b
	Laying hens	Reducing *S. Enteritidis* contaminated eggs rate in experimentally infected hens	Gurtler *et al.*, 2004
		Elimination of *S. Enteritidis* shedding in faeces	Kassaify *et al.*, 2004a
	Broiler chickens	Failure in the elimination of the intestinal colonization by *S. Enteritidis*	Wilkie, 2006
		Reducing caecal colonization and faecal shedding in *S. Enteritidis* challenged chickens	Rahimi *et al.*, 2007
Campylobacter jejuni	Broiler chickens	Inhibiting the faecal shedding of *C. jejuni*	Tsubokura *et al.*, 1997
		Reducing the faecal shedding of *C. jejuni*	Tsubokura *et al.*, 1997
		Failure in the elimination of the intestinal colonization by *C. jejuni*	Wilkie, 2006
Escherichia coli	Pigs	Curing diarrhoea affected piglets in a field study	Wiedemann *et al.*, 1991
		Preventing K88+, K99+, 987P+ ETEC infection in neonatal piglets	Yokoyama *et al.*, 1992
		Protecting pigs challenged with K88+ ETEC from *E. coli*-induced enterotoxernia	Yokoyama *et al.*, 1993
		Inhibiting shedding of F18+ *E. coli* in infected piglets	Imberechts *et al.*, 1997
		Reducing intestinal colonization by F18 + ETEC in infected weaned pigs	Zúniga *et al.*, 1997
		Prevention of ETEC K88+ infection in neonatal and early weaned piglets	Marquardt *et al.*, 1999
	Calves	Protecting neonatal calves from fatal enteric colibacillosis by K99-piliated ETEC	Ikemori *et al.*, 1992
	Rabbits	Preventing diarrhoea in rabbits challenged with ETEC	O'Farrelly *et al.*, 1992
Rotavirus	Mice	Preventing murine rotavirus (MRV)	Yolken *et al.*, 1988
		Preventing human rotavirus (HRV)-induced gastroenteritis in mice	Ebina *et al.*, 1990
		Preventing mice from HRV-induced diarrhoea	Hatta *et al.*, 1993
		Protecting mice from bovine rotavirus (BRV)-induced diarrhoea	Kuroki *et al.*, 1993
		Preventing HRV-induced gastroenteritis in mice	Ebina *et al.*, 1996
	Calves	Protecting calves from BRV disease	Kuroki *et al.*, 1994
		Preventing BRV-induced diarrhoea in neonatal calves	Ozpinar *et al.*, 1996
Coronavirus	Calves	Protecting neonatal calves from bovine Coronavirus (BCV)-induced diarrhoea	Ikemori *et al.*, 1997

Note: *ETEC* - Enterotoxigenic *Escherichia coli*

2.4.1 Passive immunization of anti-chicken coccidia IgY

Lee *et al.* (2009) have investigated the efficacy of inducing passive immunity against coccidiosis by oral feeding hyperimmune IgY antibodies. For each feeding study, a commercially available egg yolk powder, Supracox (SC), a purified IgY fraction of egg yolk prepared from hens hyperimmunized with three major species of *Eimeria* oocysts, were continuously fed to young chicks from hatch. Two groups (uninfected and infected controls) were fed ad libitum with a standard diet alone or a standard diet supplemented with 0.01% SC, 0.02% SC, or 0.05% SC (v/v). These broiler chicks except the uninfected control chicks were orally infected with 1.0×10^4 sporulated *E. tenella or E. maxima* oocysts at 1 week of age. It was found that 0.05% SC-fed chicks showed significantly higher body weight gains ($P < 0.05$) compared to the untreated controls. Furthermore, treated chicks showed significantly less intestinal lesions and reduced fecal oocyst output compared to the untreated controls. These results provide clear evidence that passive immunization of chicks with hyperimmune egg yolk IgY antibodies provide significant protection against *E. tenella or E. maxima* infections.

In another study, the same group of researcher has evaluated the protective effect of IgY fraction of egg yolk against *Eimeria acervulina* in young broiler chickens. Chickens were continuously fed from hatch with a standard diet containing IgY egg yolk powder or a nonsupplemented control diet and orally challenged at day 7 posthatch with 5.0×10^3 sporulated *Eimeria acervulina* oocysts. Body weight gain (BW) between day 0 and 10 and fecal oocyst shedding between days 5 and 10 postinfection were determined as parameters of protective immunity. Chickens given 10 or 20% hyperimmune IgY egg yolk powder showed significantly increased BW gain and reduced fecal oocyst shedding compared with control chickens fed the nonsupplemented diet. Additionally, lower IgY concentrations (0.01, 0.02, and 0.05% concentrations) were used to treat birds with 1.0×10^4 oocysts of *E. acervulina*. Total oocyst shedding was significantly ($P < 0.05$) reduced in chickens fed with 0.02 and 0.05% hyperimmune IgY supplemented-diets compared with animals fed the nonsupplemented diet. Similarly, chickens fed with 0.5% of hyperimmune IgY egg yolk powder diet and challenged with 1.0×10^4 oocysts exhibited reduced oocyst shedding in comparison with the control birds given 0.5% of IgY from nonimmunized hen eggs.

2.5 Techniques used for characterization and determination of IgY

2.5.1 Indirect ELISA

Enzyme-linked immunosorbent assay (ELISA), is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. ELISA can provide a quick and useful measurement of the concentration of an unknown antigen or antibody by combining the specificity of antibodies with the sensitivity of simple enzyme assay. Currently, there are three major types of ELISA assays commonly used by researchers depending on how the test is designed. They are: indirect ELISA, typically used for screening antibodies; sandwich ELISA (or antigen capture), for analysis of antigen present; and competitive ELISA, for antigen specificity.

Indirect ELISA is the most commonly used method for antibody detection. There are four main steps to perform indirect ELISA including: coating of antigen onto the microplate, blocking of the unbound sites, incubation with primary antibody and secondary antibody, and detecting the reaction. The schematic of Indirect ELISA is shown in Figure 2.9.

Antigen coating to microplate: ELISAs are performed in 96-well plates which permits high throughput results. A buffered solution or other carbonate buffer solution containing antigen to be tested for is added to each well of a microtiter plate, where it is given time to adhere to the surface through charge interactions.

Blocking the unbound sites: A solution of non-reacting protein, such as bovine serum albumin (BSA), or casein is added to block any plastic surface of the well that remains uncoated by the test antigen.

Incubation with primary antibody and secondary antibody: The sample, which contains a mixture of unknown concentration of the donor's antibodies is added. Only antibody against the test antigen (primary antibody) will bind specifically to the antigen that is coated on the well surface, not to other serum proteins or the blocking proteins. After incubation, the wells are washed to get rid of unbound antibodies. Secondary antibody, capable of binding to any remaining primary antibody, is added to the wells and incubated. The secondary antibody is conjugated to the substrate-specific enzyme such

as peroxidase or alkaline phosphatase. The wells are washed again to remove the excess unbound enzyme-antibody conjugates.

Detecting the reaction: A substrate for the enzyme is then added. The enzyme can metabolize colorless substrates (sometimes called chromagens) into colored products. When the enzymatic reaction is complete, the ELISA plate is placed into a plate reader and the optical density (the amount of colored product) is determined for each well. The amount of color produced is proportional to the amount of primary antibody bound to the test antigens on the bottom of the wells.

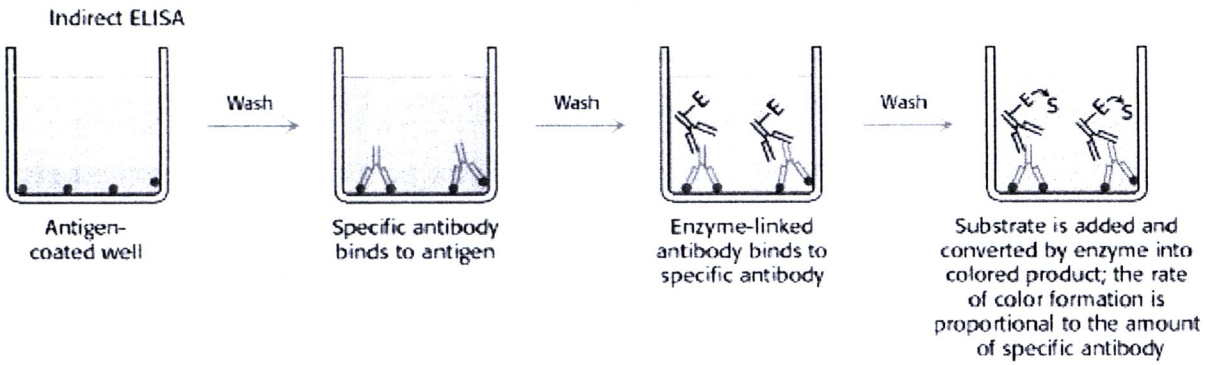


Figure 2.9 Indirect ELISA schematic.

2.5.2 Immunofluorescence

Immunofluorescence, a technique whereby an antibody labeled with a fluorescent molecule, is used to detect the presence of an antigen in or on a cell or tissue by the fluorescence emitted by the bound antibody.

Most commonly, immunofluorescence employs two sets of antibodies: (i) a primary antibody used against the antigen of interest; and (ii) secondary dye-coupled antibody introduced to recognize the primary antibody. Immunofluorescence can be performed on cells, tissue, or some other substances containing antigens fixed on slides. A small amount of sample containing antibodies is placed over the cells or tissue, allowing the antibodies that are specific to the particular tissue or cellular antigens to bind. The non specific antibodies and other materials are washed away, and a second antibody that binds to specific antibodies or primary antibodies is applied to the slide. This second antibody chemically linked with a fluorescent dye such as fluorescein isothiocyanate (FITC). If the sample contains antibodies bound to the tissue or cells, a bright fluorescence can be observed under a fluorescence microscope or confocal microscope. The schematic of immunofluorescence is shown in Figure 2.10.

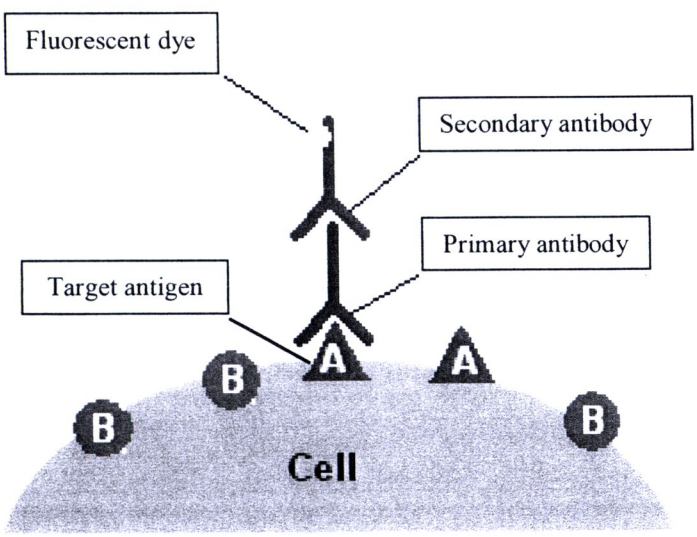


Figure 2.10 Immunofluorescence schematic.

2.6 Quality control of egg product

Microbial contamination of eggs is a well-established phenomenon and has important economic implications to the poultry industry. Contamination of hatching eggs may reduce hatchability, be responsible for transmission of poultry pathogens and impair the quality of chicks produced. In case of table eggs, contamination may cause not only spoilage but also spreading of diseases if the eggs contaminated with pathogenic microorganisms. There are two ways in which eggs can become contaminated, namely, by the transovarian and trans-shell routes. In transovarian contamination, the egg becomes contaminated prior to oviposition (before laying), with the source of contamination originating in the egg-laying apparatus of the chicken. In the case of trans-shell contamination organisms gain access to the egg after oviposition (after laying) by penetration of the shell (Bruce and Drysdale, 1994).

It is generally accepted that microorganisms are absent from the vast majority of eggs while in the oviduct of healthy hens (Brooks and Taylor, 1955; Boad, 1968). The microbiology of the hen's oviduct has been studied by several authors (Harry, 1963; Jacobs *et al.,* 1978; Blankenship *et al.,* 1982; Bruce and Drysdale, 1991). In these studies, the bacterial flora recovered from the oviducts was found to differ markedly from that found on the eggs indicating that contamination occurs most frequently after oviposition. There are several poultry and human diseases of bacterial and viral origin which can be disseminated via the egg. *Mycoplasma* spp. including *M. synoviae, M. gallisepticum and M. meliagridis* are examples of microorganisms capable of undergoing this vertical mode of transmission. Other organisms such as *Staphylococcus aureus*, *Salmonella* spp. and *Pasteurella* spp. are also known to produce infection (Mayes and Takeballi, 1983). The recent outbreaks of *Salmonella enteritidis* PT4 infection of eggs occurred as a result of the organism's ability to infect the reproductive tract. These invasive strains can infect the ovary or oviduct and certain instances can contaminate the contents of egg before it is laid.

As most eggs contain no viable organisms prior to oviposition the first major opportunity for contamination to take place is at the point of lay. The shell probably receives its first load of microorganisms when passing through the cloaca. From that time until the eggs is used, the opportunities exist for the shell to acquire microorganisms from every surface with which it makes contact. Several reports available in literature indicated that the level of contamination on the shells of eggs produced under different conditions (Haines, 1938; Harry, 1963; Board, 1969; Quarles *et al.*, 1970; Metwally *et al.*, 1984). The level of contamination ranges from 10^3 -10^5 cfu per egg in clean condition to 10^7 – 10^8 cfu per egg under dirty conditions (Baxter-Jones, 1991) which suggests that contamination on eggs is related to the environment in which the egg is laid. However, external contamination levels need not relate directly to incidence of spoilage or hatchability levels as other factors determine whether the organisms will actually penetrate the shell, grow and cause problems.

The importance of the type of microbial flora challenged to the egg has to be respected as not all organisms are capable of penetrating the outer structures of the egg and surviving the adverse conditions in the albumen. A number of workers have reported on the flora present on eggshells (Table 2.5). These observations have been summarized and compared with the types of bacteria isolated from spoiled eggs (Board, 1966; Mayes and Takeballi.1983).

Table 2.5 Comparison of the microflora on the surface of the egg shell and within spoiled eggs (Mayes and Takeballi, 1983)

Type of organism	Frequency of occurrence	
	On the shell	In rotten eggs
Micrococcus	+++	+
Achromobacter	++	+
Aerobacter	++	-
Alcaligenes	++	+++
Arthrobacter	++	+
Bacillus	++	+
Cytophaga	++	+
Escherichia	++	+++
Flavobacterium	++	+
Pseudomonas	++	+++
Staphylococcus	++	-
Aeromonas	+	++
Proteus	+	+++
Sarcina	+	-
Serratia	+	-
Streptococcus	+	+

The more the plus signs, the more frequent the occurrence

To prevent egg contamination, the control methods are described as follows. It has been shown that eggs handle with gloved hands have a lower level of surface contamination compared with eggs handle normally (Rosser, 1942). In addition, the length and the temperature of storage are also of significant importance. If infected eggs are kept at room temperature, microorganisms such as *Salmonellas* can multiply rapidly and reach high levels inside the egg, whereas cold storage has been shown to completely inhibit the growth of *Salmonella* in both yolk and the albumen (Board *et al.,* 1989).

The majority of reports on the effect of egg washing have established that various egg washing practices can result in an increase in the number of infected or spoiled eggs (Lorenz and Starr, 1952; Miller and Crawford, 1953; March, 1969; Moats, 1978, 1981). Several factors are regarded as being important in influencing the penetration process. The bacteriological quality of the wash water is of prime importance given that in general spray systems which employ fresh water are better in this respect than immersion systems in which the water tends to become contaminated. It is also important to ensure that the wash water temperature is higher than that of the eggs. However, care should be taken to ensure that the temperature difference is not too great or the shells may crack. Thus, allow microorganisms to penetrate rapidly (Bruce and Drysdale, 1994).

The washing of eggs on a large scale began in the United State in the 1940s. Before that time, washing of eggs entailed either soaking very dirty eggs in water for several hours and then wiping the dirty off the shells with a cloth or using a wet cloth to get rid of the dirt. The water used for soaking was usually colder than the eggs which caused the eggs to contract and thus allowed bacteria to be pulled across the shell, resulting in contamination of some of the washed eggs. The washing process was improved once it was recognized that it was essential to use water with a temperature higher than that of the eggs. The warmer water caused the eggs to expand thereby creating an outward pressure and the bacteria were not sucked in (Baker and Bruce, 1994). Generally, alkaline products are added to the washwater in order to increase the pH thus making microorganisms such as *Salmonella* spp. more susceptible to the heat (Humphrey *et al.*, 1981; Kinner and Moats, 1981). The eggshell is more sensitive to acidic conditions, therefore the pH of egg washwater is usually adjust upwards. Holly and Proulx (1986) emphasized the importance of monitoring the conditions of egg washwater. The authors found that *Salmonella* would not survive very long in egg washwater with pH > 10 and with temperatures in excess of 38°C. It was found that washwater at pH 10 and 42°C had a greater lethal effect than washwater at pH 7 and 60°C. It is recommended that egg washwater should have a minimum temperature of 32°C or 11°C warmer than the eggs being washed (IAMFES, 1976).