"Nature is inexorable and immutable; she never transgresses the laws imposed upon her, or cares a whit whether her abstruse reasons and methods of operation are understandable to men"

Galileo Galilei (1564 – 1642)

"As my father's daughter, I felt I had a duty to get involved"

Aung San Suu Kyi (1945 -)

University of Alberta

Yolk Sac Infections in Broiler Chicks: Studies on *Escherichia coli*, Chick Acquired Immunity, and Barn Microbiology

by

Ana Milena Ulmer Franco

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Animal Science

Department of Agricultural, Food and Nutritional Science

Fall 2011

Edmonton, Alberta

To God, the Almighty Father

To my beloved husband Lance, for his unlimited support at all times

To my Colombian and Canadian families, for believing in me

Abstract

The avian yolk sac is a well vascularised membrane that surrounds the yolk of an embryonated egg and functions as a placenta-like structure transferring yolk nutrients including maternal antibodies, to the embryo. The absorption of the yolk sac content during the first days post-hatching is essential for chick growth and development. However, the infection of the yolk sac is the main cause of chick mortality accounting for large economic losses to the poultry industry. The overall goal of this thesis was to study the epidemiological triad of yolk sac infections: the pathogen: *Escherichia coli*, the susceptible host: the chick, and the environment: the chicken barn. In the first experiment a strain of avian pathogenic *E. coli* was transformed with a plasmid carrying a green fluorescent protein. Using fluorescence microscopy it was observed that *E. coli* entered the yolk sac via the chick navel. In the second experiment, the effects of breeder flock age on the total IgY content of egg yolk and yolk sac was determined. It was concluded that IgY increased with breeder flock age in eggs and yolk sacs. The consequences of these results on chick health are unknown. In the third experiment, the effects of cleaning and disinfection methods of the chicken barn on microbial counts were analyzed in barn swabs and in pooled organ and yolk sac samples. It was surprising to observe that 4 days after placing chicks in the barn, samples of chicks from

"cleaner" pens had more bacteria than those of chicks from "dirtier" pens. In the fourth experiment, all *E. coli* isolated from barn and chick samples from the previous experiment were typified using the RAPD method. It was determined that "cleaner" pens had greater *E. coli* variability than "dirtier" pens. Also, more *E. coli* types were shared between chicks and the environment in "cleaner" pens suggesting that chicks that are placed in very clean environments acquired more environmental *E. coli* than chicks placed in environments with greater bacterial loads. The long term consequences of environmental sanitation on chick growth and development, disease susceptibility, and broiler performance should be studied.

Acknowledgements

At the end of the tortuous and thorny road, this doctoral thesis will only have one author. However, this project could have never been completed without the support, help, advice, and experience of several people and institutions. I hope no one is left behind in this list.

I am most especially grateful to my beloved husband Lance, for his support throughout this arduous and sometimes frustrating process.

First and foremost: to my supervisor, Dr. Lynn McMullen, for "adopting" me and my project into her research team, and for supporting me at all times.

To my parents Luis Eduardo and Elida, for nurturing my love for learning, for respecting my decisions (the good and the bad) and for believing in me. To my abuelita (grandma) Carmen, the wisest and most loving woman. I miss you all.

To Dr. Gaylene Fasenko, for giving me the opportunity to enter this path.

To my doctoral committee members, Drs. Leluo Guan and Jianping Wu, for their assistance and recommendations.

To Dr. Kathy Magor, for introducing me to the fascinating world of avian immunology and for her unconditional and constant advice.

To the University of Alberta Poultry Research Centre staff for their co-operation with barn and hatchery duties.

To the former incubation research group at the University of Alberta: Erin Christopher, Jacob Hamidu, Margaret MacKenzie, Thania Moraes and Josue Romao, for all their help during sample collection.

To the Biological Sciences Animal Facility staff at the University of Alberta, especially to Simmone Kerswell, for their careful management of chicks.

To the Biological Sciences Advanced Microscopy Unit, especially to Arlene Oatway, Randy Mandryk and Dr. Rakesh Bhatnagar, for their assistance with sample preparation for fluorescence microscopy.

To Dr. Gita Cherian and Ms. Nathalie Quezada, from Oregon State University, for their training and assistance with IgY determination.

To the AFNS Food Microbiology lab members, for their guidance and their help during sample processing (special thanks to Brenna Black, Elena Dluskaya, Melissa Haveroen, Christine Liu, Aaron Pleitner, Kathleen Vail, Heather Vandertol-Vanier, and Patrick Ward).

To Emma Hernandez-Sanabria for her coaching with molecular techniques and statistical analysis of bacteria.

The financial support for this research was provided by the Alberta Livestock and Meat Agency, the Natural Sciences and Engineering Research Council of Canada, and the Saskatchewan Chicken Industry Development Fund. The donation of broiler chicks from Maple Leaf Hatchery is always appreciated.

On a personal note, I'd like to thank Dr. Francisco Henao (Professor, Universidad de Caldas, Colombia), Dr. Alberto Botero (Scientific Manager Avidesa Mac Pollo, Colombia), and Dr. Juan Carlos Rodriguez-Lecompte (Assistant Professor, University of Manitoba) for being mentors at different stages of my life. Your support encouraged me to pursue this dream.

Life would not be the same without the company of our furry family: Argos, Havoc (alias Monkey) and Leo, our hiking partners, and Split and Sparta, our purring lap warmers.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AMP – antimicrobial peptides

APEC – avian pathogenic *Escherichia coli*

BG – brilliant green agar

BSA – bovine serum albumin

C – "Clean" cleaning and disinfection treatment applied in the barn

CFU – colony forming units

CpG-ODN – Cytosine Guanine olygodeoxynucleotides

D – "Dirty" cleaning and disinfection treatment applied in the barn

E. coli – *Escherichia coli*

eGFP – enhanced green fluorescent protein

Ig – immunoglobulin

LB – Luria Bertani broth or agar

LPS – lipopolysaccharide

OFFSAP – On-Farm Food Safety Assurance Program

PBS – phosphate buffered saline

PC – principal component

PCA – plate count agar

RAPD – random amplification of polymorphic DNA

RIR – Rhode Island red

SCWL – single comb white leghorn

SEM – standard error of the means

VC – “Very Clean” cleaning and disinfection treatment applied in the barn

VD – “Very Dirty” cleaning and disinfection treatment applied in the barn

VRBG – violet red bile agar with 1% added glucose

WSF – water soluble fraction

1. Introduction and Literature Review:

Avian Pathogenic *Escherichia coli (APEC)*

1.1. Introduction

Broiler chick mortality during the first week post-hatching is mainly a consequence of omphalitis / yolk sac infection (Rai et al., 2005) and Avian Pathogenic *Escherichia coli* (APEC) are the most frequently isolated bacteria from infected yolk sacs (Rosario Cortés et al., 2004). Colibacillosis or *E. coli* infection, the most common bacterial disease affecting poultry, accounts for huge economic losses to the poultry industry (La Ragione and Woodward, 2002). Direct economic losses are a consequence of mortality, cost of antimicrobial treatments, and condemnations at slaughter; indirect losses are attributed to poor performance and stunted growth of surviving birds (Barnes et al., 2003). Most avian species are susceptible to being affected by colibacillosis (Barnes, 2003).

The main objective of this chapter is to provide a comprehensive review of *E. coli* with a final emphasis on APEC. Basic concepts as well as current information on these bacteria will be presented. The ultimate goal of this introduction is to set the grounds for a more detailed description of factors related to omphalitis / yolk sac infections in broiler chicks.

1.2. History

Since the first description of *E. coli* 125 years ago, these have become the most studied organisms currently known (Bettelheim, 1994). It was in 1885 when the Austrian paediatrician Theodor Escherich described that certain bacteria, which he called *Bacterium coli commune*, appeared in the bacteriologically sterile meconium of new-born babies shortly after normal feeding of breast milk started (Bettelheim, 1986). Initially, *E. coli* were considered commensal, harmless saprophytes (Bettelheim, 1994) although they are similar to *Salmonella typhi* (Sojka, 1965). Shortly after Escherich's description of *E. coli*, Laurelle (1889) published his studies on peritonitis by intestinal perforation in different animals. He was the first to suggest the possible pathogenicity of *E. coli* and their involvement in enteric disease, hepatic abscesses, and endocarditis (Laurelle, 1889). Since then *E. coli* have been given great attention and have been confirmed as causative and as opportunistic agents of multiple diseases in animals and humans (Sojka, 1965). The first available report of disease caused by *E. coli* in poultry dates back to 1894; Lignieres was the first to report mortality of fowls on a poultry farm associated with isolation of "*Bacterium coli*-like organisms" in blood, liver, and spleen (Palmer and Baker, 1923). During the years following Lignieres' report, multiple cases of disease outbreak

affecting USA's poultry with high mortality rates, lesions of infectious enteritis and multi-systemic infection plus the isolation of a "small, motile, Gram-negative, rod-shaped organism with rounded ends, and even filaments" were observed (Palmer and Baker, 1923). *Bacterium coli commune* was given its present name, *Escherichia coli*, by Castellani and Chalmers in 1919 (Barnes et al., 2003).

1.3. Classification

E. coli is a Gram-negative non-sporing bacillus, 2-3 µm in length, 0.6 µm in breadth with parallel sides, rounded ends, and peritrichous flagella (Sojka, 1965). *E. coli* are members of the *Enterobacteriaceae* family which are able to grow aerobically and anaerobically (Bettelheim, 1994). Sojka (1965) summarized the description provided by the 1958 *Enterobacteriaceae* Subcommittee as follows: "The family *Enterobacteriaceae* is composed of Gram-negative rod-shaped bacteria which are motile with peritricous flagella, or non motile. They ferment glucose rapidly with or without gas production, reduce nitrates to nitrites and grow on ordinary media". Other well studied members of the *Enterobacteriaceae* family include *Salmonella, Shigella, Klebsiella*, and *Proteus* (Sojka, 1965). Based on biochemical and serological characteristics, members of the *Enterobacteriaceae* family are subdivided in groups, subgroups, serotypes,

and biotypes (Sojka, 1965). The description of the antigenic structure of *E. coli* provided below will help to better understand this concept.

1.4. Biochemical Properties

Since the first isolation by Escherich diverse fermentation tests have been used to differentiate bacterial groups within the *Enterobacteriaceae* family (Sojka, 1965). Initially, lactose-fermenters were considered normal inhabitants of the intestinal tract whereas lactose non-fermenters such as *Salmonella* and *Shigella* were considered pathogenic organisms (Sojka, 1965). This is not the rule for *E. coli:* they are lactose fermenters and many strains of *E. coli* are pathogenic for animals and humans (Barnes et al., 2003). Furthermore, strains of *E. coli* which do not ferment lactose are occasionally isolated; these non-fermenter strains should be differentiated from *Salmonella* (Barnes et al., 2003). In most cases, *E. coli* are able to ferment a number of sugars including arabinose, glucose, galactose, fructose, saccharose, and maltose (Sojka, 1965). The ability to ferment glucose with the production of acid and gas is a basic characteristic of *E. coli*: these bacteria are methyl red positive, which means that they reduce the pH enough for methyl red to change in colour. *E. coli* also produce a mixture of almost equal amounts of hydrogen and carbon dioxide (Bettelheim, 1994) as well as indole (Sojka, 1965).

The reduction of nitrate to nitrite is characteristic of *E. coli*, which allows them to grow anaerobically on nitrate and utilize the ammonia formed by its anaerobic reduction as a nitrogen source (Bettelheim, 1994). *E. coli* do not grow in the presence of potassium cyanide, do not hydrolyze urea, liquefy gelatin or grow in citrate medium (Barnes et al., 2003). Detailed descriptions of the biochemical properties of *E. coli* have been provided elsewhere for the reader's interest (Sojka, 1965; Bettelheim, 1994).

The susceptibility of *E. coli* to different chemical and physical agents is equivalent to that of other Gram-negative bacteria (Barnes et al., 2003). The majority of strains will be inactivated at temperatures between 60°C for 30 min and 70°C for 2 min; however, they survive freezing temperatures for extended periods of time (Barnes et al., 2003). A pH below 4.5 or above 9 will inhibit although it will not kill the replication of most *E. coli* strains (Barnes et al., 2003). Organic acids are more effective at inhibiting bacterial growth than inorganic acids (Barnes et al., 2003).

1.5. Growth Requirements and Colony Morphology

E. coli are able to utilize simple carbon and nitrogen sources for their metabolic needs, thus they can grow on simple medium containing only glucose as preferred carbon source, ammonium sulphate as preferred

nitrogen source, and mineral salts (Bettelheim, 1994). However, *E. coli* are "unsaturated systems designed for rapid growth", and their growth rate will increase if the medium is supplemented with amino acids, nucleosides, bases and vitamins (Bettelheim, 1994). Since the 1950's a commonly used medium for molecular biology procedures involving *E. coli* is Luria-Bertani (LB) Miller (Luria and Burrous, 1957). This medium is nutritionally enriched with tryptone to provide nitrogen and carbon, yeast extract to provides vitamins, and sodium chloride for transport and osmotic balance (Luria and Burrous, 1957). *E. coli* growth is enhanced as they do not need to use their available energy and carbon sources for synthesizing these important compounds (Bettelheim, 1994).

After incubating at 37°C for 24 h, low, convex, smooth and colorless colonies can be observed on agar plates (Barnes et al., 2003). On MacConkey agar *E. coli* colonies are bright pink surrounded by a precipitate, and on eosin-methylene blue agar *E. coli* colonies have a dark green-black metallic sheen (Barnes et al., 2003). In contrast to the frequent hemolysis observed on blood agar by mammalian pathogenic *E. coli*, this is not common of avian pathogenic *E. coli* (Barnes et al., 2003).

1.6. Serological Characteristics of *E. coli*

Kauffmann (1947) was the first to successfully use serological methods to classify *E. coli* (Ørskov et al., 1977). In 1947 he published an antigenic schema with 25 'O' antigens, 55 'K' antigens and 19 'H' antigens (Lior, 1994). Currently, 167 O, 74 K, 53 H, and 17 F *E. coli* antigens are recognized (Barnes et al., 2003).

1.6.1. Somatic Antigens

'O' Antigens (lipopolysaccharide (LPS) antigens).

The O antigens are thermostable surface antigens found in all smooth or S forms of *Enterobacteriaceae* (Ørskov et al., 1977). They are composed of phospholipid-polysaccharide complexes with a heat resistant protein fraction (Lior, 1994). These antigens retain their immunogenic, agglutinating and binding capacity even after boiling (Ørskov et al., 1977).

The differences among O antigens are defined by their terminal group, mainly by its nature and by the order of the repeating units within the chain (Lior, 1994). However, due to common LPS and outer membrane proteins in Gram-negative bacteria, cross-reactivity among the O antigens of *E. coli, Salmonella* and *Shigella* is common (Ørskov et al., 1977). Based on their mobility during electrophoresis O antigens are divided into 2 groups:

1) Mobile O antigens, which contain acidic components in the LPS moiety and are usually associated with strains causing dysentery-like disease; 2) Immobile O antigens, which lack acidic components and are mostly found in extraintestinal disease (Lior, 1994). Additionally, mutations on specific LPS chains are responsible for the variation of O antigens from smooth (S) O^+ to rough (R) O^- strains; these R strains, which have lost antigenic specificity, autoagglutinate in saline and cannot be serotyped (Lior, 1994).

'K' Antigens (acidic polysaccharide factors)

In his extensive investigations of the serology of *E. coli*, Kauffman (1947) noted two interesting phenomena: first, some live bacteria were not agglutinated by the corresponding O antiserum, and second, when the bacterial suspension was heated, the inhibition of agglutination was resolved. The term 'K' antigen, from Kapsel the German word for capsule, was then introduced by Kauffman et al. (Kauffman, 1947) as well as the division of this antigen into three classes: L, A, and B. In general, K antigens are polymeric acids on the cell surface, they contain 2% reducing sugars and are associated with virulence (Barnes et al., 2003). Lior (1994) hypothesized that the inability to agglutinate might have been caused by flagella, fimbriae or other surface structures.

1.6.2. Protein Antigens

'H' (flagellar) Antigens.

Flagellar antigens are thermolabile determinants contained on flagellin, the constituent protein of flagella in motile organisms (Lior, 1994). Some *E. coli* do not possess flagella and are non-motile while others need to be provided with a semisolid or fluid media until motility is developed (Wray and Woodward, 1994). H antigens may be associated with any of the O antigens, so in human medicine the OH combination, i.e. the serotype, should be determined in cases of diarrhoeal and extraintestinal disease caused by pathogenic *E. coli* (Lior, 1994).

'F' (fimbriae of pili) Antigens.

Fimbriae or pili are thread-like structures that project from the surface of many Gram-negative bacteria (Lior, 1994). They play an important role in bacterial adherence to epithelial cell surfaces, especially in the intestinal and urinary tracts (Lior, 1994). Fimbrial adhesion is classified as mannose sensitive or mannose resistant depending on inhibition of agglutination by the presence of D-mannose, (Lior, 1994).

Certain enterotoxigenic *E. coli* strains associated with diarrhoea in pigs, calves, and lambs possess many of the characteristics of the KL antigens;

however, because these antigens are fimbriae, they have been re-classified as F antigens; for example K88 and K99 are now classified as F4 and F5 respectively (Wray and Woodward, 1994).

Curli.

Curli are thin, coiled filaments present on the surface of *E. coli* and *Salmonella* spp. (Olsen et al., 1989). Curli mediate bacterial binding to the extracellular matrix and to serum proteins such as fibronectin, laminin, plasminogen and plasminogen activator protein, thus contributing to bacterial adherence and colonization (Olsen et al., 1989).

1.7. *E. coli* virulence factors

Once bacteria penetrate the intestinal mucosa and gain access to the bloodstream they have to resist the bacteriostatic and bactericidal properties of the serum carried on by immunoglobulins, lysozyme, transferrin, and complement (Waters and Crosa, 1991). Resistance to serum killing is multifactorial and complex and has been attributed to various bacterial cell surface components including LPS, capsules and chromosomally encoded surface proteins (Waters and Crosa, 1991).

1.7.1. Colicins.

The first report about the Colicin V phenomenon was provided by Andre Gratia in 1925; he described how certain strains of *E. coli* were "inhibited" by pathogenic strains (Waters and Crosa, 1991). Colicins are defined as antibiotic substances or complexes of antibiotic substances produced by certain *Enterobacteriaceae*; these substances are lethal to related sensitive strains (Fredericq, 1957). Different groups of colicins have been described; colicins from each group may be chemically different but they have one common characteristic: they bind to the same receptor on the sensitive strain (Fredericq, 1957). Colicins are bactericidal but not bacteriolytic (Fredericq, 1957). The lethal action of colicins is comparable to that of virulent bacteriophages; however, colicins are inert chemical substances that completely suppress cell synthesis and kill the cell, whereas bacteriophages are biological entities which partially suppress cell synthesis, reproduce within the cells, and finally kill them to be released (Waters and Crosa, 1991). Most colicins range in molecular mass from 27,000 to 80,000 Da; however, Colicin V is a small molecule (4,000 Da) that is not released from the bacteria by lysis like most of the other colicins but instead is exported (Waters and Crosa, 1991).

Colicin V is encoded in a single self-replicating and transferable plasmid. Colicin V plasmids are found among virulent enteric bacteria and provide an array of additional virulence-related factors (Waters and Crosa, 1991). These provide a competitive advantage in the colonization of the gut and enhance the pathogenicity of *E coli* (Waters and Crosa, 1991).

1.7.2. Aerobactins.

The Colicin V plasmid encodes for an iron chelator known as aerobactin iron uptake system. Although most *E. coli* strains produce the iron chelator enterobactin, the aerobactin system permits the acquisition of iron from serum transferrin thereby enhancing bacterial growth and replication (Waters and Crosa, 1991).

1.7.3. Toxins.

In addition to *E. coli* endotoxin, a structural component of *E. coli* cell wall, pathogenicity is increased by the production of several toxins with cytopathic effects (Barnes et al., 2003). Specific toxins will be described later.

1.7.4. Adhesins.

These virulence factors can be fimbrial or non-fimbrial. A special non-fimbrial adhesin is intimin, which mediates bacterial adhesion to the

enterocyte surface in cases of human enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) (Nataro and Kaper, 1998). Other adhesins will be described for each *E. coli* pathotype.

1.8. Strain classification

In human medicine, a broad classification of *E. coli* based on their genetics and clinical importance divides them in commensal strains, intestinal pathogenic, enteric or diarrheagenic, and extraintestinal pathogenic strains (Russo and Johnson, 2000). Commensal strains of *E. coli* are the main component of the fecal flora in most healthy humans and animals including birds (Russo and Johnson, 2000). They lack the virulence factors carried by pathogenic intestinal and extraintestinal strains, thus they do not normally cause disease within the intestinal tract or outside it (Russo and Johnson, 2000).

Intestinal pathogenic *E. coli* strains are not present in the fecal flora of healthy hosts; their presence is associated with gastroenteritis or colitis (Russo and Johnson, 2000). The intestinal pathogenic *E. coli* strains have been grouped in six pathotypes: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998). The different

pathotypes of *E. coli* are composed by clonal groups sharing O and H antigens (Nataro and Kaper, 1998). To cause disease in animals, EPEC, EHEC, and ETEC use the same virulence factors found in human strains but have specific colonization factors for animal species not found in human strains (Kaper et al., 2004). A brief description of the main human enteric *E. coli* pathotypes is provided below.

Even though extraintestinal *E. coli* may affect almost any organ or anatomical site the most common extraintestinal infections caused by pathogenic *E. coli* strains in humans are urinary tract infections and bacterial meningitis (Kaper et al., 2004). These *E. coli* strains were initially classified according to infection site as uropathogenic *E. coli* (UPEC) and bacterial meningitis *E. coli* (BMEC) but now are grouped together as Extraintestinal Pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000). Diverse *E. coli* strains causing pneumonia, peritonitis, osteomyelitis, soft tissue infection, etc., are also included within this pathotype (Russo and Johnson, 2000). An additional animal pathotype known as Avian Pathogenic *E. coli* (APEC) causes extraintestinal infections in poultry species (Barnes et al., 2003).

1.9. *E. coli* infections in humans

1.9.1. Enteric disease.

The following is a brief description of the six human diarrheagenic *E. coli* pathotypes.

Enterotoxigenic* E. coli *(ETEC). These strains were first described as cause of diarrheal disease in newborn piglets (Gonzalez and Blanco, 1986). ETEC causes diverse levels of watery diarrhoea without blood, mucus or pus in humans; they are the main cause of the traveler's diarrhoea and of childhood diarrhoea in developing countries (Kaper et al., 2004).

Enteropathogenic* E. coli *(EPEC). The description of a syndrome of watery diarrhoea, vomiting and fever associated with *E. coli* infection in infants less than 2 years of age was first provided by Neter (1959). Most EPEC infections are associated with outbreaks of neonatal diarrhoea in infants from developing countries with clinical presentations that can range from self-limiting diarrhoea to chronic enteritis and wasting (Nataro and Levine, 1994).

Enterohaemorrhagic* E. coli *(EHEC). The main reservoir for EHEC is the intestinal tract of both healthy and symptomatic (diarrheic) ruminants (Blanco et al., 1997b). Initial outbreaks of haemorrhagic colitis were related

to the consumption of uncooked ground beef hamburgers, but diverse food sources including unpasteurized milk, cantaloupe melon, apple juice, and radish sprouts, have also been linked to disease by EHEC contamination (Kaper et al., 2004). Shiga toxins produced by EHEC induce local damage in the colon resulting in haemorrhagic diarrhoea, necrosis, and intestinal perforation (Kaper et al., 2004). When Shiga toxins reach the bloodstream they target the kidneys damaging the renal endothelial cells and occluding the microvasculature. Resulting nephritis leads to haemolytic uremic syndrome consisting of acute renal failure, thrombocytopenia, and microangiopathic haemolytic anaemia (Kaper et al., 2004). A month prior to submission of this thesis, Germany was dealing with the "deadliest outbreak of EHEC of modern history". According to the International Society for Infectious Diseases this outbreak had affected more than 3300 people leading to more than 800 cases of HUS and 38 deaths. Bean and seed sprouts contaminated with an unusual EAggEC STEC/VTEC O104:H4 were the source of the outbreak (www.promedmail.org, archive number 20110615.1823, June 15th, 2011).

Enteroinvasive* E. coli *(EIEC). These *E. coli* strains are biochemically and pathogenically identical to *Shigella* spp. (Nataro and Kaper, 1998). They normally cause watery diarrhoea but in a small percentage of

patients EIEC may invade the colonic epithelium causing dysentery with fever, tenesmus, abdominal cramps, and diarrhoea containing blood, mucus and leukocytes (Kaper et al., 2004).

Enteroaggregative* E. coli *(EAEC). It is recognized as a cause of diarrhoea persisting for more than 14 days in children and adults in both developing and developed countries (Kaper et al., 2004).

Diffusely adherent* E. coli *(DAEC). Is reported as a cause of watery diarrhoea affecting children between 1 and 5 years of age (Nataro and Kaper, 1998).

1.9.2. Human extraintestinal disease

Extraintestinal Pathogenic* E. coli *(ExPEC). *E. coli* are the most common bacteria associated with urinary tract infections in humans (Kaper et al., 2004). Urinary tract infections generally start as a bladder infection (cystitis) but uropathogenic *E. coli* can ascend to the kidneys resulting in pyelonephritis, renal failure and urosepsis if bacteria reach the bloodstream (Vejborg et al., 2011). A human's own intestinal content is the source of *E. coli* strains, however, uropathogenic strains are different from *E. coli* strains normally present in the human lower intestinal tract (Kaper et al., 2004). *E. coli* producing urinary tract infections are characterized by

the expression of P fimbriae necessary for adherence to uroepithelial cells, haemolysin which damages the renal epithelium, aerobactin, serum resistance, and encapsulation pathogenicity mechanisms (Kaper et al., 2004). Highly uropathogenic E. coli strains possess more pathogenicity islands than less uropathogenic strains; these pathogenicity islands are not present in the chromosome of faecal *E coli* strains (Vejborg et al., 2011). Uropathogenic *E. coli* isolated from clinical cases express metabolic genes for rapid replication and active iron scavenging, and multiple virulence genes for motility and adherence (Hagan et al., 2010). The marked accumulation of virulence genes such as *hly*, *cdt*1, *clb*, *pap*, *sfa/foc*, *fyuA*, *iroN*, *kpsMT*, and *traT* is characteristic of highly virulent ExPEC (Krieger et al., 2011).

ExPEC are the most common Gram-negative bacteria causing neonatal meningitis characterized by severe neurological damage and fatality rates up to 40% (Dawson et al., 1999). Strains that cause meningitis are limited to a small number of serogroups and 80% of these belong to the K1 capsule type (Kaper et al., 2004). Meningitis-causing ExPEC are not transmitted by faeces or urine; these infections spread haematogenously: ExPEC present in the bloodstream translocate through the blood-brain barrier reaching the central nervous system (Kaper et al., 2004)

1.10. *E. coli* infections in domestic animals

E. coli produce intestinal and systemic disease in domestic animals. Detailed descriptions are available for the readers' interest (Gyles, 1994).

Early exposure to pathogenic *E. coli* along with low levels of circulating immunoglobulins make neonatal calves and lambs susceptible to colisepticaemia, an acute fatal infection of dairy calves under 1 week of age (Gay and Besser, 1994) and neonatal lambs within 2 days of birth (Hodgson, 1994). The clinical course of the acute infection is short (3 to 8 h) with no diagnostic clinical findings; initial signs of depression and responsiveness are rapidly followed by recumbency, coma and cardiovascular collapse with nearly 100% mortality rate (Sojka, 1965). Acute or chronic neonatal diarrhoea commonly affects calves during the first 4 wk after birth (Butler and Clarke, 1994). Calves affected by enteric colibacillosis produce large amounts of foul-smelling pasty to watery faeces, with colours varying from pale yellow to white, which produce characteristic soiling of the buttocks known as "white scours" (Butler and Clarke, 1994). Severe diarrhoea in piglets less than 15 days of age can be caused by enterotoxins produced by ETEC (Gonzalez and Blanco, 1986). *E. coli* also cause post-weaning diarrhoea and oedema disease in young pigs

characterized by vascular lesions in the intestine, subcutis and brain, leading to oedema and neurological signs (Frydendahl, 2002).

Cattle are a reservoir for EHEC O157:H7 but this serotype is not associated with disease in cattle (Butler and Clarke, 1994). In 1997, Blanco et al. characterized EHEC strains recovered from more than 350 healthy animals from 19 different farms in Spain; they concluded that 95% of the farms examined were positive for EHEC strains not only in adult cows but also in calves (Blanco et al., 1997b). In cases of mastitis caused by *E. coli* clinical signs may vary from fatal peracute mastitis, through acute, chronic and subclinical mastitis (Hill, 1994). In the peracute infection, soon after parturition cows will show signs of fever, anorexia, shivering, rumen stasis and diarrhoea; and within hours, hypotension and dehydration lead to disseminated intravascular coagulopathy, multisystemic failure and death (Hill, 1994). In the acute form there might be transient fever, the affected udder quarter becomes swollen and hard and the milk shows discoloration and clots (Sojka, 1965). The chronically infected animals show recurrent clinical episodes of mastitis with intervals of "normal" appearance, while in the subclinical cases of mastitis, the latent infection within the udder can last for extended periods of time without inflammatory signs (Hill, 1994).

1.11. *E. coli* infections in poultry

Contrary to *E. coli* infections in mammals which are a primary enteric disease, colibacillosis in poultry is an extraintestinal infection, either localized or systemic (Barnes et al., 2003). Localized forms of colibacillosis are described according to the affected tissue/organ and include coliform omphalitis/yolk sac infection, coliform cellulitis, swollen-head syndrome, venereal colibacillosis, and coliform salpingitis/peritonitis (Barnes et al., 2003). Except for cellulitis and yolk sac infection, these conditions generally represent different manifestations of the same disease known as avian septicaemic colibacillosis (Gross, 1994).

In general, inhalation of bacteria with further airsacculitis has been proven to play an important role in the pathogenesis of avian colibacillosis (Pourbakhsh et al., 1997). After bacterial invasion of *E. coli* colonization of vital organs lead to colisepticaemia (Ramirez et al., 2009). Colisepticaemia or systemic colibacillosis constitute the main clinical form of colibacillosis specially affecting growing broiler chickens (Stordeur and Mainil, 2002). According to age and type of bird, and depending on how the microorganisms gained access to the bloodstream, colisepticaemia can be classified as: neonatal, of respiratory origin, of enteric origin, acute septicaemia of layers, and coliform septicaemia of ducks (Barnes et al.,

2003). Even though death is the most common outcome of colisepticaemia, survivors will commonly develop sequelae when *E. coli* localize in sites such as the eyes, brain, synovial tissues and bones (Barnes et al., 2003). Coliform panophthalmitis, coliform meningitis/encephalitis, coliform synovitis/arthritis, and coliform osteomyelitis are common consequences of systemic *E. coli* infections (Barnes et al., 2003). All these syndromes diminish bird health and performance.

Coliform cellulitis, also known as necrotic dermatitis, inflammatory process or infectious process, leads to important economic losses to the poultry industry due to carcass condemnation during processing (Vaillancourt and Barnes, 2003). Lesions due to coliform cellulitis are referred to as plaques; these sheets of serosanguineous to caseated, fibrinopurulent exudate locate in the subcutaneous tissue of the lower abdomen and thighs of broiler chickens (Vaillancourt and Barnes, 2003). Although living birds do not display clinical signs specific to coliform cellulitis, 35% of broilers condemned for cellulitis during processing had pericarditis, airsacculitis, perihepatitis or osteomyelitis (Gomis et al., 2001). Scratches and minor injuries on the skin increase the incidence of coliform cellulitis, thus, stocking density, aggressiveness or nervousness of

the birds, and feather coverage are considered predisposing factors for this condition (Elfadil et al., 1996).

The main interest of this thesis is to study predisposing factors to coliform omphalitis/yolk sac infections in broiler chicks. Multiple studies regarding other clinical forms of localized and systemic colibacillosis in poultry are available for the reader's interest (Sojka, 1965; Gross, 1994; Barnes et al., 2003).

1.11.1. Omphalitis / yolk sac infections.

Omphalitis, by definition, is an inflammation of the navel; however, in birds it usually involves the yolk sac due to its close anatomic location (Barnes et al., 2003). The avian yolk sac develops as a well vascularised extraembryonic membrane that surrounds the yolk of an embryonated egg (Romanoff, 1960). The yolk sac membrane is essential for the transfer of nutrients (including IgY, the maternal antibodies) from the yolk to the developing embryo (Romanoff, 1960). However, the infection of the yolk sac is the main infectious cause of chick mortality during the first week of the post-hatching period (Rai et al., 2005), and APEC are the most common bacteria isolated from infected yolk sacs (Rosario Cortés et al., 2004).

In ovo contamination with *E. coli* may occur prior to oviposition if hens are suffering from oophoritis and / or salpingitis, and as a consequence of poor hygiene during artificial insemination of breeder hens (Montgomery et al., 1999). After oviposition, *in ovo* contamination with *E. coli* is due to fecal contamination of egg shells in dirty nest boxes or floor eggs (Gross, 1994). Embryonic mortality during the last stages of incubation as well as mortality shortly after hatching are common in cases of *in ovo* infection (Barnes et al., 2003). Contamination of unhealed navels has been suggested as a cause of omphalitis (Fasenko and O'Dea, 2008).

Barnes et al. (2003) described the clinical findings of omphalitis as characterized by inflammation, oedema, and small abscesses of the navel accompanied by abdominal distension and hyperaemic abdominal blood vessels. In severe cases of omphalitis lysis of the abdominal wall and exudation will moisten the chick's abdomen; these chicks are known as "mushy chicks" (Randall, 1991). The lack of yolk sac absorption is due to changes in the yolk sac content composition as a consequence of nutrient breakdown by bacteria (Sander et al., 1998). The yolk sac membrane is oedematous and inflamed, and the vitelline blood vessels are hyperaemic (Randall, 1991). Chicks surviving yolk sac infections show stunted growth and poor performance; they may also suffer from systemic infections and

their common consequences: pericarditis, peritonitis, and perihepatitis (Barnes et al., 2003). The impaired absorption of the yolk sac content deprives chicks of maternal nutrients and antibodies, all essential for normal chick development (Noy and Sklan, 2001). Furthermore, important pathogens such as *Salmonella* spp. and *Campylobacter* spp. have been isolated from unabsorbed yolk sac in market-age broilers, extending the relevance of omphalitis from a bird health issue to a possible food safety concern (Cox et al., 2006).

1.11.2. Reservoirs of avian pathogenic E. coli

The most important reservoir of *E. coli* is the intestinal tract of animals including poultry; in the chicken, concentrations of 10^6 bacterium per gram of faecal matter are common (Stordeur and Mainil, 2002). From this population, 10 to 15% of the *E. coli* belongs to potentially pathogenic serotypes (Stordeur and Mainil, 2002). As with mammalian *E. coli* serotypes, APEC serotypes vary according to geographic location and have changed overtime. Relatively few clonal types are responsible for different forms of colibacillosis in chickens and turkeys in extensive geographic areas (Barnes et al., 2003). In 1965 Sojka reported O1, O2, O35, and O78 as the most common serogroups affecting poultry. According to Barnes et al. (2003) more recent isolates from diseased birds belonged to

the O18, O81, O115, O116, and O132 serogroups, which were not previously considered pathogenic suggesting the emergence of new pathogenic serotypes. In Canada, Allan et al. (1993) characterized 44 *E. coli* isolates from clinical cases of colibacillosis in turkeys and chickens. They reported that 23% belonged to serogroups O1, O2 or O78; 39% were non-typeable, and remaining isolates belonged to 10 different serogroups (Allan et al., 1993).

1.11.3. Virulence factors of avian pathogenic E. coli

Several virulence factors have been identified in APEC. These include colonization factors such as fimbrial or non-fimbrial adhesins, invasive factors such as survival to macrophage activity, survival factors such as resistance to bactericidal action of complement, the iron acquisition system aerobactin, production of toxins, and haemagglutination (Vandekerchove et al., 2005; Dziva and Stevens, 2008). However, to distinguish between pathogenic and non pathogenic *E. coli* no single virulence factor has been identified (Wooley et al., 2000).

Adhesiveness to the epithelial respiratory cells and ability to grow under iron-limiting conditions are common in microorganisms that cause systemic infections (Lafont et al., 1987). Aerobactin iron uptake systems compete with transferrin and allow *E. coli* to overcome growth limitations

caused by insufficient free iron in the body fluids (Lafont et al., 1987). This system is present and expressed in APEC and absent from most non-virulent isolates (Lafont et al., 1987). After characterising 45 *E. coli* strains isolated from clinical cases of colibacillosis in Brazil, Vidoto (1990) concluded that 60% of strains were pathogenic for 1-day-old chicks. From these pathogenic strains, 57.7% produced colicin V, 68.8% were resistant to rabbit and chicken serum, and 66.6% produced aerobactin (Vidoto, 1990). Allan et al. (2003) confirmed the presence of the aerobactin iron uptake system in 95% of the 44 strains studied in Canada. It has also been recognized that APEC tend to be less toxigenic than mammalian pathogenic *E. coli* (Blanco et al., 1997a). In summary, virulence of APEC is strongly based on the ability of these strains to invade the host, to escape its immune system, and to easily replicate (Vidotto et al., 1990; Mellata et al., 2003).

The pathogenicity of certain strains without defined virulence determinants has been attributed to host-dependent factors, which means that susceptibility to infection is most likely linked to an underlying disease or predisposing factors to bacterial invasion (Picard et al., 1999). Pathogens such as Newcastle Disease virus, Infectious Bursal Disease virus, and *Mycoplasma gallisepticum,* as well as increased environmental

pressure increase the risk of *E. coli* infections (Kabir, 2010). It is prudent to highlight the importance of appropriate flock management procedures including providing the newly hatched chick with adequate temperature, stocking density, air quality, clean water and feed to avoid unnecessary stress.

1.11.4. Protection of chicks against E. coli *infections*

During the first week post hatching the immune system of the broiler chick is not fully developed and the antigen-specific protection is totally dependent on maternal antibodies (Brambell, 1970). In contrast to mammals who after birth may obtain maternal antibodies in the colostrums, all the maternal immunoglobulins needed to protect the newly hatched chick must be deposited in the egg before it is laid (Rose et al., 1974).

To protect the chick by passive antibody transfer vast amount of research on maternal vaccination has been carried out. The major challenge in the developments of *E. coli* bacterins, live vaccines and subunit vaccines is that the protection achieved is serotype-specific (Dziva and Stevens, 2008). Live vaccines are cheaper than purified proteins and can be used for mass immunization via aerosol, feed, or drinking water (Peighambari et al., 2002). The *car*AB mutants of the virulent APEC

serogroups O1, O2, and O78 constructed and characterized by Kwaga et al. (1994) were attenuated, stable and immunogenic. The mutant constructed from serotype O2 protected turkeys after an intratracheal challenge with the homologous wild-type strains when used as an oral vaccine (Kwaga et al., 1994). Live attenuated *cya* and *crp* mutants from O2 and O78 strains developed by Peighambari et al (2002) induced protection against airsacculitis when birds were challenged with the homologous *E. coli* virulent strains.

A novel approach to the development of bacterins against APEC involves using those antigens that are conserved among APEC strains (Kariyawasam et al., 2002). These antigens include: LPS, F1 pilus adhesion (*FimH*), P pilus adhesion (*PapG*), and aerobactin receptor protein (*lutA*) (Kariyawasam et al., 2002). Research involving IgY, the avian analog of mammalian IgG, purified from eggs laid by hens immunized with *PapG* or *lutA* reported successful protection of broiler chickens against respiratory infections and septicaemia when used as an intramuscular vaccine (Vandemaele et al., 2006). However, natural transfer of antibodies from broiler breeders vaccinated with *PapG* did not protect the offspring against bacterial challenge (Vandemaele et al., 2006). In a different experiment, a recombinant vaccine against the *Iss* (Increased Serum Survival) protein

was able to elicit an immune response and protect against heterologous *E. coli* challenge in chickens, making it a possible candidate for future use (Lynne et al., 2006).

Another approach to enhancing vaccination response is through the development of new vaccine adjuvants. As an example Gomis et al (2007) improved the immune response against APEC by using synthetic oligodeoxynucleotides (ODN) containing cytosine–phosphodiester-guanine (CpG) as an *E. coli* vaccine adjuvant (Gomis et al., 2007). The APEC strains tested are known to cause respiratory infections and septicaemia in chickens. The development of novel vaccines / bacterins for hen immunization to provide protection against APEC in chicks would be of great benefit if they could not only prevent respiratory infections and septicaemia but also early yolk sac infections.

To date, the only available bacterin in Canada for passive immunization of broilers by vaccination of breeders against *E. coli* is Nobilis *E. coli*, commercialized by Intervet. This is an *E. coli* inactivated sub-unit vaccine based on the fimbrial antigen F11 variant plus the flagellar antigen FT. In Denmark, this vaccine proved reduction in *E. coli*-associated mortality in broiler breeders but showed no significant effect on first week mortality of broilers (Gregersen et al., 2010). Two other patents

held by Wyeth/Fort Dodge on a live genetic deletion *aro*A mutant *E. coli* vaccine offer more possibilities for using this type of preventative measure in the future (Fan and Kumar, 2004; Fan et al., 2008). Garavax®-T a commercial Schering-Plough product is available in the US. This is a live *E. coli* vaccine for prevention of airsacculitis in turkeys, but its use has not been approved in chickens.

1.12. APEC: a new foodborne pathogen?

The transfer of antibiotic resistance plasmids among chicken bacteria and from chicken to human bacteria has been described since the 1970's. Initial studies on the potential hazard of APEC for human health were concerned with the transfer of antibiotic resistant bacteria to personal directly involved in bird handling (Levy et al., 1976). Greater concerns on antibiotic resistance have been raised on the European Union due to the fact that most fecal *E. coli* of food animals are resistance to antibiotics used on veterinary prescriptions (van den Bogaard et al., 2001). In intensive poultry production operations antibiotic treatments are administered to whole flocks rather than individual animals; in addition, antimicrobial agents may be continuously fed to broiler chickens and turkeys as antimicrobial growth promoters (van den Bogaard et al., 2001). In a study conducted in the Netherlands, van den Bogaard et al. (2001) compared the

antibiotic resistance patterns of faecal *E. coli* from meat birds (broiler chickens and turkeys), laying hens, their respective farmers, and personnel working at slaughter plants. Their results described higher prevalence of multiple antibiotic resistant *E. coli* in fecal samples from both turkey and chicken farmers and slaughterers compared with those from laying-hen farmers (van den Bogaard et al., 2001). An explanation for this is that under commercial conditions laying hens are less exposed to antibiotics as compared to meat birds. Furthermore, the same antibiotic resistance patterns were found in *E. coli* from meat birds and in those from farmers suggesting the dissemination of resistant bacteria and/or resistance plasmids from bird to human bacteria (van den Bogaard et al., 2001).

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), a program from the Public Health Agency of Canada, examines the relationship between antimicrobials used in food-animals and humans. It monitors antimicrobial use and antimicrobial resistance in humans, animals (cattle, swine, horses, and poultry), and food sources across Canada. For 2009 CIPARS reported *E. coli* resistance patterns to 15 different antibiotics from chicken abattoir samples and retail

chicken meat samples. Resistance ranged from 0% for ciprofloxacin and amikacin, to 50% for ampicillin and streptomycin.

Aside from the transmission of multiple antibiotic resistance plasmids between poultry and human *E. coli* most APEC were thought to represent a low risk of disease for people or other animals and they were only considered pathogenic for birds (Caya et al., 1999). These conclusions were based on certain differences between APEC and human *E. coli*. In 1999 Caya et al. compared *E. coli* isolates from humans with diarrhoea and urinary tract infections (UTI) vs. *E. coli* isolates from chickens with airsacculitis and colisepticaemia. They reported little to no relatedness between human and avian *E. coli* strains and suggested that avian isolates recovered from diseased birds possessed very few of the attributes required to cause diseases in humans (Caya et al., 1999). The lack of the virulence genes associated with human intestinal *E. coli* pathogens was also confirmed in APEC by Janßen et al. (2001).

As molecular diagnostic methods advanced the concept that APEC were not capable of causing disease in humans changed. The availability of complete genome sequences of multiple strains of *E. coli*, the focus of the study of APEC vs. UPEC has been redirected over the last 5 years. It is known that the immediate source of UPEC causing human UTIs is an

individual's own colonic flora; however, it is not completely understood how these virulent clones come to inhabit the human colon (Johnson et al., 2005). An interesting and concerning hypothesis was stated by Johnson et al. (2005) to answer this question: they suggested that retail poultry harbouring APEC was a foodborne source of the *E. coli* clones capable of causing human UTIs (Johnson et al., 2005). A similar hypothesis had been postulated by van den Bogaard et al. (2001) who reported the possibility that at slaughter, multiple antibiotic resistant *E. coli* strains from the avian intestine may soil poultry carcasses, infect humans both directly (slaughter personnel) and via food, colonize the human intestinal tract and contribute multiple antibiotic resistance genes to human endogenous flora (van den Bogaard et al., 2001).

Current concerns are based on the confirmation that UPEC and APEC both cause extraintestinal disease, share virulence-associated traits, and have overlapping O serogroups and phylogenetic types (Rodriguez-Siek et al., 2005). Their ability to cause extraintestinal diseases relates to possession of multiple virulence factors including adhesins, iron acquisition systems, toxins, protectins, and invasins that enable them to adapt to an extraintestinal lifestyle (Johnson et al., 2007). If some human and avian ExPEC strains are highly similar to one another, it is possible

that a foodborne link between some APEC and UPEC strains exists (Johnson et al., 2007).

APEC plasmids can contribute to the urovirulence of *E. coli* for mammalian hosts. Skyberg et al. (2006) reported that acquisition of APEC plasmids (pAPEC-O2-ColV and pAPEC-O2-R) by an avian fecal *E. coli* commensal strain enhanced its ability to kill chicken embryos, grow in human urine, and colonize the murine kidney. They concluded that these results along with the documented ability of APEC plasmids to transfer to humans provided further reasons to think that APEC plasmids could serve as reservoirs of urovirulence genes for UPEC (Skyberg et al., 2006). To validate this hypothesis the ability of APEC to survive and persist on retail poultry still needs to be assessed, as well as the possibility of colonization of the human intestinal and urinary tracts by APEC (Skyberg et al., 2006).

1.13. Economic Impact of APEC Infections

Colibacillosis is the most common cause of poultry disease and condemnations during processing leading to important economic losses to the poultry industry worldwide (Barnes et al., 2003). According to Kumor et al. (1998) condemnations for coliform cellulitis in Canada increased a 12

fold between 1986 and 1996; 30% of total condemnations at slaughter were due to coliform cellulitis.

An exact figure for the incidence of yolk sac infections in broiler chicks across Canada could not be found. However, the 2007 Western Poultry Disease Report included yolk sac infections in the top 5 diseases problems in broiler chickens in Saskatchewan and Manitoba (Annett, 2007). An increase in the number of cases of omphalitis / yolk sac infection in broiler chicks was reported in British Columbia and Manitoba; Alberta reported no change in the number of cases and Saskatchewan reported less cases (Annett, 2007). For the same year Alberta was the only Canadian western province to report an increase in the number of yolk sac infection cases in broiler breeder chicks (Annett, 2007). Canadian hatcheries include additional 2% chicks to broiler chicken producers to account for possible mortality in the early post-hatching period. This leads to important economic losses for the non-integrated Canadian poultry industry. The following industry parameters will provide a better perspective to this problem.

According to Agriculture and AgriFood Canada 791.7 million hatching eggs were set for broiler chicken production across the country in 2010 (Agriculture and Agri-Food Canada, 2011). Canadian hatcheries hatched a

total of 647.9 million broiler chicks bringing the broiler hatching egg hatchability to an average of 81.8% for the year 2010. Canada exported over 5.8 million chicks (worth over $13 million) to 26 countries including the US, Japan, Colombia, the Philippines, Algeria and Taiwan (Canadian Hatching Egg Producers, 2010). Canadian hatching egg producers are paid based on saleable chicks (i.e. chicks of optimum quality). At the 2010 set price of ¢45.07 per saleable chick, farm cash receipts for hatching egg producers totalled $232 million (Canadian Hatching Egg Producers, 2010).

In 2010 each one of the 2,800 registered Canadian chicken farmers raised an average of 215,000 chickens per year which yielded an average of 365 Tons of chicken meat per farm for a national total of 1.02 billion Kg of eviscerated chicken. Domestic consumption of chicken reached 31.7 Kg per person (Chicken Farmers of Canada, 2010). The negative impact of yolk sac infections is evident if we consider that 2% of the 600 million chickens produced in Canada per year may die during the first week post-hatching.

1.14. Conclusion

In summary, infections caused by *E. coli* in poultry have a detrimental effect on bird welfare and have negative consequences to the poultry industry due to increased mortality, retarded growth, increased

medication costs, and increased condemnation at slaughter. Current research on the similarities between avian pathogenic *E. coli* and human uropathogenic *E. coli* pose a concern on the food-borne potential of APEC. The approach of this thesis to the study of yolk sac infections is based on Snieszko's (1974) epidemiological triad (Figure 1-1): "An infectious disease (yolk sac infection) occurs when a susceptible host (the broiler chick) is exposed to a virulent pathogen (APEC) under proper environmental conditions (the broiler chicken barn)".

The main objectives of this study were to: 1) Determine whether *E. coli* can reach the YS by entering through the chick navel; 2) Evaluate the effect of broiler breeder flock age on the immunoglobulin content of egg yolk and YS; 3) Evaluate the effects of cleaning and disinfection of the chicken barn on bacteria numbers from barn swabs, chick organs and YS samples; and 4) To compare *E. coli* diversity among samples collected from the barn and from chicks in order to determine how many bacterial types are shared between them. This first chapter offered a literature review on APEC; following chapters will guide the reader through the experimental approach used to integrate the three above mentioned factors in a comprehensive study of yolk sac infections of broiler chicks.

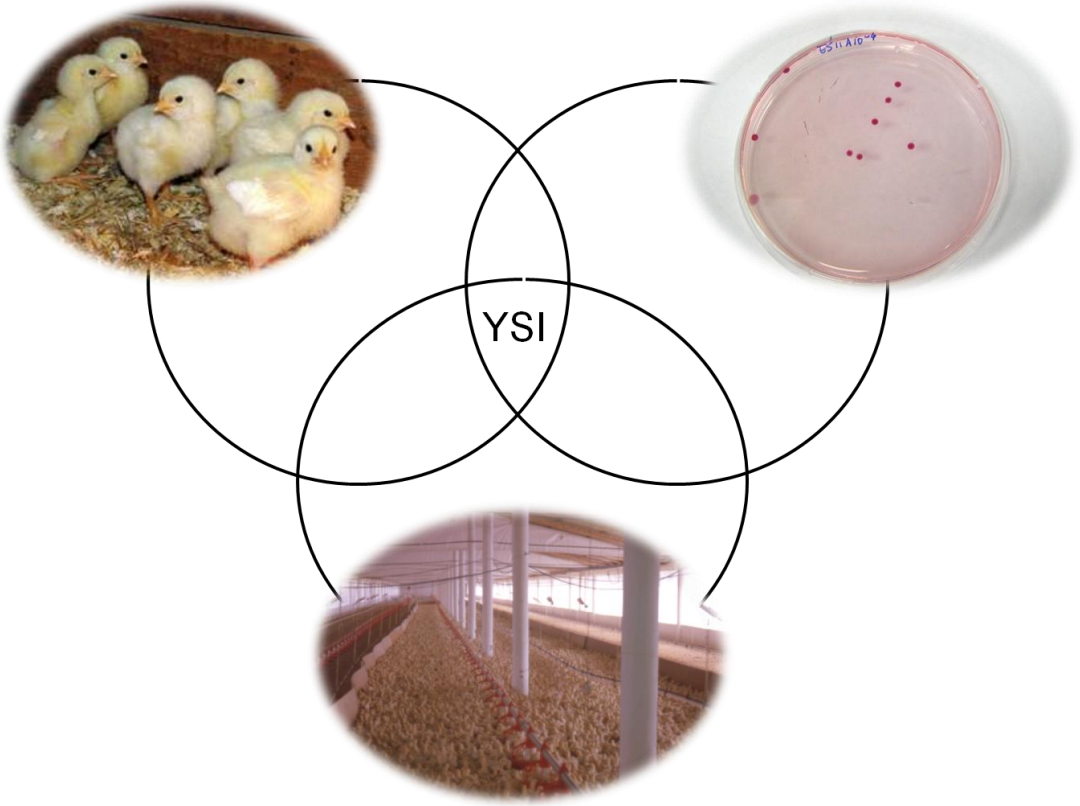


Figure 1-1. Epidemiological triad (Snieszko, 1974) for yolk sac infections.

1.15. References

Acres, S.D., 1985. Enterotoxigenic *Escherichia coli* infections in newborn calves: A Review. J. Dairy Sci. 68, 229-256.

Agriculture and Agri-Food Canada, 2010. The poultry marketplace: chicken at a glance.

http://www.agr.gc.ca/poultry-volaille/hatc-couv_eng.htm

Last accessed: April 8, 2011.

Allan, B.J., van den Hurk, J.V., Potter, A.A., 1993. Characterization of *Escherichia coli* isolated from cases of avian colibacillosis. Can. J. Vet. Res. 57, 146-151.

Barnes, J.H., Vaillancourt, J.P., Gross, W.B., 2003. Colibacillosis. In: Saif, Y.M., Barnes, H.J., Fadly, A.M. et al (Eds.), Diseases of poultry. Blackwell Publishing, Ames, Iowa, USA, pp. 631-652.

Bettelheim, K.A., 1994. Biochemical characteristics of *Escherichia coli*. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Wallingford, UK, pp. 3-30.

Bettelheim, K.A., 1986. Commemoration of the publication 100 years ago of the papers by Dr. Th. Escherich in which are described for the first time the organisms that bear his name. Zentralbl. Bakteriol. Orig. A 261, 255-265.

Blanco, J.E., Blanco, M., Mora, A., Blanco, J., 1997a. Production of toxins (enterotoxins, verotoxins, and necrotoxins) and colicins by *Escherichia coli* strains isolated from septicemic and healthy chickens: relationship with in vivo pathogenicity. J. Clin. Microbiol. 35, 2953-2957.

Blanco, M., Blanco, J.E., Blanco, J., Mora, A., Prado, C., Alonso, M.P., Mouriño, M., Madrid, C., Balsalobre, C., Juárez, A., 1997b. Distribution

and characterization of faecal Verotoxin-Producing *Escherichia coli* (VTEC) isolated from healthy cattle. Vet. Microbiol. 54, 309-319.

Brambell, R., 1970. Transmission of immunity in birds. In: Neuberger, A., Tatum, E.L. (Eds.), The transmission of passive immunity from mother to young. North-Holland Publishing Company, Amsterdam, the Netherlands, pp. 20-41.

Butler, D.G., Clarke, R.C., 1994. Diarrhoea and dysentery in calves. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Wallingford, UK, pp. 91-116.

Canadian Hatching Egg Producers, 2010 Annual report.
http://chep-poic.ca/pdf/Annual_Reports/2010/CHEP10AR_e.pdf
Last accessed: April 8, 2011.

Canadian Integrated Program for Antimicrobial Resistance Surveillance
http://phac-aspc.gc.ca/cipars/index-eng.php
Last accessed: September 20, 2011.

CIPARS, 2009 Preliminary results.
http://phac-aspc.gc.ca/cipars-picra/2009/pdf/2009-eng.pdf
Last accessed: September 20, 2011

Caya, F., Fairbrother, J.M., Lessard, L., Quessy, S., 1999. Characterization of the risk to human health of pathogenic *Escherichia coli* isolates from chicken carcasses. J. Food Prot. 62, 741-746.

Chicken Farmers of Canada, 2010 Annual report. http://chicken.ca/upload/Documents/2010-AnnualReport.pdf Last accessed: April 8, 2011.

Correa, M.G.P., Marin, J.M., 2002. O-Serogroups, *Eae* gene and EAF plasmid in *Escherichia coli* isolates from cases of bovine mastitis in Brazil. Vet. Microbiol. 85, 125-132.

Cox, N.A., Richardson, L.J., Buhr, R.J., Northcutt, J.K., Fairchild, B.D., Mauldin, J.M., 2006. Presence of inoculated *Campylobacter* and *Salmonella* in unabsorbed yolks of male breeders raised as broilers. Avian Dis. 50, 430-433.

Dawson, K.G., Emerson, J.C., Burns, J.L., 1999. Fifteen years of experience with bacterial meningitis. Pediatr. Infect. Dis. J. 18, 816-822.

Dziva, F., Stevens, M.P., 2008. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathol. 37, 355-366.

Elfadil, A.A., Vaillancourt, J.P., Meek, A.H., 1996. Impact of stocking density, breed, and feathering on the prevalence of abdominal skin scratches in broiler chickens. Avian Dis. 40, 546-552.

Fan, H.H., Kumar, M., 2004. Avian vaccine composition for the protection of poultry against disease and infection caused by *E. coli* and *Salmonella*. PCT/US2004/014836, 1-17.

Fan, H.H., Kumar, M., La Ragione, R.M., Woodward, M.J., 2008. Avian *E. coli* vaccine for protection against colibacillosis. PCT 10/837,793, 1-13.

Fasenko, G.M., O'Dea, E.E., 2008. Evaluating broiler growth and mortality in chicks with minor navel conditions at hatching. Poult. Sci. 87, 594-597.

Fredericq, P., 1957. Colicins. Annu. Rev. Microbiol. 11, 7-22.

Frydendahl, K., 2002. Prevalence of serogroups and virulence genes in *Escherichia coli* associated with postweaning diarrhoea and edema disease in pigs and a comparison of diagnostic approaches. Vet. Microbiol. 85, 169-182.

Gay, C.C., Besser, T.E., 1994. *Escherichia coli* septicaemia in calves. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Wallingford, UK, pp. 75-116.

Gomis, S., Riddell, C., Potter, A., Allan, B., 2001. Phenotypic and genotypic characterization of virulence factors of *Escherichia coli* isolated from broiler chickens with simultaneous occurrence of cellulitis and other colibacillosis lesions. Can. J. Vet. Res. 65, 1-6.

Gomis, S., Babiuk, L., Allan, B., Willson, P., Waters, E., Hecker, R., Potter, A., 2007. Protection of chickens against a lethal challenge of *Escherichia coli* by a vaccine containing CpG oligodeoxynucleotides as an adjuvant. Avian Dis. , 78-83.

Gonzalez, E.A., Blanco, J., 1986. Colonization antigens, antibiotic resistance and plasmid content of enterotoxigenic *Escherichia coli* isolated from piglets with diarrhoea in Galicia (North-Western Spain). Vet. Microbiol. 11, 271-283.

Gregersen, R.H., Christensen, H., Ewers, C., Bisgaard, M., 2010. Impact of *Escherichia coli* vaccine on parent stock mortality, first week mortality of broilers and population diversity of *E. coli* in vaccinated flocks. Avian Path. 39, 287-295.

Gross, W.G., 1994. Diseases due to *Escherichia coli* in poultry. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Willingford, UK, pp. 237-259.

Gyles, C.L. 1994. *Escherichia coli* in domestic animals and humans. CAB International, Willingford, UK.

Hagan, E.C., Lloyd, A.L., Rasko, D.A., Faerber, G.J., 2010. *Escherichia coli* global gene expression from women with urinary tract infection. PLOS Pathogens, 6, 1-19.

Hill, A.W., 1994. *Escherichia coli* mastitis. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Willingford, UK, pp. 117-133.

Hodgson, J.C., 1994. Diseases due to *Escherichia coli* in sheep. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Willingford, UK, pp. 135-150.

Janβen, T.; Schwarz, C.; Preikschat, P.; Voss, M.; Philipp, H-C.; Wieler, L., 2001. Virulence-associated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis. Int. J. Med. Microbiol. 291, 371-378.

Johnson, J.R., Delavari, P., O'Bryan, T.T., Smith, K.E., Tatini, S., 2005. Contamination of retail foods, particularly turkey, from community markets (Minnesota, 1999-2000) with Antimicrobial - Resistant and

Extraintestinal Pathogenic *Escherichia coli*. Foodborne Pathog Dis. 2, 38-49.

Johnson, T.J., Kariyawasam, S., Wannemuehler, Y., Mangiamele, P., Johnson, A.J., Doetkott, C., Skyberg, J.A., Lynne, A.M., Johnson, J.R., Nolan, L.K., 2007. The Genome sequence of avian pathogenic *Escherichia coli* strain O1:K1H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. J. Bacteriol. 189, 3228-3236.

Kabir, S.M.L., 2010. Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control, and public health concerns. Int. J. Environ. Res. Public Health 7, 89-114.

Kaper, J.B., Nataro, J.P., Mobley, H.L.T., 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2, 123-141.

Kariyawasam, S., Wilkie, B.N., Hunter, D.B., Gyles, C.L., 2002. Systemic and mucosal antibody responses to selected cell surface antigens of avian pathogenic *Escherichia coli* in experimentally infected chickens. Avian Dis. 46, 668-678.

Kauffman, F., 1947. The Serology of the c*oli* Group. J. Immunol. 57, 71-100.

Krieger, J.N., Dobrindt, U., Riley, D.E., Oswald, E., 2011. Acute *Escherichia coli* prostatitis in previously healthy young men: bacterial virulence

factors, antimicrobial resistance, and clinical outcomes. Urology, 77, 1420-1425.

Kumor, L.W., Olkowski, A.A., Gomis, S.M., Allan, B.J., 1998. Cellulitis in broiler chickens: epidemiological trends, meat hygiene, and possible human health implications. Avian Dis. 42: 285-291.

Kwaga, J.K.P., Allan, B.J., van den Hurk, J.V., Seida, H., Potter, A.A., 1994. A *car*AB mutant of avian pathogenic *Escherichia coli* serogroup O2 is attenuated and effective as a live oral vaccine against colibacillosis in turkeys. Infetc. Immun. 62, 3766-3772.

La Ragione, R.M., Woodward, M.J., 2002. Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia. Res. Vet. Sci 73, 27-35.

Lafont, J.P., Dho, M., D'hauteville, H.M., Bree, A., Sansonetti, P.J., 1987. Presence and expression of aerobactin genes in virulent avian strains of *Escherichia coli*. Infect. Immun. 55, 193-197.

Laurelle, L., 1889. Étude bactériologique sur les péritonites par perforation. In: Carnay, J.B., Gilson, G., Denys, J. (Eds.), La Cellule: Recueil de cytologie et histologie générale. L'Université de Louvain, Louvain, Belgique, pp. 77-119.

Levy, S.B., FitzGerald, G.B., Macone, A.B., 1976. Spread of antibiotic-resistant plasmids from chicken to chicken and from chicken to man. Nature 260, 40-42.

Lior, H., 1994. Classification of *Escherichia coli*. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Wallingford, UK, pp. 31-72.

Luria, S.E., Burrous, J.W., 1957. Hybridization between *Escherichia coli* and *Shigella*. J. Bacteriol. 74, 461-476.

Lynne, A.M., Foley, S.L., Nolan, L.K., 2006. Immune response to recombinant *Escherichia coli* Iss protein in poultry. Avian Dis. 50, 273-276.

Mellata, M., Dho-Moulin, M., Dozois, C.M., Curtiss III, R., Brown, P.K., Arne, P., Bree, A., Desautels, C., Fairbrother, J.M., 2003. role of virulence factors in resistance of avian pathogenic *Escherichia coli* to serum and in pathogenicity. Infect. Immun. 71, 536-540.

Montgomery, R.D., Boyle, C.R., Lenarduzzi, T.A., Jones, L.S., 1999. consequences to chicks hatched from *Escherichia coli* - inoculated embryos. Avian Dis. 43, 553-563.

Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11, 142-201.

Nataro, J.P., Levine, M.M., 1994. *Escherichia coli* diseases in humans. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Wallingford, UK, pp. 285-333.

Neter, E., 1959. Enteritis due to enteropathogenic *Escherichia coli*. J. Pediatr. 55, 223-239.

Noy, Y., Sklan, D., 2001. Yolk and exogenous feed utilization in the posthatch chick. Poult. Sci. 80, 1490-1495.

Olsen , A., Jonsson, A. Normark, S., 1989. Fibronectin binding mediated by a novel class of surface organelles on Escherichia coli. Nature 338, 652-655.

Ørskov, I., Ørskov, F., Jann, B., Jann, K., 1977. Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. Bacteriol. Rev. 41, 667-710.

Palmer, C.C., Baker, H.R., 1923. Studies on infectious enteritis of poultry caused by *Bacterium coli* Communis. J. Am. Vet. Med. Assoc. 63, 85-96.

Peighambari, S.M., Hunter, D.B., Shewen, P.E., Gyles, C.L., 2002. Safety, immunogenicity, and efficacy of two *Escherichia coli* cya crp mutants as vaccines for broilers. Avian Dis. 46: 287-297.

Picard, B., Garcia, J.S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., Elion, J., Denamur, E., 1999. The Link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infect. Immun. 67, 546-553.

Pourbakhsh, S.A., Boulianne, M., Martineau-Doizé, B., Dozois, C.M., Desautels, C., Fairbrother, J.M., 1997. Dynamics of *Escherichia coli* infection in experimentally inoculated chickens. Avian Dis. 41, 221-233.

Rai, M.F., Khan, S.A., Aslam, A., Saeed, K., 2005. Effects of yolk sac infection in chicken. Avian Poult. Biol. Rev. 16, 87-93.

Ramirez, R.M., Almanza, Y., Garcia, S., Heredia, N., 2009. Adherence and invasion of avian pathogenic *Escherichia coli* to avian tracheal epithelial cells. World J. Microbiol. Biotechnol 25, 1019-1023.

Randall, C.J., 1991. Omphalitis / yolk sac infections. In: Diseases of the domestic fowl and turkey. Mosby Intl. Ltd., Barcelona, Spain, pp 21-24.

Rodriguez-Siek, K.E., Giddings, C.W., Doetkott, C., Johnson, T.J., Fakhr, M.K., Nolan, L.K., 2005. Comparison of *Escherichia coli* isolates

implicated in human urinary tract infection and avian colibacillosis. Microbiology 151, 2097-2110.

Rosario Cortés, C., Téllez, I.G., López Cuello, C., Villaseca Flores, J.M., Anderson, R.C., Eslava Campos, C., 2004. Bacterial isolation rate from fertile eggs, hatching eggs and neonatal broilers with yolk sac infection. Rev. Latinoam. Microbiol. 46, 12-16.

Rose, M.A., Orlans, E., Buttress, M., 1974. Immunoglobulin classes in the hen's egg: their separation in yolk and white. Eur. J. Immunol. 4, 521-523.

Russo, T., Johnson, J.R., 2000. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. J. Infect. Dis. 181, 1753-1754.

Sander, J.E., Willinghan, E.M., Wilson, J.L., Thayer, S.G., 1998. The effect of inoculating *Enterococcus faecalis* into the yolk sac on chick quality and maternal antibody absorption. Avian Dis. 42, 359-363.

Skyberg, J.A., Johnson, T.J., Johnson, J.R., Clabots, C., Logue, C.M., Nolan, L.K., 2006. Acquisition of avian pathogenic *Escherichia coli* plasmids by a commensal *E. coli* isolate enhances its abilities to kill chicken embryos,

grow in human urine, and colonize the murine kidney. Infect. Immun. 74, 6287-6292.

Snieszko, S. F., 1974. The effects of environmental stress on outbreaks of infectious diseases of fishes. J. Fish Biol. 6, 197-198.

Sojka, W.J., 1965. *Escherichia coli* infection in domestic animals and poultry. the Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, England.

Stordeur, P., Mainil, J., 2002. La colibacillose aviaire. Ann. Méd. Vét. 146, 11-18.

Vaillancourt, J.P., Barnes, J.H., 2003. Coliform cellulitis (inflammatory process). In: Saif, Y.M., Barnes, H.J., Fadly, A.M. et al (Eds.), Diseases of poultry. Blackwell Publishing, Ames, Iowa, USA, pp. 652-656.

van den Bogaard, A.E., London, N., Driessen, C., Stobberingh, E.E., 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. J. Antimicrob. Chemother 47, 763-771.

Vandekerchove, D., Vandemaele, F., Adriaensen, C., Zaleska, M., Hernalsteens, J.-., Baets, L.D., Butaye, P., Immerseel, F.V., Wattiau, P., Laevens, H., Mast, J., Goddeeris, B., Pasmans, F., 2005. Virulence-associated traits in avian *Escherichia coli*: comparison between isolates

from colibacillosis-affected and clinically healthy layer flocks. Vet. Microbiol. 108, 75-87.

Vandemaele, F., Bleyen, N., Abuaboud, O., vandeerMeer, E., Jacobs, A., Goddeeris, B.M., 2006. Immunization with the biologically active lectin domain of PapGII induces strong adhesion-inhibiting antibody responses but no protection against avian pathogenic *Escherichia coli*. Avian Pathol. 35, 238-249.

Vejborg, R.M., Hancock, V., Schembri, M.A., Klemm, P., 2011. Comparative genomics of *Escherichia coli* strains causing urinary tract infections. Appl. Environ. Microbiol. 77, 3268-3278.

Vidotto, M.C., Müller, E.E., de Freitas, J.C., Alfieri, A.A., Guimarães, I.G., Santos, D.D., 1990. Virulence factors of avian *Escherichia coli*. Avian. Dis. 34, 531-538.

Waters, V.L., Crosa, J.H., 1991. Colicin V virulence plasmids. Microbiol. Rev. 55, 437-450.

Wooley, R.E., Gibbs, P.S., Brown, T.P., Maurer, J.J., 2000. Chicken embryo lethality assay for determining the virulence of avian *Escherichia coli* isolates. Avian Dis. 44, 318-324.

Wray, C., Woodward, M.J., 1994. Laboratory diagnosis of *Escherichia coli* infections. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Wallingford, UK, pp. 595-628.

2. The Use of peGFP *Escherichia coli* to Establish that Yolk Sac Infection Occurs Via the Broiler Chick Navel[1]

2-1. Introduction

Colibacillosis is the main cause of morbidity and mortality in young chicks and accounts for huge economic losses to the poultry industry (La Ragione and Woodward, 2002). In contrast to *Escherichia coli* infections in mammals, a primary enteric disease, colibacillosis in poultry is an extraintestinal infection either localized or systemic (Barnes et al., 2003). In general, inhalation of bacteria with subsequent airsacculitis has been proven to play an important role in the pathogenesis of avian colibacillosis (Pourbakhsh et al., 1997). Salpingitis, oophoritis, and contamination during artificial insemination lead to contamination of eggs prior to oviposition (Barnes et al., 2003). Fecal contamination of the surface of eggs due to dirty nest boxes or to collection of floor eggs leads to contamination of embryos (Gross, 1994). In addition, contamination of unhealed navels has been suggested as a cause of omphalitis and yolk sac infections in newly hatched chicks (Fasenko and O'Dea, 2008). After initial bacterial

[1] A version of this chapter has been submitted for publication. Ulmer-Franco et al., 2011. Veterinary Microbiology # VETMIC-D-11-5839.

invasion, colonization of vital organs leads to septicaemia (Ramirez et al., 2009).

Omphalitis, by definition, is an inflammation of the navel; however, in birds it usually involves the yolk sac due to its close anatomic location (Barnes et al., 2003). Yolk sac infection is the main infectious cause of chick mortality during the first week of the post-hatching period (Rai et al., 2005), and Avian Pathogenic *E. coli* (APEC) are the most common bacteria isolated from infected yolk sacs (Rosario Cortés et al., 2004). Chicks with unhealed navels have lower body weights and higher mortality at 41 days of age (Fasenko and O'Dea, 2008) as well as delayed absorption of the yolk sac content and shorter intestinal villi in the first 5 days post hatching (Kawalilak et al., 2010) than chicks with properly healed navels. Whether APEC entered the chick through the unhealed navels, infected the yolk sac and lead to colisepticaemia was not proven in these reports.

Since the late 1990's green fluorescent protein (GFP) derived from the jellyfish *Aequorea victoria* has been used as a biomarker for detection of specific bacteria (Andersen et al., 1998). A modification on the genetic characteristics of GFP resulted in the brighter and more stable enhanced green fluorescent protein (eGFP) (Cormack et al., 1996) which maintains its properties at temperatures up to 65 ^{o}C and up to a pH of 11 (Cubitt et

al., 1995). The fluorescence properties of eGFP are expressed in the absence of an enzymatic substrate or a cofactor making it the most frequently used reporter in prokaryotes and eukaryotes (Brazelton and Blau, 2005). A recent study used GFP to monitor *Lactobacillus* in the gastro-intestinal tract of chickens after oral administration (Yu et al., 2007). However, to the author's knowledge, fluorescent proteins have not been used to directly examine *E. coli* infections in broiler chicks.

The objectives of this study were to: 1) Determine whether *E. coli* with a plasmid for eGFP could be used to scientifically prove that bacterial infection occurs via the chick navel into the yolk sac; 2) Establish if a lack of navel healing promotes the infiltration of peGFP *E. coli* into the yolk sac during the first 5 days post-hatching. We hypothesized that 1) Through the use of fluorescence microscopy we would confirm that *E. coli* peGFP would enter the chick navel and reach the yolk sac; 2) A greater percentage of chicks with unhealed navels than chicks with healed navels would be positive to *E. coli* peGFP infection.

2.2. Materials and Methods

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies (Canadian Council on

Animal Care, 2009) with approval from the Animal Care and Use Committee: LIVESTOCK for the University of Alberta.

2.2.1. Preparation of peGFP-tagged avian pathogenic **E. coli**

Escherichia coli Top10 (Invitrogen Canada Inc., Burlington, ON, Canada) carrying a plasmid for eGFP were used for plasmid extraction. These transformed bacteria were obtained from Pierce (2009). The peGFP contained the *Lac* promoter and ampicillin resistance gene (Clontech Laboratories Inc., Mountain View, CA, USA). Experimentation started with two APEC strains: EC317 and EC234 (kindly provided by Dr. Brenda Allan, Vaccine and Infectious Disease Organization, University of Saskatchewan, Canada) as the recipient strains. These strains were selected because of the availability of information on characteristics, pathogenicity and their presence in Canada. EC317 a wild-type strain isolated from a diseased turkey by Dr. Ridell (Western College of Veterinary Medicine, Saskatoon, SK, Canada) was characterized by Allan et al. (1993) as a septicemic strain, serotype O2:NM (Allan et al., 1993; Kwaga et al., 1994; Ngeleka et al., 1996). EC234 a wild-type strain was initially isolated by Dr. L. Arp (Iowa State University). Serotyping and pathogenicity studies on EC234 in Canada have been performed by

Kwaga et al. (1994) and van den Hurk et al. (1994). EC234 serotype is O78:K80:H9.

Electrocompetent cells were prepared following the methods of Sambrook and Russell (2001) and preserved at -80 °C. *Escherichia coli* Top10 peGFP transformants were selected on Luria-Bertani (LB) agar plates (BD Difco, Mississauga, ON, Canada) containing 60 µg/mL ampicillin. Plasmid DNA was purified from *E. coli* Top10 *p*eGFP using a plasmid mini kit following the manufacturer's specifications (QIAGEN Inc., Mississauga, ON, Canada). A 99 µL cell suspension of electrocompetent cells was electroporated using a Gene Pulser electroporator (Bio-Rad laboratories, Hercules, CA, USA) in 0.2 cm electrode gap cuvettes with 1 µL plasmid DNA (161 ng/µL). The following electroporation parameters were used: 2.5 kV, 200 Ω parallel resistance, and 25 µF capacitance. Immediately after electroporation, cells were allowed to recover in 1 mL LB broth for 1 hour at 37 °C. Cells (200 µL) were plated onto LB agar containing 60 µg/mL ampicillin and incubated overnight at 37 °C. Sixteen transformant colonies were streaked on LB agar containing 60 µg/mL of ampicillin and incubated overnight at 37 °C. Single colonies exhibiting green fluorescence (Figure 2-1) were

picked and inoculated in LB broth with ampicillin and incubated overnight at 37 °C with shaking at 350 rpm.

Figure 2-1. Green fluorescent *E. coli* colonies on LB agar. *E coli* colonies (in this case, EC234) transformed with eGFP plasmid exhibit strong green fluorescence.

Overnight cultures were mixed with 50% sterile glycerol solution (v:v) in a 2:1 ratio and stored at -80 °C. EC234 without peGFP were used as negative control. One day prior to chick collection, cultures to be used for inoculation were grown in 5 mL LB broth containing 60 µg/mL of ampicillin at 37 °C with shaking at 350 rpm. Overnight cultures which had an absorbance of ~0.6 at OD_{600} were centrifuged at 4 °C for 10 min at 2,516 x g. To avoid using LB media with ampicillin on the chick navel, supernatants were discarded and pellets suspended in 5 mL sterile brain heart infusion (BHI) broth (BD Difco, Mississauga, ON, Canada) 1 to 2 hours prior to infection.

2.2.2. Chick collection and infection

Broiler chicks were collected from a commercial hatchery on the day of hatching. Equal numbers of chicks were collected on three separate occasions from the same Cobb 500 broiler breeder flock when the flock was 32, 40, and 55 wk of age, for three trials. Chicks were selected according to navel condition at hatching in two groups: healed/closed navel and unhealed/open navel with a scab smaller than 3 mm in diameter covering the navel (n= 36 chicks per navel group) (Figure 2-2). Regardless of navel condition all chicks were standing, alert, healthy in appearance, and had no visible physical defects. Upon collection chicks were transported to the Biological Sciences Animal Facility at the University of Alberta and randomly assigned to one of 3 treatment groups: 1) uninoculated control; 2) inoculated with *E. coli* Top 10 peGFP; and 3) inoculated with EC234 peGFP.

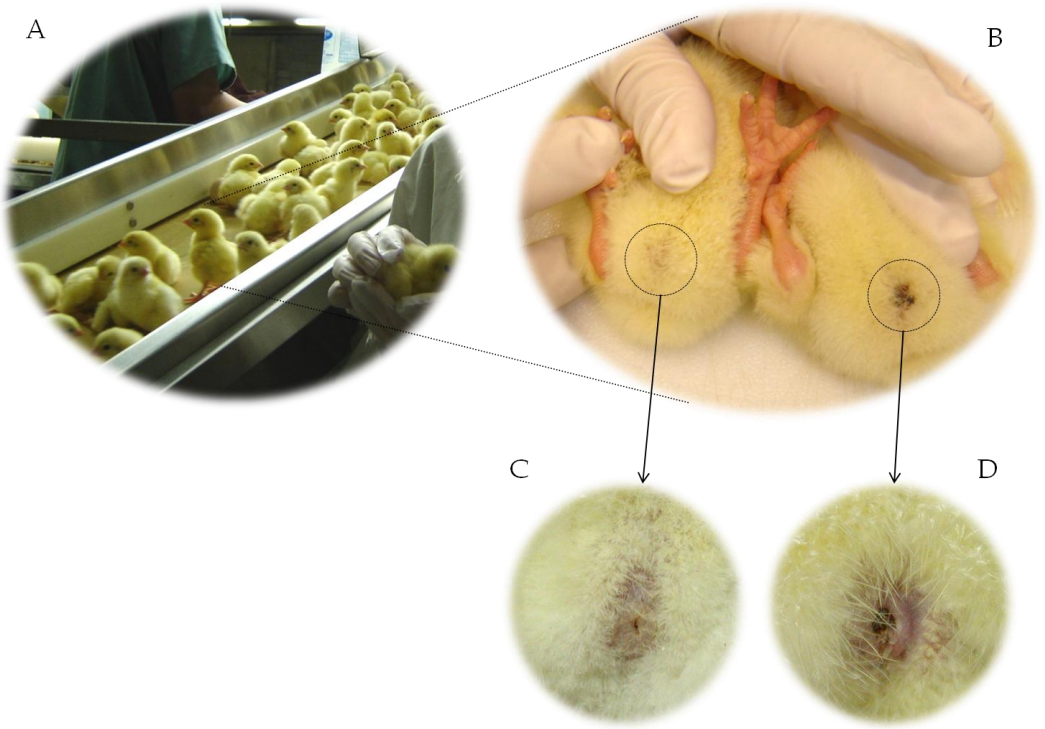


Figure 2-2. A) Newly hatched broiler chicks on a commercial hatchery line. B) Ventral view of the abdomens of two newly hatched chicks; their navel areas have been circled. C) Close-up of a healed navel. D) Close-up of an unhealed navel with a small scab covering the navel opening

All animal procedures were conducted in a NuAire NU-602-500 class II type A2 laminar airflow biological safety cabinet (NuAire Laboratory Equipment Supply, Plymouth, MN, USA). Twelve chicks per navel treatment were individually held with the navel clearly visible and had 100 µL the reconstituted *E. coli* Top 10 peGFP or EC234 peGFP on BHI applied on the navel area with a pipette. Control chicks had 100 µL of sterile BHI applied on their navels. Post inoculation, chicks were held on

their backs for a few seconds after which they were placed inside plastic micro-isolator rat cages (25.9 cm x 47.6 cm x 20.9 cm, Allentown Inc., Allentown, NJ, USA). Each cage was covered with microbarrier tops containing HEPA filters. An individual cage housed 6 chicks belonging to the same navel class and infection treatment; in total, each trial consisted of 12 cages containing 6 chicks (Figure 2-3).

Figure 2-3. Chick housing set up. Each one of the three trials started with 12 micro-isolator chambers housing 6 chicks each. Infra-red heating lamps were used to maintain a constant cage temperature of 30°C.

Each cage bottom was covered with sterile wood shavings. Chicks were fed a Purina® Laboratory Chick Diet S-G 5065 (Purina LabDiet®, PMI Nutrition International, Mulberry, FL, USA) containing 3.02 Kcal/g metabolizable energy, 21.6% crude protein, and 3.0% crude fat. Feed and water were provided *ad libitum.* Four 175W Philiphs 364034 IR-175R-PAR heat lamps (Philiphs, Eindhoven, the Netherlands) were used to maintain an average cage temperature of 30 °C. All cage litter, feed and water were refreshed daily.

2.2.3. Tissue collection and processing

One chick per cage was randomly selected for tissue collection after 8, 24, 48, 72, 96 and 120 hours post-inoculation. Chicks were euthanized by cervical dislocation, dipped in a 1% Virkon® solution (DuPont Animal Health Solutions, Suffolk, United Kingdom) and dissected. A sagittal section of the skin peripheral to the navel plus the yolk sac were collected and fixed in 6% paraformaldehyde for 48 h, and then transferred to phosphate buffered saline solution (PBS; pH 7.4) for a week until processing. Prior to processing, a sagital section of the navel skin and yolk sac of about 0.7 cm x 0.5 cm x 0.2 cm was placed into an imbedding cassette and rinsed in running tap water for 30 minutes. One of the main concerns was whether eGFP may lose its fluorescence during tissue

fixation and subsequent processing. Different studies involving eGFP, mostly in mice, have raised the same type of concern when tracking eGFP in formaldehyde fixed-paraffin embedded tissues. To maintain membrane integrity and prevent the rapid diffusion of GFP out of the cell, tissues must be fixed prior to preservation and sectioning (Brazelton and Blau, 2005; Drakaki et al., 2007). Prior to starting animal experiments, it was determined if *E. coli* peGFP were tolerant to fixatives as well as solutions and methods used during tissue processing. A few drops of a fresh culture of *E. coli* Top 10 peGFP were placed on a glass slide and analyzed by fluorescence microscopy to test the effects of 6% paraformaldehyde, 30%, 50%, and 70% ethanol and toluene solutions as well as the effects of 65 ^{o}C for 4 hours on fluorescence. The only negative effect observed was a slight decrease in the intensity of the green fluorescence. Despite this decrease in intensity, green fluorescent bacteria could be easily observed confirming these procedures could be used for tissue fixation and processing.

Tissue samples were imbedded in paraffin using a Fisher 166 MP Histomatic Tissue Processor (Fisher Scientific, Pittsburgh, PA, USA) for trials 1 and 2 when broiler breeders were 32 and 40 wk of age, respectively. A Leica TP1020 tissue processor (Leica Microsystems Inc, Richmond Hill, ON, Canada) was used for trial 3 when breeders were 55

wk of age. Serial sections of 7 μm were obtained using an AO-820 rotary microtome (American Optical Corporation, Buffalo, NY, USA). The set of sections for fluorescence microscopy was mounted on SuperFrost® Plus glass slides (Fisher Scientific Company, Ottawa, ON, Canada); other set of sections used for histological analysis was mounted normal glass slides. Sections were dewaxed using a series of toluene solutions followed by ethanol solutions. For fluorescence microscopy, unstained sections were covered with #0 coverslips using Fluoromount G (Electron Microscopy Sciences, Hatfield, PA, USA). These slides were kept at 4 °C in the dark until observation. For histological analysis, slides were stained with haematoxylin – eosin and covered with #1 coverslips using neutral DPX mounting media (Leica Microsystems Inc, Richmond Hill, ON, Canada).

Even though the techniques used in this study provided positive results, the paraffin embedding method is more labour-intensive, more time-consuming, and it uses a greater amount of organic solvents. As an alternative, cryopreservation after tissues fixation in paraformaldehyde is recommended.

2.2.4. Slide analysis

All slides were randomly labelled with a number prior to observation; this was done in order to prevent bias due to identification of infection

treatments. Blind evaluation of slides in search for the presence of eGFP-tagged *E. coli* was performed by the same observer using a Zeiss Axio Imager 2 M2 microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada). Absorption and emission wavelengths were 488 and 507 nm respectively. Each slide was observed for approximately 10 min under 20X and 100X immersion objectives using Immersol™ 518 F (Carl Zeiss Canada Ltd., Toronto, ON, Canada). Images were acquired with a Zeiss HRm camera and AxioVision 4.6 software (Carl Zeiss Canada Ltd., Toronto, ON, Canada). Results were recorded as presence or absence of fluorescent bacteria.

2.2.5. Statistical analysis

The experimental unit for this experiment was each microisolator chamber. To analyze the association between navel condition and the variables time post-inoculation, broiler breeder age and inoculation treatment, a categorical model based on maximum likelihood was created in SAS (SAS Institute Inc. 2002-2003). The effect of each variable on the presence of fluorescent *E. coli* was determined based on the transformation of the cell probabilities. In SAS proc catmod a data matrix containing the number of positive samples per navel condition was analyzed. Proc freq was used to produce two-way contingency tables containing the frequency of positive samples for each navel condition.

2.3. Results

Plasmid DNA for eGFP was successfully extracted from *E. coli* Top10 peGFP and electroporated into the transformed APEC strain EC234 but not into EC317. Freshly transformed EC234 peGFP cultures as well as *E. coli* Top10 peGFP and non transformed EC234 were evaluated by fluorescence microscopy at 488 nm absorption and 507 nm emission wavelengths. Bright green fluorescence was observed in both EC234 peGFP and *E. coli* Top10 peGFP; no fluorescence was observed in the negative control or in the electroporated EC317.

During careful dissection of the navel area, one of the most interesting findings was the presence of an anatomical connection between the navel skin and the yolk sac (Figure 2-4). This "yolk sac attachment" appeared as an elastic tube-like structure of 2 to 4 mm long and 0.5 to 1 mm in diameter originating from the yolk sac membrane distal to the yolk sac stalk. It was observed in almost every chick dissected on days 0, 1, and 2 post-hatching regardless of infection treatment or navel condition. The diameter of the "yolk sac attachment" decreased as chick age increased and the yolk sac content was absorbed. At 4 days post hatching this structure was absent in most chicks and it was not observed in any chick dissected on day 5, when only a small vestige of the yolk sac remained.

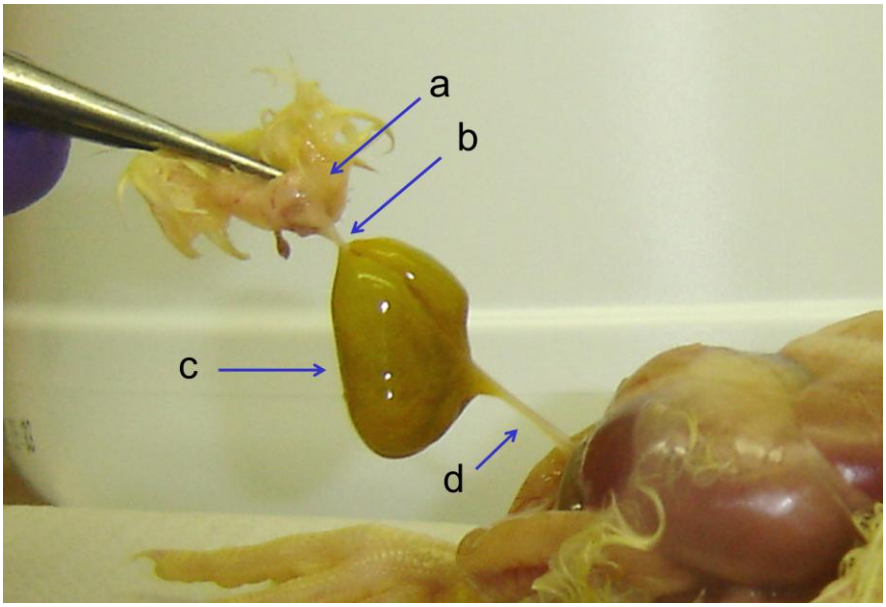


Figure 2-4. Dissection of the navel area and yolk sac of a 1 day old chick. The navel skin (a) is connected to the yolk sac (c) by an attachment (b). The yolk sac is connected to the ileo-jejunal portion of the intestine by the yolk stalk (d).

In the present experiment, two chicks were found dead in their cages. One chick belonged to unhealed navel group inoculated with peGFP *E. coli* Top10 from the 40 wk old flock. The chick's abdomen was filled with clear yellowish liquid, the heart was hypertrophic and organs were cyanotic. It was determined that this chick had died of ascites. The other chick belonged to the unhealed navel group of the control treatment from the 52 wk old flock, no changes were observed at necropsy, this was considered to be a case of sudden death syndrome.

In every skin and yolk sac sample multiple fluorescent signals of different shapes and sizes were observed; this fluorescence was considered to be a consequence of autofluorescent compounds. Strong autofluorescence was observed in the stratum corneum, the outermost layer of the skin. Down feather follicles were especially bright. Abundant fluorescent elements within the connective tissue of skin, "yolk sac attachment" tissue, yolk sac membrane and yolk sac content were observed under 20X and 40X magnifications (Fig 2-5 A, B). The single layer of cylindrical epithelial cells from the yolk sac membrane was easily recognized projecting into the yolk sac content in multiple folds

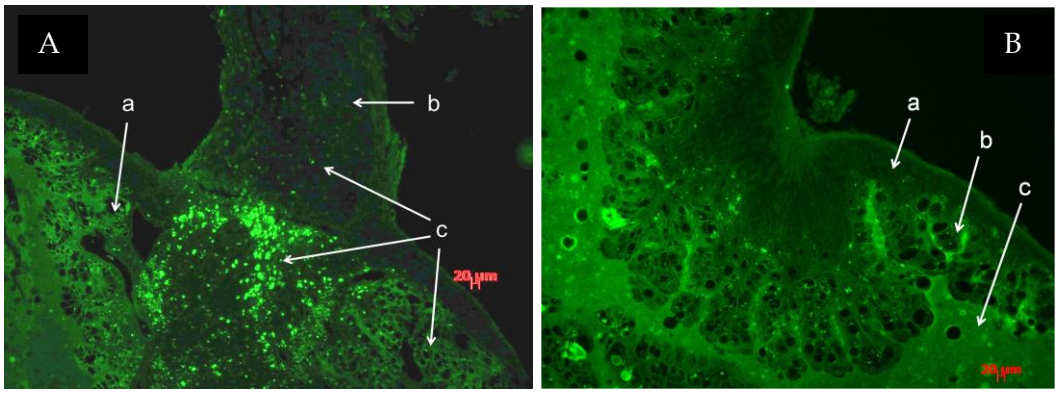


Figure 2-5. Fluorescence microscopy pictures. A) Yolk sac (a) and yolk sac attachment (b) of a 1 day old broiler chick (20X, unhealed navel, *E. coli* Top 10 peGFP). Multiple fluorescent elements are present (c). B) Yolk sac of a 2 day old broiler chick (40X, healed navel, control). (a) Membrane folds, (b) single cylindrical epithelium, (c) highly fluorescent yolk sac content.

When slides were analyzed under 100X magnification, large cells containing intracellular fluorescent bodies were observed. The rod-like fluorescent elements 2 to 3 µm long observed within these large cells were considered to be peGFP *E. coli* (Fig 2-6). Erythrocytes, abundant in chicken skin and yolk sac membrane, also emitted green fluorescence; however, their oval shape, greater size (~ 7 µm in length), and presence of a nucleus made them easily recognizable from surrounding cells (Figure 2-6).

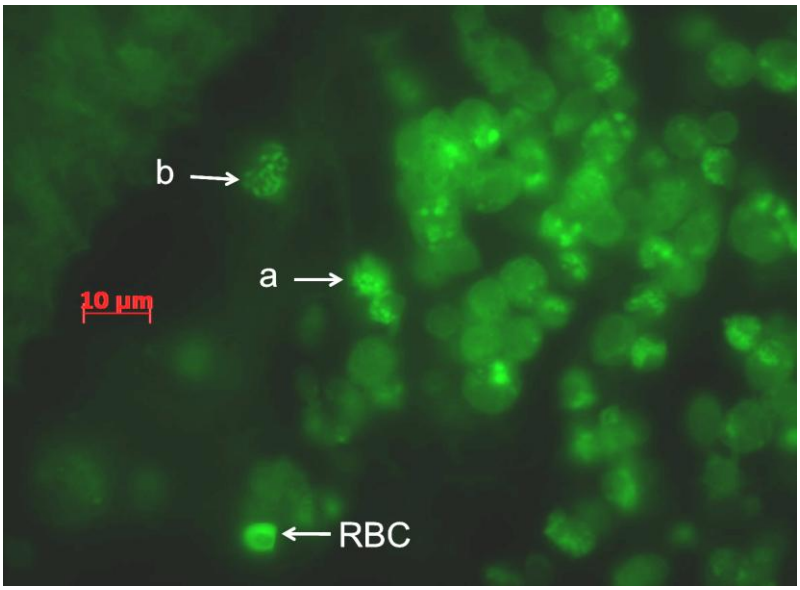


Figure 2-6. Fluorescence microscopy picture of the yolk sac attachment in a 4 day old broiler chick (100X, healed navel, *E. coli* Top 10 peGFP). Large cells contain fluorescence bodies of different shapes and sizes (a). One large cell contains rod-shape fluorescent bodies (b). An avian red blood cell (RBC) can be easily identified.

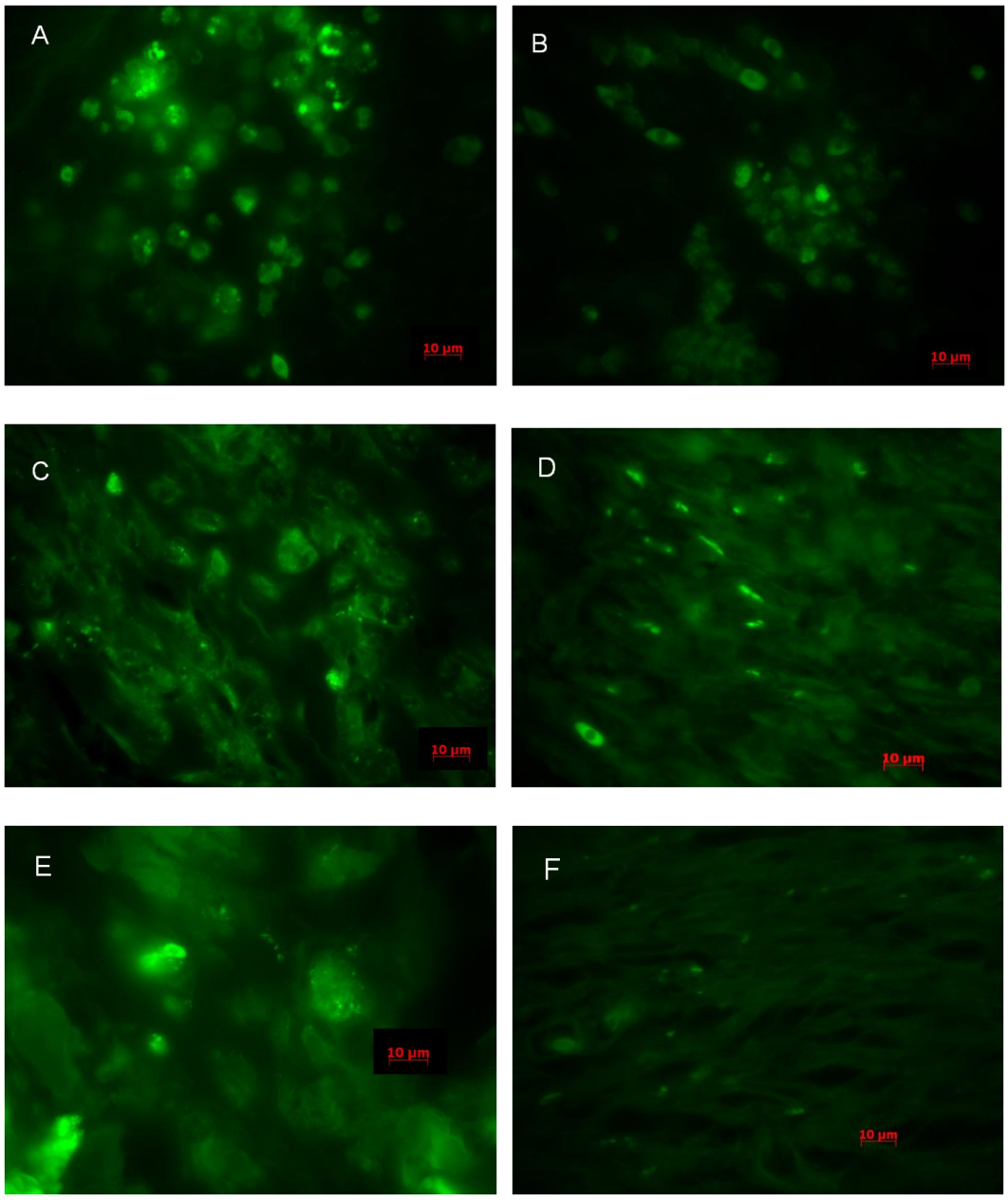


Figure 2-7. Fluorescence microscopy pictures obtained from chicks with healed (A, C, E) and unhealed navels (B, D, F) belonging to controls (A, B), inoculated with peGFP *E. coli* Top10 (C, D), or inoculated with peGFP EC234 (E, F) treatments. Pictures were taken under 100X magnification.

The statistical analysis after blind evaluation of slides determined that the presence of fluorescent *E. coli* in chick navel skin or YS was not significantly affected by broiler breeder age (P = 0.351), time post-inoculation (P = 0.780), or infection treatment (P= 0.238) (Table 2-1).

Table 2-1. Frequency counts of fluorescent E. coli according to time post-inoculation, navel healing at hatching, breeder flock age and inoculation treatment.

Hours post-Inoculation (P=0.780)	Navel (P=0.027)	Breeder Flock age (P=0.351)			Inoculation treatment (P=0.238)		
		32 wk (n=72)	40 wk (n=72)	55 wk (n=72)	Control (n=72)	peGFP *E. coli* Top10 (n=72)	peGFP EC234 (n=72)
8 hours	Healed (n=6)	2 (33.3%) P=1.0000	2 (33.3%) P=0.2577	4 (66.7%) P=0.5603	1 (16.7%) P=0.2388	3 (50.0%) P=0.5603	4 (66.7%) P=0.2577
	Unhealed (n=6)	2 (33.3%) P=1.0000	4 (66.6%) P=0.2577	3 (50.0%) P=0.5603	3 (50.0%) P=0.2388	4 (66.7%) P=0.5603	2 (33.3%) P=0.2577
24 hours	Healed (n=6)	0 (0.0%) P=0.3033	2 (33.3%) P=0.0992	1 (16.7%) P=0.0377	1 (16.7%) P=0.2388	2 (33.3%) P=0.2577	0 (0.0%) P=0.0076
	Unhealed (n=6)	2 (33.3%) P=0.3033	5 (83.3%) P=0.0992	5 (83.3%) P=0.0377	3 (50.0%) P=0.2388	4 (66.6%) P=0.2577	5 (83.3%) P=0.0076
48 hours	Healed (n=6)	3 (50.0%) P=0.2388	0 (0.0%) P=0.3033	3 (50.0%) P=0.5603	1 (16.7%) P=0.5117	2 (33.3%) P=0.2577	3 (50.0%) P=0.2388
	Unhealed (n=6)	1 (16.7%) P=0.2388	2 (33.3%) P=0.3033	4 (66.7%) P=0.5603	2 (33.3%) P=0.5117	4 (66.6%) P=0.2577	1 (16.7%) P=0.2388
72 hours	Healed (n=6)	2 (33.3%) P=0.2577	0 (0.0%) P=0.2135	1 (16.7%) P=1.0000	0 (0.0%) P=0.4611	1 (16.6%) P=0.2388	2 (33.3%) P=0.2577
	Unhealed (n=6)	4 (66.7%) P=0.2577	3 (50.0%) P=0.2135	1 (16.7%) P=1.0000	1 (16.7%) P=0.4611	3 (50.0%) P=0.2388	4 (66.7%) P=0.2577
96 hours	Healed (n=6)	1 (16.7%) P=0.5117	2 (33.3%) P=0.1743	3 (50.0%) P=0.2388	1 (16.7%) P=0.5117	3 (50.0%) P=1.0000	2 (33.3%) P=0.2577
	Unhealed (n=6)	2 (33.3%) P=0.5117	6 (100%) P=0.1743	1 (16.7%) P=0.2388	2 (33.3%) P=0.5117	3 (50.0%) P=1.0000	4 (66.7%) P=0.2577
120 hours	Healed (n=6)	2 (33.3%) P=0.5117	4 (66.6%) P=0.2577	4 (66.7%) P=1.0000	3 (50.0%) P=0.5603	2 (33.3%) P=0.5603	5 (83.3%) P=0.0992
	Unhealed (n=6)	1 (16.7%) P=0.5117	2 (33.3%) P=0.2577	4 (66.7%) P=1.0000	3 (50.0%) P=0.5603	3 (50.0%) P=0.5603	2 (33.3%) P=0.0992

It was very interesting to confirm that navel health at hatching (healed vs. unhealed navel) had a significant effect on the number of positive cases of *E. coli* peGFP (P = 0.027). Fluorescent *E. coli* were observed in the connective tissue of the skin and yolk sac in a greater percentage of chicks with unhealed navels (51.5%) than in chicks with healed navels (36.0%).

It was of interest to study the histological characteristics of the YS attachment. There was no information available in the published literature on the macroscopic or microscopic description of this structure. The following pictures (Figure 2-8), show the daily evolution of the yolk sac attachment at (A) 8 h post-hatching, (B) 24 h, (C) 48 h, (D) 72 h, and (D) at 96 h post-hatching. The sagittal sections show the connective tissue of the dermis in the chick's navel area attaching with the yolk sac membrane. From these sagittal sections it can be observed that the YS attachment tissue is similar to the connective tissue of the dermis. The YS attachment decreased in diameter as the chicks grew older, disappearing by 5 d post-hatching.

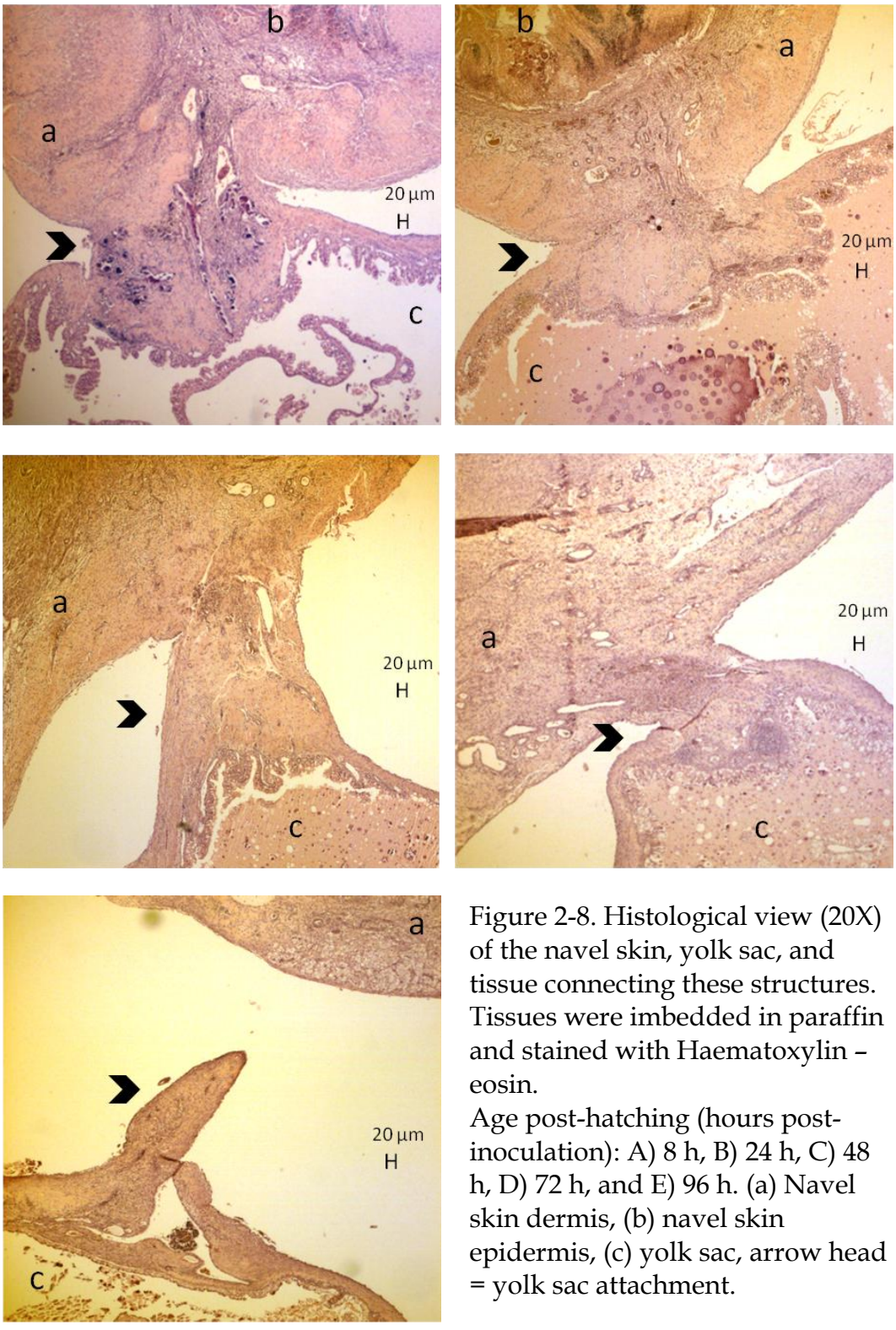


Figure 2-8. Histological view (20X) of the navel skin, yolk sac, and tissue connecting these structures. Tissues were imbedded in paraffin and stained with Haematoxylin – eosin.
Age post-hatching (hours post-inoculation): A) 8 h, B) 24 h, C) 48 h, D) 72 h, and E) 96 h. (a) Navel skin dermis, (b) navel skin epidermis, (c) yolk sac, arrow head = yolk sac attachment.

2.4. Discussion

In this study *E. coli* with a plasmid for eGFP was used to prove that bacteria could reach the yolk sac via the chick navel. One of the objectives was to determine if navel healing at hatching affected the incidence of yolk sac colonization by *E. coli* peGFP.

With regards to animal housing the rat cages were a good option for this experiment. They allowed isolation of chick groups according to inoculation treatments, they were easy to transport between the shelf and the biosafety cabinet for either daily cleaning or daily dissection, and they allowed for easy monitoring of chicks for any sign of distress. Even though 6 chicks were placed per cage on the day of hatching, this chick density of 0.02 m2 per bird is not recommended for longer trials. Eight hours post-infection and then daily, one chick was removed from each cage for tissue collection thus reducing chick density.

One of the first observations during fluorescence microscopy analysis was the presence of some background fluorescence and autofluorescence. Background fluorescence is of concern because it could mask the presence of fluorescent markers increasing the incidence of false negative results (Billinton and Knight, 2001). The preventative measures taken against the emission of excessive background fluorescence were: 1) Tissues were

transferred from 6% PF to PBS (pH 7.4) to decrease background fluorescence due to fixatives (Andersson et al., 1998). 2) Prior to processing, tissues were rinsed with running tap water for 30 minutes to remove all traces of chemicals that could interfere with eGFP (Billinton and Knight, 2001). 3) Slides were not stained to prevent background fluorescence produced by chemical dyes (Bhatnagar, R. Personal communication). By following these steps we were able to reduce the background fluorescence that could interfere with peGFP *E. coli* detection.

Intrinsic autofluorescence from tissue components and autofluorescence induced by fixation media and tissue processing techniques have been reported in formaldehyde-fixed and paraffin-embedded tissues (Baschong et al., 2001). Even though GFP is known to tolerate fixatives such as formaldehyde (Chalfie et al., 1994), an increase in autofluorescence from flavins in the presence of formaldehyde has been reported (Andersson et al., 1998). Fluorescence microscopy studies on mouse skin (Kollias et al., 1998) and human skin (Masters et al., 1997; König and Riemann, 2003) are available; however, information on fluorescence of chicken skin and yolk sac is scarce. In 2007, Drakaki et al. published a study on laser-induced autofluorescence of mouse, pig, and chicken vs. human skin. Even though a description of fluorescent elements

within chicken skin was not provided, chicken skin was reported to have the greatest variety of fluorescence spectral shapes in the 400–550 nm region regardless of sample location (Drakaki et al., 2007). We could compare the fluorescent elements previously described in human skin vs. those observed in this study, keeping in mind that some differences exist between mammalian and avian skin. In avian species the epidermis is divided into 4 stratums: corneum, transitivum, intermedium, and basale (Samuelson, 2007). Keratinocytes within avian epidermis are known as sebokeratocytes; these cells produce, in addition to keratin proteins, a lipid emulsion that fills the intercellular space (Samuelson, 2007). The presence of neutral lipids produced by sebokeratocytes within the avian epidermis could explain the abundance of droplet-like fluorescent images observed in this study (Figure 2-5). In human connective single fluorescent long fibres of elastin and collagen have also been reported (Masters et al., 1997). Chicken skin is mainly formed by type I (75%) and type III (15%) collagen (Cliche et al., 2003) which are known to be autofluorescent molecules (Drakaki et al., 2007). In the present study, abundant fluorescent long fibres were observed in the connective tissue of the navel skin and yolk sac attachment (Figure 2-7).

In addition, mitochondria and lysosomes in different mammalian cell types also exhibit autofluorescence (Andersson et al., 1998). In human skin, punctuated fluorescence within the cytoplasm of large cells has been reported to be mitochondria with a high concentration of NAD(P)H (Masters et al., 1997). Whether the small intracellular fluorescent bodies observed in this study were mitochondria or lysosomes contained within metabolically active cells, is unknown. It was hypothesised that the 2 to 3 μm long rod-like fluorescent elements observed within large cells (Figure 2-6) could be *E. coli* peGFP which had been phagocytized by macrophages or dendritic cells. However, this possibility was not tested in the present study.

The bright fluorescence observed in the nucleated avian erythrocytes in this experiment (Figures 2-6 and 2-7) is known to be due to haemoglobin, another endogenous autofluorescent pigment (Billinton and Knight, 2001).

By using fluorescent *E. coli* the hypothesis that bacteria can access the yolk sac via the chick the navel has been proven. The importance of navel health at hatching as a predisposing factor to bacterial contamination of the yolk sac has also been demonstrated. It was expected that more positive samples of fluorescent bacteria would be observed in chicks

produced at 55 wk of age because the incidence of navel problems and yolk sac infections has been reported to increase with parent flock age (Yassin et al., 2009). However, in this experiment, equal number of healthy chicks with healed vs. unhealed navels were collected thus eliminating the detrimental effect of breeder age on chick quality due to navel healing.

It was also expected to observe a greater number of positive samples in chicks infected with peGFP EC234, an APEC, than in those infected with peGFP *E. coli* Top10, a harmless laboratory strain. Previous research has demonstrated that transformation of bacteria with GFP, a high-copy plasmid, resulted in decreased doubling time in the harmless *E. coli* K-12 (Oscar et al., 2006) and in important gastrointestinal pathogens such as enterohaemorrhagic *E. coli, Salmonella typhi* and *Shigella flexnerii* (Rang et al., 2003). Whether peGFP had a negative effect on the overall fitness of *E. coli* Top 10 or EC234 was not analyzed in this study, but this hypothesis could explain the lack of difference in the number of positive samples observed in chicks infected by these 2 transformed *E. coli* strains. The difference in infection vigour between strains could have been masked by the large number of bacteria placed on the navel area. The presence of ~10% false positive samples obtained from the uninoculated control chicks

is likely due to the abundant autofluorescent elements previously described

The histological evaluation of the yolk sac attachment revealed that this structure was similar to that of the connective tissue from the dermis. Because only sagittal sections of the anatomical pieces were evaluated, it was not possible to determine if this structure was in fact a hollow tube that could act as an open connection between the navel opening and the yolk sac. Transverse sections of the structure were not prepared because all anatomical pieces were fixed and processed at the same time for both, fluorescence and light microscopy. Sagittal sections were necessary to allow for fluorescence microscopy evaluation of the entire anatomical piece from the navel skin to the yolk sac.

2.5. Conclusion

In summary, *E. coli* transformed to express eGFP were used to confirm that bacterial infection occurs from the surface of the chick navel into the chick yolk sac. Some of the concerns regarding tissue preparation for fluorescence microscopy and presence of autofluorescent elements were summarized in this paper. This study provided a fluorescence microscopy description of the chicken skin and yolk sac not available in the literature. Finally, the importance of navel health at hatching was confirmed by

demonstrating that a greater percentage of chicks with unhealed navels than those with healed navels had been colonized by fluorescent *E. coli*.

2.6. References

Allan, B.J., van den Hurk, J.V., Potter, A.A., 1993. Characterization of *Escherichia coli* isolated from cases of avian colibacillosis. Can. J. Vet. Res. 57, 146-151.

Andersen, J.B., Sternberg, C., Kongsbak Poulsen, L., Petersen Bjørn, S., Givskov, M., Molin, S., 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl. Environ. Microbiol 64, 2240-2246.

Andersson, h., Baechi, T., Hoechl, M., Richter, C., 1998. Autofluorescence of living cells. J. Microsc. 191, 1-7.

Barnes, J.H., Vaillancourt, J.P., Gross, W.B., 2003. Colibacillosis. In: Saif, Y.M., Barnes, H.J., Fadly, A.M. (Eds.), Diseases of poultry. Blackwell Publishing, Ames, Iowa, USA, pp. 631-656.

Baschong, W., Suetterlin, R., Laeng, R.H., 2001. Control of autofluorescence of archival formaldehyde-fixed, paraffin-embedded tissue in confocal laser scanning. J. Hystochem. Cytochem. 49, 1565-1571.

Billinton, N., Knight, A.W., 2001. Seeing the wood through the trees: a review of techniques for distinguishing green fluorescent protein from endogenous autofluorescence. Anal. Biochem. 291, 175-197.

Brazelton, T., Blau, H.M., 2005. Optimizing techniques for tracking transplanted stem cells in vivo. Stem Cells 23, 1251-1265.

Canadian Council on Animal Care, 2009. Guidelines on the care and use of farm animals in research, teaching and testing. Available at: http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/PDFs/Farm_Animals.pdf (Accessed, 21 May 2010).

Chalfie, M., Tu, G., Euskirchen, G., Ward, W.W., Prasher, D.C., 1994. Green fluorescent protein as a marker for gene expression. Science 263, 802-805.

Cliche, S., Amiot, J., Averzard, C., Gariépy, C., 2003. Extraction and characterization of collagen with or without telopeptides from chicken skin. Poult. Sci. 82, 503-509.

Cormack, B.P., Valdivia, R.H., Falkow, S., 1996. FACS-Optimized mutants of the Green Fluorescent Protein . Gene 173, 33-38.

Cubitt, a., Heim, R., Adams, S.R., Boyd, A.E., Gorss, L.A., Tsien, R.Y., 1995. Understanding, improving and using Green Fluorescent Proteins. Trends Biochem. Sci. 20, 448-455.

Drakaki, E., Borisova, E., Makropoulou, M., Avramov, L., Serafetinides, A.A., Angelov, I., 2007. Laser induced autofluorescence studies of animal skin used in modeling of human cutaneous tissue spectroscopic measurements. Skin Res. Technol. 13, 350-359.

Fasenko, G.M., O'Dea, E.E., 2008. Evaluating broiler growth and mortality in chicks with minor navel conditions at hatching. Poult. Sci. 87, 594-597.

Gross, W.G., 1994. Diseases due to *Escherichia coli* in poultry. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Willingford, UK, pp. 237-259.

Kawalilak, L.T., Ulmer-Franco, A.M., Fasenko, G.M., 2010. Impaired intestinal villi growth in broiler chicks with unhealed navels. Poult. Sci. 89, 82-87.

Kollias, N., Gillies, R., Moran, M., Kochevar, I.E., Anderson, R.R., 1998. Endogenous skin fluorescence includes bands that may serve as

quantitative markers of aging and photoaging. J. Invest. Dermatol. 111, 776-780.

König, K., Riemann, I., 2003. High resolution multiphoton tomography of human skin with subcellular spatial resolution and picosecond time resolution. J. Biomed. Optics 8, 432-439.

Kwaga, J.K.P., Allan, B.J., van den Hurk, J.V., Seida, H., Potter, A.A., 1994. A *car*AB mutant of avian pathogenic *Escherichia coli* serogroup O2 is attenuated and effective as a live oral vaccine against colibacillosis in turkeys. Infetc. Immun. 62, 3766-3772.

La Ragione, R.M., Woodward, M.J., 2002. Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia. Res. Vet. Sci 73, 27-35.

Masters, B.R., So, P.T., Gratton, E., 1997. Multiphoton excitation fluorescence microscopy and spectroscopy of in vivo human skin. Biophys. J. 72, 2405-2412.

Ngeleka, M., Kwaga, J.K.P., White, D.G., Whittam, T.S., Riddell, C., Goodhope, R., Potter, A.A., Allan, B., 1996. *Escherichia coli* cellulitis in broiler chickens: clonal relationships among strains and analysis of

virulence-associated factors of isolates from diseased birds. Infect. Immun. 64, 3118-3126.

Oscar, T.P., Dulal, K., Boucaud, D., 2006. Transformation of *Escherichia coli* K-12 with a high-copy plasmid encoding the green fluorescent protein reduces growth: implications for predictive microbiology. J. Food Prot. 69, 276-281.

Pierce, D., 2009. The effect of meat and fat surface properties, steam and acid treatments on *Escherichia coli*. M.Sc. Thesis, Department or Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada. T6G2P5.

Pourbakhsh, S.A., Boulianne, M., Martineau-Doizé, B., Dozois, C.M., Desautels, C., Fairbrother, J.M., 1997. Dynamics of *Escherichia coli* infection in experimentally inoculated chickens. Avian Dis. 41, 221-233.

Rai, M.F., Khan, S.A., Aslam, A., Saeed, K., 2005. Effects of yolk sac infection in chicken. Avian Poult. Biol. Rev. 16, 87-93.

Ramirez, R.M., Almanza, Y., Garcia, S., Heredia, N., 2009. Adherence and invasion of avian pathogenic *Escherichia coli* to avian tracheal epithelial cells. World J. Microbiol. Biotechnol 25, 1019-1023.

Rang, C., Galen, J.E., Kaper, J.B., Chao, L., 2003. Fitness cost of the green fluorescent protein in gastrointestinal bacteria. Can. J. Microbiol. 49, 531-537.

Rosario Cortés, C., Téllez, I.G., López Cuello, C., Villaseca Flores, J.M., Anderson, R.C., Eslava Campos, C., 2004. Bacterial isolation rate from fertile eggs, hatching eggs and neonatal broilers with yolk sac infection. Rev. Latinoam. Microbiol. 46, 12-16.

Sambrook, R., Russell, D.W., 2001. Plasmids and their usefulness in molecular cloning. In: Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y., pp. 119-122.

Samuelson, D.A., 2007. Integument. In: Textbook of veterinary histology. Saunders - Elsevier, St. Louis, Missouri, pp. 299-302.

SAS Institute Inc., 2002-2003. SAS User's Guide Version 9.1.

van den Hurk, Jan V., Allan, B.J., Riddell, C., Watts, T., Potter, A.A., 1994. Effect of infection with hemorrhagic enteritis virus on susceptibility of turkeys to *Escherichia coli*. Avian Dis. 38, 708-716.

Yassin, H., Velthuis, A.G.J., Boerjan , van Rielt, J., 2009. Field study on broilers' first-week mortality. Poult. Sci. 88, 798-804.

Yu, Q., Dong, S., Zhu, W., Yang, Q., 2007. Use of Green Fluorescent Protein to monitor *Lactobacillus* in the gastro-intestinal tract of chicken. FEMS Microbiol. Lett. 275, 207-213.

3. Hatching Egg Yolk and Newly Hatched Chick Yolk Sac Total IgY Content at Three Broiler Breeder Flock Ages[2]

3.1. Introduction

The newly hatched broiler chick relies on two basic mechanisms of defence against disease: its innate immune system, especially functional at the enteric mucosa level (Bar-Shira and Friedman, 2006) and the antigen specific protection received from maternal antibodies (Brambell, 1970; Yasuda et al., 1998). Studies on the development of the chick's innate immune system and its protective role during early infectious challenges have been made available recently (Derache et al., 2009). In contrast, the importance of vertical transmission of immunity to provide specific pathogen protection during the early post-hatching period has long been recognized (Rose and Orlans, 1981). Immunoglobulin-secreting B cells of chick origin have been detected in circulation after 6 days post-hatching (Lawrence et al., 1981) meaning that during the first days of the post-hatching period humoral immunity is totally dependent on maternal transfer of immunoglobulins. In contrast to mammals who after birth may obtain maternal antibodies in the colostrums, all the maternal

[2] A version of this chapter has been submitted for publication. Ulmer-Franco et al., 2011. Poultry Science # PS-11-01757.

immunoglobulins needed to protect the newly hatched chick must be incorporated into the egg before it is laid (Rose et al., 1974).

In the domestic chicken three classes of immunoglobulins (Ig) have been identified as the homologues of mammalian IgM, IgA, and IgG (Leslie and Clem, 1969). Avian IgY is the evolutionary ancestor of mammalian IgG and IgE and it combines their separate functions: IgY is the main defence mechanism against systemic infections similar to IgG. IgY also acts as a skin-sensitizing antibody that can mediate anaphylactic reactions similar to IgE (Warr et al., 1995).

The transfer of IgY from the hen to the chicks is a two-step process: First, circulating IgY must be transferred from the hen's bloodstream into the ovarian follicle (i.e. the egg yolk); then, IgY must be transferred from the egg yolk to the embryo (Patterson et al., 1962; Orlans, 1967). The transfer of IgY into the egg yolk is a demanding process for the hen and is related to IgY serum concentration (Morrison et al., 2001). The yolk sac, formed by a highly vascularised placenta-like membrane that surrounds the yolk during embryonic development, is known to transfer egg yolk nutrients to the chicken embryo (Romanoff, 1960). By the time of hatching a broiler chick carries in its abdominal cavity a residual yolk sac of approximately 12% of its BW (Kawalilak et al., 2010). The absorption of

the yolk sac content during the first week after hatching is essential for chick growth (Murakami et al., 1992) and intestinal development (Noy and Sklan, 1999).

Over the last 20 years the chicken egg has been extensively studied as an important source of commercial antibodies. Antibody harvesting from eggs is easier, more convenient less labour-intensive and more animal-welfare friendly as there is no need for constant blood collection, than antibody recovery from mammalian species (Shimizu et al., 1992). As a result, multiple research projects using hyper-immunization of hens to produce avian (Gómez-Verduzco et al., 2010) and inter-species (Machado Leal et al., 2005) specific egg Ig has been carried out. The main role of egg IgY is to protect the offspring against disease; differences in antibody transfer in modern chicken strains have been described by Hamal et al. (2006) and Carlander et al. (2003). However, the physiological consequences of aging on total egg yolk and yolk sac IgY content on broiler birds have not been reported.

The main objective of this research was to evaluate the effect of broiler breeder flock age (32, 40 and 55 wk) on the total IgY content of egg yolk and newly hatched chick yolk sacs. It was hypothesized that the total IgY content per gram of egg yolk and yolk sac would decrease with hen age.

3.2. Materials and methods

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies (Canadian Council on Animal Care, 2009) with approval from the Animal Care and Use Committee: LIVESTOCK for the University of Alberta.

3.2.1. Egg collection for analysis and incubation

Hatching eggs produced by a commercial Cobb 500 broiler breeder flock reared and managed under standard production practices were used for this experiment. Eggs from the same breeder flock at 32, 40 and 55 wk of age (n = 84 eggs per age) were collected from a commercial hatchery where they had been stored for 3 to 4 d at 18°C and 60 to 70% relative humidity. At each collection time the average egg weight (wt) for that flock age was determined by randomly weighing 72 eggs (the equivalent of two hatching egg trays). Eggs used for the experiment were within ± 0.5 g from the average egg wt. Thirty eggs were randomly selected for yolk total IgY determination and the remaining 54 eggs were set for incubation in a Jamesway AVN single stage incubator (Jamesway Incubator Company Inc., Cambridge, ON, Canada). Eggs flats were placed on the same location in the incubator for the three flock ages. Plastic eggs were used to fill the incubator to its 1,152 egg capacity. Eggs were incubated for

18 days at a dry bulb and wet bulb temperature of 37.5°C and 29.4°C, respectively, and then transferred (along with the plastic eggs) to a Jamesway AVN hatcher (Jamesway Incubator Company, Cambridge, ON, Canada) where they were further incubated for an additional 3.5 days at a dry bulb temperature of 35.2°C and a wet bulb temperature of 29.4°C.

3.2.2. Egg yolk and yolk sac sampling

For each flock age, 30 eggs were individually weighed and broken open. Yolks were separated from the albumen, carefully rolled on scientific cleaning wipes to remove excess albumen and wet yolk wt were recorded. Equal volumes (8 mL) of five egg yolks per flock age were pooled in 50 mL plastic capped tubes, mixed thoroughly and stored at -20°C. After 21.5 days of incubation all hatched chicks were individually weighed, humanely euthanized via cervical dislocation and dissected. The yolk sacs of 24 chicks from the 32 wk old flock and of 30 chicks from the 40 and 55 wk old flock were collected, individually weighed, pooled in groups of four or five yolk sacs, mixed thoroughly and stored at -20°C.

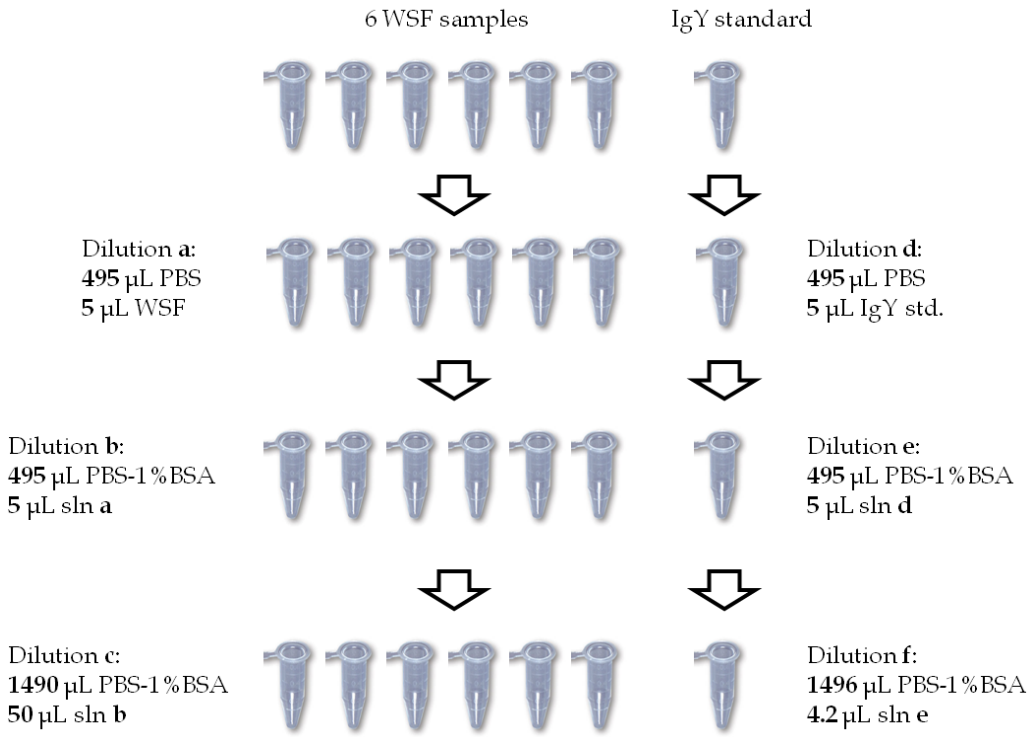
3.2.3. IgY isolation and quantification

Chicken IgY is one of the soluble proteins contained in the water soluble fraction (WSF) of the egg yolk (Williams, 1962). To extract the WSF, 150 to 200 mg of pooled egg yolk or yolk sac were diluted 1:6

vol/vol with acidified deionized water pH 2.5, vortexed well and stored at 4°C. After overnight refrigeration samples were centrifuged at 10,062 x *g* at 4°C for 15 min, supernatants (WSF) were collected and used immediately (Wang et al., 2004).

Egg yolk and yolk sac total IgY contents were quantified by ELISA as described by Selvaraj and Cherian (2004). Briefly, 96-well microtiter plates were coated with 100 ng/well of rabbit anti-chicken IgG (Rockland Inc., Gilbertsville, PA, USA) by adding 150 µL/well (1:5000 vol/vol) anti-IgG in coating buffer (50 mM carbonate/bicarbonate, pH 9.6) and incubating for 2 h at 37°C. After washing twice with 200 µL/well 0.1% Tween 20 (Fisher Scientific, Pittsburgh, PA, USA) in PBS pH 7.4 (PBST), wells were blocked overnight at 4°C with 200 µL/well 1% BSA (Sigma Chemical Co., St. Louis, MO, USA) in PBS. Wells were washed twice with PBST prior to adding samples and standards. Aliquots of 150 µL of chicken IgG standard (Rockland Inc., Gilbertsville, PA, USA) diluted to 0.004 µg/mL in 1% BSA in PBS were prepared (four standards per plate). Aliquots of 150 µL of the WSF diluted in 1% BSA in PBS (1:330,000 vol/vol) were prepared (Figure 3-1). Aliquots of 150 µL 1% BSA in PBS were used as blanks. When the microtiter plate was loaded (4 standards, 6 WSF in triplicates, and 2 blanks), solutions were serially diluted (1:2 vol/vol) up

to three dilutions and plates were incubated for 2 h at room temperature in a shaker.



	1	2	3	4	5	6	7	8	9	10	11	12
A	St1	St2	S1	S1	S1	S2	S2	S2	S3	S3	S3	Blk
B	St1	St2	S1	S1	S1	S2	S2	S2	S3	S3	S3	Blk
C	St1	St2	S1	S1	S1	S2	S2	S2	S3	S3	S3	Blk
D	St1	St2	S1	S1	S1	S2	S2	S2	S3	S3	S3	Blk
E	St 3	St 4	S4	S4	S4	S5	S5	S5	S6	S6	S6	Blk
F	St 3	St 4	S4	S4	S4	S5	S5	S5	S6	S6	S6	Blk
G	St 3	St 4	S4	S4	S4	S5	S5	S5	S6	S6	S6	Blk
H	St 3	St 4	S4	S4	S4	S5	S5	S5	S6	S6	S6	Blk

Figure 3-1. Top: Dilutions performed to obtain final samples and standard for IgY determination. Aliquots of 150 µL per well for dilutions c (samples) and f (standard) were loaded in rows A and E of the microtiter plate. Bottom: Finished plate with 4 standards (St1 to St4), 6 samples (S1 to S6) in triplicates and 2 blanks (Blk). All solutions were serially diluted (2:1 vol/vol) 3 times.

Plates were washed three times with PBST and 150 µL of rabbit anti - chicken IgG peroxidase conjugate (Rockland Inc., Gilbertsville, PA, USA) diluted to 214 µg/mL in 1% BSA in PBS (1:7000 vol/vol) were incubated for 2 h at room temperature. Plates were washed four times with PBST prior to incubating with 150 µL of peroxidase substrate solution, 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) [ABTS] (Sigma Chemical Co., St. Louis, MO, USA) in 0.1 M citrate-phosphate buffer containing 0.01% hydrogen peroxide. After overnight incubation at 4 °C absorbance was measured at 405 nm using an ELISA microplate reader (BioTek EL 800, BioTek Instruments Inc., Winooski, VT, USA) and KC junior software (BioTek Instruments Inc., Winooski, VT, USA). Absorbance was corrected in reference to the blanks, and the standards were used to calculate total IgY concentration of each sample. Egg yolk and yolk sac weights were used to calculate total IgY (mg) per gram of egg yolk or gram of yolk sac and total IgY (mg) per egg yolk or yolk sac.

3.2.4. Statistical analysis

The effects of broiler breeder age on egg and yolk sac weights and on total IgY content of egg yolk and yolk sacs were analysed by 1-way ANOVA using the mixed model of SAS software (SAS Institute Inc., 2002-2003). The experimental unit for egg and yolk sac weights was each

individual egg or yolk sac. For total IgY contents the experimental unit was each pooled sample of 5 eggs or 4 or 5 yolk sacs. Pooled sample number was set as the random factor. The probability level was set at $P \leq 0.05$. Where the model indicated significance, the LSmeans were separated using the pdiff procedure of SAS.

3.3. Results

3.3.1. Egg, yolk, chick, and yolk sac weights

Egg collection was based on average egg weight for each flock age. Egg weight and wet egg yolk weight significantly increased with flock age (Table 3-1). Eggs and yolks from eggs laid at 32 wk were lighter than those laid at 40 wk which in turn were lighter than eggs and yolks from eggs laid when the breeder flock reached 55 wk of age. Flock age did not have a significant effect on wet egg yolk weight as a percentage of egg weight.

Table 3-1. Average egg and yolk weights of eggs produced by the same broiler breeder flock at 32, 40 and 55 weeks of age.

Broiler breeder age (wk)	n[1]	Egg wt (g)	Wet yolk wt (g)	Wet yolk wt (%)
32 wk	30	58.0 ± 0.19[c]	16.8 ± 0.38[c]	28.9 ± 0.65
40 wk	30	63.1 ± 0.47[b]	19.5 ± 0.92[b]	30.8 ± 1.60
55 wk	30	68.4 ± 0.57[a]	22.8 ± 1.49[a]	33.3 ± 2.58
P value		< 0.0001	0.0182	0.3530

[1]Number of experimental units. Each experimental unit = one egg.
[a-c]Means within a column lacking a common superscript differ significantly ($P \leq 0.05$).
Mean values are given ± the standard error of the means (SEM)

There were no significant differences between chick wt at different flock ages (Table 3-2). However, YS weight and YS weight as a percentage of chick wt were affected by flock age: YS of chicks hatching from 55 wk old breeders were the heaviest, followed by those of chicks from 32 wk old breeders; YS of chicks from the 40 wk old breeders were the lightest.

Table 3-2. Average body weight (BW) and yolk sac (YS) weight of chicks hatching from the same broiler breeder flock at 32, 40 and 55 weeks of age.

Broiler breeder age (wk)	n[1]	Chick BW (g)	Wet YS wt (g)	Wet YS wt (%)
32 wk	24	43.81 ± 0.47	6.48 ± 0.20[b]	14.76 ± 0.42[b]
40 wk	30	45.11 ± 1.14	5.61 ± 0.18[c]	12.37 ± 0.37[c]
55 wk	30	50.41 ± 1.85	8.50 ± 0.18[a]	16.74 ± 0.37[a]
P value		0.1033	<0.0001	<0.0001

[1]Number of experimental units. Each experimental unit = one chick.
[a-c]Means within a column lacking a common superscript differ significantly ($P \leq 0.05$).
Mean values are given ± the standard error of the means (SEM)

In this experiment eggs from the same breeder flock were collected at three flock ages and incubated under the same conditions for a total of 516 hours. When the hatch from the 32 wk old flock was pulled only 46% chicks had hatched, an additional 26% had externally pipped (eggshell had been perforated and at least the beak was visible), and 13% were still alive but had not started to hatch. For this reason, only 26 yolk sacs were collected and not 30 yolk sacs as it has been planned.

3.3.2. Total egg yolk IgY content

Total egg yolk IgY content expressed in mg of IgY per gram of yolk increased significantly with flock age ($P < 0.0001$). Eggs from the 32 wk old flock had less IgY than eggs from the 40 wk old flock, which in turn had less IgY than eggs from the 55 wk old flock (8.1 vs. 9.3 vs. 11.3 mg/g, respectively; SEM ± 0.37) (Figure 3-2 A). The calculated total IgY contained by the entire egg yolk also increased with flock age ($P < 0.0001$, SEM ± 7.7) accordingly with the increase of egg yolk weight (Figure 3-2 B).

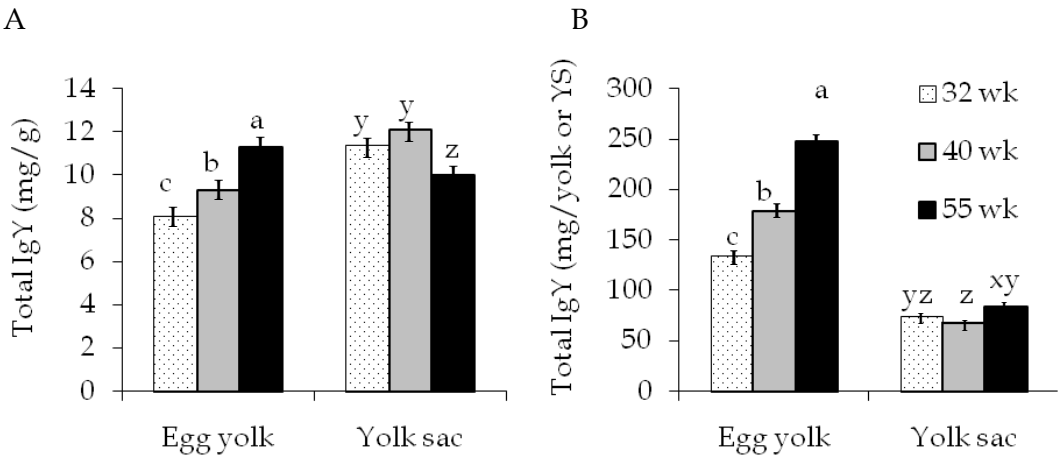


Figure 3-2. Average IgY content of egg yolks and yolk sacs of eggs and chicks collected from the same broiler breeder flock at 32, 40, and 55 wk of age. A) Average IgY content per gram of egg yolk and gram of yolk sac; B) Total IgY contained per egg yolk and yolk sac. Significant differences ($P \leq 0.05$) are indicated by different letters within sample type, error bars represent the standard error of the means (SEM)

3.3.3. Total yolk sac IgY content

Broiler breeder age significantly affected the total IgY content per gram of yolk sac (Figure 3-2 A). Yolk sacs of chicks obtained when breeders were 55 wk of age had significantly less IgY per gram of yolk sac ($P < 0.0080$) than yolk sacs of chicks obtained at 32 and 40 wk of age, which did not differ from each other (10.0 ± 0.42 (55 wk) vs. 11.5 ± 0.42 (32 wk) vs. 12.1 ± 0.38 (40 wk) mg/g, respectively). Total yolk sac IgY content, calculated according to yolk sac weight, was also affected by breeder flock age (Figure 3-2 B). The yolk sac of chicks from 32 wk old parents contained 74.3 ± 3.52 mg total IgY, this amount did not differ from that of yolk sacs from chicks of 40 wk old hens (67.9 ± 3.85 mg) and from that of yolk sacs from chicks of 55 wk old hens (84 ± 3.85 mg). Total IgY contained by the yolk sac of chicks from 55 wk old breeders was significantly larger than that contained by the yolk sac of chicks from 40 wk old breeders.

Total yolk sac IgY content was also calculated as a percentage of total egg yolk IgY content; it was determined that the total IgY contained in the yolk sac of chicks from 32 wk old breeders corresponded to 55.4 ± 2.1% (SEM) of the total egg yolk IgY for that breeder age. This value was significantly greater ($P < 0.0001$) than those calculated for 40 wk old

breeders (39.1 ± 2.3%) and 55 wk old breeders (33.3 ± 2.3%), which did not differ from each other.

3.4. Discussion

The main objective of this study was to determine the effects of broiler breeder age (32, 40 and 55 wk) on total IgY content of egg yolks and newly hatched chick yolk sac. The effects of breeder age on egg and yolk sac weights were also quantified.

3.4.1. Egg, yolk, chick, and yolk sac weights

Egg weight increased with flock age, this was expected because a positive correlation between these two factors has been demonstrated since the 1950's (Wiley, 1950). The observation that wet egg yolk wt increased with flock age is in agreement with previous studies on this genetic strain of chicken (Ulmer-Franco et al., 2010); however, as it was previously reported, it was expected that wet egg yolk wt as a percentage of egg wt would also increase with flock age. In this experiment, there were no differences in wet egg yolk weight as a percentage of egg weight, perhaps as a consequence of the small sample size (Table 3-1).

It was expected to obtain heavier chicks as the breeders grew older, as a consequence of heavier eggs (Suarez et al., 1997; O'Dea et al., 2004);

however, in this experiment there were no differences (Table 3-2). Eggs laid by 32 wk old hens had the smallest yolks so it was expected that the yolk sacs of chicks produced at this age would be smaller than the yolk sacs of chicks hatching from older parents. However the yolk sacs of chicks from the 32 wk old breeders were smaller than the yolk sacs of chicks from 55 wk old breeders, but they were also heavier than the yolk sacs of chicks from the 40 wk old flock (Table 3-2).

A number of studies have reported late hatching of chicks from young breeder hens compared to chicks from older hens (Hudson, et al., 2004; Hamidu et al., 2007) suggesting their need for either a longer incubation period or different incubation conditions. The yolk sacs of chicks from 32 wk old breeders were heavier than those obtained when breeders reached 40 wk age (Table 3-2). It is unknown if this was a consequence of the late hatch and lack of maturity or if differences exist in the rates of yolk sac absorption between chicks from different breeder ages.

3.4.2. Total Egg Yolk IgY Content

The total egg yolk IgY determined in this study is higher than the 5.2 mg/mL and 42 to 105 mg/yolk reported by Carlander et al. (2001) in 50 wk old single comb white leghorn (SCWL) hens. That study followed the daily total IgY content of egg yolks for 28 straight days reporting minor

differences in daily IgY amounts (Carlander et al., 2001). The differences observed between the results could be a consequence of the different IgY extraction and quantification methods used in each study (Deignan et al., 2000). Genetic variations could also explain the differences observed: SCWL hens are laying-type birds and Cobb 500 hens are meat-type birds, meaning that egg production is higher in SCWL than in Cobb 500, perhaps leading to dilution of the IgY amount deposited per egg for the SCWL. In a study comparing three strains of laying-type birds (SCWL, Rhode Island Red (RIR), and their cross) Carlander et al. (2003) reported different levels of IgY/mL of egg yolk among strains, although egg yolk weight and bird age were not indicated in this study. Li et al. (1998) compared egg yolk IgY levels after immunization of 35 wk old SCWL and RIR hens; they reported no differences in the percentage of IgY/mL of yolk between strains concluding that egg yolk weight was a deciding factor on the total egg yolk IgY content (Li et al., 1998). Results from the present experiment in meat-type birds are in agreement with that study in that egg yolk weight influences total yolk IgY content. In 2009, Pauly et al. described how egg IgY contents changed between 25 wk and 24 months of age in ISA brown and Lohmann hens (laying-type birds) immunized against ricin or *Clostridium botulinum* neurotoxin. They reported levels of IgY of 30 mg/egg at the beginning of the laying phase and of 80 mg/egg during the

second year of the study (Pauly et al., 2009). The authors suggested that older hens maintained a stable IgY yield by increasing egg IgY contents to compensate for the physiological decline in egg production observed with hen aging. Results from the present experiment are in agreement with Pauly et al. (2009) showing that total egg yolk IgY increased with hen age.

To the author's knowledge, there are no available reports comparing the total IgY content of eggs produced by breeders of meat type birds under standard commercial conditions. This is most likely due to the fact that most antibody research has been performed on laying type birds used for industrial antibody production.

3.4.3. Total Yolk Sac IgY Content

Total egg yolk IgY content increased with flock age, thus, it was expected that total yolk sac IgY would follow the same pattern. However, no clear pattern was observed and the yolk sac of chicks obtained when breeders were 55 wk of age had significantly less IgY per gram of yolk sac than that of chicks obtained at 32 and 40 wk of age (Figure 3-2). It was interesting to observe that the total IgY content per gram of yolk sac may be greater than that obtained per gram of egg yolk at 32 and 40 wk of age but not at 55 wk of age. Whether embryos from the 55 wk old breeders were absorbing their yolk sac IgY at a faster rate than embryos from the 32

and 40 wk old breeders was not determined, but it could explain these results. Kowalczyk et al. (1985) compared egg yolk IgY and hen and chick serum IgY (hen age not specified) and reported that only around 10% of egg IgY could be detected in the newly hatched chick's serum (Kowalczyk et al., 1985). Even though Kowalczyk et al. (1985) did not measure yolk sac IgY and in this study serum IgY was not determined, it is possible that the IgY not observed in chick serum could be contained in the newly hatched chick yolk sac. If there is indeed a delay in yolk sac IgY absorption in chicks from 32 and 40 wk old breeders, it could mean that these chicks are more susceptible to specific pathogen invasion during the early post-hatching period than chicks hatching from 55 wk old breeders. This possibility should be examined. The hypothesis that the apparent delay in yolk sac IgY absorption would be compensated by rapid post hatching absorption in preparation for antigenic challenge should also be tested.

Newly hatched chicks from the 32 wk old breeders carried 55.4% of egg IgY in their yolk sac, chicks from 40 wk old breeders carried 39.1% and chicks from 55 wk old breeders had 33.3% of egg IgY in their yolk sac. It is possible that egg yolk IgY was not absorbed at the same rate as other yolk nutrients during embryonic development in eggs from each flock age. Post-hatching absorption of the yolk sac content is essential for chick

growth and development; any event leading to decreased yolk sac absorption would negatively affect chick survival and performance. It is known that bacterial yolk sac infections change the composition of the yolk sac content impairing its normal absorption and decreasing circulating IgY levels (Sander et al., 1998). Chicks hatching from old breeder flocks are known to have a higher incidence of yolk sac infections than chicks from young flocks (Yassin et al., 2009). Whether the delayed absorption of yolk sac IgY in chicks from young breeders is protecting them from a localized yolk sac infection is unknown.

3.5. Conclusion

The hypothesis that total egg and yolk sac IgY concentration would decrease with flock age has not been accepted. It was hypothesised that vaccination-induced IgY levels would be higher in younger birds because vaccination programs for broiler breeders under commercial conditions are intense up to around 20 weeks of age; after this age breeders are only given a few more boosters every 10 weeks. In 2008, Gharaibeh et al. compared serum IgY concentrations against 10 different avian pathogens in chicks and their breeders (Lohman hens) at 37, 40, and 45 wk of age. They proposed that breeder serum titres could be used to predict disease susceptibility in chicks (Gharaibeh et al., 2008). In the current experiment

total IgY was determined but pathogen - specific IgY was not measured. It was thought that circulating levels of vaccination-induced IgY would decrease in older hens due to natural turnover; instead, total IgY per gram of yolk increased with flock age. In 2004 Parmentier et al. reported the presence of antibodies binding antigens to which chickens had not been previously immunized against; they referred to these as "natural antibodies". They determined that 15 month old chickens had higher plasma levels of natural antibodies than 37 day old birds (Parmentier et al., 2004). In the present study, all eggs and chicks were produced by the same broiler breeder flock at different flock ages. However it is possible that the differences observed in the total egg yolk IgY contents were a consequence of differences in natural antibodies of hens that had been exposed to the environment for longer time.

In summary, total egg yolk IgY per gram of yolk increased with flock age; this fact plus the natural increase in egg and egg yolk wt observed in older birds, dramatically increased the total egg yolk IgY content. Conversely to that observed in egg yolks, the total yolk sac IgY in chicks from 55 wk old hens was the lowest. Further research to determine the physiological mechanisms and consequences of this observation is required.

3.6. References

Bar-Shira, E., Friedman, A., 2006. Development and adaptations of innate immunity in the gastrointestinal tract of the newly hatched chick. Developmental & Comparative Immunology 30, 930-941.

Brambell, R., 1970. Transmission of immunity in birds. In: Neuberger, A., Tatum, E.L. (Eds.), The transmission of passive immunity from mother to young. North-Holland Publishing Company, Amsterdam, the Netherlands, pp. 20-41.

Canadian Council on Animal Care, 2009. Guidelines on the care and use of farm animals in research, teaching and testing. Available at: http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/PDFs/Farm_Animals.pdf (Accessed, 21 May 2010).

Carlander, D., Wilhelmson, M., Larsson, A., 2003. Immunoglobulin Y levels in egg yolk from three chicken genotypes. Food Agric. Immunol. 15, 35-40.

Carlander, D., Wilhelmson, M., Larsson, A., 2001. Limited day to day variation of igy levels in eggs from individual laying hens. Food Agric. Immunol. 13, 87-92.

Deignan, T., Kelly, J., Alwan, A., Gert, H.J., 2000. Comparative analysis of methods of purification of egg yolk immunoglobulin. Food Agric. Immunol. 12, 77-85.

Derache, C., Esnault, E., Bonsergent, C., Le Vern, Y., Quéré, P., Lalmanach, A., 2009. Differential modulation of β-Defensin gene expression by *Salmonella* Enteritidis in intestinal epithelial cells from resistant and susceptible chickens. Dev. Comp. Immunol. 33, 959-966.

Gharaibeh, S., Mahmoud, K., Al-Natour, M., 2008. Field evaluation of maternal antibody transfer to a group of pathogens in meat-type chickens. Poult. Sci. 87, 1550-1555.

Gómez-Verduzco, G., Téllez, G., Quintana, A.L., Isibasi, A., Ortiz-Navarrete, V., 2010. Humoral immune response in breeding hens and protective immunity provided by administration of purified *Samonella* Gallinarum proteins. Poult. Sci. 89, 495-500.

Hamal, K.R., Burgess, S.C., Pevzner, I.Y., Erf, G.F., 2006. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. Poult. Sci. 85, 1364-1372.

Hamidu, J.A., Fasenko, G.M., Feddes, J.J.R., O'Dea, E.E., Ouellette, C.A., Wineland, M.J., Christensen, V.L., 2007. The effect of broiler breeder

genetic strain and parent flock age on eggshell conductance and embryonic metabolism. Poult. Sci. 86, 2420-2432.

Hudson, B. P., B. D. Fairchild, J. L. Wilson, W. A. Dozier, III, and R. J. Buhr, 2004. Breeder age and zinc source in broiler breeder hen diets on progeny characteristics at hatching. J. Appl. Poult. Res. 13, 55-64.

Kawalilak, L.T., Ulmer-Franco, A.M., Fasenko, G.M., 2010. Impaired intestinal villi growth in broiler chicks with unhealed navels. Poult. Sci. 89, 82-87.

Kowalczyk, K., Daiss, J., Halpern, J., Roth, T.F., 1985. Quantitation of maternal-fetal IgG transport in the chicken. Immunology 54, 755-762.

Lawrence, E.C., Arnaud-Battandier, F., Grayson, J., Koski, I.R., Dooley, N.J., Muchmore, A.V., Blaese, R.M., 1981. Ontogeny of humoral immune function in normal chickens: a comparison of immunoglobulin-secreting cells in bone marrow, spleen, lungs and intestine. Clin Exp Immunol 43, 450-457.

Leslie, G.A., Clem, L.W., 1969. Phylogeny of immunoglobulin structure and function: III Immunoglobulins of the chicken. J. Exp. Med. 130, 1337-1352.

Li, X., Nakano, T., Sunwoo, H.H., Paik, B.H., Chae, H.S., Sim, J.S., 1998. Effects of egg and yolk weights on yolk antibody (IgY) production in laying chickens. Poult. Sci. 77, 266-270.

Machado Leal Ribeiro, A., Rudnik, L., Wageck Canal, C., Ribeiro Kratz, L., Farias, C., 2005. Uso de gemas de ovos de aves hiperimunizadas contra *Escherichia coli* suína no controle da diarréia neonatal de leitões. R. Bras. Zootec. 34, 1234-1239.

Morrison, S.L., Mohammed, M.S., Wims, L.A., Trinh, R., Etches, R., 2001. Sequences in antibody molecules important for receptor-mediated transport into the chicken egg yolk. Mol. Immunol. 38, 619-625.

Murakami, H., Akiba, Y., Horiguchi, M., 1992. Growth and utilization of nutrients in newly-hatched chick with or without removal of residual yolk. Growth Dev. Aging 56, 75-84.

Noy, Y., Sklan, D., 1999. Energy utilization in newly hatched chicks. Poult. Sci. 78, 1750-1756.

O'Dea, E.E., Fasenko, G.M., Feddes, J.J., Robinson, F.E., Segura, J.C., Ouellette, C.A., van Middelkoop, J.H., 2004. Investigating the eggshell conductance and embryonic metabolism of modern and unselected

domestic avian genetic strains at two flock ages. Poult. Sci. 83, 2059-2070.

Orlans, E., 1967. Fowl Antibody VIII. A Comparison of natural, primary and secondary erythrocytes in hen sera; their transmission to yolk and chick. Immunology 12, 27-37.

Parmentier, H.K., Lammers, A., Hoekman, J.J., de Vries Reilingh, G., Zaanen, I.T., Savelkoul, H.F., 2004. Different levels of natural antibodies in chickens divergently selected for specific antibody responses. Dev. Comp. Immunol. 28, 39-49.

Patterson, R., Youngner, J.S., Weigle, W.O., Dixon, F.J., 1962. The metabolism of serum proteins in the hen and chick and secretion of serum proteins by the ovary of the hen. J. Gen. Physiol. 45, 501-513.

Pauly, D., Dorner, M., Zhang, X., Hlinak, A., Dorner, B., Schade, R., 2009. Monitoring of laying capacity, immunoglobulin Y concentration, and antibody titer development in chickens immunized with ricin and botulinum toxins over a two-year period. Poult. Sci. 88, 281-290.

Romanoff, A.L., 1960. The extraembryonic membranes. The avian embryo; structural and functional development. Macmillan, New York, pp. 1042-1081.

Rose, M.A., Orlans, E., Buttress, M., 1974. Immunoglobulin classes in the hen's egg: their separation in yolk and white. Eur. J. Immunol. 4, 521-523.

Rose, M.E., Orlans, E., 1981. Immunoglobulins in the egg, embryo and young chick. Dev. Comp. Immunol. 5, 15-20.

Sander, J.E., Willinghan, E.M., Wilson, J.L., Thayer, S.G., 1998. The effect of inoculating *Enterococcus faecalis* into the yolk sac on chick quality and maternal antibody absorption. Avian Dis. 42, 359-363.

SAS Institute Inc., 2002-2003. SAS User's Guide Version 9.1.

Selvaraj, R.K.; Cherian, G., 2004. Changes in delayed type hypersensitivity, egg antibody content and immune cell fatty acid composition of layer birds fed conjugated linoleic acid, n-6 or n-3 fatty acids. Can. J. Anim. Sci. 84:221-228.

Shimizu, M., Nagashima, H., Sano, K., Hashimoto, K., Ozeki, M., Tsuda, K., Hatta, H., 1992. Molecular stability of chicken and rabbit immunoglobulin G. Biosci. Biotechnol. Biochem. 56, 270-274.

Suarez, M.E., Wilson, H.R., Mather, F.B., Wilcox, C.J., McPherson, B.N., 1997. Effect of strain and age of the broiler breeder female on incubation time and chick weight. Poult. Sci. 76, 1029-1036.

Ulmer-Franco, A.M., Fasenko, G.M., O'Dea Christopher, E.E., 2010. Hatching egg characteristics, chick quality, and broiler performance at 2 breeder flock ages and from 3 egg weights. Poult. Sci. 89, 2735-2742.

Wang, Y.W., Sunwoo, H., Cherian, G., Sim, J.S., 2004. Maternal dietary ratio of linoleic acid to α-linolenic acid affects the passive immunity of hatching chicks. Poult. Sci. 83, 2039-2043.

Warr, G.W., Magor, K.E., Higgins, D.A., 1995. IgY: Clues to the origins of modern antibodies. Immunol. Today 16, 392-398.

Wijga, S., Parmentier, H.K., Nieuwland, M.G.B., Bovenhuis, H., 2009. Genetic parameters for levels of natural antibodies in chicken lines divergently selected for specific antibody response. Poult. Sci. 88, 1805-1810.

Wiley, W., 1950. The Influence of egg weight on the pre-hatching and post-hatching growth rate in the fowl. Poult. Sci. 29, 595-604.

Williams, J., 1962. Serum proteins and the livetins in hen's-egg yolk. Biochem. J. 83, 346-355.

Yassin, H., Velthuis, A.G.J., Boerjan, M., van Rielt, J., 2009. Field study on broilers' first-week mortality. Poult. Sci. 88, 798-804.

Yasuda, M., Furusawa, S., Matsuda, H., Taura, Y., Urano, T., Yokomizo, Y., Ekino, S., 1998. Development of maternal IgG-free chick obtained from surgically bursectomized hen. Comp. Immunol. Microbiol. Infect. Dis. 21, 191-200.

4. Bacterial Counts of Yolk Sacs and Organs in Broiler Chicks are Affected by Cleaning and Disinfection of Chicken Barns

4.1. Introduction

The Canadian poultry industry has been implementing and operating a certification process for the On-Farm Food Safety Assurance Program (OFFSAP) since 2000. The OFFSAP is focused on reducing infections in live animals and thus minimize the risks of pathogens in end products sold as food. The broiler chicken OFFSAP program called "*Safe, Safer, Safest*", combines good production practices and Hazard Analysis Critical Control Point (HACCP) principles into chicken production (Chicken Farmers of Canada, 2010). The main objective of HACCP programs is to identify potential risks to food safety with the goal of preventing food contamination at every level of the production chain. The "*Safe, Safer, Safest*" program is mandatory in each of the Canadian provinces and over 97% of chicken farms are certified (Chicken Farmers of Canada, 2010). Certification by the OFFSAP program offers an important benefit to the non-integrated Canadian chicken industry by helping maintain a common food safety standard.

The importance of environment sanitation in broiler chick health has been greatly studied and emphasized. Since the 1960's a higher risk of bacterial contamination leading to disease and mortality in chicks has been linked to poor sanitation practices (Fuller and Jayne-Wiliams, 1968). Multiple reports comparing different cleaning and disinfection protocols for poultry barns are available in the literature. The in vitro efficacy of disinfectants such as glutaraldehyde, formaldehyde, hydrogen peroxide, quaternary ammonium, phenols and iodine against barn bacterial suspensions was tested by Ruano et al. (2001). This research team concluded that disinfectants were effective within the first 10 minutes of contact if manufacturer's recommendations were followed and in absence of organic matter (Ruano et al., 2001). An experiment using low vs. high application rates of phenol, potassium peroxymonosulfate, nascent oxygen and quaternary ammonia was carried out by Payne et al. (2005) to compare bacterial reduction on the barn floor. No reduction on aerobic bacteria numbers was observed when disinfectants were used at the low application rate (i.e. just covering the surface), further confirming the importance of following manufacturer's recommendations (Payne et al., 2005). Other experiments have tested the efficacy of different disinfectants on different surfaces in the absence of organic matter. Rathgeber et al. (2009) compared bacterial counts on plywood, metal and plastic resin after

disinfection with iodine, sodium hypochlorite, or sodium hypochlorite plus potassium hydroxide. They concluded that metal and plastic surfaces were easier to sanitize than plywood, and that sodium hypochlorite-based disinfectants were more effective than iodine in reducing bacterial counts (Rathgeber et al., 2009). Ward et al. (2006) studied the effects of washing and disinfecting after litter removal on bacterial counts of wood and metal barns. They reported further reduction of *Enterobacteriaceae* counts on wood barns after both washing and disinfecting. On metal barns a significant reduction in *Enterobacteriaceae* counts was observed after washing but subsequent disinfection did not further reduce these counts (Ward et al., 2006).

The hatchery is an important factor to take into account when considering environmental exposure of broiler chicks. The hatchery must provide appropriate environmental conditions for chicken embryonic development; however, the warm and humid environment maintained at the hatchery also offers adequate growth conditions for microorganisms. Improper hatchery sanitation has been linked to chick contamination and omphalitis (Fuller and Jayne-Wiliams, 1968; Walker and Sander, 2004). Transmission of hatchery bacteria to newly hatched chicks increased during the pipping stage, when chicks are first exposed to the

environment outside the egg (Walker and Sander, 2004). Hatchery disinfection has also been studied by multiple research teams. Sander et al. (2002) demonstrated that different disinfectants varied on their bactericidal efficacy against *Staphylococcus, Enterococcus, Salmonella, Proteus, Pseudomonas* and *E. coli*. They also reported that even individual bacteria within the same genus (e.g. *E. coli*) exhibited different resistance levels, suggesting that hatcheries should select disinfectants based on their own bacterial challenges (Sander et al., 2002). In the present study, chicks were collected from a commercial hatchery, thus simulating commercial conditions for Canadian broiler producers who have no direct influence on the hatchery environment.

The objective of this research was to evaluate the effects of cleaning and disinfection protocols on the total numbers of aerobic bacteria, *Enterobacteriaceae* and *E. coli* in the broiler barn and in organs and yolk sacs of broiler chicks during the first week post-hatching. Four different cleaning and disinfection protocols were evaluated. These protocols differed by the cumulative removal of one step at the time. The hypothesis tested was that a lower number of bacteria would be isolated from pens receiving the complete (OFFSAP standard) cleaning and disinfection treatment than in those pens receiving incomplete cleaning and

disinfection. It was also hypothesized that fewer bacteria would be isolated from chicks placed in "cleaner" pens than from those placed in "dirtier" pens.

4.2. Materials and methods

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies (Canadian Council on Animal Care, 2009) with approval from the Animal Care and Use Committee: LIVESTOCK for the University of Alberta.

4.2.1 Cleaning and Disinfection Protocols

This experiment was conducted in the Environmental Chambers of the University of Alberta Poultry Research Centre. The barn consisted of 8 individual rooms[3]: 4 adjacent rooms on the north and 4 on the south side of the barn separated by a 3.9 m hallway. Each room had cement floor, solid walls, and solid metal doors, and was completely isolated from neighbouring rooms to prevent cross contamination at all times. The experiment started immediately after a broiler grow-out period of 56 days. These broilers belonged to a different research experiment and had been housed at a density of 0.2 m^2 per bird. Immediately after bird removal one

[3] Pen dimensions: 4.4 m deep x 3.9 m wide = 17.16 m^2

of the following four cleaning and disinfection treatments was applied to two replicate rooms based on previous research (Ward et al., 2006).

- Treatment 1 ("very clean", VC): Litter material (wood shavings) removed, pens washed with high pressure chlorinated water (city water), pens disinfected with 1% Virkon®[4] solution (DuPont Animal Health Solutions, Suffolk, United Kingdom), and new litter material (wood shavings) placed.

- Treatment 2 ("Clean", C): Litter material (wood shavings) removed, pens not washed, pens disinfected with 1% Virkon® solution, and new litter material (wood shavings) placed.

- Treatment 3 ("Dirty", D): Litter material (wood shavings) removed, pens not washed, pens not disinfected, but new litter material (wood shavings) placed.

- Treatment 4 ("Very dirty", VD): Litter material (wood shavings) not removed, pens not washed, pens not disinfected, new litter material (wood shavings) placed on top of used litter.

[4] Virkon® composition: 40-60% potassium peroxymonsulfate; 10-20% sodium dodecylbenzen-sulphonate; 1-10% sulfamic acid

All galvanized metal 25 lb capacity feeders and plastic 1 gallon drinkers were washed and disinfected with 1% Virkon® solution prior to use. Cleaning and disinfection of the barn took place during December of 2009 when temperatures in Edmonton, Canada, ranged between -36.7°C and 0.5°C. After cleaning and disinfection of the barn, each room was divided in half with a wire fence to obtain a total of 16 pens. Withdrawal time before placement of new chicks was 21 days.

To assure biosecurity-wise movement of personnel inside the barn VD treatment was applied to pens located at the opposite end of the barn door. Treatments D and C were applied to middle pens and VC treatment was applied to those pens located next to the barn door. Boot dips containing a 1% Virkon® solution were place outside each pen. Any activity taking place in the barn followed a VC - C - D - VD flow, and boots were dipped in the disinfectant solution before entering and after leaving all pens (Figure 4-1).

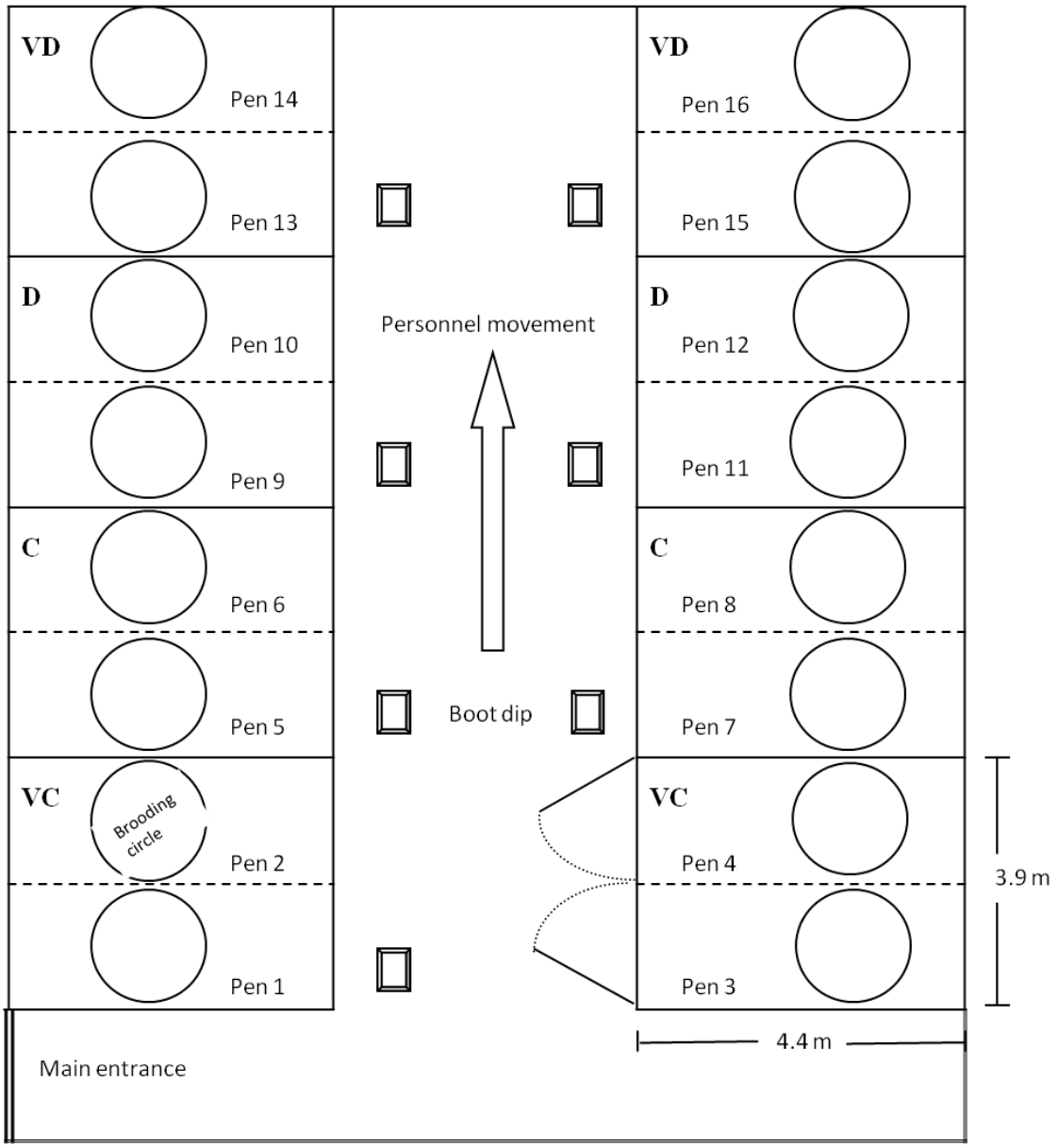


Figure 4-1. Schematic representation of the broiler barn and distribution of cleaning and disinfection treatments. Eight individual rooms were divided in half to obtain 16 pens. Cleaning and disinfection treatments: VC (very clean), C (clean), D (dirty), VD (very dirty). Chicks were kept inside the brooding circles. Daily activities started in VC pens and followed the direction of the arrow. Boots were dipped in disinfectant solution before entering and after leaving each pen. Stepping inside the brooding circles was avoided at all times.

The day before chick collection 30.5 cm high corrugated cardboard was used to build rearing circles inside each pen. Chick density was set at 0.07 m^2 per bird. One circle of 2.1 m in diameter, which provided adequate area for 50 chicks, was set in the same location for each pen. Stepping inside rearing circles was avoided at all times in this trial. Temperature in the barn was set at 32°C.

4.2.2 Microbiological Sampling of Environment

At a commercial hatchery two swab samples of 10 x 10 cm areas from two hatcher trays on the day of hatching were collected using sterile gauze pads (10 cm x 10 cm, 12 ply) (Safe Cross First Aid Ltd., Toronto, ON, Canada) moistened in sterile Luria Bertani (LB) broth (Difco & BD, Mississauga, ON, Canada) (Figure 4-2, A).

A B

Figure 4-2. Hatchery environmental sample collection. A) hatcher tray swabs, B) chick fluff collection.

Two additional samples of both chick fluff and eggshell (approx. 15 g) were collected with sterile plastic 75 cc scoops (Fisher Scientific Company, Ottawa, ON, Canada) (Figure 4-2 B). Immediately after collection, samples were placed into sterile 532 mL (18-oz) Whirl-Pak® Write-On bags (Nasco Canada, Newmarket, ON, Canada) and placed in a cooler for transportation to the laboratory.

The day of hatching and prior to chick placement, two drag swabs were collected from each brooding circle using sterile gauze pads moistened in sterile LB broth. The entire area of each brooding circle (3.5 m^2) was swabbed for approximately 20 seconds by two handlers wearing examination gloves. Gloves were disinfected with 70% ethanol before and after each sampling and new gloves were used when sampling brooding circles belonging to different cleaning and disinfection treatments. Immediately after sampling, gauze pads were placed into sterile 532 mL (18-oz) Whirl-Pak® Write-On bags and placed in a cooler for transportation to the laboratory. Sampling from the brooding circles was repeated on days 4 and 8 after chick placement.

4.2.3 Chick Collection and Placement

A total of 896 unvaccinated and unsexed broiler chicks produced by one 48 wk old Cobb 500 breeder flock were selected from the hatching line

in a commercial hatchery on the day of hatching. Chicks were transported to the University of Alberta Poultry Research Centre Environmental Chambers[5], randomly divided in 16 groups of 50 chicks (n = 800), and placed inside the rearing circles in each pen. Temperature, humidity and ventilation were controlled in all pens to be the same. Chicks were kept for a maximum of 8 days at 30-32°C with a photoperiod of 23 h of light and 1 h of darkness. Water and a crumbled starter diet (23.0% crude protein and 3,067 kcal of ME/kg) were provided *ad libitum*.

4.2.4 Tissue Collection

Chicks (n = 96) were randomly selected for tissue collection on the day of hatching (day 0). These chicks were kept in the hatchery cardboard boxes provided for transportation while remaining chicks were placed in the barn, thus, any contact with the outside environment was prevented. Chicks were transported to the dissection room, divided into 8 groups of 12 chicks, humanely euthanized via cervical dislocation and dissected for tissue collection. On day zero, two types of samples were collected: yolk sacs and internal organs (heart, liver and spleen) (Figure 4-3). The following strategies were used to prevent cross contamination between samples: 1) Chicks were dissected on stainless steel tables which surfaces

[5] Approximate distance between hatchery and farm: 60 Km. Approximate temperature: -18°C.

were washed and disinfected with 1% Virkon® solution; 2) Chicks were dipped in 1% Virkon® solution prior to dissection; 3) One person was assigned to collect only one type of sample per day, and was given a set of dissection tools to be used only on the specific type of sample; 4) Dissection tools were washed and disinfected with Virkon® between groups of chicks; 5) gloves were disposed after dissecting all chicks from the same treatment. Organs and yolk sacs from each group were pooled in separate sterile 532 mL (18-oz) Whirl-Pak® Write-On bags and placed in a cooler for transportation to the laboratory.

A B

Figure 4-3. Sample collection from newly hatched broiler chicks. A) yolk sac dissection. B) organ dissection (heart in this case).

On day four post-hatching, two groups of 12 chicks per pen were selected for tissue collection. Dissections were carried out exactly as on day zero. Similarly, on day eight post-hatching, two replicate groups of 12

chicks per pen were dissected but on this day only organs were collected. Also on day eight, individual sterile blood samples of approx. 2 mL per chick were collected from five chicks per pen via cardiac puncture using 4.0 mL vacutainer tubes containing 68 USP units of Lithium heparin (BD, Franklin Lakes, NJ, USA). Upon collection, samples were placed in a cooler and transported to the microbiology lab.

4.2.5 Microbiological Analysis

Environmental swab samples were added 20 mL of LB broth and then shaken 20 times through the arc of one foot. Tenfold serial dilutions were prepared using sterile 0.1% peptone water (Difco & BD) and 100 µL were plated on plate count agar (PCA) (Difco & BD), MacConkey agar (Difco & BD), and violet red bile agar (Difco & BD) with 1% added glucose [(Fisher Scientific Company, Ottawa, ON, Canada) (VRBG)]. After overnight incubation at 37°C colonies of total aerobic bacteria were enumerated on PCA, presumptive *Enterobacteriaceae* on VRBG agar, and presumptive *E. coli* on MacConkey agar. All colony-forming unit (CFU) counts were converted to $\log_{10}$ CFU per 100 cm^2.

Pooled organs and yolk sac samples were stomached for 2 min. Ten gram samples were weighed into sterile Whirl-Pak® Write-On bags, 90 mL of LB broth were added, and bags were stomached for an additional

minute. Tenfold serial dilutions were prepared using sterile 0.1% peptone water and 100 µL of each dilution were plated on PCA, VRBG, and MacConkey agars (Figure 4-4). Bacterial colonies were enumerated after overnight incubation at 37°C and CFU counts were converted to $\log_{10}$ CFU per gram of pooled tissue

A B C

Figure 4-4. A) Aerobic bacteria colonies on plate count agar B) *Enterobacteriaceae* colonies on violet red bile agar plus glucose, C) *Escherichia coli* colonies on MacConkey agar (dark fuchsia colonies).

From the individual blood samples collected on day 8 post hatching, 0.5 mL were pipetted into 4.5 mL brain-heart infusion broth (Difco & BD); after samples were gently agitated 100 µL were plated on MacConkey agar and incubated overnight at 37°C.

4.2.6 Statistical analysis

Bacterial counts from barn samples were analyzed as repeated measures using the Mixed procedure of SAS (SAS Institute Inc., 2002-2003) and the compound symmetry variance-covariance structure for the "split-plot in time" data structure. Day of sample collection was the repeated factor, brooding circle was the experimental unit and random effect. One-way analysis of variance of bacterial counts from hatchery samples was done separately with each hatching tray as the experimental unit. Bacterial counts from organ samples collected at days 4 and 8 were analysed in a 2 x 4 factorial design with day of sample collection and cleaning treatment as the main factors, the experimental unit was each pooled sample obtained from 12 chicks, and the random factor was the pooled sample identification number. The probability level for statistical significance set at $P \leq 0.05$. When interactions were significant the main effects were not discussed. Where the model indicated significance, the LSmeans were separated using the pdiff procedure of SAS.

4.3. Results

Average chick mortality recorded up to 8 days post-hatching was 1.5%. No differences in mortality values were observed between cleaning and disinfection treatments: VC 0.5%, C 2.5%, D 2.0%, and VD 1.0%.

Dead birds were collected by the barn staff each day and placed in a 4°C fridge until necropsy on days 4 and 8 (sampling days). Mortality during this experiment was mostly due to runting and stunting syndrome; dead chicks were dehydrated, emaciated, and had visible urate deposits in the ureters and cloacae. A number of chicks in good body condition were found dead on dorsal recumbency, no specific lesions were observed during necropsy leading to the conclusion that these chicks had succumbed to sudden death syndrome. Because mortality was only collected during the morning, some chicks had an advanced degree of decomposition when they were placed in the fridge. The cause of death in these chicks could not be determined. No samples were collected from microbiological analysis.

4.3.1 Bacterial counts in the environment.

The interactions of sampling day with cleaning treatment on bacterial counts in the broiler barn were significant. Total aerobic bacteria (P = 0.0022; SEM = 0.23), estimated *Enterobacteriaceae* (P = 0.0002; SEM = 0.75), and estimated *E. coli* (P = 0.0009; SEM = 0.48), counts were greater in VD pens than in VC, C and D pens on day 0 (i.e. before chick placement) (Figure 4-5 A). On day 4 after chick placement total aerobic bacteria and estimated *E. coli* counts were not significantly different between cleaning

treatments; however VD pens had fewer *Enterobacteriaceae* than VC and C, and counts did not differ from those of D pens (Figure 4-5 B).

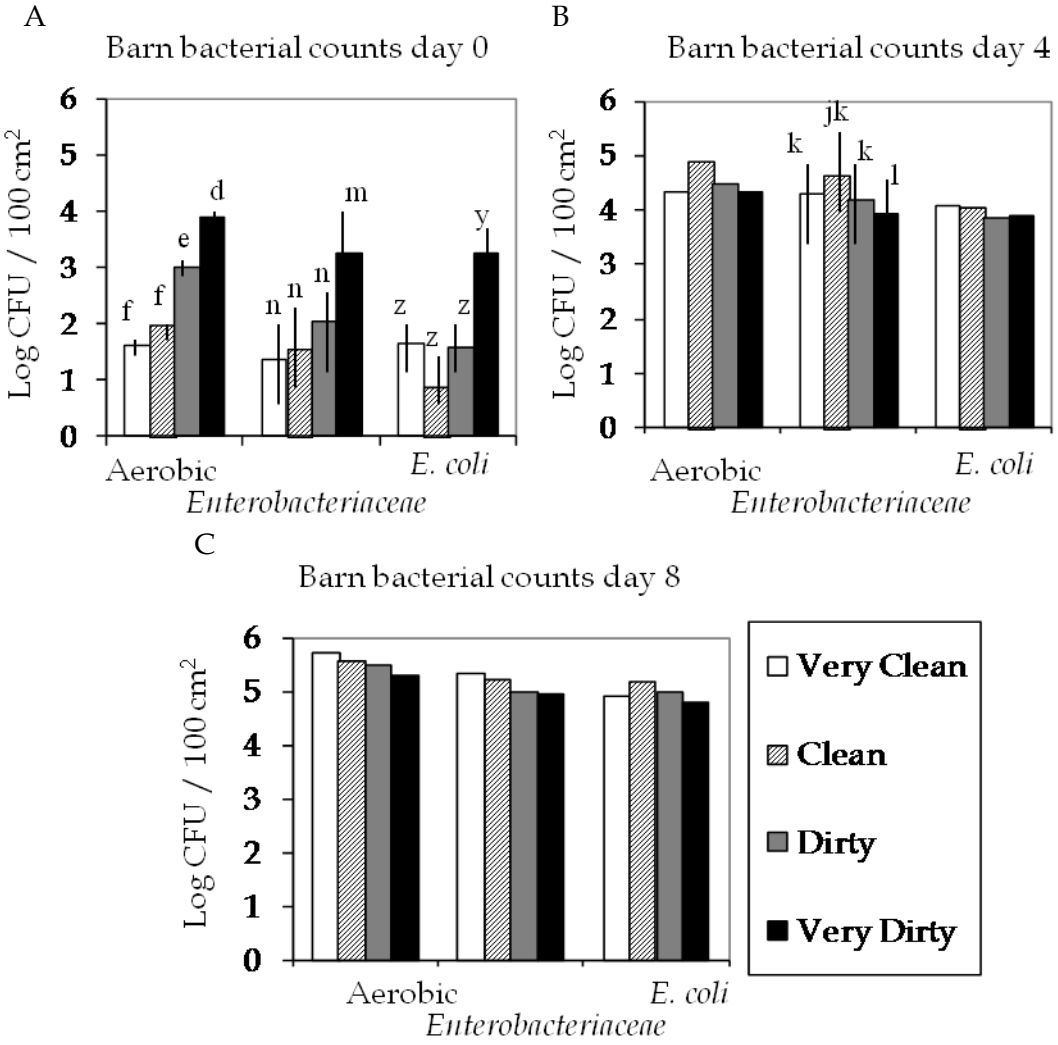


Figure 4-5. Bacterial counts (log CFU/100 cm^2) of barn swabs at 0, 4, and 8 days. Pens received one of four different cleaning and disinfection treatments: Very Clean, Clean, Dirty, or Very Dirty. A) bacterial counts at day 0 (swabs collected prior to chick placement). B) bacterial counts at day 4 (swabs collected 4 d after chick placement). C) bacterial counts at day 8 (swabs collected 8 d after chick placement). n = 8 swabs per cleaning treatment per day. Significant differences ($P \leq 0.05$) are represented by different letters within a bacterial type. Error bars = Standard error of the means (SEM)

Regardless of cleaning and disinfection method the highest bacterial counts in the barn were observed 8 days post hatching indicating that bacterial colonization was time-dependent (Figure 4-5 C).

Average counts of total aerobic bacteria, estimated *Enterobacteriaceae* and *E. coli* in hatchery swabs and chick fluff were 5.94, 6.00 and 5.59 log CFU / 100 cm^2; and 4.89, 1.00 and 2.92 log CFU / g, respectively.

4.3.2 Bacterial counts in organs and yolk sacs

To analyze the impact of environment cleanliness during the early post-hatching period, bacterial counts from pooled organs and pooled yolk sacs collected from hatchery chicks on day 0 and from barn chicks on day 4 were compared. The interaction between sample type and environment was significant. In pooled organs there were no significant differences in total aerobic bacteria between different environments (P = 0.1713). However, organs from 4 day old chicks that had been placed in VC and C (i.e. "cleaner") pens had more estimated *Enterobacteriaceae* than organs from hatchery chicks and from 4 d old chicks placed in D and VD (i.e. "dirtier") pens, which did not differ between each other (P = 0.0011; SEM = 0.49). There were no significant differences (P = 0.076) in *E. coli* counts from pooled organs (Figure 4-6 A).

Results of bacterial counts in pooled yolk sacs (Figure 4-6 B) were also unanticipated: yolk sacs of 4 day old chicks that had been placed in VC and C pens had the same number of total aerobic bacteria as yolk sacs of hatchery chicks, but had more total aerobic bacteria than yolk sacs of 4 day old chicks placed in D and VD pens, which did not differ between each other ($P = 0.0243$, SEM = 0.48). Similarly, yolk sacs of 4 day old chicks placed in VC and C pens had equal numbers of *Enterobacteriaceae* than yolk sacs of chicks from hatchery environment, but more *Enterobacteriaceae* than yolk sacs of 4 day old chicks from D and VD pens. *Enterobacteriaceae* counts between yolk sacs of hatchery chicks and yolk sacs of chicks from D pens were the same, as were counts between yolk sacs of chicks from D and VD pens ($P = 0.0005$, SEM = 0.63). Yolk sacs from 4 day old chicks from VC pens had equal numbers of *E. coli* to yolk sacs from hatchery chicks and chicks from C pens, but more *E. coli* than yolk sacs from chicks from D and VD pens. Yolk sacs of chicks placed in these 2 types of pens had equal numbers of *E. coli* ($P = 0.0324$, SEM = 0.85).

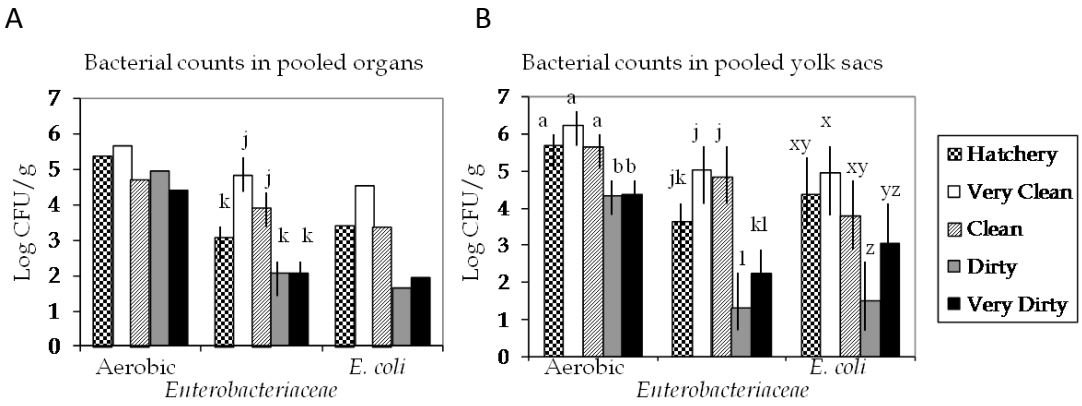


Figure 4-6. Bacterial counts (log CFU/g) of A) pooled organs (heart, liver, and spleen) and B) pooled yolk sacs. Tissues were collected from hatchery chicks on day 0 and from 4 day old chicks placed in pens receiving one of four cleaning and disinfection treatments: Very Clean, Clean, Dirty, or Very Dirty. Tissues collected from 12 chicks were pooled in one sample (n = 8 per cleaning treatment). Significant differences ($P \leq 0.05$) within bacterial types are represented by different letters. Error bars = standard error of the means (SEM).

At 8 days post-hatching only organs were analyzed as normally by this time the yolk sac has been absorbed. Results were compared to bacterial counts obtained from organs of 4 day old chicks. Differences were not significant for total aerobic bacteria due to chick age, cleaning and disinfection treatment or their interactions ($P = 0.2436$) (Figure 4-7 A). The interaction of age by cleaning treatment was significant for counts of *Enterobacteriaceae* and *E. coli* ($P = 0.0006$, SEM = 0.56). The highest *Enterobacteriaceae* counts were observed in organs from 4 day old chicks from VC and C pens as well as 8 day old chicks from C pens. Lower

counts were found in organs from 4 day old chicks in D and VD pens and 8 day old chicks from VC, C and VD pens (Figure 4-7 B).

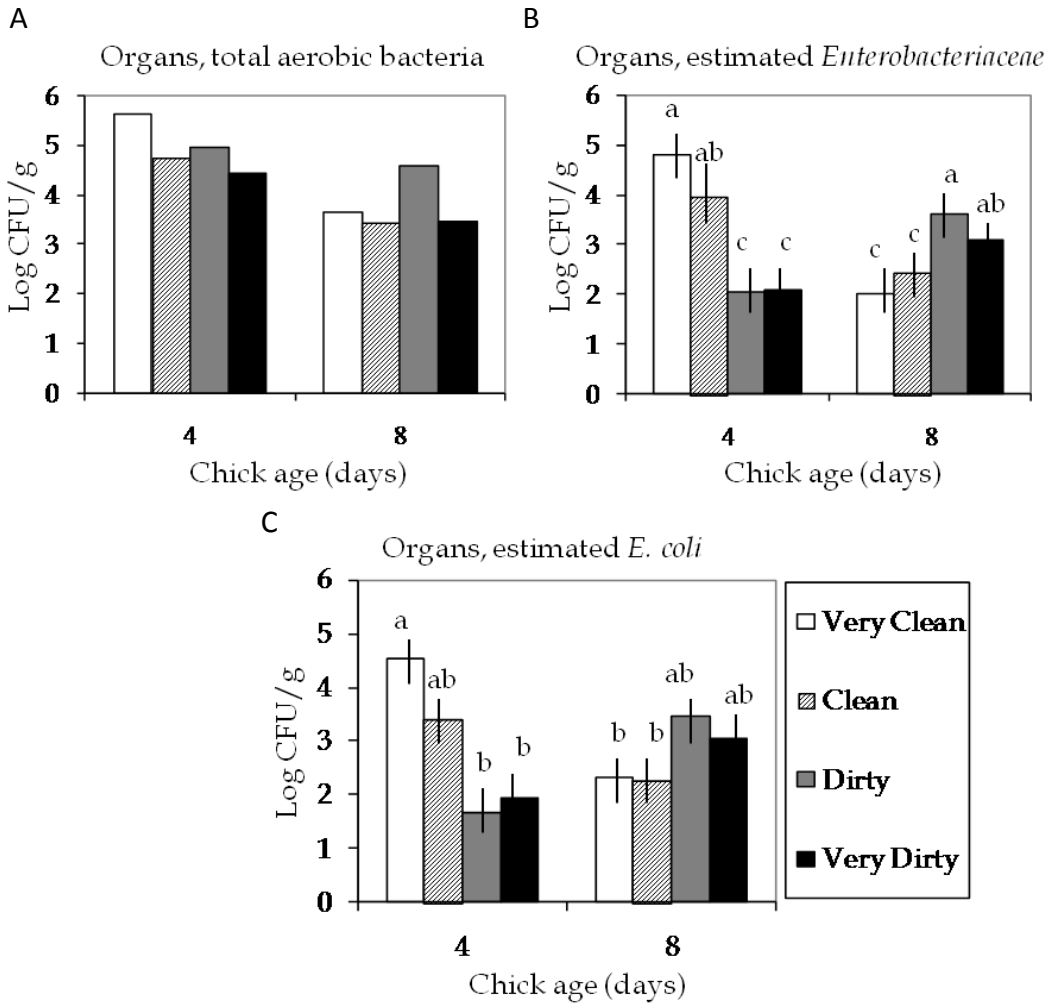


Figure 4-7. Bacterial counts (log CFU/g) of pooled organs (heart, liver, and spleen) collected from 4 and 8 day old broiler chicks placed in pens receiving one of four different cleaning and disinfection treatments: Very Clean, Clean, Dirty, Very Dirty. A) Total aerobic bacteria, B) *Enterobacteriaceae*, C) *E. coli*. Organs collected from 12 chicks were pooled per sample (n = 8 samples per cleaning treatment). Significant differences ($P \leq 0.05$) are represented by different letters. Error bars = standard error of the means (SEM).

Organs of 4 day old chicks from VC pens had more *E. coli* than those from 4 day old chicks from D and VD pens and 8 day old chicks from VC and C pens, but were not significantly different to organs of 4 day old chicks from C pens and 8 day old chicks from D and VD pens (P = 0.0056, SEM = 0.60). Except for the 4 day – VC group there were no significant differences between *E. coli* counts of organs in chicks from other groups (Figure 4-7 C).

All blood samples collected at 8 d post-hatching were negative for *E. coli* suggesting that none of the chicks randomly selected for blood collection were bacteremic.

4.4. Discussion

4.4.1 Bacterial counts in the environment

Control of environmental contaminants at the farm level is considered important in order to reduce food-borne pathogens (Doyle and Erickson, 2006). Common on-farm food safety practices include single-species farms, one-age farms, all-in all-out practices, as well as manure removal, washing and disinfecting, use of fresh clean litter, ventilation manage, vermin control, visitors control, mortality disposal, water management, and prevention of feed contamination (Proudfoot et al., 1991). In Canada, all chicken manure is removed and barns are cleaned and disinfected after

each production cycle; in the United States, removal of used litter material followed by cleaning and disinfection takes place after the fifth or sixth flock cycle, usually once per year (Payne et al., 2005). Previous research teams have tested bacterial populations after different cleaning and disinfection treatments providing information on the *in vitro* efficiency of disinfectants or on their efficiency on the poultry barn prior to chick placement (Ruano et al., 2001; Rathgeber et al., 2009). To the author's knowledge, the effect of cleaning and disinfection on microbial counts after chick placement has not been reported before. In this experiment the effect of four cleaning and disinfection treatments on bacterial counts in the broiler barn before and after chick placement were compared. Prior to chick placement, pens in which manure was removed, were washed, disinfected, or both, had fewer total aerobic bacteria, *Enterobacteriaceae* and *E. coli* than pens in which neither was manure removed nor were washed or disinfected. However, 4 days after chick placement very clean pens had more *Enterobacteriaceae* counts than very dirty pens; and 8 days after placement there were no differences in bacterial loads between pens (Figure 4-5).

4.4.2 Bacterial counts in organs and yolk sacs

The presence of microorganisms in the peritoneum and yolk sacs of apparently healthy newly hatched chicks has been reported since the late 1960's. Fuller and Jane-Williams (1968) hypothesized that bacteria reach the abdominal cavity of the chick by translocation from the intestine to the peritoneum and from there to the yolk sac. Streptococci were isolated in the yolk sac of 60% of chicks, micrococci in 50% and coliforms in 25% of newly hatched chicks (Fuller and Jayne-Wiliams, 1968). In more recent studies newly hatched chick liver and yolk sac have been reported to contain up to 1.7×10^3 and 4.09×10^4 CFU / g *E. coli* respectively (Kizerwetter-Świda and Binek, 2008). Tankson et al. (2002) reported the presence of *Staphylococcus, Bacillus,* and *Corynebacterium* in hearts of 12% newly hatched chicks and of those same bacteria plus *Enterococcus,* and *E. coli* in hearts of 17% 8 day old chicks. All organisms, except for *Enterococcus faecalis*, were considered transient rather than permanent (Tankson et al., 2002). This experiment provided bacterial counts of total aerobic bacteria, *Enterobacteriaceae* and *E. coli* in pooled organs (heart, liver, and spleen) and yolk sacs of newly hatched, 4 day old and 8 day old chicks exposed to environments with different bacterial loads. It should be noted that bacterial enumeration was done in pooled samples, for this

reason the percentage of chicks carrying bacteria in their organs or yolk sacs cannot be provided.

It was of great interest to observe that organs from chicks that had been placed in pens with fewer bacteria on the day of hatching had more *Enterobacteriaceae* on day 4 than chicks placed in pens with greater bacterial loads (Figure 4-6 A). Even more, the yolk sacs of chicks from "cleaner pens" had more total aerobic bacteria, *Enterobacteriaceae* and *E. coli* on day 4 than their "dirtier pen" counterparts (Figure 4-6 B). Whether there were differences in the numbers of fecal bacteria shedding among chicks from the different cleaning and disinfection treatments was not tested. However, it is possible that a greater fecal shedding of *Enterobacteriaceae* and *E. coli* in chicks from "cleaner" pens during the first 4 days post-hatching evened the differences in barn bacterial counts due to cleaning and disinfection treatments. The mechanisms by which bacterial numbers decrease in organs and yolk sacs from "dirtier" pens are unknown. It has been reported that age-associated changes in susceptibility to infections reflect the development of gut immune competence (Smith and Beal, 2008). Such changes are first targeted at reducing bacterial translocation restricting the majority of bacteria to the

intestinal lumen, and then involve bacterial clearance (Smith and Beal, 2008).

In broiler chicks immune protection during the first week post-hatching is provided by maternal antibodies (IgY) and by innate effector mechanisms active along all mucosal surfaces (Bar-Shira and Friedman, 2006). Because all chicks collected for this experiment were produced by the same broiler breeder flock at the same age it was considered that maternal antibody levels would have been the same for all chicks and that the differences observed in bacterial counts at 4 days post-hatching would have been the result of different mechanisms. Furthermore, IgY-secreting B cells of chick origin have only been detected after 6 days post-hatching (Lawrence et al., 1981) meaning that the specific antigen-mediated protection of chick immunoglobulins was not causing these differences. Even though chicks possess the same mechanisms of innate immunity at hatching, it is possible that a stimulation of innate immunity controlled bacterial numbers as early as 4 days post-hatching period. Innate responses are important in the initial phases of microbial invasion not only by limiting pathogen spread but also by initiating events that lead to induction and modulation of the adaptive immune system (Juul-Madsen et al., 2008). Cellular components of the innate immune system include

granulocytes, thrombocytes, natural killer cells, dendritic cells, macrophages, and avian heterophils, the counterpart of mammalian neutrophils. Some of these cells act directly through phagocytosis and antigen presentation, indirectly by producing effector molecules, or both (Juul-Madsen et al., 2008). After inflammatory stimuli, peritoneal exudates in the chicken are known to contain macrophages, responsible for bacterial clearance of the peritoneal cavity (Rose and Hesketh, 1974). Macrophage production and activation were not determined in this research but it would be of interest to investigate if early bacterial contact stimulates these events in peritoneal macrophages of broiler chicks from modern strains.

The possibility that bacterial clearance was a consequence of antimicrobial peptide (AMP) production could also be considered. Antimicrobial peptides are important components of the innate immune system of many vertebrate species; the first AMP described in chickens were isolated from avian heterophils (Evans et al., 1994). Avian heterophils have phagocytic capability but, in contrast to mammalian neutrophils, they lack myeloperoxidase and depend on non-oxidative microbicidal mechanisms (Harmon, 1998). Avian AMP have direct antibacterial and antifungal activities and can influence inflammation,

proliferation, release of cytokines and chemotaxis (Juul-Madsen et al., 2008). Avian AMP create pores in the lipopolysaccharide (LPS) membrane of Gram-negative bacteria leading to membrane permeabilization and cell death (Powers and Hancock, 2003). Two classes of AMP have been identified in chickens: β-defensins also known as Gallinacins and cathelicidin-like proteins known as fowlicidins (Juul-Madsen et al., 2008).

The hypothesis that innate immunity plays a role in bacterial clearance was based on recent research reports: *In vitro* studies of embryonic enterocytes as early as 17 d of incubation determined that the sole contact between *Salmonella* Enteritidis' membrane components and intestinal epithelial cells was enough to induce gene expression of Avian β-defensins 1 and 2 (Derache et al., 2009). He et al. (2005) demonstrated *in vitro* that avian heterophils from 2 d old chicks recognized bacterial DNA (namely, CpG-oligodeoxynucleotides [ODN]) stimulating degranulation and release of AMP. When an intra-peritoneal injection of CpG-ODN was given to newly hatched chicks, invasion and mortality caused by *Salmonella* Enteritidis injected by the same route was reduced. They concluded that CpG-ODN activated the innate immune functions of neonatal chicken immune cells (He et al., 2005). Bar-Shira and Friedman (2006) described a pro-inflammatory, cytokine-mediated and chemokine-

mediated innate response in the intestine of newly hatched chicks upon exposure to feed and the environment (Bar-Shira and Friedman, 2006). They believed this response was linked to bacterial colonization that triggered the recruitment of heterophils to the developing intestine (Bar-Shira and Friedman, 2006). Interestingly, β-defensins were reported to be elevated throughout the intestine on the day of hatching (Bar-Shira and Friedman, 2006). Because chicks from the current experiment were fed the same type of feed, the differences in bacterial numbers should be considered to be a consequence of environmental exposure. In the hypothetical case that β-defensins were active on the intestine of the newly hatched chicks, exposure to a greater bacterial load on the early post-hatching period could have triggered the innate response which decreased bacterial numbers in organs and yolk sacs by day 4 post-hatching. A decrease in bacterial numbers in chicks placed on "clean" pens was observed at 8 days of life suggesting that their bacterial clearance mechanisms were delayed compared to those of chicks that were "boosted" from the day of hatching.

Because this experiment was finalized at 8 days post hatching the long term consequences of this hypothetical stimulation of an early innate immune response due to bacterial exposure could not be determined.

However, van Dijk et al. (2007) examined the chicken β-defensin Gal-6 and reported that it was highly expressed in the digestive tract of 6 wk old Ross 308 chickens a commercial broiler chicken line. Gal-6 was found to have strong bactericidal activity against different types of foodborne pathogens including Gram-negative such as *Campylobacter jejuni, Salmonella* Typhimurium, and *E. coli*; Gram-positives such as *Clostridium perfringens, Staphylococcus aureus* and *Bacillus cereus*, and fungi such as *Candida albicans* and *Saccharomyces cerevisiae* (van Dijk et al., 2007). It would be of great interest to examine *in vivo* if early bacterial exposure in the barn affects the expression of AMP and has a significant effect on broiler performance, pathogen colonization, and disease susceptibility.

4.5. Conclusion

The objective of this research was to evaluate how different cleaning and disinfection protocols affected total aerobic bacteria, *Enterobacteriaceae* and *E. coli* numbers in broiler chicken barns prior to and after chick placement. The main interest was to determine if cleaning and disinfection methods had an effect on bacterial colonization of the yolk sac and organs of broiler chicks during the first week post-hatching. As expected, bacterial counts prior to chick placement were higher in pens that were not cleaned and disinfected than in those which received complete

cleaning and disinfection. However, unexpected results were obtained when analyzing bacterial counts of chicks placed in these pens. Four day old chicks that had been placed in "dirty" pens had fewer bacteria in their yolk sac and organs than those placed in "clean" pens; most differences in bacterial counts had disappeared at 8 days post hatching.

In summary, recent research on early stimulation of the chick's innate immune system was discussed; it was suggested that the greater bacterial load present in "dirty" barns helped to promptly establish the gut microflora and served as a stimulant of the innate response. As a consequence, chicks placed in "dirty" pens were able to decrease their bacterial loads earlier than chicks placed in "clean" pens. The impact of an early innate stimulation on broiler performance, mortality or disease susceptibility was not determined.

At this point it is prudent to advise the reader to cautiously interpret the statements provided in this chapter. Cleaning and disinfection of poultry houses is a crucial step to control any infectious disease chain. It should be mentioned that this experiment was carried out in a research facility that follows the OFFSAP program and has a very low disease challenge; thus, it is possible that bacteria encountered by these chicks were not of a highly pathogenic nature. Furthermore, cleaning and

disinfection has proven to be an effective tool to prevent the spread of viral infections. It is not the intention to advise against this component of general biosecurity programs. However, these results offer new and interesting possibilities for the prevention of disease and bacterial contamination in the absence of antibiotics. Further research on the use of "environmental probiotics" or on early, controlled, environmental bacterial stimulation could provide more answers.

4.6. References

Bar-Shira, E., Friedman, A., 2006. Development and adaptations of innate immunity in the gastrointestinal tract of the newly hatched chick. Dev. Comp. Immunol. 30, 930-941.

Canadian Council on Animal Care, 2009. Guidelines on the care and use of farm animals in research, teaching and testing. Available at: http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/PDFs/Farm_Animals.pdf (Accessed, 21 May 2010).

Chicken Farmers of Canada, 2010. On-Farm Food Safety Assurance Program: Safe, Safer, Safest. Available at: http://chicken.ca/upload/Documents/OFFSAP_Pamphlet_-_EN.pdf. (Accessed, 25 September 2010).

Derache, C., Esnault, E., Bonsergent, C., Le Vern, Y., Quéré, P., Lalmanach, A., 2009. Differential modulation of β-Defensin gene expression by *Salmonella* Enteritidis in intestinal epithelial cells from resistant and susceptible chickens. Dev. Comp. Immunol. 33, 959-966.

Doyle, M.P., Erickson, M.C., 2006. Reducing the carriage of foodborne pathogens in livestock and poultry. Poult. Sci. 85, 960-973.

Evans, E.W., Beach, G.G., Wunderlich, J., Harmon, B.G., 1994. Isolation of antimicrobial peptides from avian heterophils. J. Leuk. Biol. 56, 661-665.

Fuller, R., Jayne-Wiliams, D.J., 1968. The origin of bacteria recovered from the peritoneum and yolk sac of healthy chickens. Br. Poult. Sci. 9, 159-163.

Harmon, B.G., 1998. Avian heterophils in inflammation and disease resistance. Poult. Sci. 77, 972-977.

He, H., Lowry, V.K., Swaggerty, C.L., Ferro, P.J., Kogut, M.H., 2005. In vitro activation of chicken leukocytes and in vivo protection against *Salmonella* Enteritidis organ invasion and peritoneal *S.* Enteritidis infection-induced mortality in neonatal chickens by immunostimulatory CpG Oligodeoxynucleotide. FEMS Immunol. Med. Microbiol. 43, 81-89.

Juul-Madsen, H.R., Viertlboeck, B., Smith, A.L., Göbel, T.W.F., 2008. Avian innate immune responses. In: Davison, F., Kaspers, B., Chat, K.A. (Eds.), Avian Immunology. Academic Press, London, UK, pp. 129-158.

Kizerwetter-Świda, M., Binek, M., 2008. Bacterial microflora of the chicken embryos and newly hatched chicken. Journal of Animal and Feed Sciences 17, 224-232.

Lawrence, E.C., Arnaud-Battandier, F., Grayson, J., Koski, I.R., Dooley, N.J., Muchmore, A.V., Blaese, R.M., 1981. Ontogeny of humoral immune function in normal chickens: a comparison of immunoglobulin-secreting cells in bone marrow, spleen, lungs and intestine. Clin Exp Immunol 43, 450-457.

Payne, J.B., Kroger, E.C., Watkins, S.E., 2005. Evaluation of disinfectant efficacy when applied to the floor of poultry grow-out facilities. J. Appl. Poult. Res. 14, 322-329.

Powers, J.S., Hancock, R.E.W., 2003. The relationship between peptide structure and antibacterial activity. Peptides 24, 1681-1691.

Proudfoot, F.G., Hamilton, R.M.G., DeWitt, W.F., Jansen, H.N., 1991. Raising chicken and turkey broilers in Canada. Agriculture Canada, Ottawa, ON, 1-64.

Rathgeber, B.M., Thompson, K.L., Ronalds, C.M., Budgell, K.L., 2009. Microbiological evaluation of poultry house wall materials and industrial cleaning agents. J. Appl. Poult. Res. 18, 579-582.

Rose, M.E., Hesketh, P., 1974. Fowl peritoneal exudate cells: collection and use for the macrophage migration inhibition test. Avian Pathol. 3, 297-300.

Ruano, M., El-Attrache, J., Villegas, P., 2001. Efficacy comparisons of disinfectants used by the commercial poultry industry. Avian Dis. 45, 972-977.

Sander, J.E., Hofacre, C.L., Cheng, I., Wyatt, R.D., 2002. Investigation of resistance of bacteria from commercial poultry sources to commercial disinfectants. Avian Dis. 46, 997-1000.

SAS Institute Inc., 2002-2003. SAS User's Guide Version 9.1.

Smith, A.L., Beal, R., 2008. The avian enteric immune system in health and disease. In: Davison, F., Kaspers, B., Schat, K.A. (Eds.), Avian Immunology. Academic Press, London, UK, pp. 243-271.

Tankson, J.D., Thaxton, J.P., Vizzier-Thaxton, Y., 2002. Bacteria in the heart and lungs of young chicks. J. Appl. Microbiol. 92, 443-450.

van Dijk, A., Veldhuizen, E.J.A., Kalkhove, S.I.C., Tjeerdsma-van Bokhoven, J.L.M., Romijn, R.A., Haagsman, H.P., 2007. The β-Defensin Gallinacin-6 is expressed in the chicken digestive tract and has antimicrobial activity against food-borne pathogens. Antimicrob. Agents Chemother. 51, 912-922.

Walker, S.E., Sander, J.E., 2004. Effect of BioSentry 904 and Ethylenediaminetetraacetic Acid-Tris disinfecting during incubation of chicken eggs on microbial levels and productivity of poultry. Avian Dis. 48, 238-243.

Ward, P., Fasenko, G.M., Gibson, S., McMullen, L.M., 2006. A microbiological assessment of on-farm food safety cleaning methods in broiler farms. J. Appl. Poult. Res. 15, 1-9.

5. Molecular Typing of *Escherichia coli* and *Salmonella* spp. Isolated from Broiler Chicks and Chicken Pens that Received One of Four Cleaning and Disinfection Treatments

5.1. Introduction

In addition to errors in management practices, first week chick mortality is mainly caused by omphalitis, the infection of the chick's navel and yolk sac (Rai et al., 2005). Avian Pathogenic *Escherichia coli* (APEC) are the most commonly isolated bacteria in the yolk sac of broiler chicks with omphalitis (Rosario Cortés et al., 2004).

Poor sanitation practices have, for a long time, been linked to an increased risk of bacterial contamination leading to disease and mortality of broiler chicks (Fuller and Jayne-Wiliams, 1968). *E. coli in ovo* infection of chicks is possible if breeder hens are affected by salpingitis, oophoritis, or both, and through contamination during artificial insemination (Montgomery et al., 1999). Contamination of newly hatched chicks with *E. coli* has also been shown to be a consequence of fecal contamination of egg shells (Gross, 1994). In addition, penetration of *E. coli* through the unhealed navels of broiler chicks has been demonstrated (Chapter 2 of this thesis). After bacterial invasion APEC spread and colonize vital organs

leading to colisepticaemia with pericarditis, peritonitis, and perihepatitis (Ramirez et al., 2009). The normal absorption of the yolk sac is impaired as a consequence of changes to the yolk sac content due to bacterial infection (Sander et al., 1998) thus depriving the chick of nutrients and maternal antibodies, essential for normal development (Noy and Sklan, 2001). In addition, important pathogens such as *Salmonella* spp. and *Campylobacter* spp. have been isolated from unabsorbed yolk sacs in market-age broilers, extending the relevance of omphalitis from a bird health and production problem to a possible food safety concern (Cox et al., 2006).

The main objectives of this research were 1) To type *E. coli* isolated from broiler chick and environmental samples using the Random Amplification of Polymorphic DNA (RAPD) method, 2) To compare bacterial diversity among different cleaning and disinfection treatments applied to the broiler barn, and 3) To determine how many bacterial types are shared between chicks and the environment. It was hypothesized that 1) Bacterial diversity after chick placement would be greater in pens that received through cleaning and disinfection, and 2) More bacterial types would be shared between chicks and pens belonging to the "cleaner" treatments.

5.2. Materials and methods

All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies (Canadian Council on Animal Care, 2009) with approval from the Animal Care and Use Committee: LIVESTOCK for the University of Alberta.

5.2.1. Sample collection

Experimental procedures for sample collection were described in the previous chapter, section 4.2. Additional to the sampling already described, two 200 g feed samples were collected from the feed bin on the day of chick arrival. In addition to organs and yolk sacs, newly hatched chicks had their intestines removed and pooled.

5.2.2. Isolation of **E. coli** *and* **Salmonella** *spp.*

E. coli from organs, yolk sacs, and the environment were isolated and enumerated on MacConkey agar as previously described. Isolation of *Salmonellas* spp. was performed according to Waltman and Gast (2008). Briefly: 1) Pooled samples of yolk sacs, organs, and intestines were each stomached for two minutes, 10 g samples were weighed into sterile 532 mL (18-oz) Whirl-Pak® Write-On bags (Nasco Canada, Newmarket, ON, Canada), added 90 mL of Luria Bertani (LB) broth (Difco & BD, Mississauga, ON, Canada) and stomached for one additional minute. 2)

Two 25 g feed samples were added 225 mL LB broth and then shaken 20 times through the arc of one foot. 3) Drag swabs (Kingston, 1981) were added 20 mL LB broth and then shaken likewise. For *Salmonella* isolation a non-selective pre-enrichment was carried out at 37°C for 24 h. Selective enrichment was done by inoculating 1 mL of the pre-enriched cultures into 9 mL of tetrathionate (TT) broth (Difco & BD, Mississauga, ON, Canada) and incubating at 37°C for additional 24 h. A 100 µL volume was plated on brilliant green (BG) agar (Difco & BD, Mississauga, ON, Canada) and incubated at 37°C for 24 h (Waltman and Gast, 2008). *Salmonella* spp. colonies were identified by their pink colour on BG agar (Figure 5-1).

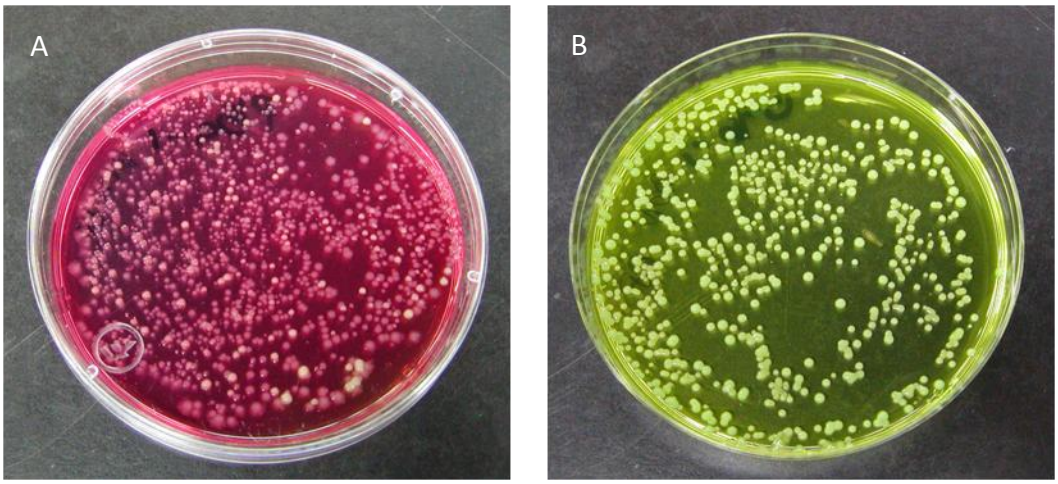


Figure 5-1. Brilliant green agar plates. A) Pink colonies growing on pink agar are suggestive of *Salmonella* spp. B) The presence of bright green colonies and the change in agar colour from red to green is suggestive of *E. coli* growth.

To isolate strains of *E. coli*, the square root of colony counts were purified by randomly picking from MacConkey agar plates used for enumeration. To isolate strains of *Salmonella*, bacterial colonies were purified by randomly picking from BG agar plates used for identification. Single colonies were inoculated in LB broth, incubated overnight at 37°C, streaked on LB (*E. coli*) or BG (*Salmonella*) agars and incubated overnight at 37°C. Purification steps were performed twice. Pure colonies were stored at -80°C in LB with 15% glycerol for further molecular analysis.

5.2.3. Analysis by Random Amplification of Polymorphic DNA (RAPD)

DNA extraction. Due to the large number of samples, the development and standardization of a RAPD protocol using colony PCR was necessary. Frozen colony stocks were grown overnight at 37°C in 96-well microtiter plates containing 300 µL LB broth per well (Figure 5-2 A). Ninety colonies were inoculated in each microtiter plate; four non-inoculated wells containing LB broth were used as negative controls. Two positive controls were included in each plate: *E. coli* K12 and EC234, an APEC strain serotype O78:K80:H9 (Kwaga et al., 1994; van den Hurk et al., 1994) donated by Dr. Brenda Allan (VIDO Vaccine and Infectious Disease Organization, Saskatoon, SK, Canada). *Salmonella enterica* subspecies *enterica* serovar Enteritidis PT8 and *Salmonella enterica* subspecies *enterica*

serovar Hadar PT11 were used as positive controls for *Salmonella* isolates. Sterile wooden toothpicks were used to streak LB agar and obtain single colonies (n= 30 samples per 15 cm agar plate, Figure 5-2 B). Agar plates were incubated overnight at 37°C. A small portion of each bacterial colony was picked with a 10 µL sterile pipette tip and re-suspended in chilled 96-well PCR microplates (Progene®, Ulti-Dent Scientific, St. Laurent, QC, Canada) containing 20 µL cold sterile deionized water per well (Figure 5-2 C). Tips were left in each well (on ice) for approximately 30 min; each colony was stirred in the cold water for about 3 seconds prior to disposing off the tips.

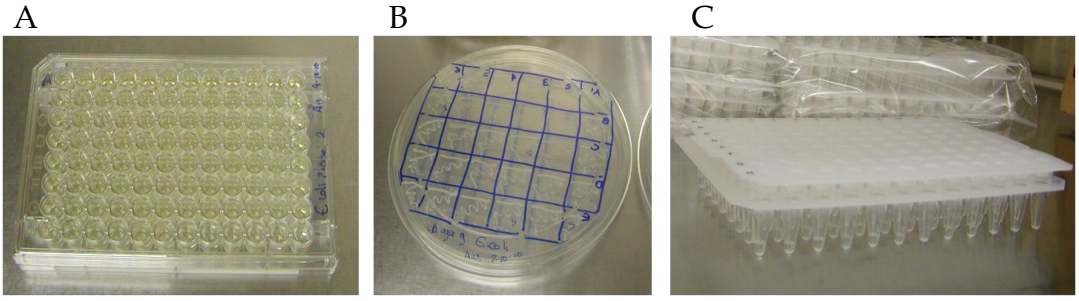


Figure 5-2. Steps involved in colony DNA extraction. A) 90 colonies were grown in 96-well microtiter plates containing 300 µL LB broth per well. B) cultures per 15-cm dish were streaked on LB agar. C) 90 single colonies were inoculated in 96-well PCR microplates containing 20 µL cold sterile deionized water per well.

DNA concentration was determined in 30 random samples using an ND 1000 spectrophotometer (NanoDrop products) at optical densities of 260 and 280 nm. Concentrations of colony DNA ranged between 90 and

120 ng/µL (data not shown). PCR microplates with inoculated single colonies were stored at 4°C for no longer than 3 weeks prior to analysis.

RAPD – PCR conditions. Each PCR master mix was prepared using the Platinum® Taq DNA polymerase kit (Invitrogen Canada, Burlington, ON, Canada) in a final reaction volume of 10 µL. Final concentrations were 1X PCR buffer minus Magnesium, 1.5 mM Magnesium Chloride, 0.2 mM each dNTP, 1.0 unit Platinum® Taq polymerase, and 0.2 µM each primer. The following oligonucleotide primers from Integrated DNA Technologies, Canada, were used: *E. coli* 1254 (5'- CCG CAG CCA A -3) (Madico et al., 1995) and *Salmonella* primer 3 (5'- AAC GCG CAA C -3') (Chansiripornchai et al., 2000). Master mix was prepared for 94 reactions per plate: 90 samples, two positive and one negative control; 9 µL master mix were aliquoted on each well of a chilled 96-well PCR microplates (Progene®) and 1 µL of colony DNA was added as template. In negative control reactions the DNA template was replaced by 1 µL of sterile deionized water. Bacterial DNA was amplified in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using the following programs: For *E. coli*, 2 cycles of 94°C for 5 min, 36°C for 5 min, and 72°C for 5 min; 10 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min; and 20 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2

min; with a final extension of 7 min at 72°C. For *Salmonella* an initial denaturation step of 10 min at 94°C was followed by 40 cycles of: 94°C for 30 s, 36°C for 30 s, and 72°C for 30 s, and a final extension of 7 min at 72°C.

The PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with 1:10000 solution SYBR® Safe DNA gel stain in DMSO (Invitrogen). A volume of 2.5 µL of 6X loading dye (Invitrogen) was aliquoted in each well of a chilled 96-well PCR microplate and mixed with 4 µL PCR product; 6 µL of this mix were pipetted in each well. A 3.5µL volume of 100 bp DNA ladder (Invitrogen) was loaded as reference for each lane. A total of 90 samples, two positive controls and one negative control were run on each gel at 175 V for 70 min on 0.5X TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA pH 8.0). After electrophoresis, gels were scanned in a Typhoon Trio + Imager (General Electrics Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and images were saved.

5.2.4. Similarity analysis of RAPD profiles

The first step in the RAPD profile similarity analysis was to visually identify RAPD types within each one of the 90-sample gels. Six gels for *E. coli* and two gels for *Salmonella* were analyzed. One of each RAPD type per gel was randomly selected and corresponding bacterial colonies were

used to carry out one new 90-sample PCR reaction for each *E. coli* and *Salmonella* samples using the protocols described above. RAPD band profiles within these last two gels were analyzed separately using BioNumerics software version 6.0 (Applied Maths, Austin, TX, USA). All images were normalized using the internal control samples (100 bp ladder). Profile comparisons were performed using the Dice similarity coefficient which allowed generation of dendograms using the unweighted pairwise grouping with mathematical averages (UPGMA) method. Optimization and tolerance were determined for each dendogram using the BioNumeric 6.0 tools. Optimization for *E. coli* profiles was set at 1.8% and for *Salmonella* at 0.3%. Tolerance parameters were set at 1.0% and 1.4% for *E. coli* and *Salmonella*, respectively.

5.2.5. Sequencing analysis

To confirm that colonies obtained on BG agar were *Salmonella* spp., 23 isolates were randomly selected for sequencing based on their RAPD profiles. Frozen cultures were grown at 37° C in 1 mL LB broth, streaked on LB agar and incubated overnight at 37° C to obtain single colonies. DNA from 21 single colonies (two cultures didn't grow) was purified with a DNeasy blood & tissue kit following the manufacturer's specifications (QIAGEN Inc., Mississauga, ON, Canada). PCR amplification of 16S

bacterial DNA was performed using primer combination 616V (AGA GTT TGA TYM TGG CTC AG) and 630R (CAK AAA GGA GGT GAT CC) (Lehner et al., 2004). PCR master mix was prepared using the Platinum® Taq DNA polymerase kit (Invitrogen) as explained above in a final reaction volume of 25 µL. Bacterial DNA was amplified in a GeneAmp PCR System 9700 (Applied Biosystems) with an initial denaturation step of 5 min at 94 °C followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, and 1:30 min at 72°C; with a final extension of 4 min at 72°C. Presence of the 16S DNA band was confirmed on agarose gel electrophoresis using 4 µL of each PCR product. Remaining 21 µL were purified according to manufacturer's instructions of the QIAquick PCR purification kit (QIAGEN Inc.). Final DNA concentrations were measured using a ND 1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA) prior to submitting samples for sequencing (Macrogen Corp., Rockville, MD, USA). All sequences were subjected to BLAST® search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Basic Local Alignment Search Tool) to identify resembling microbial library sequences (Zhang et al., 2000).

5.2.6. Statistical analysis

To examine the structure of the *E. coli* data, principal component (PC) analysis was applied to the *E. coli* counts. In the case of chick samples the following eight variables were included in the analysis: VC day 4, VC day 8, C day 4, C day 8, D day 4, D day 8, VD day 4, VD day 8. For the analysis of barn data, the same eight plus four more variables (VC day 0, C day 0, D day 0, VD day 0) were included. Principal component analysis was performed using the Princomp procedure of SAS (SAS Institute Inc. 2002-2003). The following criteria were used to determine which meaningful components to retain: 1) Any component with an eigenvalue greater than 1.00, 2) Analysis of scree plots in search for an obvious break between eigenvalues, 3) Individual components accounting for at least 10% variance or combined components which cumulative variance accounted for at least 70% of total variance (SAS Institute Inc. 2002-2003).

To analyze the association between samples and RAPD types a categorical model based on maximum likelihood was created in SAS as previously described by Hernandez-Sanabria et al. (2010). Samples from each of the variables mentioned above were first classified as being of chick or barn origin. The effect of each variable on the prevalence of each RAPD type was determined based on the transformation of the cell

probabilities (response function). In SAS proc Catmod a data matrix containing the number of positive samples for a given RAPD type (or zero) was analyzed. After this analysis proc FREQ was used to produce two-way contingency tables containing the frequency of samples per each RAPD type and results were graphed.

5.3. Results

5.3.1. Correlation between variables and **E. coli** *counts*

A total of 493 *E. coli* colonies were purified and used for analysis. Samples collected from the hatchery and from chicks dissected on day 0 accounted for 40% of total *E. coli* isolates (n= 200 colonies). To prevent skewed results in the statistical analyses these 200 samples were analysed separately. We used PC analysis in the remaining 293 samples to identify the structure of the data and their correlation with bacterial counts. For chick samples, two PC accounting for 66% of total variance were retained. The first component (PC1) accounted for 39% of total variance and was associated with the following variables: VC4, C4, D4, and VD4. The second component (PC2) accounted for 27% of total variance and involved VC8, C8, D8, and VD8. Even though cumulative variance of these 2 components did not reach 70%, subsequent components only accounted for 10% variance or less, for this reason only PC1 and PC2 were retained.

When PC1 and PC2 were plotted it was revealed that bacterial counts of chick samples clustered according to sampling time (Figure 5-3).

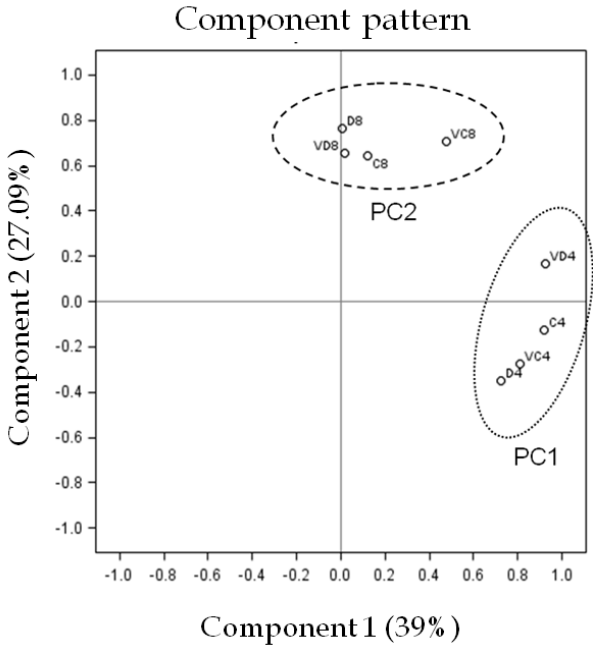


Figure 5-3. Principal component (PC) analysis of bacterial counts from broiler chick samples. The plot of PC1 and PC2 describes the relationship among bacterial counts and sampling variables: cleaning treatment (VC, very clean; C, clean; D, dirty; VD, very dirty) plus collection time (days 4 and 8) in broiler chicks.

For swabs samples collected from the barn the three retained components accounted for 41, 21 and 18% of variance, respectively (cumulative variance 80%). The structure of barn swab data did not reveal a clear clustering pattern: PC1 was associated with VC0 and D0; PC2 was associated with D4 and VD0 (which were orthogonal), and PC3 with C4 and VD4. Not all variables had significant loading in these 3 components (Fig 5-4).

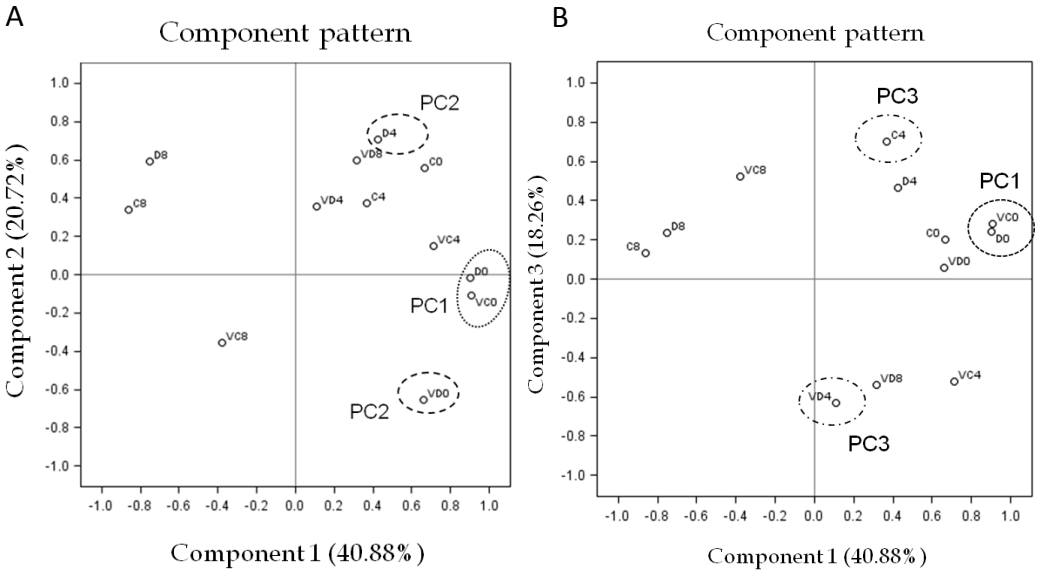


Figure 5-4. Principal component (PC) analysis of bacterial counts from broiler barn swabs. The plots of PC1 vs. PC2 (A) and PC1 vs. PC3 (B) describe the relationship between bacterial counts and sampling variables: cleaning treatment (VC, very clean; C, clean; D, dirty; VD, very dirty) plus collection time (days 0, 4 and 8) in barn swabs.

5.3.2. Sequencing analysis

A total of 199 colonies were suggestive of *Salmonella* spp. based on colony morphology on BG agar; however, only 150 colonies were successfully amplified by RAPD-PCR using *Salmonella* primer 3 (Chansiripornchai et al., 2000) (Figure 5-5, Table 5-1).

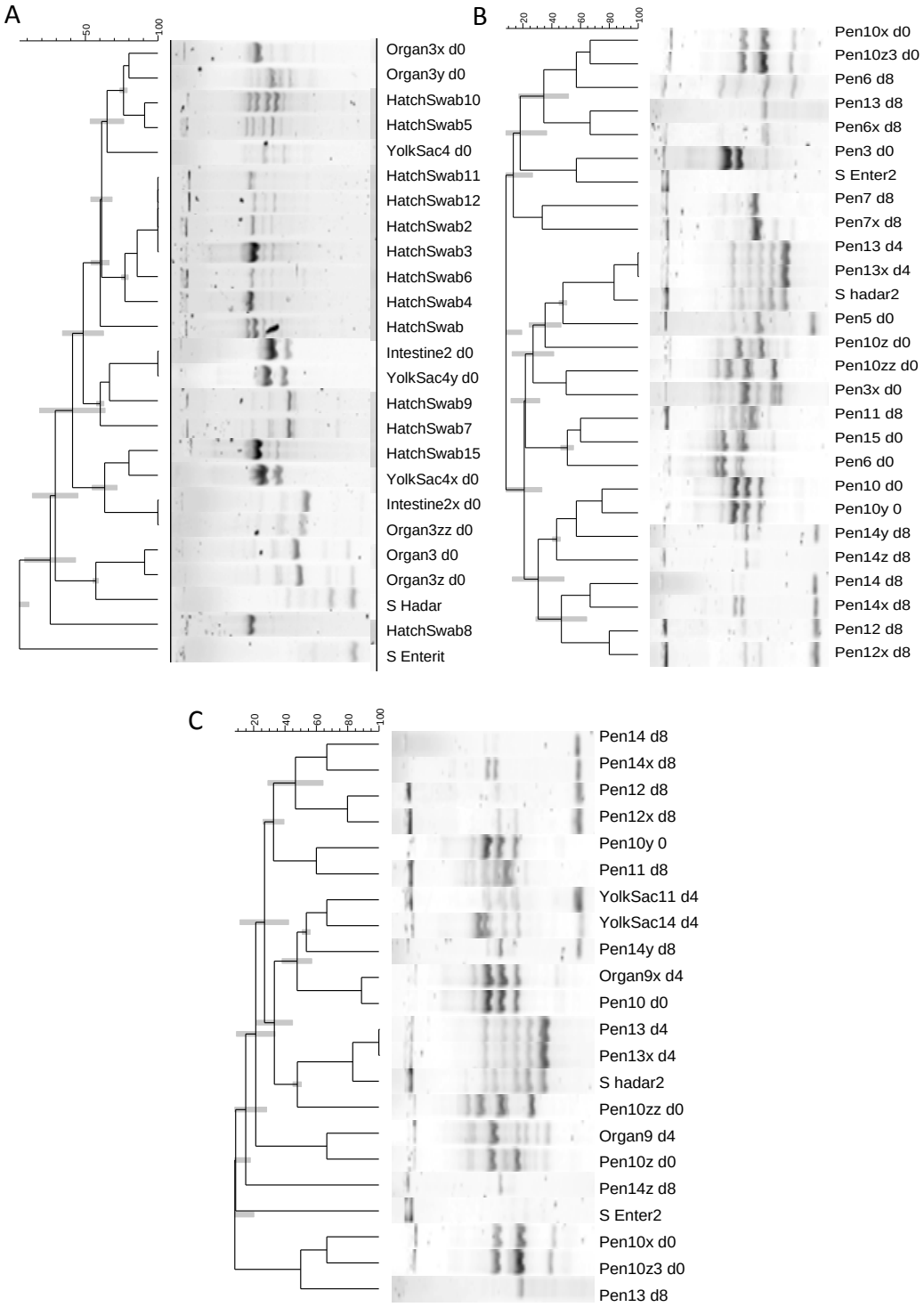


Figure 5-5. RAPD-PCR profiles of 150 *Salmonella*-suggestive isolates. A) Samples isolated on day 0, B) Samples isolated from the barn, C) Samples isolated from chicks and the barn on days 4 and 8. Comparison of RAPD profiles and dendograms were generated by BioNumerics 6.0 software.

Table 5-1. Sample distribution of 150 *Salmonella*-suggestive isolates obtained by culture techniques on brilliant green agar (BGA).

Sample ID	# isolates on BGA	% of total isolates	# samples for sequencing
Hatchery swabs	26	17.3	5
Feed samples	0	0.0	0
Chicks			
Day 0			
Intestines	19	12.7	2
Organs	20	13.3	2
Yolk sacs	3	2.0	1
Day 4			
Organs			
Very Clean	0	0.0	0
Clean	0	0.0	0
Dirty	5	3.3	1
Very Dirty	3	2.0	1
Yolk sacs			
Very Clean	0	0.0	0
Clean	0	0.0	0
Dirty	3	2.0	0
Very Dirty	9	6.0	1
Day 8			
Organs			
Very Clean	0	0.0	0
Clean	0	0.0	0
Dirty	0	0.0	0
Very Dirty	0	0.0	0
Barn swabs			
Day 0			
Very Clean	1	0.7	0
Clean	4	2.7	1
Dirty	12	8.0	1
Very Dirty	2	1.3	1
Day 4			
Very Clean	0	0.0	0
Clean	0	0.0	0
Dirty	0	0.0	0
Very Dirty	7	4.7	1
Day 8			
Very Clean	2	1.3	1
Clean	4	2.7	0
Dirty	9	6.0	1
Very Dirty	21	14	2

Based on the RAPD profiles, 21 samples were sequenced to confirm identity. Based on sequences, all samples collected from the hatchery were

identified as *Pseudomonas putida* a common environmental bacterium. Similarly, none of the samples of chick origin were confirmed as *Salmonella* spp.; instead, they were *Enterobacter* spp. or *Klebsiella pneumoniae*. Three samples of barn origin (C day 0, VD day 4, and D day 8) were confirmed as *S.* Paratyphi (98% identity), *S.* Hadar (99% identity) and *S.* Gallinarum (98 % identity), respectively (Table 5-2).

Table 5-2. Taxonomic identification and GenBank accession numbers of 21 *Salmonella*-suggestive isolates subjected to sequencing. ★= *Salmonella* spp.

Sample ID	Taxonomy (GenBank accession number)	Identity (%)
Hatchery swab 1	*Pseudomonas putida* (NC_010322.1)	99
Hatchery swab 2	*Pseudomonas putida* (NC_010322.1)	99
Hatchery swab 3	*Pseudomonas putida* (NC_010322.1)	99
Hatchery swab 4	*Pseudomonas putida* (NC_010322.1)	99
Hatchery swab 5	*Pseudomonas putida* (NC_010322.1)	99
Yolk Sacs day 0	*Pseudomonas putida* (NC_010322.1)	99
Organs day 0	*Pseudomonas* sp. TJI-51 Scaffold032 (AEWE01000048.1)	98
Organs day 0	*Enterobacter* sp. 638 (NC_009436.1)	93
Intestines day 0	*Enterobacter* sp. 638 (NC_009436.1)	93
Intestines day 0	*Enterobacter* sp. 638 (NC_009436.1)	93
★ Pen "Clean" day 0	*Salmonella enterica* subsp. *enterica* serovar Paratyphi C strain RKS4594 (NC_012125.1)	98
Pen "Dirty" day 0	*Enterobacter cloacae* subsp. *cloacae* ATCC 13047 (NC_014121.1)	96
Pen "Very Dirty" day 0	*Escherichia coli* 97.0264 ctg1125157569984 (AEZP01000124.1)	96
Organs "Dirty" day 4	*Klebsiella pneumoniae* subsp. *rhinoscleromatis* ATCC 13884 contig00038 (ACZD01000038.1)	99
Organs "Very Dirty" day 4	*Klebsiella pneumoniae* subsp. *rhinoscleromatis* ATCC 13884 contig00038 (ACZD01000038.1)	99
★ Pen "Very Dirty" day 4	*Salmonella enterica* subsp. *enterica* serovar Hadar str. RI_05P066 gcontig_1106390391968 (ABFG01000019.1)	99
Yolk Sacs "Very Dirty" 4	*Klebsiella pneumoniae* NTUH-K2044 (NC_012731.1)	99
Pen "Very Clean" day 8	*Pseudomonas putida* (NC_010322.1)	97
★ Pen "Dirty" day 8	*Salmonella enterica* subsp. *enterica* serovar Gallinarum str. SG9 strain 9 SG9_genome_3 (AEUL01000002.1)	98
Pen "Very Dirty" day 8	*Enterobacter* sp. 638 (NC_009436.1)	98
Pen "Very Dirty" day 8	*Enterobacter* sp. 638 (NC_009436.1)	98

5.3.3. Similarity analysis of **E. coli** *RAPD profiles*

The 493 *E. coli* isolates were re-classified by BioNumerics 6.0 into 55 types based on their RAPD profiles (Figure 5-6).

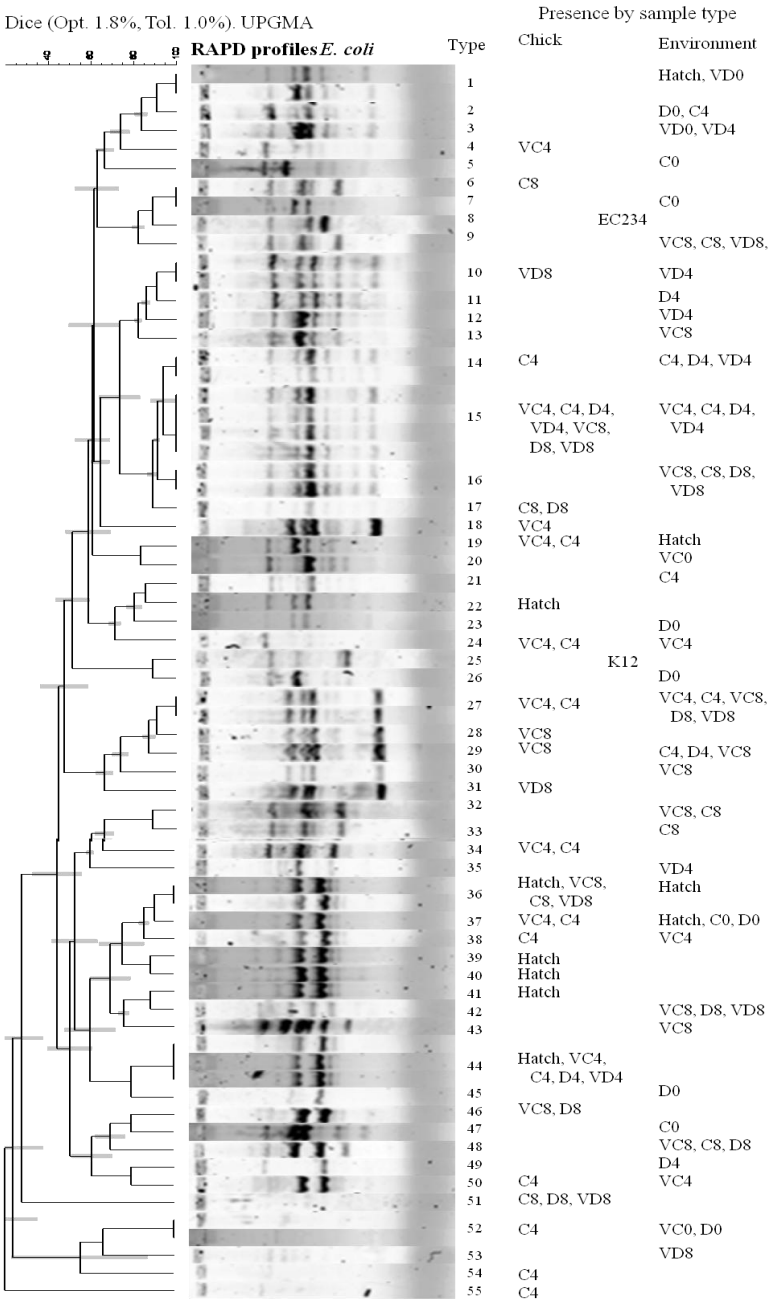


Figure 5-6. RAPD-PCR profiles of 493 *E. coli* colonies isolated from organs and yolk sacs of broiler chicks and from hatchery and barn swabs.

To study the relationship between a specific RAPD type and a particular variable, a maximum likelihood analysis was performed. As noted above, the statistical analysis of hatchery and chick samples collected on day 0 was performed separately. From the nine *E. coli* RAPD types identified from hatchery and chick samples from day 0 three types were significantly associated with chick origin: RAPD type #37 ($P < 0.0001$), RAPD type #40 ($P = 0.0062$) and RAPD #41 ($P = 0.0092$).

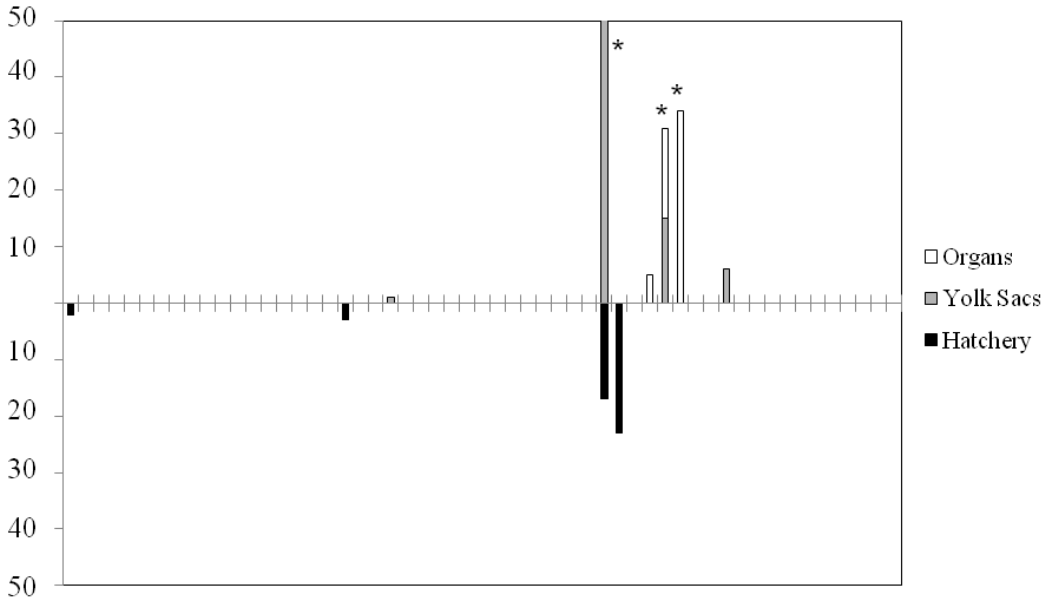


Figure 5-7. Frequency counts of RAPD types of 200 *E. coli* colonies isolated from organs and yolk sacs of broiler chicks (top columns) and from the hatchery samples (bottom columns). The x axis represents 55 RAPD types identified by BioNumerics 6.0. The y axis represents number of isolated colonies (n= 200). Significant associations between RAPD type and sample type are identified by a star.

No significant associations were observed between RAPD type and the interaction of sampling day by sampling source (data not shown). RAPD types were then analyzed according to cleaning treatment irrespective of sampling day with the following results: *E. coli* from the VC treatment were divided into 25 RAPD types; one RAPD type (#36) was associated with chick origin (P = 0.050) (Figure 5-8 A). In the C treatment *E. coli* belonged to 28 RAPD types; two RAPD types were associated with chick origin (#36, P = 0.0155; and #44, P =0.050) (Figure 5-8 B). Nineteen RAPD types of *E. coli* were isolated from the D treatment but no RAPD type was associated with a specific sample source (Figure 5-8 C). Likewise, 16 RAPD types of *E. coli* were determined for the VD treatment with no association between type and sample source (Figure 5-8 D).

Our main interest was to determine how many RAPD types were shared between chick and environment samples. One RAPD type (# 37) was shared between yolk sacs and hatchery environment, and one RAPD type (#40) was shared between organs and yolk sacs (Figure 5-7). In the VC treatment two RAPD types (#24 and #27) were shared between organs and the barn, one between yolk sac and the barn (# 29), and one RAPD type (#15) was shared between organs, YS, and the barn (Figure 5-8 A). In the C treatment two RAPD types (#14 and #36) were shared between

organs and the barn, one RAPD type (# 26) was shared between yolk sacs and the barn, one RAPD type (# 43) was shared between yolk sacs and organs, and one RAPD type (#15) was shared between yolk sacs, organs, and the barn Figure 5-8 B).

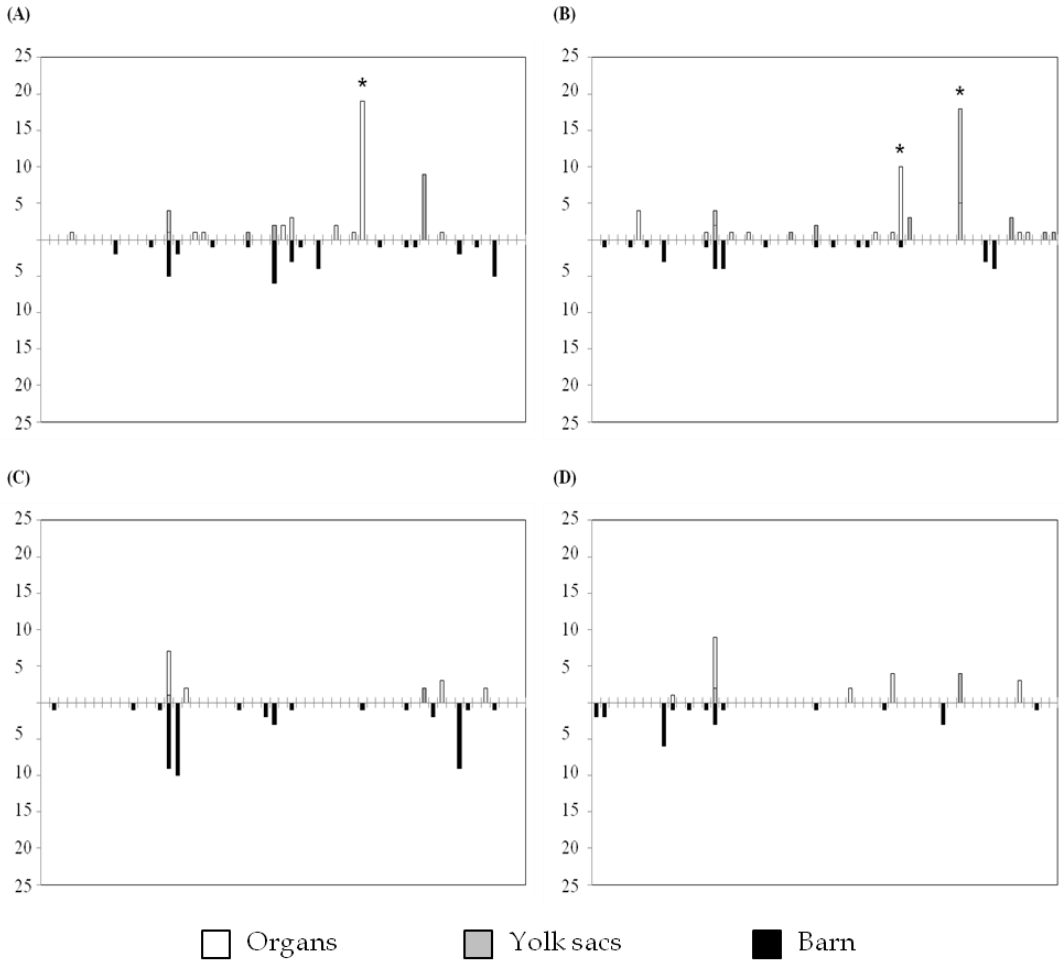


Figure 5-8. Frequency counts of RAPD types of 293 *E. coli* colonies isolated from chicks organs and yolk sacs (top columns) and from barn swabs (bottom columns) compared according to cleaning and disinfection treatment: A) Very clean, B) Clean, C) Dirty, and D) Very dirty. The x axis represents 55 RAPD types identified and localized by BioNumerics 6.0. The y axis represents number of isolated colonies (n = 293). Significant associations between RAPD type and sample type are identified by a star.

Except for *E. coli* RAPD type #15 which was shared between barn, yolk sacs and organs regardless of cleaning and disinfection treatment, no *E. coli* RAPD types were shared between chick samples and barn samples in the D or VD treatments (Figures 5-8 C, D).

The main findings of this study were: 1) Six *E. coli* RAPD types (10.9%) had a significant association with a given sample type, remaining 49 RAPD types were randomly present. 2) Nine *E. coli* RAPD types were isolated from hatchery and newly hatched chicks, 25 types from the VC treatment, 28 from the C treatment, 19 from the D treatment and 16 *E. coli* RAPD types from the VD treatment. 3) A specific *E. coli* RAPD type was shared between environment and chick samples in 11 cases: one at the hatchery, four in the VC treatment, three in the C treatment, one in the D treatment and two in the VD treatment. These results suggest that chick and environment are linked to particular *E. coli* RAPD types and that the cleaning and disinfection process reduced the *E. coli* diversity in the barn.

E. coli RAPD #36 and #15 were sequenced and identified with 99% identity as *Escherichia coli* 9.1649 (GenBank accession number AEZY01000031.1) and *Escherichia coli* 95.0941 (GenBank accession number AEZN01000046.1), respectively.

A total of 53 RAPD profiles were obtained with primer *Salmonella* 3 on the 150 BGA-positive isolates (dendogram not shown). There were no significant associations between RAPD type and sample type. Three RAPD types were shared between chick and barn samples: two from the VD treatment and one from the D treatment (Figure 5-9).

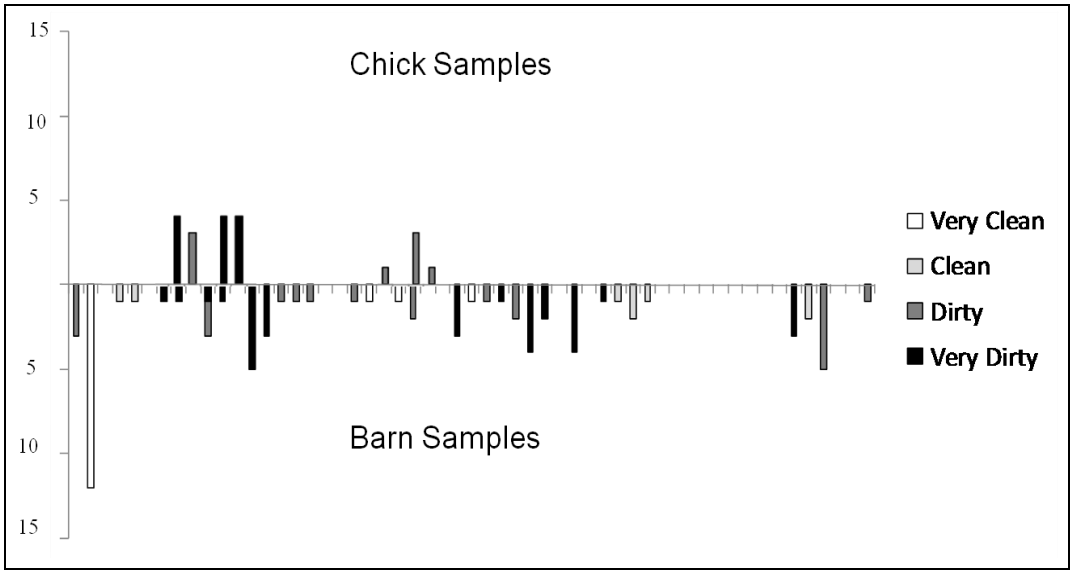


Figure 5-9. Frequency counts of RAPD types of 124 *Salmonella*-suggestive colonies isolated from chick samples and barn swabs. Chick samples (organs, intestines and yolk sacs) are represented by the top columns and barns swabs by the bottom columns. Cleaning and disinfection treatments are identified by different shades of grey. The x axis represents 55 RAPD types identified and localized by BioNumerics 6.0. The y axis represents number of isolated colonies (n = 124).

5.4. Discussion

The main objective of this research project was to evaluate if barn sanitation affected the diversity of *E. coli* present in pooled yolk sacs and pooled organs (heart, liver and spleen) of broiler chicks up to eight days post-hatching. The RAPD method was used to characterize *E. coli* isolated from chick and environmental samples to determine how many bacterial types were shared between them. Characterization options for APEC strains include serotyping by agglutination against O antigens O1, O2 and O78 (Dho-Moulin and Fairbrother, 1999), multilocus enzyme electrophoresis (Ngeleka et al., 1996), pathogenicity tests (Dias da Silveira et al., 2002), PCR amplification of specific genes (Amabile de Campos et al., 2005), and pulsed-field gel electrophoresis (PFGE) (Ewers et al., 2004). Additionally, RAPD analysis has been used as an effective method for typing *E. coli* isolates and determine strain relationships (Maurer et al., 1998; Vogel et al., 2000). To characterize the large number of *E. coli* isolates obtained from environmental and chick samples we decided to use the RAPD method with a short unspecific primer instead of targeting specific virulence genes of APEC. The greatest difficulty encountered was the standardization of a colony PCR protocol. The rationale behind using colony DNA for the PCR reactions was to save on time and resources necessary for standard DNA extraction.

The first hypothesis tested and confirmed was that *E. coli* diversity after chick placement would be greater in pens receiving through cleaning and disinfection. This hypothesis was based on the concept of bacterial competition: it was expected that "cleaner" pens would have greater variability of *E. coli* types after 4 and 8 days of chick placement because *E. coli* populations of these pens would be in the process of being established. In turn, "dirtier" pens at chick placement would have already had an established *E. coli* population that would compete with entering microorganisms. The study of bacterial competition is outside the scope of this project, detailed reviews on this subject are available elsewhere for the reader's interest (Hibbing et al., 2010).

The second hypothesis tested and also confirmed was that more *E. coli* types would be shared between chicks and pens belonging to the "cleaner" treatments. This hypothesis was based on observations described on the previous chapter that chicks reared on very clean environments carried more *E. coli* in their organs and yolk sac at 4 days of age than chicks reared in "dirtier" environments. If the previous hypothesis that chicks placed in very clean environments are slower at preventing translocation and at clearing bacteria from their peritoneal cavity proves accurate, microbial flora of these chicks (specifically *E. coli*)

would be more influenced by the environment's microbial flora in the early post-hatching period.

Colonization of chicks with *E. coli* is known to start in the first hours after hatching leading to rapid replication in the intestine (Dho-Moulin and Fairbrother, 1999). The role of hatchery transfer of *E. coli* to the newly hatched chick has been previously studied as *E. coli* colonization will start when chicks are still in the hatching baskets (Johnson et al., 2001). The possibility of vertical transfer of APEC from broiler breeders to newly hatched chicks leading to yolk sac infections and colisepticaemia has also been shown (Giovanardi et al., 2005). Since the late 1960's it has been suggested that bacteria translocate from the intestine of apparently healthy chicks to the peritoneum and from there to the yolk sac (Fuller and Jayne-Wiliams, 1968). In the present study a significant association between one *E. coli* RAPD type and the yolk sac of newly hatched chicks was found.

In the present study it was also determined if cleaning and disinfection treatments were related to *Salmonella* isolation. Out of the initial 199 *Salmonella*-suggestive isolates obtained by culture methods only 150 colonies were successfully amplified by RAPD-PCR. Out of the 21 sequenced samples only three samples of barn origin (C day 0, VD day 4,

and D day 8) were confirmed as *S.* Paratyphi (98% idendity), *S.* Hadar (99% identity) and *S.* Gallinarum (98 % identity), respectively. Bacteria isolated from hatchery swabs were identified as *Pseudomonas putida* (99% identity), a common non-pathogenic organism previously isolated from commercial hatcheries (Tymczyna et al., 2007). Bacteria isolated from chick samples were either *Enterobacter, Klebsiella* or *Pseudomonas* spp. These Gram-negative bacteria were not isolated by Tankson et al. (2002) in their microbiological analysis of hearts and lungs collected between 17 days of incubation and 21 days post-hatching (Tankson et al., 2002). However, their results should not be compared with the present study because in the present experiment chick samples consisted of pooled yolk sacs, intestines, or organs (hearts, livers and spleens) collected the day of hatching and at 4 and 8 days post-hatching. From the current experiment it cannot be determined whether chicks acquired *Enterobacter, Klebsiella* and *Pseudomonas* spp. from the hatchery environment.

From this portion of the experiment it can be concluded that the culture methods chosen for isolation of *Salmonella* were not accurate when used alone and molecular confirmation was necessary. Furthermore, the laboratory methods used for recovery and culture of *Salmonella* spp. were complicated and time-consuming. Besides the use of RAPD analysis

(Chansiripornchai et al., 2000), real-time PCR (qRT-PCR) has been used as a screening tool for *Salmonella* spp. when dealing with large numbers of samples with promising results (Szmolka et al., 2006; Mainali et al., 2011). Future applications of qRT-PCR may increase the detection sensitivity of this pathogen in environmental and chick samples. It is important to point out that *Salmonella* spp. were not isolated from VC pens. Although results obtained from the chosen culture methods were not accurate and not all isolates were sequenced, it should be remembered that these pens had been thoroughly washed and disinfected suggesting that a comprehensive and strict cleaning and disinfection regime is still an essential tool for controlling this pathogen (Fris and Van den Bos, 1995).

Under natural conditions microbial colonization of the newly hatched chick's intestine starts with the ingestion of the hen's feces, however, in commercial poultry operations, chicks do not have contact with the breeder hen. In the early 1970's Nurmi and Rantala made the observation that the "abnormally hygienic conditions" in which commercial broiler chickens are produced hinder the development of the intestinal microflora necessary to prevent colonization by pathogenic bacteria. By administering normal adult chicken crop and intestinal contents to newly hatched chicks they were able to prevent intestinal colonization by

Salmonella Infantis (Nurmi and Rantala, 1973), this was the first ever published experiment on competitive exclusion in poultry. Ever since this publication, multiple studies have investigated into different options for administering competitive exclusion bacteria in commercial poultry operations with the objective of preventing *Salmonella* infections. These options include administration of competitive exclusion "cocktails" in drinking water, spray application at the hatchery (Mead and Barrow, 1990), and competitive exclusion cocktail administration *in ovo* (Cox and Bailey, 1993).

The use of competitive exclusion in poultry was initially aimed at preventing intestinal colonization by the foodborne pathogen *Salmonella* Enteritidis. However, Hofacre (2002) proved that oral administration of a competitive exclusion cocktail to day-old broiler chickens decreased intestinal colonization of an antibiotic-resistant APEC strain at 7 and 14 days of age (Hofacre et al., 2002). In the present experiment, fewer *E. coli* types were shared between chicks and the environment when chicks were placed in "dirtier" pens. Whether this effect was due to competitive exclusion is unknown.

5.5. Conclusion

The main objective of this research was to evaluate if barn sanitation affected environmental and chick *E. coli* diversity up to eight days post-hatching. By typifying *E. coli* isolated from environmental and broiler chick samples using the RAPD method it was determined how many *E. coli* types were shared between them. It was confirmed that *E. coli* diversity after chick placement was greater in pens receiving throughout cleaning and disinfection than in pens that were not thoroughly cleaned, perhaps due to bacterial competition. It was also determined that chicks placed in "cleaner" pens shared more *E. coli* types with the environment than chicks placed in "dirtier" pens. Even though it seemed that throughout cleaning and disinfection could be increasing the rate of *E. coli* transfer from the barn to the chicks (by unknown mechanisms), a strict cleaning and disinfection regime seemed to prevent the presence of *Salmonella* spp. in the environment.

In summary, throughout cleaning and disinfection of chicken houses is an essential component of the biosecurity program to prevent disease outbreaks in poultry operations. Raising broiler chicks in very clean environments has been an obligate practice for modern commercial poultry even though it goes against chicken nature and impairs the

establishment of a desired intestinal microflora (Nurmi and Rantala, 1973). More research is needed in order to find ways to expose newly hatched chicks to environmental microorganisms and achieve controlled bacterial intestinal colonization. This could be a promising option to protect chicks against different pathogens, especially avian and foodborne pathogens.

5.6. References

Amabile de Campos, T., Stehling, E.G., Ferreira, A., Pestaña de Castro, A.F., Brocchi, M., Dias da Silveira, W., 2005. Adhesion properties, fimbrial expression and PCR detection of adhesin-related genes of avian *Escherichia coli* strains. Vet. Microbiol. 106, 275-285.

Barnes, J.H., Vaillancourt, J.P., Gross, W.B., 2003. Colibacillosis. In: Saif, Y.M., Barnes, H.J., Fadly, A.M. et al (Eds.), Diseases of poultry. Blackwell Publishing, Ames, Iowa, USA, pp. 631-656.

Canadian Council on Animal Care, 2009. Guidelines on the care and use of farm animals in research, teaching and testing. Available at: http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/PDFs/Farm_Animals.pdf (Accessed, 21 May 2010).

Chansiripornchai, N., Ramasoota, P., Bangtrakulnonth, A., Sasipreeyajan, J., Svenson, S.B., 2000. Application of Randomly Amplified

Polymorphic DNA (RAPD) analysis for typing avian *Salmonella enterica* subsp. *enterica*. FEMS Immunol. Med. Microbiol. 29, 221-225.

Cox, N.A., Bailey, J.S., 1993. Introduction of bacteria in ovo. Patent # 362635.

Cox, N.A., Richardson, L.J., Buhr, R.J., Northcutt, J.K., Fairchild, B.D., Mauldin, J.M., 2006. Presence of inoculated *Campylobacter* and *Salmonella* in unabsorbed yolks of male breeders raised as broilers. Avian Dis. 50, 430-433.

Dho-Moulin, M., Fairbrother, J.M., 1999. Avian Pathogenic *Escherichia coli* (APEC). Vet. Res. 30, 299-316.

Dias da Silveira, W., Ferreira, A., Brocchi, M., Maria de Hollanda, L., Pestaña de Castro, A.F., Tatsumi Yamada, A., Lancellotti, M., 2002. Biological characteristics and pathogenicity of avian *Escherichia coli* strains. Vet. Microbiol. 85, 47-53.

Ewers, C., Janßen, T., Kießling, S., Philipp, H., Wieler, L.H., 2004. Molecular epidemiology of avian pathogenic *Escherichia coli* (APEC) isolated from colisepticemia in poultry. Vet. Microbiol. 104, 91-101.

Fris, C., Van den Bos, J., 1995. A retrospective case-control study of risk factors associated with *Salmonella enterica* subsp. *enterica* serovar

Enteritidis infections on Dutch broiler breeder farms. Avian Pathol. 24, 255-272.

Fuller, R., Jayne-Wiliams, D.J., 1968. The origin of bacteria recovered from the peritoneum and yolk sac of healthy chickens. Br. Poult. Sci. 9, 159-163.

Giovanardi, D., Campagnari, E., Ruffoni, L.S., Pesente, P., Ortali, G., Furlattini, V., 2005. Avian pathogenic *Escherichia coli* transmission from broiler breeders to their progeny in an integrated poultry production chain. Avian Pathol. 34, 313-318.

Gross, W.G., 1994. Diseases due to *Escherichia coli* in poultry. In: Gyles, C.L. (Ed.), *Escherichia coli* in domestic animals and humans. CAB International, Willingford, UK, pp. 237-259.

Hernandez-Sanabria, E., Guan, L.L., Goonewardene, L.A., Li, M., Mujibi, D.F., Stothard, P., Moore, S.S., Leon-Quintero, M.C., 2010. Correlation of particular bacterial PCR-Denaturing Gradient Gel Electrophoresis patterns with bovine ruminal fermentation parameters and feed efficiency traits. Appl. Environm. Microbiol. 76, 6338-6350.

Hibbing, M.E., Fuqua, C., Parsek, M.R., Peterson, S.B., 2010. Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. 8, 15-25.

Hofacre, C.L., Johnson, A.C., Kelly, B.J., Froyman, R., 2002. Effect of a commercial competitive exclusion culture on reduction of colonization of an antibiotic-resistant pathogenic *Escherichia coli* in day-old broiler chickens. Avian Dis. 46, 198-202.

Johnson, L.C., Bilgili, S.F., Hoerr, F.J., McMurtrey, B.L., Norton, R.A., 2001. The effects of early exposure of cellulitis-associated *Escherichia coli* in 1-day-old broiler chickens. Avian Pathol. 30, 175-178.

Kingston, D.J., 1981. A comparison of culturing drag swabs and litter for identification of infections with *Salmonella* spp. in commercial chicken flocks. Avian Dis. 25, 513-516.

Kwaga, J.K.P., Allan, B.J., van den Hurk, J.V., Seida, H., Potter, A.A., 1994. A *car*AB mutant of avian pathogenic *Escherichia coli* serogroup O2 is attenuated and effective as a live oral vaccine against colibacillosis in turkeys. Infetc. Immun. 62, 3766-3772.

Lehner, A., Tasara, T., Stephan, R, 2004. 16S rRNA gene based analysis of *Enterobacter sakazakii* strains from different sources and development of a PCR assay for identification. BMC Microbiol. 4, 1-7.

Madico, G., Akopyants, N.S., Berg, D.E., 1995. Arbitrary primer PCR DNA fingerprinting of *Escherichia coli* O157:H7 strains by using templates from boiled cultures. J. Clin. Microbiol. 33, 1534-1536.

Mainali, C., McFall, M.E., King, R.K., 2011. Validation of a Real-Time Polymerase Chain Reaction assay for the detection of *Salmonella* in crops of broiler chickens. Poult. Sci. 90, 660-664.

Maurer, J.J., Lee, M.D., Lobsinger, C., Brown, T., Maier, M., Thayer, S., 1998. Molecular typing of avian *Escherichia coli* isolates by Random Amplification of Polimorphic DNA. Avian Dis. 42, 431-451.

Mead, G.G., Barrow, P.A., 1990. *Salmonella* control in poultry by 'competitive exclusion' or immunization. Letters Appl. Microbiol. 10, 221-227.

Montgomery, R.D., Boyle, C.R., Lenarduzzi, T.A., Jones, L.S., 1999. Consequences to chicks hatched from Escherichia coli-inoculated embryos. Avian Dis. 43, 553-563

Ngeleka, M., Kwaga, J.K.P., White, D.G., Whittam, T.S., Riddell, C., Goodhope, R., Potter, A.A., Allan, B., 1996. *Escherichia coli* cellulitis in broiler chickens: clonal relationships among strains and analysis of virulence-associated factors of isolates from diseased birds. Infect. Immun. 64, 3118-3126.

Noy, Y., Sklan, D., 2001. Yolk and exogenous feed utilization in the posthatch chick. Poult. Sci. 80, 1490-1495.

Nurmi, E., Rantala, M., 1973. New aspects of *Salmonella* infection in broiler production. Nature 241, 210-211.

Rai, M.F., Khan, S.A., Aslam, A., Saeed, K., 2005. Effects of yolk sac infection in chicken. Avian Poult. Biol. Rev. 16, 87-93.

Ramírez, R.M., Almanza, Y., García, S., Heredia, N., 2009. Adherence and invasion of avian pathogenic *Escherichia coli* to avian tracheal epithelial cells. World J. Microbiol. Biotechnol. 25, 1019-1023.

Rosario Cortés, C., Téllez, I.G., López Cuello, C., Villaseca Flores, J.M., Anderson, R.C., Eslava Campos, C., 2004. Bacterial isolation rate from fertile eggs, hatching eggs and neonatal broilers with yolk sac infection. Rev. Latinoam. Microbiol. 46, 12-16.

Sander, J.E., Willinghan, E.M., Wilson, J.L., Thayer, S.G., 1998. The effect of inoculating *Enterococcus faecalis* into the yolk sac on chick quality and maternal antibody absorption. Avian Dis. 42, 359-363.

SAS Institute Inc., 2002-2003. SAS User's Guide Version 9.1.

Szmolka, A., Kaszanyitzky, É., Nagy, B., 2006. Improved diagnostic and Real-Time PCR in rapid screening for *Salmonella* in the poultry food chain. Acta Vet. Hung. 54, 297-312.

Tankson, J.D., Thaxton, J.P., Vizzier-Thaxton, Y., 2002. Bacteria in the heart and lungs of young chicks. J. Appl. Microbiol. 92, 443-450.

Tymczyna, L., Chmielowiec-Korzeniowska, A., Drabik, A., 2007. The effectiveness of various biofiltration substrates in removing bacteria, endotoxins, and dust from ventilation system exhaust from a chicken hatchery. Poult. Sci 86, 2095-2100.

van den Hurk, Jan V., Allan, B.J., Riddell, C., Watts, T., Potter, A.A., 1994. Effect of infection with hemorrhagic enteritis virus on susceptibility of turkeys to *Escherichia coli*. Avian Dis. 38, 708-716.

Vogel, L., van Oorschot, E., Maas, H.M.E., Minderhoud, B., Dijkshoorn, L., 2000. Epidemiologic typing of *Escherichia coli* using RAPD analysis, ribotyping and serotyping. Clin. Microbiol. Infect. 6, 82-87.

Waltman, W.D., Gast, R.K., 2008. Salmonellosis. In: Dufour-Zavala, L., Swayne, D.E., Glisson, J., R. et al (Eds.), A laboratory manual for the isolation, identification and characterization of avian pathogens. American Association of Avian Pathologists, Athens, GA, pp. 3-9.

Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7, 203-214.

6. Yolk Sac Infections in Broiler Chicks: Conclusion

Why study yolk sac infections? The avian yolk sac develops as a well vascularised membrane that surrounds the yolk of an embryonated egg (Romanoff, 1960). This extraembryonic membrane is essential for the transfer of nutrients including IgY, the maternal antibodies, from the yolk to the developing embryo (Romanoff, 1960). However, the infection of the yolk sac and the navel during the first week of the post-hatching period is the main infectious cause of broiler chick mortality (Rai et al., 2005). After a localized *E. coli* infection, spread and colonization of vital organs lead to colisepticaemia (Ramirez et al., 2009). Death is the most common outcome of yolk sac infections, however, stunted growth and poor performance are sequelae observed in chicks surviving an infection of the yolk sac (Barnes et al., 2003) leading to further economic losses. The impaired absorption of the yolk sac content as a consequence of yolk sac infections deprives the chick of nutrients and maternal antibodies essential for normal chick development (Noy and Sklan, 2001). Recently, zoonotic pathogens such as *Salmonella* spp. and *Campylobacter* spp. have been isolated from unabsorbed yolk sacs in market-age broiler chickens (Cox et al., 2006). This suggests that yolk sac infections not only affect bird health but may

also pose a risk to food safety if there is cross contamination during processing (Cox et al., 2006). Current research on the similarities between avian pathogenic *E. coli* (APEC) and human uropathogenic *E. coli* (UPEC) raised concerns on the foodborne potential of APEC (Skyberg et al., 2006; Johnson et al., 2007).

The overall objective of this thesis was to provide a comprehensive study of yolk sac infections in broiler chicks. The approach to this problem was based on Snieszko's epidemiological triad (1974) which considered that an infectious disease affecting humans, animals, or both, was determined by the interactions between three factors: a virulent pathogen, a susceptible host, and the environment (Snieszko, 1974).

The introductory chapter provided an extensive literature review on the main pathogen involved in yolk sac infections: *Escherichia coli* (Rosario Cortés et al., 2004). Subsequent chapters offered research results for four different experiments.

In the experiment from the second chapter a strain of APEC was transformed with a plasmid carrying a green fluorescence protein. The objective of this experiment was to scientifically prove that *E. coli* can enter the chick navel and colonize the yolk sac. Fluorescent *E. coli* were placed on the navel area of newly hatched chicks and tracked using fluorescent

microscopy in samples collected from day 0 to 5 post-hatching. Results from this experiment concluded that *E. coli* were able to reach the yolk sac via the chick navel. Also, it was observed that a greater percentage of chicks hatching with unhealed navels vs. chicks with healed navels were positive for fluorescent *E. coli* in their yolk sacs.

The third chapter dealt with the susceptible host of yolk sac infections, in this case, the broiler chick. This experiment studied whether there were differences in maternal antibodies present in egg yolk and in the yolk sac of chicks. The objective was to determine if broiler breeder flock age had an effect on the total IgY content of egg yolks and yolk sacs. The rationale behind this experiment was that newly hatched chicks totally depend on maternal antibodies for their antibody-mediated immune response during the early post-hatching period (Brambell, 1970). It was concluded that the total IgY content of egg yolk and yolk sacs increased with breeder flock age, perhaps as a consequence of longer antigenic exposure in older hens. It would be interesting to test whether these findings would be related to a chick's ability to fight yolk sac infections.

The fourth and fifth chapters of this thesis studied the last factor of the epidemiological triad: a suitable environment, in this case the broiler chicken barn. The experiment from chapter 4 compared microbial counts

for total aerobic bacteria, *Enterobacteriaceae* and *E. coli* obtained from broiler pens that had received one of four cleaning and disinfection treatments based on Canadian On Farm Food Safety Assurance Program standards. Microbial counts were also determined in pooled organs and pooled yolk sacs collected from chicks that came directly from the hatchery, and in chicks that were placed in those pens after 4 and 8 days. Interesting and unexpected results were obtained from this experiment: after 4 days of having placed the chicks in the barn, the organs and yolk sacs of chicks from the "cleaner" pens had more bacteria than those of chicks from "dirtier" pens. This was a transient effect: at 8 days post-hatching there were no differences in bacterial counts among chicks of different cleaning and disinfection treatments. It will be of great interest to determine what mechanisms are allowing chicks placed in dirtier pens to clear bacteria faster than chicks placed in cleaner pens. Also, it should be investigated if broiler performance, diseases susceptibility and mortality would be affected by cleaning and disinfection of the chicken barn.

In the fifth chapter, the RAPD (Random Amplification of Polymorphic DNA) method was used to typify all *E. coli* isolated from chick and environmental samples described in chapter four. As expected, *E. coli* variability was greater in "cleaner" than in "dirtier" pens, most likely due

to less bacterial competition in populations that are being established in clean environments. Also, more *E. coli* types were shared between the environment and the chicks in "cleaner" pens suggesting that chicks that were placed in very clean environments were exposed to greater *E. coli* diversity and, perhaps as a consequence, acquired more types of environmental *E. coli* than chicks placed in "dirtier" environments. The same comment provided in the previous paragraph applies for this chapter. No recommendations on cleaning and disinfection methods of chicken barns can be provided before studying the consequences of these research findings on important bird health, welfare and production parameters.

In summary, yolk sac infections in broiler chicks, as any other infectious disease, is determined by the interactions between pathogen - host – and environment. This thesis studied some factors related to *E. coli*, the broiler chick, and the broiler barn. It was determined that 1) *E. coli* can enter the navel and reach the yolk sac, and that navel healing at hatching had a significant effect on the number of positive cases of *E. coli*. 2) Maternal antibody content of the yolk sac increased with breeder flock age, and 3) Thorough cleaning and disinfection of the chicken barn decreased *E. coli* counts in the environment prior to chick placement in the

barn but increased *E. coli* numbers and *E. coli* variability of organs and yolk sacs collected from chicks exposed to the clean environment after 4 days.

Yolk sac infections in broiler chicks have a multifactorial aetiology, the analysis of clinical cases of yolk sac infections should include the health status of the broiler breeder flock, management of hatching eggs prior to and during incubation, chick quality at hatching with special emphasis on navel healing, and finally, management practices at the broiler barn with special emphasis on environmental conditions. Further research on the effects of environmental sanitation on chick health with special emphasis on the development of an early innate immune response is advised.

6.1. References

Barnes, J.H., Vaillancourt, J.P., Gross, W.B., 2003. Colibacillosis. In: Saif, Y.M., Barnes, H.J., Fadly, A.M. et al (Eds.), Diseases of poultry. Blackwell Publishing, Ames, Iowa, USA, pp. 631-656.

Brambell, R., 1970. Transmission of immunity in birds. In: Neuberger, A., Tatum, E.L. (Eds.), The transmission of passive immunity from mother to young. North-Holland Publishing Company, Amsterdam, the Netherlands, pp. 20-41.

Cox, N.A., Richardson, L.J., Buhr, R.J., Northcutt, J.K., Fairchild, B.D., Mauldin, J.M., 2006. Presence of inoculated *Campylobacter* and *Salmonella* in unabsorbed yolks of male breeders raised as broilers. Avian Dis. 50, 430-433.

Johnson, T.J., Kariyawasam, S., Wannemuehler, Y., Mangiamele, P., Johnson, A.J., Doetkott, C., Skyberg, J.A., Lynne, A.M., Johnson, J.R., Nolan, L.K., 2007. The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. J. Bacteriol. 189, 3228-3236.

Noy, Y., Sklan, D., 2001. Yolk and exogenous feed utilization in the posthatch chick. Poult. Sci. 80, 1490-1495.

Rai, M.F., Khan, S.A., Aslam, A., Saeed, K., 2005. Effects of yolk sac infection in chicken. Avian Poult. Biol. Rev. 16, 87-93.

Ramirez, R.M., Almanza, Y., Garcia, S., Heredia, N., 2009. Adherence and invasion of avian pathogenic *Escherichia coli* to avian tracheal epithelial cells. World J. Microbiol. Biotechnol 25, 1019-1023.

Romanoff, A.L., 1960. The extraembryonic membranes. The avian embryo; structural and functional development. Macmillan, New York, pp. 1042-1081.

Rosario Cortés, C., Téllez, I.G., López Cuello, C., Villaseca Flores, J.M., Anderson, R.C., Eslava Campos, C., 2004. Bacterial isolation rate from fertile eggs, hatching eggs and neonatal broilers with yolk sac infection. Rev. Latinoam. Microbiol. 46, 12-16.

Skyberg, J.A., Johnson, T.J., Johnson, J.R., Clabots, C., Logue, C.M., Nolan, L.K., 2006. Acquisition of avian pathogenic *Escherichia coli* plasmids by a commensal *E. coli* isolate enhances its abilities to kill chicken embryos, grow in human urine, and colonize the murine kidney. Infect. Immun. 74, 6287-6292.

Snieszko, S.F., 1974. The effects of environmental stress on outbreaks of infectious diseases of fishes. J. Fish Biol. 6, 197-208.