An investigation of the cause of enteritis in ostrich (*Struthio camelus*) chicks in the Western Cape Province, South Africa

By

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Declaration

I hereby declare that this dissertation is my own work and no part of it has been previously submitted for any degree at this or any other university.

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Abbreviations

AIDA-1	Adhesin involved in diffuse adherence
ARC-OVI	Agricultural Research Council's Onderstepoort Veterinary Institute
bp	Base pairs
^{0}C	Degrees celcius
DNA	Deoxyribonucleic acid
DVTD	Department of Veterinary Tropical Diseases
EAST1	Enteroaggregative *E. coli* heat stable enterotoxin 1
EM	Electron microscopy
ELISA	Enzyme-linked immunosorbent assay
FVS	Faculty of Veterinary Science
g	Force of gravity
h	Hour
HEp-2	Human epithelial cells
kb	kilo base pairs
km	Kilometre
LEYMA	Lactose egg yolk milk agar
LT	Heat labile enterotoxin
MLST	Multilocus sequence typing
MLVA	Multilocus variable number of tandem repeat analysis
µl	Microlitre
µM	Micromolar
min	Minute
ml	Milliliter
mm	Millimeter
ng	Nanogram
%	Percentage
Paa	Porcine attaching and effacing-associated factor
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
RT-PCR	Real-time PCR
rt-PCR	Reverse transcription PCR
s	Seconds

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SAOBC	South African Ostrich Business Chamber
ST	Heat stable enterotoxin
Stx	Shiga toxin
UP	University of Pretoria
V	Volts
XLD	Xylose lysine desoxycholate

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Dissertation Summary

An investigation of the cause of enteritis in ostrich (*Struthio camelus*) chicks in the Western Cape Province, South Africa

by

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Ostrich (*Struthio camelus*) chicks less than three months of age are observed to experience a high mortality rate of 30-40% that is often associated with enteritis. In the event of a disease outbreak mortality can reach 80-100%. Morbidity in those individuals that survive an episode of enteritis, leads to poor growth rate.

Enteritis is a multifactorial syndrome that is seen in different animal species. Various infectious agents have been mentioned in relation to the syndrome but no clear cause of the condition has yet been identified. *Clostridium perfringens* especially in broiler chickens is recognised as a cause of necrotic enteritis. *Salmonella* spp. and *Escherichia coli* are recognised factors of enteritis in chickens and turkeys, where damage to the intestinal mucosa has occurred as a result of a co-existing viral infection. This study was undertaken to investigate different infectious agents implicated in enteritis in ostrich chicks.

Post mortems were performed on 122 ostrich chicks aged from one day to three months of age. Small and large intestines were kept on ice or at 4°C for bacterial culture and collected in 10% formalin for histopathological examination. Fresh intestinal contents were collected for electron microscopy and rectal faeces collected

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for faecal flotation test for helminthic or coccidial eggs and staining by safranin counterstained with methylene blue to detect *Cryptosporidium* oocysts.

Escherichia coli (49%) was the most frequently isolated from the samples followed by *C. perfringens* (20%), *Enterococcus* spp. (16%) and *Salmonella* spp. (7%). Eight percent was made up of other less significant bacteria and samples where no bacteria were isolated. Of the *E. coli*, 39% were categorised as enteropathogenic *E. coli*, 4% enterotoxigenic *E. coli* and no enterohaemmorrhagic *E. coli* were found. For the purposes of this study, those *E. coli* that could not be categorised were considered as non-pathogenic. The majority (93%) of *C. perfringens* were type A and only 7% were type E. *Clostridium perfringens* type B, C and D were not present. The *netB* toxin gene was identified from 16% of the *C. perfringens*. All the *C. perfringens* type E harboured the *netB* toxin gene and just 10% of the *C. perfringens* type A had this gene. Three *Salmonella* serotypes were identified, *S.* Muenchen (80%), *S.* Hayindongo (13%) and *S.* Othmarschen (7%).

Necrotic enteritis, non-specific necrosis and sloughing lesions of the intestines were identified. Different bacteria mainly; *E. coli*, *C. perfringens* and *Salmonella* spp. were isolated in association with these lesions. Eight enteroviruses or enterovirus-like particles, one reovirus, one birnavirus and one unidentified viral particles were identified from 76 samples. No helminths, no coccidia and no *Cryptosporidia* were identified from the samples.

The findings suggest that viruses and parasites do not play a significant role in the occurrence of enteritis in ostrich chicks. The indication is that the cause of enteritis in ostrich chicks is bacterial involving; enteropathogenic *E. coli* and enterotoxigenic *E. coli*; *C. perfringens* type A and E (with the possible influence of *netB* toxin gene) and *S.* Muenchen, *S.* Hayindongo and *S.* Othmarschen. The farm management and degree of biosecurity play an important role in the onset of disease as they determine the level of stress on the chicks and hygiene on the farm.

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CHAPTER 1

Literature Review

1.1 Introduction

Ostrich farming began in the Little Karoo region of South Africa and dates back to about 1860 where it was concerned primarily with the production of feathers (Shanawany *et al.* 1999). In the early 20th century ostrich feathers were South Africa's fourth largest export industry after gold, diamonds and wool (Stables 2011). Around the time of the First World War in 1914 and with a change in fashion trends, the ostrich feather industry collapsed (Black 2001). Currently the market for ostrich meat and hides has significantly surpassed that for feathers. Ostrich meat contains much less fat and cholesterol and this health aspect of ostrich meat has been exploited in the promotion of the ostrich industry. Ostrich leather is a highly sort commodity for clothing and footwear fashion the world over. Previous estimates were that, of the total income from ostrich products, 75% was from hides, 15-20% from meat and 5-10% from feathers (Smith *et al.* 1995). Annual export income, for South Africa, from ostrich products (mainly meat and hides) is now estimated at about R1.2 billion annually (South African Ostrich Business Chamber 2004).

From the mid-1980s there has been renewed interest in ostrich farming that expanded within South Africa and in southern Africa (Botswana, Namibia, Zimbabwe), North America, Europe, Australia and parts of the Middle East and Asia including China. This expansion was driven mainly by the anticipation of international demand for high quality leather and meat (More 1996; Verwoerd *et al.* 1998; Black 2001; Glatz *et al.* 2008b).

1.1.1 Ostrich farming practices in South Africa

The rearing of ostriches involves three stages from hatching to slaughter, these are: immediately post-hatch, rearing to 4–6 months of age and finishing (Verwoerd *et al.* 1999). Breeding birds, from which eggs are produced, are held on different properties or a different section of the same farm and a designated hatchery handles the

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artificial incubation and hatching of the eggs (Shanawany *et al.* 1999). Chicks are kept in the hatchery immediately post-hatch before being transferred to a farm for rearing usually after 24-48 h (Glatz *et al.* 2008b).

Artificial rearing is usually practised rather than foster rearing which utilises foster parents on an open paddock. With artificial rearing, chicks are kept inside a brooder house at a stocking rate of about two to three chicks/m^2. An outside run is provided where chicks can be allowed outside to exercise during the day and then returned inside at night. Temperature inside the brooder house is controlled, especially by providing heating at night and ventilation is normally passive through slats or windows. Brooding quarters are cleaned regularly (can be daily to weekly) and an all-in-all-out system is usually practised between batches of similar aged chicks. A formulated ration and water are provided ad lib in the brooder house and in the outside run (Shanawany *et al.* 1999; Verwoerd *et al*. 1999; Glatz *et al.* 2008b).

The experience and knowledge of the farm manager and the degree of development of the ostrich industry are factors which determine the overall quality of management of the ostrich farm. The implementation of biosecurity measures, hygiene and production practices will be based on these factors (Shane *et al.* 1996; Black, 2001).

1.1.2 Enteritis of ostrich chicks (< 3 months of age)

Enteritis describes a condition where the intestinal mucosa is inflamed. Diarrhoea, dysentery, abdominal pain, dehydration and acid-base imbalance may occur depending on the cause, the severity and the location of the lesion (Radostits *et al.* 2007). Enteritis is a significant contributing factor to the high mortality observed in chicks in the ostrich farming industry. High morbidity associated with enteritis leads to reduced growth rate. Therefore, improved chick growth rates and improved chick survival rates will have a positive impact on the profitability of the ostrich industry (Samson 1997).

Naturally, the newly born or newly hatched animal acquires its microbial gut flora from the immediate surroundings that harbour bacteria from the mother and other adult individuals (Fuller 1989; Huchzermeyer 1998). Artificially hatched and raised ostrich chicks do not get the opportunity to establish the normal gastrointestinal

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microflora as they originate from a disinfected hatchery environment and are transferred to a cleaned and disinfected brooder house (Huchzermeyer 1998).

The normal intestinal flora acts to prevent the establishment of pathogenic bacteria by: competing for nutrients, competing for binding sites to intestinal epithelium, producing antimicrobial compounds (e.g. volatile fatty acids and bacteriocins), stimulating the immune system or a synergistic combination of any of these effects (Patterson *et al.* 2003; Callaway *et al*. 2008). This function of the normal intestinal flora is known as competitive exclusion and the failure to establish this flora soon after birth is instrumental in the development of enteritis in chicks (Huchzermeyer 1998).

1.2 Identification and diagnosis of enteric pathogens

Established, routine methods for the culture and isolation of pathogenic bacteria are well described (Quinn *et al.* 2011). Typing methods are used to further differentiate bacteria beyond the level of species (Olsen *et al.* 1993). The criteria for typing methods are: they should be able to differentiate between divergent strains (discriminatory power); the same result should be obtained from repeated testing of the same strain (reproducibility); all isolates should be able to be typed (typeability) (Maslow *et al.* 1993; Olsen *et al.* 1993; Olive *et al.* 1999). However, no comprehensive, standard typing method exists and this should be taken into consideration when a method is utilised (Maslow *et al.* 1993; Olsen *et al*. 1993).

Phenotypic typing methods have traditionally been used for typing bacteria; these include: serotyping, phage typing, biotyping and antibiogram typing. Molecular or genotypic typing methods are more recent and include: polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE), plasmid profiling, restriction endonuclease analysis, restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA, multilocus variable number of tandem repeat analysis (MLVA), multilocus sequence typing (MLST) and DNA sequencing (Maslow *et al*. 1993; Olsen *et al*. 1993; Olive *et al.* 1999; Maiden 2006; Van Belkum 2006b).

The PCR involves the exponential replication of a particular DNA sequence and detection of the amplified product by electrophoresis through an agarose gel to determine the presence or absence of the DNA sequence of interest (Maslow *et al.*

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1993). With RFLP the amplified PCR product is digested with a restriction enzyme and the restriction fragments which are produced are observed by agarose gel electrophoresis. Different bacterial strains will have different fragment sizes as the site of action of the restriction enzyme for a given genetic locus can be polymorphic from one strain to the other (Olive *et al.* 1999).

For the PFGE, a restriction enzyme with few digestion sites is applied to the chromosomal DNA. Fragments are produced that vary in size from 10 to 800 kb and these are separated by electrophoresis in an agarose gel where the current is periodically altered to achieve good separation of fragments. The resulting PFGE pattern is used to identify the bacterial strain (Maslow *et al.* 1993; Olive *et al.* 1999). The MLVA is based on the observation that bacterial genes or intergenic regions often carry loci of repetitive DNA and different bacterial strains may have differing numbers of repeat units or differing structural constitution of repeat units. These are termed "variable number of tandem repeat regions". The MLVA utilises the PCR amplification of multiple loci to determine the numbers and sizes of the variable number of tandem repeat regions to differentiate strains (Van Belkum 2006a). Alternatively, the MLST strives to differentiate strains by determining the nucleotide sequence of the alleles of several housekeeping genes and indexing the nucleotide sequence variation (Maiden 2006).

The diagnosis of many viral diseases can be revealed by clinical signs, post-mortem findings and histopathological changes. Nonetheless, specific laboratory methods are required to confirm the role of a particular viral pathogen (Quinn *et al.* 2011). There are tests to detect the virus, viral antigen or viral nucleic acid and those that detect viral antibody (Murphy *et al.* 1999).

Electron microscopy (EM) is a useful technique to observe the morphology and size of any virus that may be present in a diagnostic specimen and in most cases the family to which the virus belongs can be determined. Electron microscopy is useful to identify viruses that cannot be propagated by *in vitro* culture methods (Biel *et al.* 1999). However, EM is limited by low sensitivity: normally greater than 10^5 viral particles per ml should be present in the sample for virus to be detected (Hirsh *et al.* 2004). This concentration of virus is often exceeded in faeces and vesicle fluid but not in respiratory discharge (Murphy *et al.* 1999). Immune electron microscopy improves the detection of viruses by adding specific antibody to the sample (tissues,

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cells, faeces) and then observing the virus-antibody complexes under the electron microscope (Murphy *et al.* 1999; Hirsh *et al.* 2004).

Serological and molecular methods have been considered and applied for the diagnosis of enteric parasites but the microscopic examination of faeces to detect worm eggs or larvae remains the most commonly used technique (Webster *et al.* 1996; Taylor *et al.* 2007).

Serotyping methods and PCR were used to further characterise the microorganisms identified in this study and these are described under the relevant sections.

1.2.1 Bacterial enteropathogens

Available culture methods were used to isolate bacteria for this study and achievable typing methods which were considered relevant were utilised for the bacteria identified. *Clostridium perfringens* was toxin typed by PCR; *Salmonella* spp. were serotyped and *E. coli* was categorised by PCR for virulence factor genes.

a) Clostridium **spp.**

The clostridia are anaerobic, Gram-positive rods of which the majority are motile (Quinn *et al.* 2011). They produce endospores and are saprophytes which may reside in soil and freshwater sources or marine sediments. *Clostridium perfringens* and *C. difficile* are recognised for their role in inflammation of the gastrointestinal tract and enterotoxaemia (Quinn *et al.* 2011).

Clostridium perfringens is usually a constituent of the normal intestinal flora of animals and humans (Niilo 1980). Disease caused by *C. perfringens* may be precipitated by factors such as bad husbandry, sudden change in diet and environmental conditions that lead to its proliferation in the intestines (Quinn *et al.* 2011). The bacterium produces toxins (exotoxins and enterotoxin) which are attributed to its pathogenicity. Four major toxins alpha (α), beta (β), epsilon (ε) and iota (ι) are identified along with additional toxins β2-toxin, entero-toxin and NetB-toxin which are considered to play a role in diarrhoeal disease (Daube *et al.* 1994; Garmory *et al.* 2000; Albini *et al.* 2008; Keyburn *et al.* 2008).

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Clostridium perfringens is classified into five toxinotypes A, B, C, D and E based on the expression or possession of the four major toxins α, β, ε and ι (Songer 1996; Petit *et al.* 1999) as shown in Table 1.1.

Table 1.1 Criteria for toxinotyping of *Clostridium perfringens* based on the expression or possession of toxin genes

Clostridium perfringens	**Toxin/toxin gene**			
	α	β	ε	ι
Type A	+	-	-	-
Type B	+	+	+	-
Type C	+	+	-	-
Type D	+	-	+	-
Type E	+	-	-	+

+: presence of toxin/gene, -: absence of toxin/gene

The (Enzyme-linked immunosorbent assay) ELISA can be used for detection of toxins for the typing of *C. perfringens* (Quinn *et al.* 2011). Traditionally typing was done by the neutralisation test, using specific antisera to counter the toxigenic effect of each major toxin in a laboratory animal model (Sterne *et al.* 1975). Conventional and real-time PCR (RT-PCR) have now been developed for the detection of the major toxin genes for the toxinotyping of *C. perfringens* isolates (Daube *et al.* 1994; Yoo *et al.* 1997; Baums *et al.* 2004; Albini *et al.* 2008).

For the purpose of epidemiological investigations it is necessary to differentiate strains beyond the toxinotype level because although toxinotyping is useful diagnostically it is inaccurate for epidemiologic and/or phylogenetic applications (Engström *et al.* 2003; Chalmers *et al.* 2008). Molecular methods that have been utilised for the subtyping of *C. perfringens* include: PFGE, MLVA and MLST (Engström *et al.* 2003; Nauerby *et al.* 2003; Sawires *et al.* 2005; Jost *et al.* 2006; Chalmers *et al.* 2008).

Clostridium difficile is found in the environment (Brazier 1998). It can cause disease in animals and humans when the gut flora is disrupted by use of antibiotics. The normal flora is suppressed and the clostridial spores which persist germinate and

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multiply quickly, producing toxins that lead to diarrhoea and colitis (Brazier 1998; Songer 2004). Two major toxins are produced which are toxin A, an enterotoxin and toxin B, a cytotoxin (Songer 2004). Pathogenesis of the disease is still not clear because faeces of healthy animals can harbour *C. difficile* and its toxins (Quinn *et al.* 2011).

Diagnosis of *C. difficile* associated diarrhoea is by detection of toxins A and/or B in the faeces using a cell cytotoxity assay which is the "gold standard". Enzyme-linked immunosorbent assays have been developed for the detection of *C. difficile* toxins in the faeces but they are of limited use due to their low sensitivity (Barbut *et al.* 1993; Aldeen *et al.* 2000).

Sero-grouping by use of the slide agglutination test is a simple and rapid typing method against which other typing methods are often compared (Delmée *et al.* 1985; Brazier 2001). The molecular typing methods that have been applied to identify *C. difficile* include PFGE, PCR ribotyping, MLVA, and MLST (Delmée *et al.* 1986; Lemee *et al.* 2004; Van Den Berg *et al.* 2007)

b) *Salmonella* spp.

Salmonella are Gram-negative rods that belong to the family *Enterobacteriaceae*. They are facultative anaerobes that are usually motile and non-spore forming (Quinn *et al.* 2011). The genus *Salmonella* comprises two species, *S. enterica* and *S. bongori*. *Salmonella enterica* is separated into six subspecies that are allocated a Roman numeral and a name: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae* and VI, *S. enterica* subsp. *indica* (Brenner *et al.* 2000; Popoff *et al.* 2000).

The subspecies are classified into serovars/serotypes by serotyping. The antigenic composition of the organism is determined through the use of specific antisera that identify the somatic, lipopolysaccharide (O) antigens and the flagella (H) antigens by agglutination (Threlfall *et al* 1990; Wattiau *et al* 2011). Two phases of flagella antigens are present on the majority of *Salmonella* serovars and these are described as biphasic. Those that have one phase of flagella antigen are known as monophasic (Jones *et al.* 2000). The Kauffmann-White scheme now proposed to be the White-

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Kauffmann-Le Minor scheme provides a comprehensive list of the antigenic formulae of *Salmonella* serovars (Grimont *et al.* 2007).

The genus *Salmonella* has more than 2500 serotypes with a worldwide distribution and environmental contamination is from faeces. The majority of serotypes (>1200) belong to *S. enterica* subsp. I (*S. enterica* subsp. *enterica*) and these serotypes represent the most commonly identified causes of infection in humans and food animals (Threlfall *et al* 1990; Quinn *et al.* 2011). The pathogenicity of *Salmonella* serotypes is associated with their invasion of epithelial cells and replication within the cells (Quinn *et al.* 2011).

The classification of *Salmonella* into serovars, by serotyping, is useful and it is accepted that it should be maintained whilst molecular subtyping methods provide a sensitive tool for epidemiological studies (Wattiau *et al.* 2011). Typing of *Salmonella* serovars (especially those of clinical importance) has traditionally been achieved by phage-typing (Callow 1959; Gershman 1976; Anderson *et al.* 1977; Threlfall *et al.* 1990). Different molecular typing techniques have been developed recently and PFGE is considered to be the "gold standard" (Boxrud *et al.* 2007; Wattiau *et al.* 2011). The PCR, MLVA and MLST are some of the methods that have been explored for the typing of *Salmonella* serovars (Kotetishvili *et al.* 2002; Alvarez *et al.* 2004; Ramisse *et al.* 2004; Fakhr *et al* 2005; Boxrud *et al.* 2007).

c) Escherichia coli

Escherichia. coli is a member of the family *Enterobacteriaceae*. It is therefore a Gram-negative, facultative anaerobe which is usually motile (Weintraub 2007). *Escherichia coli* is a prevalent member of the normal intestinal microflora of humans, animals and birds, generally from birth (Levine 1987; Oswald *et al.* 2000; DebRoy *et al.* 2001). Contaminated environmental sources (vegetation, soil and water) contribute to exposure, soon after birth (Quinn *et al.*, 2011).

Some *E. coli* strains are pathogenic and have been associated with specific diseases in humans and animals: gastroenteritis, urogenital disease, septicaemia and pleural infections (Oswald *et al.* 2000). Neonatal animals are especially affected and significant losses are incurred (DebRoy *et al.* 2001).

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The distinct virulence properties of the pathogenic *E. coli*, which include: specific interactions with the intestinal mucosa; characteristic clinical syndromes; differing epidemiology and particular O:H serotypes are used to differentiate them into categories (Levine 1987; Nataro *et al.* 1998; DebRoy *et al.* 2001). The four principal categories of *E. coli* that were recognised to cause intestinal or diarrheagenic disease were; enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli* and enterohaemorrhagic *E. coli* (Levine 1987). Two further categories: enteroaggregative *E. coli* and diffusely adherent *E. coli* were later added (Nataro *et al* 1998).

i. Enteropathogenic *Escherichia coli*

Infection with enteropathogenic *E. coli* is initiated by adherence of bacterial adhesins to the epithelial cells. Adhesins are mostly proteins, structural components of the bacterium in the form of, outer membrane proteins, or other non-fimbrial proteins that act as adhesins of pathogenic *E. coli* (Kaper *et al.* 2004). A locus of enterocyte effacement on the chromosome of enteropathogenic *E. coli* encodes all the factors involved in the formation of the attaching and effacing lesion that is seen histologically as a result of enteropathogenic *E. coli* infection (McDaniel *et al.* 1995; Batisson *et al.* 2003). The *eae* gene (*E. coli* attaching and effacing) is found in the locus of enterocyte effacement and codes for the adhesin, intimin that is involved in the occurrence of the attaching and effacing lesion (Jerse *et al.* 1990; McDaniel *et al.* 1995). The *paa* gene (porcine attaching and effacing associated gene) is found at a different locus and codes for the Paa protein which is also associated with the attaching and effacing lesion. The *paa* gene is thought to be associated with the *eae* gene and this pairing is necessary for inflicting the attaching and effacing lesion (Batisson *et al.* 2003).

No toxins are produced by enteropathogenic *E. coli* and they rarely penetrate the intestinal mucosa. The lack of toxin production and the presence of the attaching effacing lesion are important features for confirmation of enteropathogenic *E. coli* (DebRoy *et al.* 2001). Different animal species are infected and diarrhoea occurs which is usually chronic or mucoid and affected individuals are stunted and fail to thrive (DebRoy *et al.* 2001).

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ii. Enterotoxigenic *Escherichia coli*

The enterotoxigenic *E. coli* colonise the mucosal surface of the small intestine by fimbrial adhesins and produce enterotoxins. Fimbriae (also known as pili) or fibrillae fall into different classes (e.g. K88 [F4], K99 [F5], 987P [F6], F18 and F41). Enterotoxins consist of heat labile enterotoxin (LT) and heat stable enterotoxins (STa and STb); these are extracellular proteins or peptides elaborated by the bacterium. The majority of enterotoxigenic *E. coli* strains have adhesins and they may express an LT alone, an ST alone or both LT and ST. Enterotoxigenic *E. coli* are characterised by their production of enterotoxins and the possession of adhesins (Nataro *et al.* 1998; DebRoy *et al.* 2001; Kaper *et al*. 2004).

The enterotoxins produced by enterotoxigenic *E. coli* strains affect adjacent enterocytes and villous atrophy together with enterocyte sloughing may occur. Watery diarrhoea is usually seen, especially in neonatal animals, which ranges from mild to severe (DebRoy *et al.* 2001; Kaper *et al*. 2004).

iii. Enterohaemorrhagic *Escherichia coli*

Enterohaemorrhagic *E. coli* strains are a subset of Shiga toxin (Stx)-producing *E. coli* or verotoxin-producing *E. coli*; this is due to their production of Stx, also known as verocytotoxin. Two subgroups Stx1 and Stx2 make up the Stx family and are the key virulence factors of enterohaemorrhagic *E. coli*. The Stx1 does not have variants whereas Stx2 consists of Stx2c, Stx2v and Stx2e amongst others. Enterohaemorrhagic *E. coli* colonise the mucosal surface of the colon and cause necrosis of villous tips but do not penetrate the mucosa. Bloody diarrhoea is often seen with enterohaemorrhagic *E. coli* infection. A characterising feature of enterohaemorrhagic *E. coli* strains is the production of Stx (DebRoy *et al.* 2001; Kaper *et al*. 2004). Enterohaemorrhagic *E. coli* also have the *eae* gene which facilitates their attachment to the mucosal surface and they can initiate the attaching and effacing lesion (Donnenberg *et al*. 1993).

iv. Enteroinvasive *Escherichia coli*

Enteroinvasive *E. coli* are very closely related to *Shigella* spp. They share the ability to penetrate and multiply within epithelial cells, leading to their destruction. The

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invasion-associated locus (*ial*) resides on a plasmid and consists of the invasion plasmid antigen (*ipa*) genes, *ipa*A, *ipa*B, *ipa*C and *ipa*D, the products of which are associated with the invasive nature of enteroinvasive *E. coli* (Venkatesan *et al.* 1989; Sethabutr *et al.* 1993). Another gene, *ipa*H is not associated with invasiveness but it is unique for *Shigella* spp and enteroinvasive *E. coli* (Venkatesan *et al.*, 1989). The enteroinvasive *E. coli* usually infect the mucosal surface of the colon and cause watery diarrhoea (Levine 1987; DebRoy *et al.* 2001; Kaper *et al.* 2004).

v. Enteroaggregative *Escherichia coli*

Enteroaggregative *E. coli* are defined by their ability to adhere to human epithelial (HEp-2) cells in an aggregative adherence or "stacked brick" pattern and the lack of secretion of enterotoxins LT and ST (Law *et al.* 1998; Nataro *et al.* 1998; Steiner *et al.* 1998). The aggregative adherence fimbrae (AAF/I and AAF/II) are thought to play a part in this typical pattern observed in cell culture and the adherence of enteroaggregative *E. coli* to intestinal mucosa (Nataro *et al.* 1998; Ménard *et al.* 2002).

Enteroaggregative *E. coli* elaborate heat-stable enterotoxin 1 (EAST1) which has been suggested to cause diarrhoea (Ménard *et al.* 2002). However, this toxin is not unique to enteroaggregative *E. coli* as it has been identified in enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enterohaemorrhagic *E. coli* and diffusely adherent *E. coli*. Furthermore, approximately only half of the enteroaggregative *E. coli* produce EAST1 (Savarino *et al.* 1996; Law *et al* 1998; Huang *et al.* 2004). The enteroaggregative *E. coli* are reported to cause diarrhoea in both children and adults throughout the world (Huang *et al.* 2004)

vi. Diffusely adherent *Escherichia coli*

Diffusely adherent *E. coli* are defined by the diffuse pattern of adherence which they form in cell culture with HEp-2 cells (Kaper *et al.* 2004). The adhesion involved in diffuse adherence (AIDA-1) was considered to be responsible for the pattern of diffuse adherence but was found to be only expressed by a few diffusely adherent *E. coli* (Benz *et al.* 1989). Subsequently, AIDA-1 was determined to not be indicative of any one type of *E. coli* (Pritchard *et al.* 2004). Diffusely adherent *E. coli* are thought

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to be associated with diarrhoea in children over 12 months of age (Scaletsky *et al.* 2002).

Serotyping was developed in the 1940's, by Kauffmann, for the typing of *E. coli* strains similar to the work done with *Salmonella* spp. (Ørskov *et al.* 1992). The surface antigens that facilitated serotyping were determined as somatic (O), flagella (H) and capsular (K) antigens. The number of *E. coli* serotypes given by the O:K:H serotyping is not known but it is estimated at over 50 000, very few of which cause disease (Ørskov *et al.* 1992). Serotyping can be limited as evident for enterotoxigenic *E. coli* for which no serological markers are available to differentiate them from non-toxigenic strains (Stacy-Phipps *et al.* 1995).

The PCR method has mostly been adopted in a multiplex format to characterise *E. coli* by the detection of virulence factor genes. Different categories of *E. coli* can be determinded by the selection of target genes: *eae* for enteropathogenic *E. coli*, Stx for enterohaemorrhagic *E. coli*, LT and ST for enterotoxigenic *E. coli* and *ipa*H for enteroinvasive *E. coli* (Pass *et al.* 2000; Toma *et al.* 2003). The presence of other virulence factor genes such as fimbrae (F4, F5 etc), EAST1, AIDA-1, *paa* can be included in a multiplex PCR as required (Mohlatlole *et al.* 2013). For enteroaggregative *E. coli* and diffusely adherent *E. coli* however, the HEp-2 cell adherence assay remains the method of choice as no PCR methods have been defined for them (Nataro *et al.* 1998; Toma *et al.* 2003).

d) *Campylobacter* spp.

Campylobacter spp. are microaerophilic, Gram-negative rods that tend to be slender and curved (appearing as gull-winged and spirals under the microscope). They have a polar flagella and are motile (Quinn *et al.* 2011). Warm-blooded animals harbour many *Campylobacter* spp. as commensals in their intestinal tracts and sometimes in their genital tracts. Nonetheless, intestinal infection causing diarrhoea and genital infection causing infertility or abortion can occur (Quinn *et al.* 2011). Bacterial enteritis, associated with *Campylobacter* spp. is reported frequently in humans, and animal products are implicated as the source (Nielsen *et al.* 2000; Mazurier *et al.* 2008).

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Reliable isolation and subsequent typing of *Campylobacter* spp. can be challenging, due to the fragility and fastidious nature of the organism (Chaban *et al.* 2009). A selective enrichment medium such as Skirrow's medium or Preston medium is required for the primary isolation of these organisms (Bolton *et al.* 1982). The conventional typing methods for *Campylobacter* spp. rely on phenotypic characteristics and include biotyping, serotyping and phage typing (Lior *et al.* 1982; Salama *et al.* 1990; Nielsen *et al.* 2000). The more recent genotypic methods are generally more discriminative and include PFGE, ribotyping, RFLP (using the flagellin gene), RT-PCR and random amplification of polymorphic DNA (Nielsen *et al.* 2000; Mazurier *et al.*, 2008; Chaban *et al.* 2009).

e) *Pseudomonas* spp.

Pseudomonas spp. are strict aerobic, Gram-negative rods which are motile by a single or multiple polar flagella. The majority produce pigments and the pigment pyocyanin in particular is specific to *Pseudomonas aeruginosa*, giving colonies a bluish green colour (Quinn *et al.* 2011). Biotyping and some molecular techniques such as PFGE, MLST and random amplification of polymorphic DNA can be used for the typing of these bacteria (Johnson *et al.* 2007; Quinn *et al.* 2011).

Pseudomonas spp. occur worldwide, throughout the environment in water, soil and plants. *Pseudomonas aeruginosa* causes varying opportunistic infections in different animal species and can be found on the skin, mucous membranes and faeces (Quinn *et al.* 2011).

f) *Lawsonia intracellularis*

Lawsonia intracellularis is a microaerophilic, non-motile, Gram-negative, slender and curved rod that cannot be cultured in cell-free media (McOrist *et al.* 1995; Quinn *et al.* 2011).

Lawsonia intracellularis has been associated with proliferative enteritis (a disease with worldwide distribution) in pigs and foals (McOrist *et al.* 1994; Lavoie *et al.* 2000). Hamster, deer and ostrich have also been reported to display signs of proliferative enteritis caused by this organism, or a very closely related causative agent (Cooper

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et al. 1997). Acute haemorrhagic diarrhoea and death or chronic diarrhoea with reduced growth rate are the possible clinical signs of proliferative enteritis (Guedes *et al*. 2002).

Successful culture of *L. intracellularis* is achieved only in enterocyte cell lines (Lawson *et al.* 1993). Immunohistochemistry is significantly more sensitive in identifying *L. intracellularis* infection from formalin-fixed tissues than the haematoxylin-eosin and Warthin-Starry silver stains which are routinely used (Guedes *et al*. 2002). Immunoperoxidase staining of faecal smears and an indirect fluorescent antibody test have been used to detect the organism from faeces and both have shown to be more sensitive than PCR for this purpose (Guedes *et al*. 2002).

1.2.2 Viral enteropathogens

Electron microscopy was utilised for this study to screen for the presence of any viral particles. No further characterisation of identified viral particles was pursued.

a) Rotavirus

Rotavirus is a member of the family *Reoviridae*. It is a non-enveloped virus forming an almost spherical icosahedron of 80 nm diameter. Electron microscopy and ELISA are commonly applied methods for the detection of rotaviruses from faeces. Genotypic and serologic analysis is the basis for classification of viruses in the genus *Rotavirus*. Rotaviruses are classified into major groups: Group A Rotavirus encompasses pathogens of humans, cattle and other animals; group B encompasses pathogens of humans only; groups C and E encompass pathogens of swine only and group D and F encompass pathogens of fowl only (Murphy *et al*. 1999). Serotypes are determined by serum neutralisation tests or enzyme immunoassay specifically targeting outer capsid proteins for both methods (Beards *et al.* 1980; Coulson *et al.* 1987). The reverse transcription PCR (rt-PCR) has been applied to the typing of rotaviruses (Gouvea *et al.* 1990; Herring *et al.* 2004).

Rotavirus is a significant cause of diarrhoea in intensive farming conditions all over the world. Infection may be subclinical but mild to severe enteritis and even death

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may occur. Usually young animals from 1-8 weeks of age are affected (Murphy *et al.* 1999).

b) Coronavirus

The family *Coronaviridae* has two genera, *Coronavirus* and *Torovirus*. The coronaviruses are enveloped and fairly spherical with a diameter of 80-220 nm. Large, 20 nm peplomers protrude from an icosahedral-like internal core structure that contains a helical nucleocapsid. The genome is made up of a single stranded RNA molecule (Murphy *et al.* 1999).

Virus isolation followed by EM or serological tests (e.g. ELISA) is considered the gold-standard for detection of coronaviruses (Sellers *et al.* 2004). However, in recent times molecular techniques such as the rt-PCR have been developed which can be performed more rapidly and can detect infection at an earlier stage (Sellers *et al.* 2004).

Coronaviruses cause a number of varying diseases including gastroenteritis in humans and other animal species. Transmissible gastroenteritis virus of swine is a Group I coronavirus and bovine coronavirus and turkey enteric (bluecomb) coronavirus are both Group II coronaviruses. These viruses affect mainly the young and symptoms of diarrhoea, weight loss, depression, dehydration and even death are observed (Guy *et al.* 1997; Murphy *et al.* 1999).

c) Adenovirus

The family *Adenoviridae* consists of the genus *Mastadenovirus* which affects mammalian species and the genus *Aviadenovirus* which affects birds (Murphy *et al.* 1999). They are non-enveloped viruses of 80-100 nm in diameter. They have a hexagonal shape with icosahedral symmetry and the genome is made up of one, linear, double stranded DNA molecule (Murphy *et al.* 1999).

Adenovirus infection can be diagnosed from faeces by a number of methods which include virus isolation, ELISA, EM, and PCR with restriction enzyme analysis (Johansson *et al.* 1980; Murphy *et al.* 1999; Meulemans *et al.* 2001).

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Adenoviruses have a narrow host range and can cause acute respiratory disease or gastroenteric disease (Murphy *et al*. 1999).

d) Enterovirus

The genus *Enetrovirus* is one of six genera of the family *Picornaviridae*. The virus is non-enveloped with a diameter of 27 nm and icosahedral symmetry. The genome is made up of a single, linear molecule of single-stranded RNA (Murphy *et al*. 1999).

Enterovirus-like particles have been detected from avian faeces by EM but the pathogenicity of these virus particles was not established. These enterovirus-like particles were not isolated by cell culture, and EM is the preferred method for their detection (McNulty *et al*. 1979).

e) Astrovirus

The family *Astroviridae* comprises two genera: *Mamastrovirus* (mammalian astroviruses) and *Avastrovirus* (avian astroviruses) (Pantin-Jackwood *et al.* 2006). They are non-enveloped, 28-30 nm in diameter with icosahedral symmetry (Welsh *et al.* 1997). The genome is made up of one linear, single-stranded RNA molecule (Murphy *et al*. 1999).

Astrovirus infection in animals is characterised by a self-limiting gastroenteritis, which can be seen as watery diarrhoea of up to four days duration. Young animals are most often affected and sub-clinical infection probably occurs in most cases (Murphy *et al*. 1999).

Detection of astroviruses from faeces or intestinal contents has traditionally been achieved by EM but this method has considerably low sensitivity (Koci *et al.* 2000). Immunoelectron microscopy and ELISA have been applied for this purpose but molecular methods, in particular rt-PCR are more sensitive and more specific. To identify each virus type, gene sequencing is necessary (Pantin-Jackwood *et al.* 2006).

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1.2.3 Parasitic enteropathogens

Helminth parasites are agents of gastrointestinal infection in ruminants and these include nematodes (worms) and trematodes (flukes). Infection results in inappetance and often in severe diarrhoea which may lead to impaired production and death, with obvious economic losses (Parkins *et al.* 1989).

The majority of species of coccidia which are intestinal parasites belong to two genera, *Eimeria* and *Isospora* in the family Eimeriidae. Oocysts of these coccidial spp. contained in the faeces of infected animals and released into the environment are the cause of intestinal coccidiosis worldwide (Long 1990). *Cryptosporidium* represents the sole genus of the family Cryptosporidiidae. The members of this genus are coccidial parasites that infect the gastrointestinal tract of vertebrates causing varying degrees of diarrhoea and enteritis (Long 1990; Fayer *et al* 2007; Muller 2010).

The faecal floatation test was used to identify nematode and coccidial eggs whilst a staining method was used specifically for *Cryptosporidium* eggs in this study.

1.3 Enteritis in farmed animals

1.3.1 Calf diarrhoea

Diarrhoea has been identified as one of the major causes of mortality in calves. Significant economic losses are incurred as a result of the mortality which ranges between zero and 80% and also due to retarded growth and cost of treatment (Woode *et al.* 1975; Tzipori 1981; Uhde *et al*. 2008). The syndrome which has been called neonatal calf diarrhoea or calf scours is complex and multifactorial without a clear aetiology. Various infectious agents, the environment, nutrition, immune and genetic status are thought to play a role in the occurrence of disease (Woode *et al.* 1975; Tzipori 1981; Uhde *et al*. 2008).

The most commonly encountered enteropathogens in diarrhoea of the young calf are enterotoxigenic *E. coli*, rotavirus, coronavirus and *Cryptosporidium* (Tzipori 1981). Infection and disease can be caused by a single agent (normally in the very young)

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or more commonly by a combination of infectious agents (Tzipori 1981; Uhde *et al.* 2008; Ok *et al.* 2009). The clinical signs of acute enteritis, which include anorexia, depression and diarrhoea are seen following infection with most enteropathogens, making laboratory diagnosis for the aetiological agent necessary (Tzipori 1981; Uhde *et al.* 2008).

There are microorganisms that have been found to be associated with neonatal calf diarrhoea but with uncertain roles in the aetiology of disease. Viruses that have been reported include; astrovirus, calicivirus, parvovirus and bovine viral diarrhoea virus (BVDV) (Tzipori 1981; Kelling *et al.* 2002). Bacteria include *Clostridium* spp., specifically the different toxinotypes of *C. perfringens* (A-E) (Fleming 1985; Lebrun *et al.* 2007; Ferrarezi *et al.* 2008) and also *C. difficile* (Hammitt *et al.* 2008). Four serotypes of salmonella, *S.* Typhimurium, *S.* Dublin, *S.* Muenchen and *S.* Copenhagen are considered the second most important bacteria in neonatal calf diarrhoea after *E. coli* (Rings 1985).

1.3.2 Foal enteritis

Newborn foals and young foals in general are considered to be commonly afflicted by enterocolitis and enteritis due to a number of non-infectious and infectious causes (Jones *et al.* 1987; Magdesian 2005). Some recognised non-infectious causes are: foal heat diarrhoea; asphyxia-associated gastroenteropathies; and nutritional/dietary causes. Infectious agents include, bacteria, viruses, parasites and protozoa (Magdesian, 2005). In the first week post-partum the disease can usually be fatal (Tzipori *et al.* 1982).

Of the *Clostridium* spp., which commonly cause enterocolitis in the neonatal foal, *C. perfringens* and *C. difficile* are the species most often involved. Mild to severe haemorrhagic diarrhoea, acute abdominal pain, fever and hypovolaemic and septic shock are the clinical signs that may be observed, although sometimes death may occur without the development of diarrhoea (Feary *et al.* 2006).

Clostridium perfringens types A and C are most often associated with enterocolitis in the first few days post-partum. Similarly, *C. difficile* can cause enteritis in young foals. Toxin A (enterotoxin) and Toxin B (cytotoxin) produced by *C. difficile*, are thought to

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act synergistically on the intestinal tissue to cause damage that leads to disease (Magdesian 2005; Feary *et al.* 2006).

Salmonella spp. can cause neonatal septicaemia and enterocolitis in horses of any age (Dunkel *et al.* 2004). Enterocolitis caused by *Salmonella* spp. displays clinical signs which are very similar to disease caused by other infectious or non-infectious causes (Feary *et al.* 2006). *E. coli* is commonly identified with sepsis in foals but has an unclear role in neonatal diarrhoea (Magdesian 2005).

The bacterium *L. intracellularis* causes equine proliferative enteropathy which is a transmissible enteric disease, primarily affecting weanling foals aged between 3 and 6 months (Lavoie *et al*. 2000; Feary *et al.* 2006). Equine proliferative enteropathy is characterised by weight loss, diarrhoea, colic, fever, dullness and hypoproteinaemia (Lavoie *et al*. 2000; Feary *et al.* 2006).

For a long time, rotavirus has been recognised to be a pathogen in foals (Dunkel *et al.* 2004). Equine rotaviruses belong to group A rotaviruses and they are the most common cause of viral neonatal diarrhoea (Magdesian 2005). Infections with rotavirus are often seen occurring as outbreaks on farms and affecting mainly young foals aged between 5 and 35 days. Clinical signs are similar to those of other infectious diarrhoeas but vary greatly from mild to severe diarrhoea with dehydration (Magdesian 2005). Rotavirus is highly contagious and morbidity in an outbreak can come close to 100% of neonates. Mortality however is considered low particularly with provision of supportive care (Magdesian 2005).

Coronavirus and adenovirus constitute other known causes of viral foal diarrhoea; but coronavirus enteritis in foals has been mentioned in a few studies only and a defined role of adenovirus in neonatal equine diarrhoea has not been established (Magdesian 2005).

A heavy infestation, 2000 eggs/g, of *Strongyloides westeri* is thought to be associated with diarrhoea. Foals have been found to be infested with *Strongyloides westeri* from an early age as embryonated eggs can be passed in the faeces at about 10-14 days after birth (Magdesian 2005). Infection comes primarily from the mare's milk although the percutaneous route and ingestion of infective larvae from faecal matter is possible (Dunkel *et al.* 2004). Nonetheless, the part played by *Strongyloides westeri* infection in neonatal foal diarrhoea is not clear (Magdesian 2005).

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Cryptosporidium parvum is associated with mild diarrhoea of foals in the first month post-partum. The diarrhoea is self-limiting and often lasts from 1 day to a week (Dunkel *et al.* 2004). The role of other protozoa, *Giardia* spp. and *Eimeria leukarti*, in neonatal foal diarrhoea has not been confirmed (Magdesian 2005).

1.3.3 Porcine enteritis

Pre-weaning piglet diarrhoea occurs in piglets mainly between 7 and 14 days of age and up to three weeks of age. Severe cases of diarrhoea may lead to dehydration and affected piglets often have retarded growth (Driesen *et al.* 1993). Infectious diarrhoea is a recognised major contributor to loss of income in pig production farms, affecting suckling and weaned piglets (Wieler *et al.* 2001). Infection by a single enteropathogen is more likely than a mixed infection. Commonly identified agents of diarrhoea in piglets are coronavirus, rotavirus, enterotoxigenic *E. coli*, *C. perfringens*, *Isospora suis* and *Cryptosporidium parvum* (Driesen *et al.* 1993; Wieler *et al.* 2001; Katsuda *et al.* 2006;).

Clostridium perfringens type C especially, is implicated in the cause of necrotising enteritis in piglets (Driesen *et al.* 1993; Miclard *et al.* 2009). *Clostridium difficile* is regarded as an emerging factor of importance in the cause of neonatal enteritis of pigs. *Clostridium difficile* affects piglets aged 1-7 days which show signs of diarrhoea very soon after birth (Songer *et al.* 2006). *Lawsonia intracellularis* is the bacterial cause of proliferative enteropathy, a well-recognised global intestinal disease, seen mostly in pigs after weaning between six and 20 weeks of age (Kroll *et al.* 2005).

1.3.4 Ovine/caprine enteritis

Cryptosporidium parvum was found to be the most frequently detected pathogen in lambs and goat kids with diarrhoea followed by *E. coli* which was detected less frequently. In some cases, a mixed infection of both *C. parvum* and *E. coli* was found (Munoz *et al.* 1996). Enteropathogenic *E. coli* and enterohaemorrhagic *E. coli* are implicated in diarrhoea of young sheep and goats (Cid *et al.* 2001). *Clostridium perfringens* (type B and C) is the other bacterium known to cause diarrhoea in lambs

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and goat kids (Uzal 2004). Viruses to be considered in cases of diarrhoea in lambs and goat kids include rotavirus and adenovirus (Theil *et al.* 1995; Olson *et al.* 2004).

1.4 Enteritis in farmed birds

1.4.1 Poult enteritis

The incidence of infectious intestinal disease is of significant concern in the turkey industry. Poult enteritis complex encompasses a condition that includes coronavirus enteritis, maldigestion syndrome, runting and stunting syndrome of turkeys, poult malabsorption syndrome, stunting syndrome, poult enteritis and mortality syndrome. Spiking mortality of turkeys and turkey viral enteritis of poults are terms which have also been used by various authors to describe intestinal disease in turkeys (Barnes *et al.* 2000).

Poult enteritis syndrome is an infectious disease causing diarrhoea, dullness/depression, pale intestines and watery caecal contents in turkey poults, usually aged from 1 day to 7 weeks (Jindal *et al.* 2009). Pathogenic agents detected from cases of poult enteritis syndrome include viruses; rotavirus, enterovirus, reovirus and adenovirus, bacteria; *Salmonella* spp., *E. coli* and *Enterococcus,* and species of the protozoa *Eimeria* (Jindal *et al.* 2009).

Some of the infectious agents that have been associated with poult enteritis and mortality syndrome are turkey coronavirus, reovirus, rotavirus and enterovirus, the bacteria: *Campylobacter* spp. and *E. coli,* and the protozoa: *Cryptosporidium* spp. (Guy *et al.* 1998). Primarily poults of 1-4 weeks of age are affected and show symptoms of depression, diarrhoea and marked increase in mortality of 10-60 % (Guy *et al.* 1998; Yu *et al.* 2000). Turkey coronavirus, avian rotavirus, reovirus and small round viruses (this includes, calicivirus, astrovirus and enterovirus) were detected from intestinal samples of turkey poults suffering from poult enteritis and mortality syndrome (Yu *et al.* 2000). In spite of the available data, the exact cause of enteritis in young turkeys is not known (Guy *et al.* 1998; Guy *et al.* 2000; Yu *et al.* 2000).

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In order to determine the relationship between the infectious agent and the observed clinical condition, experimental infection trials with viruses (i.e. turkey coronavirus and a small round virus) and a virus together with a bacterium (i.e. turkey coronavirus and enteropathogenic *E. coli*) were performed (Guy *et al.* 2000; Yu *et al.* 2000). It was concluded that the interaction of the selected pathogens, rather than the individual pathogens, produced an effect more closely related to the clinical signs of enteritis in turkey poults (Guy *et al.* 2000; Yu *et al.* 2000).

The aetiology of enteritis is multi-factorial, viruses and bacteria implicated in turkey enteritis, interact in a comparable way to produce the disease (Guy *et al.* 2000). However, further studies are required to examine the interaction of different bacteria identified in turkeys showing signs of poult enteritis and mortality syndrome and other enteric viruses of turkeys (Guy *et al.* 2000).

1.4.2 Broiler enteritis

A number of different viruses may be responsible for gastrointestinal tract infections in poultry (Guy 1998). The interaction of these viruses with the intestinal mucosa may lead to damage that allows infection by other pathogens such as *E. coli* and *Salmonella* spp. Viruses implicated in gastrointestinal disease of chickens, especially young birds, include rotaviruses, coronaviruses, enteroviruses, adenoviruses, astroviruses and reoviruses (Guy 1998). The cause of the disease in broiler chickens which has been referred to as runting stunting syndrome is unknown. It is believed that interactions of various infectious agents could give rise to observed clinical signs of poor growth, retarded feather development and diarrhoea (Guy 1998).

Necrotic enteritis is a more comprehensively described intestinal disease in chickens. *Clostridium perfringens* type A is known to be the primary cause of necrotic enteritis and in recent times the novel toxin, Net B has been established to be an important virulence factor of the disease in chickens (Keyburn *et al.* 2008; Keyburn *et al.* 2010). Broiler chickens aged two to six weeks are usually affected and will show signs of diarrhoea and necrosis of the intestinal mucosa. The disease can be fatal and a flock mortality rate of 1% per day and total mortalities of 30% may be reached (Dahiya *et al.* 2006).

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1.5 Enteritis in ostrich chicks

A high incidence of mortality is recognized in ostriches less than three months of age (Shivaprasad 2003). Gastrointestinal diseases are considered to be the most frequently observed and of major economic relevance (Herraez *et al*. 2005). Bacterial infections have been identified as the primary cause of gastrointestinal disease and poor management practices are a recognized contributing factor to bacterial enteritis (Herraez *et al*. 2005). Poor hygiene, overcrowding, stress due to factors such as improper temperature and excessive handling and other concomitant diseases are the major determinants (Samson 1997; Herraez *et al*. 2005). Cloacal prolapse in ostrich chicks can be seen as a secondary event occurring together with enteritis (Doneley 2006). The bacterial pathogens most frequently involved in infectious enteritis of ostriches are: *E. coli, Campylobacter jejuni, Pseudomonas aeruginosa, Salmonella* spp. and *Clostridium* spp. (Herraez *et al.* 2005).

Coronavirus has been implicated in the enteritis of ostrich chicks but there has been no further investigation to determine if this coronavirus is related to any of the known types of coronavirus (Frank *et al* 1992). Adenovirus affects the intestinal tract, similar to haemorrhagic enteritis in turkeys and was also isolated from the livers of experimentally infected ostrich chicks. Thus, during a differential diagnosis of enteritis, adenovirus infection should be considered in cases of high chick mortality in ostriches less than two months of age (Raines *et al* 1997).

Parasitic infections are not particularly recognized in the ostrich and no confirmed cases of coccidiosis have been reported (Huchzermeyer 2002). The round worm, *Libyostrongylus douglasii*, is found in the stomach of the ostrich (Huchzermeyer 2002). In domesticated, caged and wild birds, cryptosporidiosis ranks as one of the most prevalent parasitic infections (Sréter *et al.* 2000). *Cryptosporidium* spp. infect, grow and reproduce in the epithelium of the respiratory, gastrointestinal and urinary tracts of animals (Goodwin 1989).

Various infectious aetiologies have been mentioned in relation to the syndrome of enteritis in ostrich chicks but no clear cause of the condition has yet been identified. The identification of the infectious agent or agents will significantly aid in the treatment of ostrich chicks suffering from enteritis and in the development of effective management methods.

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1.6 Problem

A mortality rate of 30-40% in the first 3 months of the ostrich chick has come to be accepted as "normal" (Verwoerd *et al.* 1998). This mortality rate however, is relatively high and represents one of the weakest areas in ostrich farming (Samson 1997). In certain instances of disease outbreak, mortalities can reach 80-100% in ostrich chicks and the morbidity of those individuals that remain results in stunted growth (Verwoerd *et al.* 1998). Enteritis of unknown cause is often associated with this loss of ostrich chicks.

If an infectious cause is responsible for the occurrence of enteritis in ostrich chicks, the identification of this cause will allow the development of preventive measures that will reduce or eliminate the incidence of enteritis in ostrich chicks.

1.7 Objective

The objective of this research project was to investigate different infectious agents (bacterial, viral, parasitic) implicated in enteritis in ostrich chicks in the Klein Karoo region of the Western Cape Province of South Africa in order to identify the specific aetiological agent(s) that causes the disease.

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CHAPTER 2

Materials and Methods

2.1 Sampling area

Samples were obtained from farms in the Western Cape Province of South Africa, in particular the Klein Karoo region which is the heart of ostrich farming (South African Ostrich Business Chamber 2004). The region is semi-arid to arid with low rainfall (100-450 mm per year) and lies within a wide east-west oriented valley between the Langeberg-Outeniqua Mountains in the south and the Witteberg-Swartberg mountains in the north. Rainfall in the mountains is substantially higher with the Langeberg-Outeniqua Mountains receiving up to 1650 mm and the Witteberg-Swartberg Mountains receiving 1000 mm or more per annum (Le Maitre *et al.* 2009).

Most of the farms lay within a 50 km radius from the town of Oudtshoorn. The South African Ostrich Business Chamber (SAOBC) headquarters are located in Oudtshoorn and veterinarians associated with the SAOBC were involved in the sampling process for this study.

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Figure 2.1 Map of South Africa and the Western Cape Province. Sampling area delineated by the circle

2.2 Farm selection

Farms affected by enteritis of ostrich chicks less than three months of age, were identified with the help of the veterinarians associated with the SAOBC. Farmers reported cases of diarrhoea, lethargy, anorexia and death (symptoms of enteritis) to the veterinarian who would then do a farm visit to investigate and collect the relevant samples. Eighteen (n=18) farms in total were included in the study and one of these farms was used to source control birds. The farms were designated letters A to R and Farm Q is the one from which normal controls were obtained.

The chick season, when farmers received chicks on their farms, usually ran from August to March the following year. During the study period from January 2011 to December 2012, the investigator visited the sampling area at least twice per year in March/April and September/October. On these visits, samples were collected from

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farms experiencing enteritis and the general conditions concerning housing, husbandry and biosecurity measures were observed. Relevant information was received from consenting farm managers and owners in the normal history taking procedure of a disease investigation.

2.3 Sampling procedure

A total number of 122 ostrich chicks were sampled over the two year duration of the project. The age group of birds sampled was between one day and 12 weeks of age. These were chicks which had died from an enteritis related problem and moribund chicks showing the principal clinical sign of diarrhoea with or without depression, anorexia and lethargy.

Six control birds, aged between 4 days and 6 weeks, which displayed no signs of enteritis were sampled. They consisted of two, 4 day old chicks; two, 2 week old chicks; one, 4 week old chick and one 6 week old chick. Control birds were sourced from a farm which had not been affected by enteritis and no antibiotic treatment had been administered. Small intestine, colon and caecum were sampled from each to make a total of 18 samples. Control samples were subjected to the same tests as samples from diseased animals in order to provide an objective comparison between affected and non-affected individuals.

Postmortem examination was performed on dead birds within 8 hours (h) of death in order to minimise the effects of autolytic changes and overgrowth of opportunistic microbial organisms in the carcass. Intestinal tissues (small intestine, colon, caecum), intestinal contents and rectal faeces were collected. Intestines showing gross lesions indicative of enteritis (inflamed, haemorrhagic, distended, fluid filled, pseudomembranous) were sampled. Samples denoted as “intestine” were not characterised as a particular section of intestine at sampling.

When samples were taken, intestinal tissues for bacterial culture were prioritised. Therefore intestinal contents or rectal faeces to test for virology and parasitology and tissues in formalin for histopathology were not always taken.

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Samples were sent to the Department of Veterinary Tropical Diseases (DVTD) Faculty of Veterinary Science (FVS), University of Pretoria (UP) for analysis. Bacteriology, EM, histology and parasitology were done on these samples.

2.4 Handling of samples

The average time to send/courier samples from Oudtshoorn to the laboratories of the DVTD in Pretoria took two days but this ranged from one to six days. Samples were stored in a cooled environment (cooler box with ice packs) during transit.

Bacterial samples that were not processed on arrival at the laboratory were stored at -80ºC until they were processed. Samples for parasitology and electron microscopy remained at 4ºC and were processed within a week of receipt in the laboratory, if not immediately. Histology samples, preserved in 10% formalin-buffered saline could be processed at the earliest convenient time after sampling.

2.5 Laboratory procedures

2.5.1 Bacteriology

A total of 151 samples were cultured for the different bacterial agents using standard operating procedures that are routinely performed at the Bacteriology Laboratory of the DVTD. Small intestine, colon and caecum from ostrich chicks made up the sample complement and both small intestine and colon were sampled from some individuals. Each sample was processed for the detection of *Clostridium perfringens*, *Salmonella* spp, *E. coli*, *Campylobacter* spp. and *Pseudomonas* spp. The PCR and serotyping were used to characterise the bacterial isolates further as discussed for each bacterial species where either of the methods was performed.

a) *Clostridium perfringens*

Samples were inoculated onto blood agar and incubated anaerobically (5% CO_2, 5% H_2, 90% N_2) at 37ºC for 18–24 h, in an anaerobic cabinet, for the maintenance of optimal growth conditions. Gram-stain, catalase and gelatinase tests were performed

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on suspect colonies to further support the identification of *C. perfringens* (Quinn *et al.* 1994).

To characterise suspected *C. perfringens* isolates, lactose egg yolk milk agar (LEYMA) was used. The agar allows analysis of the lactose, lecithinase, proteolysis and lipase activity of the isolate, which can be used to discriminate between *Clostridium* spp. (Willis *et al.* 1959). The suspect colony was sub-cultured onto LEYMA and incubated anaerobically at 37ºC for 18–24 h. To prove that the bacterium was anaerobic, the isolate was subcultured onto blood agar and incubated aerobically at 37ºC for 18–24 h.

i. *Clostridium perfringens* toxin typing

DNA was extracted from a pure culture of *C. perfringens*. One to two colonies were placed in 100 µl distilled water, boiled at 97ºC for 10 min and centrifuged at 10 000 g for 10 min. The supernatant was used as template for PCR (Keyburn *et al*. 2008). The concentration of extracted DNA was measured in a spectrophotometer (PowerWave XS2, BioTek) and ranged from 53.6-443.1 ng/µl. The extracted DNA was sent to Deltamune Laboratories Pty (Ltd), where the toxin typing of *C. perfringens* was done.

A multiplex PCR modified from the method described by Yoo *et al*. 1997 was used. The PCR mixture contained 25 µl Dream Taq PCR Master mix (Thermoscientific), 20 µM of each primer (Table 2.1), 3 µl DNA template and the mixture was made up to 50 µl with nuclease free distilled water (H. Joubert, personal communication, 2012).

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Table 2.1 Primers for *Clostridium perfringens* toxin typing

Toxin	Primer	Nucleotide sequence	Amplicon size (bp)
α-toxin	CPA-Forward	5'-GTTGATAGCGCAGGACATGTTAAG-3'	402
	CPA-Reverse	5'-CATGTAGTCATCTGTTCCAGCATC-3'	
β-toxin	CPB-Forward	5'-ACTATACAGACAGATCATTCAACC-3'	236
	CPB-Reverse	5'-TTAGGAGCAGTTAGAACTACAGAC-3'	
ε-toxin	CPE-Forward	5'-ACTGCAACTACTACTCATACTGTG-3'	541
	CPE-Reverse	5'-CTGGTGCCTTAATAGAAAGACTCC-3'	
ι-toxin	CPI-Forward	5'-GCGATGAAAAGCCTACACCACTAC-3'	317
	CPI-Reverse	5'-GGTATATCCTCCACGCATATAGTC-3'	

A Veriti 96-well thermal cycler (Applied Biosystems) was used to perform the PCR. The programme was as follows: 5 min at 94ºC, 30 cycles of 1 min at 55ºC, 1 min at 72ºC and 1 min at 94ºC. Ten microliters of the amplified product was then analysed by electrophoresis using a 2% agarose gel (H. Joubert, personal communication, 2012).

ii. PCR for detection of *netB* toxin gene

The DNA which was extracted for the toxin typing of *C. perfringens* was also used as DNA template for the detection of *netB* toxin gene. The PCR mixture was composed of 12.5 µl Dream Taq Green PCR Master Mix (2x) (Thermoscientific); 0.8 µM of primers, and 5 µl of template DNA. Distilled, PCR grade water was used to make up the volume of the reaction mixture to 25 µl. The forward primer used was: AKP 78 5'-GCTGGTGCTGGAATAAATGC-3' and the reverse primer: AKP 79 5'-TCGCCATTGAGTAGTTTCCC-3', (Keyburn *et al.* 2008) synthesised by Inqaba Biotech, South Africa. A confirmed positive *netB* toxin gene sample was used as the positive control and PCR-grade water as the negative control.

The thermocycler programme consisted of denaturation at 94ºC for 2 min; 35 cycles of denaturation at 94ºC for 30 s; annealing at 55ºC for 30 s; extension at 72ºC for 1 min and final extension at 72ºC for 12 min (Keyburn *et al.* 2008). A 1.5% agarose gel was used to analyse 5 µl of the PCR product by electrophoresis at 100 V for 45 min.

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The size of positive amplicons was determined by the use of a 100 bp molecular marker (Hyperladder^{TM} IV, Bioline). A digital image of the gel was taken with a photo documentation system (Molecular Imager® ChemiDoc^{TM} XRS+ System, Bio-Rad, South Africa) for capture and storage of the results.

The positive control for the *netB* toxin gene was developed from the samples obtained in this study. Previous studies have indicated that 70% of *C. perfringens* isolates from chickens with necrotic enteritis were positive for *netB* toxin gene (Keyburn *et al*. 2010). Initial PCR testing was conducted without a positive control and the samples were screened with the anticipation that *netB* positive isolates would be encountered. Indeed, isolates that gave the expected positive result for *netB* toxin gene were identified. The amplification product of three of these positive samples and the primer AKP 78 were sent to Inqaba Biotech, South Africa for sequencing. The sequencing data was analysed on the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) with the Basic Local Alignment Search Tool (Blast) to determine the degree of similarity between the sequences obtained in this study and those published in Genbank. Thereafter, one of these established *netB* toxin gene positive samples was used as the positive control.

b) *Salmonella* spp.

Salmonella was isolated from the samples by a selective enrichment culture method. Samples were inoculated into buffered peptone water and incubated aerobically at 37ºC for 18-24 h. From buffered peptone water, samples were inoculated into Rappaport-Vassiliadis (RV) broth and incubated aerobically at 41.5ºC for 18-24 h. Thereafter, samples were cultured on xylose lysine desoxycholate (XLD) agar and incubated aerobically at 37ºC for 18-24 h. Colonies, suspected to be *Salmonella* spp., were transferred from XLD agar and grown on blood agar in an aerobic environment at 37ºC for 18-24 h. The Gram-stain, catalase, oxidase and indole tests were performed to further characterise the isolates. The API 10S (bioMérieux), miniaturised biochemical sugars test strips were used to confirm the isolates as *Salmonella*.

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i. Serotyping

Salmonella isolates were further serotyped at the Bacteriology Laboratory of the Agricultural Research Council's Onderstepoort Veterinary Institute (ARC-OVI) and identified according to the antigenic formula provided by the White-Kauffmann-Le Minor scheme (Grimont *et al.* 2007). The serotyping procedure involved the slide agglutination test. A single *Salmonella* colony, from a pure culture on blood agar, was added to a 10 µl drop on a glass plate, of first a polyvalent and after agglutination occurred a monovalent antiserum was used on a fresh preparation, to characterize the isolate further. The antiserum and bacterial culture were mixed thoroughly with a sterile bacterial loop to form a homogenous milky suspension. The suspension was mixed by gently tilting the glass plate up and down for about 30-60 s to allow the agglutination reaction to take place.

The O (somatic) antigens were detected first by use of polyvalent antisera. The most common polyvalent antisera "OMA" (containing antisera against Group A, B, D, E, L) and "OMB" (containing antisera against Group C, F, G, H) were used and only if there was no agglutination one proceeded with other polyvalent antisera ("OMC", "OMD", "OME", "OMF", "OMG") until agglutination occurred. When agglutination occurred with the polyvalent antisera the test was repeated with the constituent monovalent antisera and agglutination observed.

The detection of H (flagella) antigens took into account the possibility of a biphasic serovar. Identification of the first phase was similar to that of the O antigens. The most common polyvalent antisera "HMA" (containing a, b, c, d, i, z10, z29m antisera) and "HMB" (containing e, h: e, n, x: e, n, z15: G antisera) were tested first and if no agglutination occurred other polyvalent antisera ("HMC", "HMD", "HMIII", "HE", "H1", "HL", "HZ4", "HG") followed until agglutination occurred. Agglutination with the monovalent antiserum identified the H antigen. Reference was made to the White-Kauffmann-Le minor scheme to determine if the *Salmonella* isolate was monophasic or diphasic.

Phase inversion was used to determine the second phase of a diphasic isolate. A volume of 25 µl of the first phase antiserum was added to 9 ml of molten swarming agar at 56°C and swirled gently to mix. The swarming agar was poured into a petri

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dish and allowed to cool and solidify. Half a loop full of bacteria was picked from the blood agar plate, placed at the centre of the swarm agar plate and incubated at 37ºC for 18-24 h. Colonies from the edge of the spreading colony on the swarming agar were used to identify the second phase by the agglutination method described above. At this stage if the second phase was not identified, phase inversion was repeated for a maximum of two more times before the isolate was declared to be monophasic.

c) *Escherichia coli*

The routine culture method for the family *Enterobacteriaceae* was used to isolate *E. coli* from the samples. The samples were inoculated onto blood agar and MacConkey agar and incubated aerobically at 37ºC for 18-24 h. Suspect *E. coli* colonies were Gram stained and the catalase, oxidase, indole, xylose, urease and citrate tests were performed to confirm the identification (Quinn *et al.* 1994).

i. Virulence factor PCR

Escherichia coli isolates were sent to the ARC-OVI for virulence factor characterisation by PCR. Multiplex PCR was performed using the specific primers for: LT, STa, STb, EAST1, Stx1, Stx2, Stx2e, Paa and AIDA-1 (Mohlatlole *et al.* 2013). This multiplex PCR determined the presence of enteropathogenic *E. coli* by possession of *paa* gene; enterotoxigenic *E. coli* by possession of LT, STa, STb genes and enterohaemorrhagic *E. coli* by possession of Stx1, Stx2, Stx2e genes.

d) *Campylobacter*

A selective culture medium, Preston agar, was used for the detection of *Campylobacter* spp. Samples were inoculated onto Preston agar and incubated in a microaerophilic environment (6% O_2, 10% CO_2, 84% N_2) at 37ºC for three to five days. The incubation period was extended to five days if there was no growth on the culture medium after 3 days. The Gram-stain was performed on suspect *Campylobacter* spp. colonies.

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e) *Pseudomonas*

Standard procedures for aerobic culture were used for the detection of *Pseudomonas* spp*.* Samples were inoculated onto blood agar and MacConkey agar and incubated aerobically at 37ºC for 18-24 h. Pigment production and odour of the bacterial culture were considered; to give an indication of the species of *Pseudomonas* (i.e. *Pseudomonas aeruginosa* produces a bluish pigment and has a fruity odour). The Gram-stain, catalase and oxidase tests were performed to determine if an isolate was *Pseudomonas* spp*.* (Quinn *et al.* 1994).

2.6.2 Virology

The standard operating procedure for EM of negatively stained material, used at the Electron Microscopy Unit of the Department of Anatomy and Physiology, FVS, UP was employed for the detection of viral particles from the intestinal contents of the ostrich chicks. The total number of samples tested was 76 and these were received from 15 farms (Farms, A-O).

Briefly; a dilution of 1:5 of intestinal contents in distilled water was centrifuged at low speed (700 g for 15 min) to separate bacteria and other debris. Viral particles were concentrated by ultracentrifugation of the supernatant at 13 250 g for 45 min (Sigma 1-15 centrifuge). The pellet was rinsed with distilled water and then mixed thoroughly with one drop of distilled water. The pellet mixture was negatively stained with 3% phosphotungstic acid (Fluka) and prepared for EM on a formvar carbon coated grid. The Philips CM 10 transmission electron microscope was used to screen the samples for viral particles.

The paraffin wax block of one sample which showed the sloughing lesion when observed by microscopy of the haematoxylin-eosin stained section was submitted to the Electron Microscopy Unit. The section of interest was extracted from the paraffin wax block and examined by transmission electron microscopy, with negative staining, as described above.

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2.6.3 Parasitology

A total of 44 samples were tested for parasites at the Helminthology Section of the DVTD. The direct faecal flotation test (Fecalyser®) was used to screen the faecal samples for the presence of any helmintic or coccidial eggs.

Of the 44 samples, only 12 were tested for *Cryptosporidium* due to unavailability of sufficient faecal material. To detect *Cryptosporidium* oocysts, a faecal smear was prepared and stained using heated safranin counterstained with methylene blue (Baxby *et al.* 1984). The sample was observed under the light microscope using the 20x objective lens.

2.6.4 Histology

Histological examination was done at the Section of Pathology, FVS. Samples preserved in 10% formalin, were prepared for routine histological examination and stained with haematoxylin and eosin. A total of 79 samples were observed under the light microscope, to characterize any pathologic lesions. Findings were divided into four generalised groupings which were necrotic enteritis, non-specific necrosis, sloughing and no specific findings.

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CHAPTER 3

Results

3.1 Conditions observed on the ostrich farms

Biosecurity measures were at best, only partially implemented on the farms. Access control to the farms was not implemented and the use of footbaths when entering ostrich raising facilities was sporadic. The use of fresh protective clothing when entering the farm was mainly at the discretion of the visitor. It was common for wild birds to be seen aggregating on the feed and water provided for the ostriches; but most farms did have pest control measures for rodents in chick houses and feed sheds.

Figure 3.1 Illustration of wild birds attracted by feed for ostrich chicks

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3.1.1 Housing and handling

Farmers received ostrich chicks at one day of age from a hatchery located in Oudtshoorn. An “all in all out” policy was practiced on all the farms and the chicks were kept together as a batch separate from other age groups or batches. Premises were cleaned and disinfected with F10™SC Veterinary Disinfectant, Health and Hygiene (Pty) LTD in between batches of chicks.

Ostrich chicks were housed in an enclosed structure or building at night to protect them from sharp drops in temperature that could occur. Cylindrical holding pens with a blanket covering the top and/or artificial heating was used to maintain an ideal temperature of above 30ºC that was gradually dropped to 20-25ºC at four weeks of age (Deeming 1999; Shanawany *et al.* 1999). A minimum-maximum thermometer recorded the temperature range throughout the night. Ventilation was maintained through built in air vents or windows. The floor surface was made of a mesh (usually steel or sometimes hard plastic) to allow excreta to drop freely through the slats where it could be drained later. On a concrete surface wood chippings were used that acted as insulation and could easily be removed and replaced when soiled. Some farmers used carpets in place of wood chippings. Housing quarters were generally cleaned of particulate matter daily but only a few farms washed and disinfected on a daily basis.

During the day ostrich chicks were kept in a fenced enclosure outside with food and water provided ad lib. Enough space was provided for adequate access for each chick to food and water and for exercise. A shaded area was provided to shelter the chicks from excessive heat conditions.

Handling of the chicks was kept to a minimum. Chicks were handled in the evening when they were transferred indoors and in the morning when they were allowed outside. Other infrequent handling was occasioned by medications in the case of sick birds, or vaccination.

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Figure 3.2 Illustration of the housing facilities for ostrich chicks

3.1.2 Feed and water

Feed and water were provided ad lib in low lying troughs or drinkers for water. Multiple feeding and watering points were provided to avoid competition. The feed was either mixed on the farm from procured raw materials or bought as a ready mixed preparation. The feed formulation was one recommended by a veterinarian or an animal nutritionist. No medications were used either in the feed or water on a routine basis for prophylaxis or as growth promotants. Very few farms were connected to the municipal water supply and most sourced water from mountain streams. Purification of water, from natural sources, was achieved by chlorination with chlorine tablets added to the water reservoir.

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3.1.3 Treatments

Only one farm was observed to have dedicated housing quarters for sick birds. When a bird fell sick it was removed from its group and transferred to the sick quarters for observation and treatment. The bird would be returned to its group upon recovery. On most farms, sick birds were treated within their group. The veterinarian was consulted when signs of enteritis were observed (i.e. diarrhoea, anorexia, depression) and a course of antibiotics will usually be given. Prophylactic treatments administered were mainly water soluble probiotic (Protexin) at one day of age and vaccination (Coglamune®) against clostridial infection at three weeks of age.

3.2 Clinical signs, post-mortem findings and treatment

The principal presenting clinical sign of the 122 ostrich chicks examined was death. Five major symptoms seen prior to death were: diarrhoea (n=47); no preliminary signs/symptoms (n=40); depression/weakness (n=27); failure to thrive (n=5) and respiratory signs (n=3). Post-mortem revealed varying degrees of intestinal inflammation (indicative of enteritis) which affected different sections; small intestine, colon, caecum, sometimes in combination. In approximately 20% of cases there were no significant findings at post-mortem. Antibiotic treatment was used in 39% of the cases; no use of antibiotics in 14% of the cases and the use of antibiotics could not be determined in 47% of the cases. The clinical and post-mortem findings and the treatment for the different cases are presented in Table 3.1.

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Table 3.1 The clinical signs, post-mortem findings and treatment of cases received in this study

<table>
<tr><th>Clinical signs (prior to death)</th><th>Number of chicks</th><th>Post-mortem findings</th><th>Number of chicks</th><th>Antibiotic treatment</th><th>Number of chicks</th></tr>
<tr><td rowspan="6">Diarrhoea</td><td rowspan="6">47</td><td>Pseudomembranous enterocolitis</td><td>1</td><td rowspan="2">Yes</td><td rowspan="2">22</td></tr>
<tr><td>Enterocolitis</td><td>21</td></tr>
<tr><td>Pseudomembranous colitis</td><td>3</td><td rowspan="2">No</td><td rowspan="2">11</td></tr>
<tr><td>Pseudomembranous typhlocolitis</td><td>4</td></tr>
<tr><td>Typhlocolitis</td><td>7</td><td rowspan="2">Not stated</td><td rowspan="2">14</td></tr>
<tr><td>No significant findings</td><td>11</td></tr>
<tr><td rowspan="6">No preliminary signs</td><td rowspan="6">40</td><td>Pseudomembranous enteritis</td><td>8</td><td rowspan="2">Yes</td><td rowspan="2">14</td></tr>
<tr><td>Enterocolitis</td><td>6</td></tr>
<tr><td>Pseudomembranous colitis</td><td>4</td><td rowspan="2">No</td><td rowspan="2">5</td></tr>
<tr><td>Pseudomembranous typhlocolitis</td><td>9</td></tr>
<tr><td>Typhlocolitis</td><td>7</td><td rowspan="2">Not stated</td><td rowspan="2">21</td></tr>
<tr><td>No significant findings</td><td>6</td></tr>
<tr><td rowspan="4">Depression/weakness</td><td rowspan="4">27</td><td>Enterocolitis</td><td>12</td><td>Yes</td><td>9</td></tr>
<tr><td>Colitis</td><td>3</td><td>No</td><td>1</td></tr>
<tr><td>Pseudomembranous typhlocolitis</td><td>8</td><td>Not stated</td><td>17</td></tr>
<tr><td>No significant findings</td><td>4</td><td></td><td></td></tr>
<tr><td rowspan="3">Failure to thrive</td><td rowspan="3">5</td><td>Enterocolitis</td><td>1</td><td>Yes</td><td>0</td></tr>
<tr><td></td><td></td><td>No</td><td>0</td></tr>
<tr><td>No significant findings</td><td>4</td><td>Not stated</td><td>5</td></tr>
<tr><td rowspan="3">Respiratory signs</td><td rowspan="3">3</td><td rowspan="3">Enterocolitis</td><td rowspan="3">3</td><td>Yes</td><td>3</td></tr>
<tr><td>No</td><td>0</td></tr>
<tr><td>Not stated</td><td>0</td></tr>
</table>

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3.3 Bacteria

3.3.1 Bacteria isolated from samples

A total of 151 samples were collected from the 122 ostrich chicks and tested for bacteria. Bacterial species isolated from the samples are indicated in Table 3.2. The most frequently isolated bacterium was *E. coli* (49.1% of isolations), followed by *C. perfringens* (20.2% of isolations), *Enterococcus* spp. (16.1% of isolations), *Salmonella* spp. (6.9% of isolations), *Klebsiella* spp. (1.8% of isolations), *Enterobacter* and *Citrobacter* spp. (1.4% of isolations each), *Staphylococcus*, *Streptococcus* and *Bacillus* spp. (0.5% of isolations each). No *Campylobacter* spp. and no *Pseudomonas* spp. were isolated from the samples. There were no bacteria isolated from 1.8% of the samples.

Table 3.2 Different bacterial species isolated from samples obtained in this study

Bacteria	Number isolated	Percentage of total samples (n=151)
E. coli	107	49.1%
C. perfringens	44	20.2%
Salmonella spp.	15	6.9%
Campylobacter spp.	0	0%
Pseudomonas spp.	0	0%
Enterococcus spp.	35	16.1%
Klebsiella spp.	4	1.8%
Enterobacter spp.	3	1.4%
Citrobacter spp.	3	1.4%
Staphylococcus spp.	1	0. 5%
Streptococcus spp.	1	0.5%
Bacillus spp.	1	0.5%
No bacteria isolated	4	1.8%

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3.3.2 Combinations of bacteria isolated from samples

Escherichia coli alone (n=57; 37.7%) was the most frequently isolated from all the samples (n=151). The grouping "Other" consists of *Enterococcus* spp., *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp. and "No bacteria isolated" and this grouping was the second most frequently isolated (n=33; 21.8%). The combination of *E. coli* and *C. perfringens* (n=31; 20.5%) was the third most frequently isolated. The remaining combinations were of low frequency ranging from (n=7; 4.6%) to (n=1; 0.7%) and these in descending order were: *E. coli* and "Other"; *E. coli* and *Salmonella* spp.; *C. perfringens* and "Other"; *E. coli*, *C. perfringens* and *Salmonella* spp.; *Salmonella* spp. alone; *E. coli*, *C. perfringens* and "Other"; *Salmonella* spp. and *C. perfringens*; and *Salmonella* spp. and "Other". The combinations of bacteria isolated from the samples are indicated in Figure 3.3.

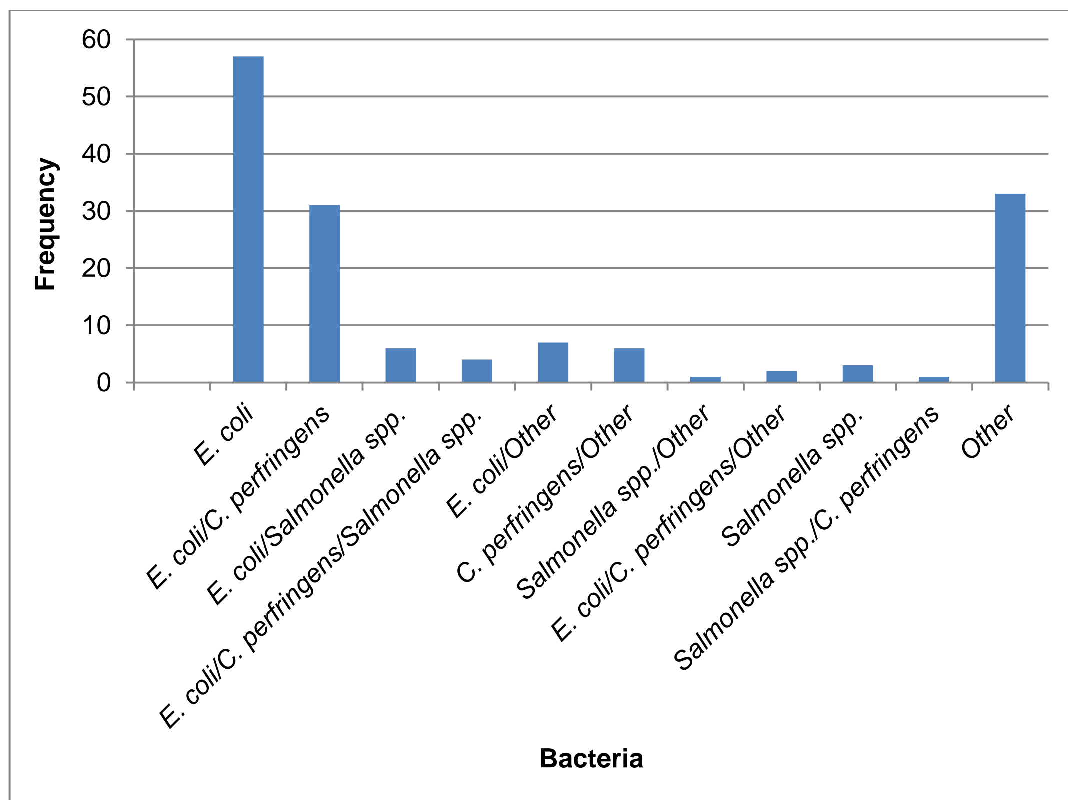

Figure 3.3 Frequency of the combinations of bacteria isolated from the samples

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3.3.3 *Clostridium perfringens* types and occurrence of *netB* toxin gene

Clostridium perfringens was isolated from the small intestine, colon and the caecum and was isolated from 12 of the 17 farms. Other bacteria were always isolated together with *C. perfringens*. The majority of the isolates (93.2%) were *C. perfringens* type A and only a few (6.8%) were *C. perfringens* type E. *Clostridium perfringens* type E was isolated from only one farm, Farm B. *Clostridium perfringens* type A was isolated from all the 12 farms (Table 3.3).

Table 3.3 *Clostridium perfringens* types and presence of *netB* toxin gene

Farm	**Tissue**	***C. perfringens* type**	***netB* toxin gene**	**Other bacteria isolated**
A	small intestine	A	Negative	*E. coli*
A	small intestine	A	Negative	*E. coli*
A	colon	A	Negative	*E. coli*, *S.* Hayindongo
A	colon	A	Negative	E. coli, *S.* Hayindongo
A	intestine	A	Negative	*E. coli*
A	intestine	A	Negative	*E. coli*
A	intestine	A	Negative	*S.* Muenchen
A	intestine	A	Negative	*E. coli*, *S.* Muenchen
A	intestine	A	Negative	E. coli, *S.* Muenchen
A	colon	A	Negative	*E. coli*
B	small intestine	A	**Positive**	*E. coli*
B	small intestine	E	**Positive**	*E. coli*
B	colon	E	**Positive**	*E. coli*
B	colon	E	**Positive**	*E. coli*
C	colon	A	Negative	*E. coli*
C	colon	A	Negative	*E. coli*
C	colon	A	Negative	*E. coli*
C	colon	A	Negative	*E. coli*
C	colon	A	Negative	*E. coli*
C	caecum	A	Negative	*E. coli*
C	caecum	A	Negative	*E. coli*
D	caecum	A	Negative	*E. coli*
E	colon	A	Negative	*E. coli*
E	colon	A	Negative	*E. coli*
E	colon	A	Negative	*E. coli*
E	colon	A	Negative	*E. coli*

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Farm	Tissue	*C. perfringens* type	*netB* toxin gene	Other bacteria isolated
E	colon	A	Negative	*E. coli*
F	small intestine	A	**Positive**	*E. coli*
F	small intestine	A	**Positive**	*E. coli*
F	small intestine	A	**Positive**	*E. coli*
F	small intestine	A	Negative	*E. coli*
G	small intestine	A	Negative	*Enterococcus* spp.
G	colon	A	Negative	*Enterococcus* spp.
G	colon	A	Negative	*E. coli*, *Enterococcus* spp.
G	colon	A	Negative	*E. coli*
G	colon	A	Negative	*E. coli*
H	colon	A	Negative	*E. coli*, *Citrobacter* spp.
H	colon	A	Negative	*E. coli*
H	colon	A	Negative	*E. coli*
I	colon	A	Negative	*E. coli*
J	colon	A	Negative	*Enterococcus* spp.
O	colon	A	Negative	*Enterococcus* spp.
R	colon	A	Negative	*Enterococcus* spp., *Citrobacter* spp.
R	colon	A	Negative	*Enterococcus* spp.

The *netB* toxin-specific PCR results showed 15.9% of the *C. perfringens* isolates were positive for *netB* toxin encoding gene. All the *C. perfringens* type E isolates possessed the *netB* toxin gene and only 9.8% of *C. perfringens* type A possessed it. The *netB* toxin gene positive isolates were found on two farms, Farm B and Farm F. The size of the PCR amplicon correlated with the 384 bp size of the *netB* toxin encoding gene fragment of the positive control sample (Figure 3.4).

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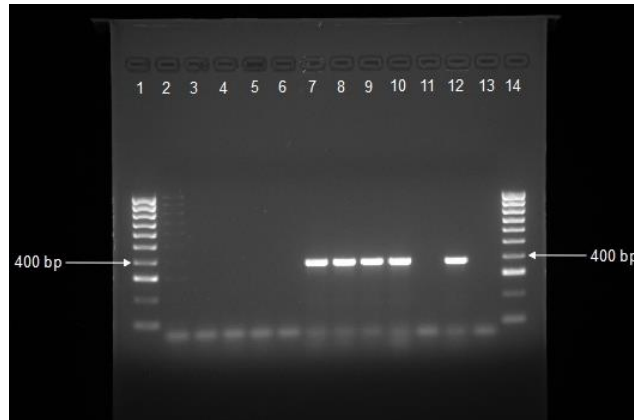

Figure 3.4 PCR amplicon results for *netB* toxin gene. Lanes 1 and 14 are the 100 bp molecular markers (Hyperladder^{TM}IV, Bioline). Lanes 2-6 and lane 11 are negative samples. Lanes 7-10 are positive samples. Lane 12 is the positive control and Lane 13 is the negative control.

3.3.4 Sequencing of the *netB* toxin gene

The BLAST results indicated that the sequence for the *netB* toxin gene had 94% similarity to the sequences available on the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The sequences of the three PCR products, that were sequenced, were identical to each other.

3.3.5 *Salmonella* serotypes

Salmonella was isolated from only three farms of the total of 17 farms sampled in this study. *Salmonella* Muenchen was the most prevalent and constituted 80% of the *Salmonella* isolates, *Salmonella* Hayindongo 13.3% and *Salmonella* Othmarschen 6.7% were the other serotypes isolated. *Salmonella* Muenchen and *S.* Hayindongo were isolated from Farm A on two separate occasions and only one type was isolated from all samples on each occasion. Farm B and K had one isolate of *S.* Muenchen and *S.* Othmarschen, respectively. *Salmonella* was mostly isolated in combination with other bacteria, mainly *E. coli* and *C. perfringens*. There were only three samples from Farm A where *S.* Muenchen was isolated without other bacteria.

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Table 3.4 Salmonella serotypes isolated from samples obtained in this study

Date sampled	Farm	Tissue	*Salmonella* serotype	Other bacteria isolated
2011/03/08	A	colon	*S.* Hayindongo	*E. coli*, *C. perfringens*
2011/03/08	A	colon	*S.* Hayindongo	*E. coli*, *C. perfringens*
2011/09/21	A	intestine	*S.* Muenchen	*E. coli*
2011/09/21	A	intestine	*S.* Muenchen	*E. coli*
2011/09/21	A	intestine	*S.* Muenchen	*C. perfringens*
2011/09/21	A	intestine	*S.* Muenchen	none
2011/09/21	A	intestine	*S.* Muenchen	none
2011/09/21	A	intestine	*S.* Muenchen	none
2011/09/21	A	intestine	*S.* Muenchen	*E. coli*
2011/09/21	A	intestine	*S.* Muenchen	*E. coli*
2011/09/21	A	intestine	*S.* Muenchen	*E. coli*
2011/09/21	A	intestine	*S.* Muenchen	*E. coli*, *C. perfringens*
2011/09/21	A	intestine	*S.* Muenchen	*E. coli*, *C. perfringens*
2011/09/22	B	intestine	*S.* Muenchen	*E. coli*
2011/10/06	K	colon	*S.* Othmarschen	*Enterobacter* spp.

3.3.6 *Escherichia coli* and virulence factor determination

Escherichia coli was isolated from 14/17 (82.4%) farms sampled. From the *E. coli* positive samples it was isolated alone from 53.3% of the samples and in combination with other bacteria from 46.7% of the samples.

The characterisation of the virulence factors from 106 of the 107 *E. coli* isolates, in this study, revealed that EAST1 was the most prevalent gene as it was identified from 63 (59.4%) *E. coli* isolates. This was followed by: *paa*, 41 (38.7%); AIDA-1, 5 (4.7%); STa, 2 (1.9%); STb, 2 (1.9%). The virulence factors LT, Stx1, Stx2 and Stx2e were not identified and 22 (20.8%) *E. coli* isolates were negative for all the virulence factors tested for.

The combination of these genes in the *E. coli* isolates was: EAST1 alone (34.9%); *paa* alone (16.0%); EAST1 and *paa* (19.8%); EAST1 and AIDA-1 (0.9%); EAST1,

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paa and AIDA-1 (2.8%); AIDA-1 alone (0.9%); STa alone (0.9%); STb alone (1.9%); STa and EAST1 (0.9%). The results are indicated in Table 3.6.

The *E. coli* isolates were categorised based on their possession of certain virulence factor genes as indicated in Table 3.5. Enteropathogenic *E. coli* made up 38.7%, enterotoxigenic *E. coli* 3.8% and no enterohaemorrhagic *E. coli* was found.

Table 3.5 Categorisation of *Escherichia coli* isolates by possession of virulence factor genes

***E. coli* category**	**Determinant**	**Total no. isolates (%)**
Enteropathogenic *E. coli*	*paa* Positive; LT, ST and Stx Negative	41 (38.7%)
Enterotoxigenic *E. coli*	LT and/or ST Positive	4 (3.8%)
Enterohaemorrhagic *E. coli*	Stx Positive	0 (0.0%)

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Table 3.6 *Escherichia coli* virulence factor gene characterisation

Farm	Type of sample	*E. coli* virulence factor gene									Other bacteria isolated
		LT	STa	STb	Stx1	Stx2e	Stx2	EAST1	Paa	AIDA-1	
A	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*C. perfringens*
A	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*C. perfringens*
A	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	*S. Hayindongo*, *C. perfringens*
A	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*S. Hayindongo*, *C. perfringens*
A	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	**Pos**	none
A	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	**Pos**	none
A	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	none
A	colon	Neg	**Pos**	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
A	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	**Pos**	none
A	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
A	intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*C. perfringens*
A	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*S. Muenchen*
A	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*S. Muenchen*
A	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*S. Muenchen*
A	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*S. Muenchen*
A	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*S. Muenchen*
A	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*S. Muenchen*, *C. perfringens*
A	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*S. Muenchen*, *C. perfrimges*
A	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
A	small intestine	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg	none
A	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
A	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*C. perfringens*
A	colon	Neg	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none

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Farm	Type of sample	*E. coli* virulence factor gene									Other bacteria isolated
		LT	**STa**	**STb**	**Stx1**	**Stx2e**	**Stx2**	**EAST1**	**Paa**	**AIDA-1**	
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*Citrobacter freundii*
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	**Pos**	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
B	intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	*S. Muenchen*
B	small inetestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
B	small inetestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
B	small inetestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
B	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
B	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
B	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
C	colon	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*C. perfringens*
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	none
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	none
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*Enterococcus* spp.
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*C. perfringens*
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	*C. perfringens*
C	caecum	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*C. perfringens*
C	caecum	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	*C. perfringens*
C	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	none

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Farm	Type of sample	*E. coli* virulence factor gene									Other bacteria isolated
		LT	**STa**	**STb**	**Stx1**	**Stx2e**	**Stx2**	**EAST1**	**Paa**	**AIDA-1**	
C	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	none
C	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	none
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	none
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	*Enterococcus* spp.
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	none
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
C	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
C	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
C	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
C	caecum	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
C	caecum	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	*Enterococcus* spp.
D	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
D	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
D	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
D	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
D	caecum	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
D	caecum	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	*C. perfringens*
E	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
E	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	*C. perfringens*
E	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	*C. perfringens*
E	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
E	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
F	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	*C. perfringens*
F	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
F	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	*C. perfringens*
F	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	*C. perfringens*
G	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*, *Enterococcus* spp.
G	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	*C. perfringens*
G	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
H	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*, *Citrobacter* spp.

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Farm	Type of sample	*E. coli* virulence factor gene									Other bacteria isolated
		LT	**STa**	**STb**	**Stx1**	**Stx2e**	**Stx2**	**EAST1**	**Paa**	**AIDA-1**	
H	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*C. perfringens*
H	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	*C. perfringens*
I	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
I	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*C. perfringens*
I	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
J	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	*Enterococcus* spp.
K	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
L	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	none
L	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	*Klebsiella* spp.
N	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
N	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
N	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
N	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*Enterococcus* spp.
P	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	none
P	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	none
P	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none

Neg: Negative

Pos: Positive

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3.3.7 Electronmicroscopy

The electronmicrograph shows an example of one sample which had the “sloughing” lesion on histopathology and from which the sole bacteria isolated was enteropathogenic *E. coli*. This was an attempt to indicate the attachment of enteropathogenic *E. coli* to the intestinal epithelium in the attaching and effacing lesion as described before (Moon *et al*. 1983). The virulence factor Paa was the only one identified from this *E. coli* isolate which was categorised as enteropathogenic *E. coli*. Rod shaped bacteria, assumed to be *E. coli* were seen attached to the intestinal epithelium and some were seen free in the intestinal lumen as shown in Figure 3.5.

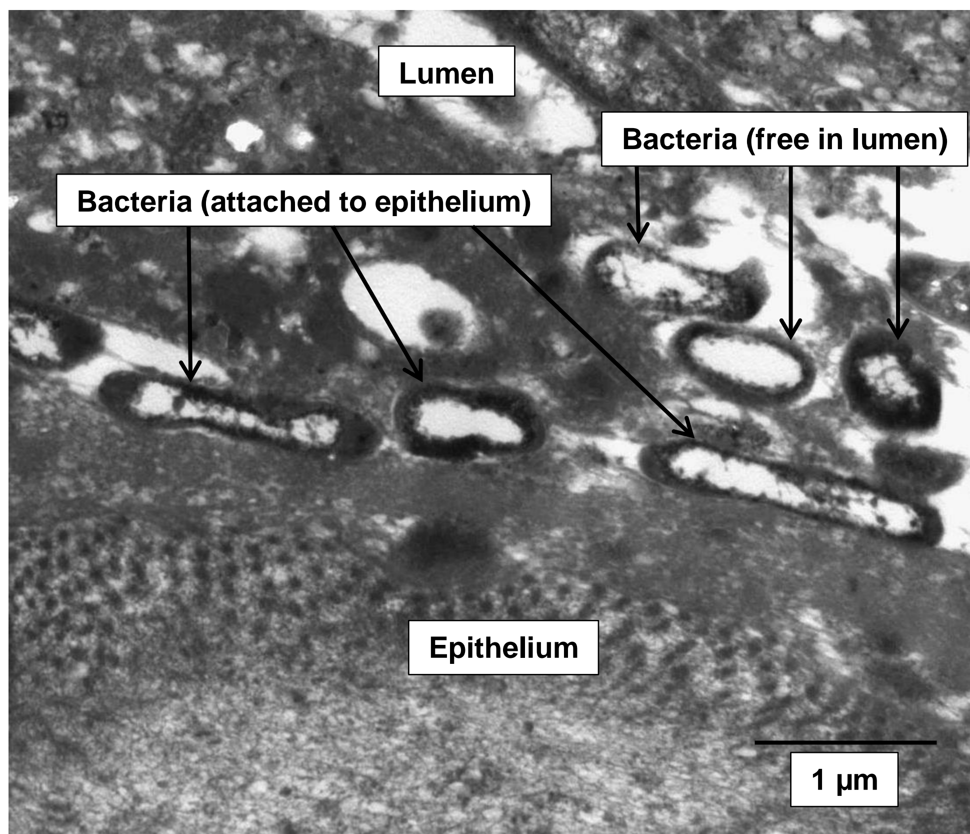

Figure 3.5 Electronmicrograph showing bacteria attached to intestinal epithelium

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3.4 Parasites

There were no coccidia and no parasite (nematode) eggs identified from the 44 samples tested. No *Cryptosporidium* spp. was identified from the 12 samples tested.

3.5 Viruses

From the 76 samples tested for viruses using EM only 11 (14.5%) were positive. Fifteen farms (A-O) were tested and samples from only four farms (A, C, F and N) were positive for viruses. There was one virus recovered from Farm A (enterovirus) and Farm F (birnavirus). There were two viruses recovered from Farm C (Enterovirus and Reovirus) from two different samples. The most viruses were recovered from Farm N with six enterovirus-like particles and one unidentified virus-like particle.

The enteroviruses and enterovirus-like particles were seen as 24-30 nm particles; the birnaviruses were 60 nm hexagonal particles; the reoviruses were 61-64 nm spherical particles and the unidentified virus-like particles were 20 nm spherical particles.

The most frequently identified virus was enterovirus or enterovirus-like particles (n=8) and only one of each of the other viruses identified, birnavirus (n=1), reovirus (n=1) and unidentified viras-like particles (n=1). From all the samples bacteria were isolated in conjunction with viruses i.e. *E. coli*, *C. perfringens*, *Enterococcus* spp. and *Bacillus* spp. The results are shown in Table 3.7 and Figures 3.6 A and B.

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Table 3.7 Viruses identified from samples by electron microscopy

Farm	Virus	Other bacteria isolated
A	Enterovirus	*E. coli*
C	Enterovirus	*E. coli*, *C. perfringens*
C	Reovirus	*E. coli*, *Enterococcus* spp.
F	Birnavirus	*E. coli*, *C. perfringens*
N	Enterovirus-like	*Enterococcus* spp.
N	Enterovirus-like	*Enterococcus* spp.
N	Enterovirus-like	*Enterococcus* spp.
N	Enterovirus-like	*Enterococcus* spp.
N	Enterovirus-like	*Enterococcus* spp., *Bacillus* spp.
N	Enterovirus-like	*E. coli*, *Enterococcus* spp.
N	Unidentified virus-like particle	*E. coli*

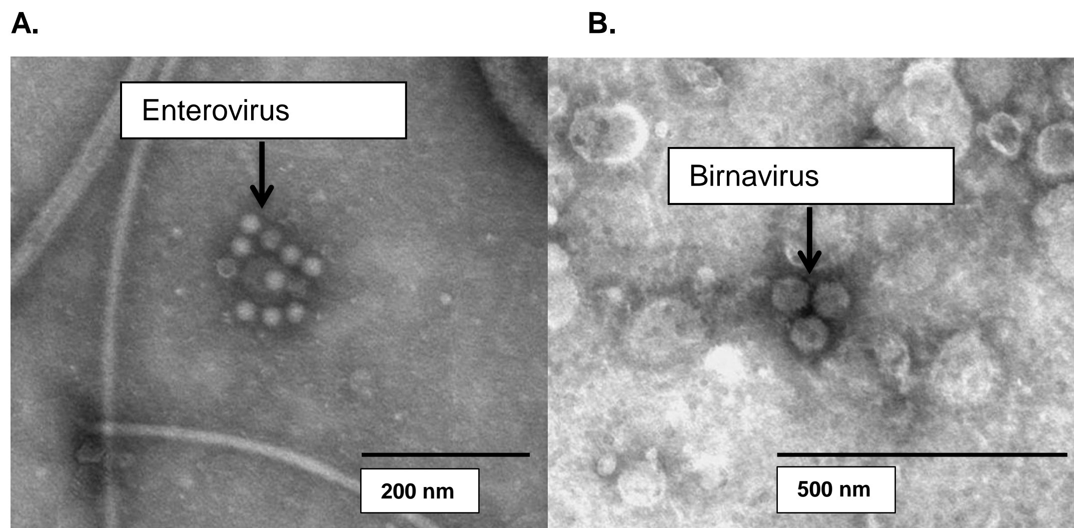

Figure 3.6 Electron micrographs of virus particles: **A.** Enterovirus particles and **B.** Birnavirus particles

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3.6 Histopathology

Only three samples had lesions regarded as necrotic enteritis. *Clostridium perfringens* was isolated from all these samples, *E. coli* from two and *Enterococcus* from one sample. Non-specific necrosis was observed in 22 samples and various bacteria including *E. coli* (n=12 samples), *C. perfringens* (n=8 samples), *Salmonella* spp. (n=9 samples), *Enterococcus* spp. (n=2 samples), *Enterobacter* spp. (n=2 samples) and *Klebsiella* spp. (n=2 samples) were isolated.

Sloughing was observed in 15 samples and bacteria isolated were *E. coli* (n=13 samples), *C. perfringens* (n=5 samples), *Enterococcus* spp. (n=3 samples) and *Citrobacter* spp. (n=1 sample). *Escherichia coli* was isolated alone from eight of the samples with sloughing lesions and in combination with other bacteria, mainly *C. perfringens* from five samples. From the other two samples *C. perfringens* in combination with *Enterococcus* spp. and *Enterococcus* spp. alone were isolated.

No specific findings were observed in 39 samples and the bacteria isolated were *E. coli* (n=26 samples), *C. perfringens* (n=2 samples), *Salmonella* spp. (n=2 samples), *Enterococcus* spp. (n=10 samples), *Klebsiella* spp. (n=1 sample), *Citrobacter* spp. (n=1 sample), *Staphylococcus* spp. (n=1 sample), *Streptococcus* spp. (n=1 sample), *Bacillus* spp. (n=1 sample) and no bacteria isolated (n=2 samples). A comparison of the lesions identified and the bacteria isolated are presented in Table 3.8.

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Table 3.8 Comparison of histopathological lesion observed in samples and bacteria isolated

Type of sample	Histopathology result	Arbitrary description	Bacteria isolated
colon	severe, extensive, necrosis of colon epithelial cells	Necrotic enteritis	*C. perfringens*, *E.coli*
caecum	severe, extensive, necrosis of caecum epithelial cells	Necrotic enteritis	*C. perfringens*, *E.coli*
colon	severe, necrotic, pseudomembranous colitis	Necrotic enteritis	*C. perfringens*, *Enterococcus spp.*
intestine	necrosis of colonic enterocytes and multifocal enterocyte necrosis of small intestinal villi	Non-specific necrosis	*E. coli*
intestine	multifocal, mild, necrosis of epithelial cells of colon	Non-specific necrosis	*E. coli*
intestine	scattered areas of necrosis of epithelial cells of colon	Non-specific necrosis	*E. coli*, *C. perfringens*
intestine	scattered areas of necrosis of colonic enterocytes	Non-specific necrosis	*E. coli*, *C. perfringens*
intestine	multifocal areas of necrosis of colonic enterocytes and bacilli present on mucosal surface	Non-specific necrosis	*E. coli*, *S.* Muenchen
intestine	scattered damage to epithelial cells of colon (loss of cellular structure)	Non-specific necrosis	*E. coli*, *S.* Muenchen
intestine	scattered damage to epithelial cells of colon (loss of cellular structure)	Non-specific necrosis	*S.* Muenchen, *C. perfringens*
intestine	scattered damage to epithelial cells of colon (loss of cellular structure)	Non-specific necrosis	*S.* Muenchen
intestine	scattered, mild, damage to colon epithelial cells (loss of cellular structure)	Non-specific necrosis	*S.* Muenchen
intestine	layer of cellular debris in the lumen adjacent to epithelial cells of colon. Damage to colon epithelial cells present	Non-specific necrosis	*S.* Muenchen
intestine	layer of cellular debris in the lumen adjacent epithelial cells of colon. Damage to colon epithelial cells present	Non-specific necrosis	*E. coli*, *S.* Muenchen

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intestine	necrosis of colon enterocytes, bacilli visible. Small intestine appears normal	Non-specific necrosis	*E. coli*, *S.* Muenchen, *C. perfringens*
colon	multifocal, moderate necrosis of colon epithelial cells	Non-specific necrosis	*C. perfringens*, *Enterococcus* spp.
colon	mild, multifocal erosions of mucosa of colon	Non-specific necrosis	*S.* Othmarschen, *Enterobacter* spp.
colon	autolytic changes present in colon. Pseudomembranous layer and multifocal necrosis of epithelial cells of colon mucosa	Non-specific necrosis	*C. perfringens*, *E. coli*
small intestine	mild, multifocal, necrosis of epithelial cells of small intestine	Non-specific necrosis	*C. perfringens*, *E.coli*
colon	mild, focal, necrosis of colon epithelial cells	Non-specific necrosis	*E.coli*
colon	mild, multifocal, necrosis of colon epithelial cells	Non-specific necrosis	*E.coli*, *C. perfringens*
colon	mild, multifocal enterocyte necrosis (intraluminal debris)	Non-specific necrosis	*Klebsiella* spp.
colon	mild, multifocal enterocyte necrosis (intraluminal debris)	Non-specific necrosis	*Enterobacter* spp.
colon	mild, multifocal enterocyte necrosis (intraluminal fibrin membrane)	Non-specific necrosis	*Klebsiella* spp., *Enterococcus* spp.
colon	mild, multifocal enterocyte necrosis (fibrinous pseudomembrane)	Non-specific necrosis	*E. coli*
intestine	damage to colon mucosal cells, sloughing from mucosal surface with evidence of bacterial involvement. Scattered, mild enterocycte necrosis	Sloughing	*E. coli*
small intestine	sloughing off, of enterocytes from epithelium	Sloughing	*C. perfringens*, *Enterococcus* spp.
small intestine	widespread autolysis, evidence of sloughing of enterocytes from epithelium	Sloughing	*Enterococcus* spp.
colon	mild, multifocal sloughing of cells of mucosa of colon	Sloughing	*E.coli*, *C. perfringens*, *Citrobacter* spp.
colon	loss (sloughing) of epithelial cells of the mucosa. Multifocal areas of autolysis and possibly necrosis of colon epithelial cells	Sloughing	*E.coli*, *C. perfringens*
colon	mild, multifocal sloughing of epithelial cells of colon. Scattered areas of necrosis not consistent with necrotic colitis	Sloughing	*E.coli*

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colon	loss (sloughing) of epithelial cells of colon mucosa	Sloughing	*E.coli*, *C. perfringens*
caecum	loss (sloughing) of epithelial cells of caecum mucosa	Sloughing	*E.coli*, *C. perfringens*
colon	mild, diffuse colitis (bacteria adherent to epithelium-sloughing off)	Sloughing	*E. coli*
colon	loss (sloughing) of epithelial cells of colon mucosa	Sloughing	*E. coli*, *Enterococcus* spp.
small intestine	loss (sloughing) of epithelial cells of mucosa	Sloughing	*E. coli*
colon	loss (sloughing) of epithelial cells of mucosa (attaching effacing lesion)	Sloughing	*E. coli*
colon	extensive, loss (sloughing) of epithelial cells of colon mucosa	Sloughing	*E. coli*
colon	extensive, loss (sloughing) of epithelial cells of colon mucosa	Sloughing	*E. coli*
colon	severe, extensive, loss (sloughing) of epithelial cells of colon mucosa	Sloughing	*E. coli*
intestine	no specific changes to enterocyte morphology	No specific findings	*E. coli*
intestine	no specific findings from the small intestine	No specific findings	*E. coli*
intestine	no specific findings from small intestine	No specific findings	*E. coli*
intestine	no specific changes to enterocyte morphology	No Specific Findings	*E. coli*, *S.* Muenchen
intestine	no specific changes to enterocyte morphology	No specific findings	*E. coli*, *S.* Muenchen
colon	no specific finding from colon	No specific findings	*E.coli*, *C. perfringens*
small intestine	no specific findings	No specific findings	*E.coli*
small intestine	no specific findings	No specific findings	*E.coli*
colon	no specific findings	No specific findings	*E.coli*
colon	no significant findings	No specific findings	*E.coli*
colon	no significant findings (but sloughing off of epithelial cells)	No specific findings	*E. coli*

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small intestine	no significant findings	No specific findings	*Enterococcus* spp.
small intestine	no significant findings	No specific findings	*Enterococcus* spp.
colon	no significant findings	No specific findings	*Enterococcus* spp.
colon	no significant findings	No specific findings	*Enterococcus* spp.
colon	no significant findings	No specific findings	*Enterococcus* spp.
colon	no significant findings	No specific findings	*Enterococcus* spp. and *Bacillus* spp.
small intestine	no significant findings	No specific findings	*E. coli*
small intestine	no significant findings	No specific findings	*Streptococcus* spp.
small intestine	no significant findings (dilatation of crypts with debris inside)	No specific findings	*Enterococcus* spp.
small intestine	no significant findings	No specific findings	*Enterococcus* spp.
colon	no significant findings	No specific findings	No significant bacterial growth
colon	no significant findings	No specific findings	*Enterococcus* spp.
small intestine	no significant findings	No specific findings	No significant bacterial growth
small intestine	no significant findings	No specific findings	*E. coli*
small intestine	no significant findings	No specific findings	*E. coli*
colon	no significant findings	No specific findings	*E. coli*
colon	(intraluminal debris) but no significant findings	No specific findings	*E. coli*, *Enterococcus* spp.
colon	no significant findings	No specific findings	*E. coli*
colon	no significant findings	No specific findings	*E. coli*
small intestine	no significant findings	No specific findings	*E. coli*

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small intestine	no significant findings	No specific findings	*E. coli*
colon	no significant findings	No specific findings	*E. coli*, *C. perfringens*
colon	no significant findings	No specific findings	*E. coli*
colon	no significant findings	No specific findings	*E. coli*, *Klebsiella* spp.
intestine	layer of necrotic debris with bacterial colonies on lumen mucosa of colon but no discernible cell damage to mucosa	No specific findings	*E. coli*, *Citrobacter* spp.
intestine	no specific findings from small intestine. Autolysis of large intestine (unable to interpret)	No specific findings	*E. coli*
intestine	severe, diffuse congestion of colon	No specific findings	*E. coli*
colon	bacteria attached to epithelial surface of colon with no visible cell damage	No specific findings	*Staphylococcus* spp.

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Micrographs showing haematoxylin and eosin stained sections of normal colon (Figure 3.7), necrotic colitis lesion (Figure 3.8) and sloughing lesion (Figure 3.9). The diphtheritic pseudomembrane refers to the adherent membrane to the mucosa made up of necrotic cells of the superficial layers of the mucosa together with an inflammatory exudate (Blood *et al.* 1999). Detached epithelial cells can be seen from the sloughing lesion.

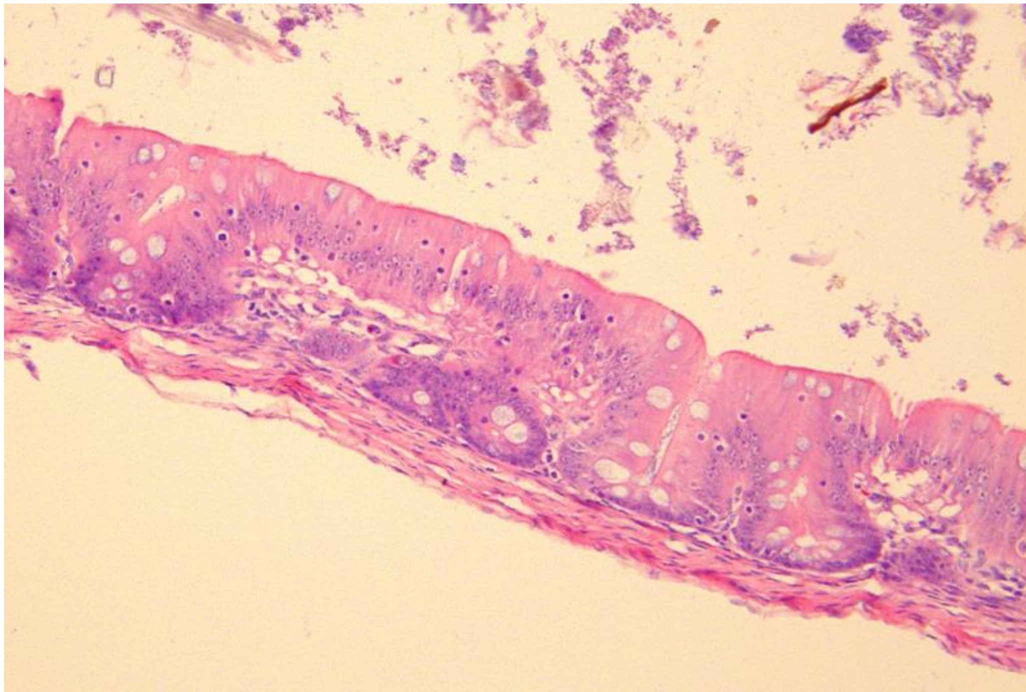

Figure 3.7 Haematoxylin and eosin section of normal colon (x200 magnification)

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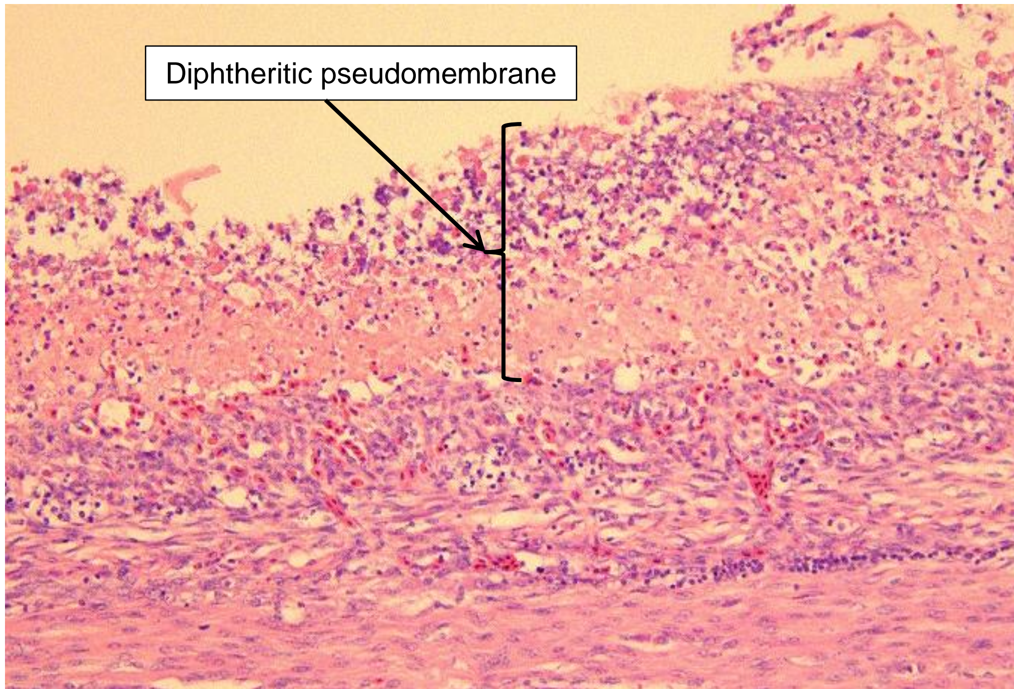

Figure 3.8 Haematoxylin and eosin section of a pseudomembranous necrotic colitis lesion (x200 magnification)

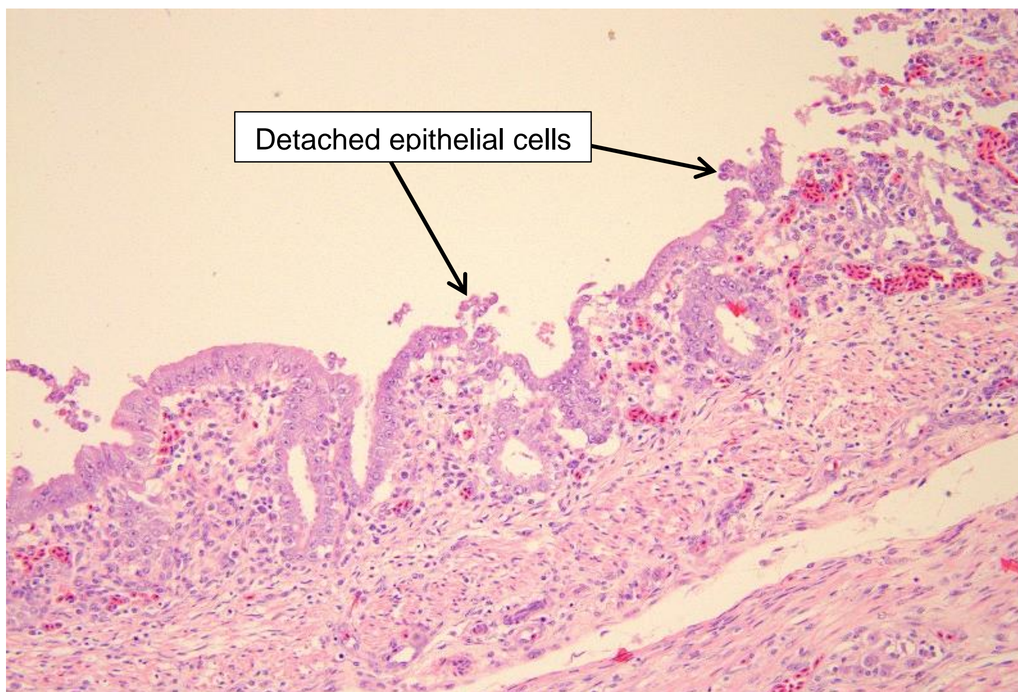

Figure 3.9 Haematoxylin and eosin section indicating the sloughing lesion with detachment of cells from the colon epithelium (x200 magnification)

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3.7 Normal controls

The normal control chicks were in good health with no clinical signs and there were no abnormal findings at post mortem. There were no viruses identified by electron microscopy from all the samples. One four day old chick was positive for sparse coccidial oocysts but there were no coccidia, no nematode and no cryptosporidial eggs identified from any of the other chicks. The histopathological appearance of all the samples was normal.

The bacteria identified from the samples (n=18) in order of decreasing frequency were *E. coli*, 13 (72.2%); *Enterococcus* spp., 11 (61.1%), *Bacillus* spp., 4 (22.2%) and *C. perfringens*, 3 (16.7%). There was only one sample, a small intestine, from which no bacteria were isolated. No *Salmonella* spp., No *Campylobacter* spp. and no *Pseudomonas* spp. were isolated from the normal control samples.

Only three of the 13 (23.1%) *E. coli* isolates harboured any of the virulence factors tested for. The combinations of these virulence factors were EAST1 alone from two *E. coli* isolates and EAST1 with *paa* from one *E. coli* isolate. The prevalence of the *E. coli* virulence factors from the normal controls (23.1%) was much less than that from sick birds (79.2%). All the *C. perfringens* isolates from the normal controls were type A and they did not carry the *netB* toxin gene. The *Enterococcus* spp. and *Bacillus* spp. are a lot more represented in the normal controls then they were from the sick birds. Bacteria isolated from the normal controls are shown in Table 3.9.

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Table 3.9 Bacteria isolated from chicks used as normal controls

Farm	Type of sample	*E. coli* virulence factor gene									Other bacteria isolated
		LT	**STa**	**STb**	**Stx1**	**Stx2e**	**Stx2**	**EAST-1**	**Paa**	**AIDA-1**	
Q	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*C. perfringens* type A, *Enterococcus* spp.
Q	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
Q	small intestine	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*Enterococcus* spp.
Q	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*Enterococcus* spp.
Q	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
Q	colon	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	none
Q	colon	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*Enterococcus* spp.
Q	caecum	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	**Pos**	Neg	*Enterococcus* spp.
Q	caecum	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*C. perfringens* type A, *Enterococcus* spp.
Q	caecum	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
Q	caecum	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	none
Q	caecum	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	*Enterococcus* spp.
Q	caecum	Neg	Neg	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	*Enterococcus* spp.
Q	small intestine	No *E. coli* isolated									none
Q	small intestine	No *E. coli* isolated									*Bacillus* spp.
Q	small intestine	No *E. coli* isolated									*Enterococcus* spp., *Bacillus* spp.
Q	colon	No *E. coli* isolated									*C. perfringens* type A, *Enterococcus* spp., *Bacillus* spp.
Q	colon	No *E. coli* isolated									*Enterococcus* spp., *Bacillus* spp.

Neg: Negative

Pos: Positive

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CHAPTER 4

Discussion and Conclusion

It is well known that ostrich chicks are susceptible to various diseases affecting the gastrointestinal system (Huchzermeyer 1998). Literature on these gastrointestinal diseases however, is particularly limited and the identification of aetiological agents has been fairly sporadic and ill defined. Field outbreaks of mortality in ostrich chicks are not adequately documented and systematic investigations are not undertaken. This leads to a lack in knowledge of the epidemiology of enteric infections (Shane *et al.* 1996).

Ostrich chick rearing continues to be plagued by high mortality in individuals up to three months of age and enteritis represents a major contributing factor to this scenario. One observation is that optimisation of the intensive farming practices has not been achieved, thus making ostrich chicks more vulnerable to infection (Shane *et al.* 1996; Huchzermeyer 2002). Solving the problem of high ostrich chick mortality will be beneficial to the industry as this will improve profitability and have a positive effect on the long term prospects (Glatz *et al.* 2008a; Glatz *et al.* 2008b).

Different factors play a role in the development of enteritis. Disease has been attributed to managerial, environmental, nutritional and infectious causes, probably in combination (Els *et al.* 1998). Ostrich chicks are considered to be exceptionally susceptible to stress, which is capable of inducing disease and even mortality (Huchzermeyer 2002). External factors (often managerial) such as temperature, poor ventilation, overcrowding and internal factors such as nutrition and presence of disease causing organisms are all implicated in the onset of stress (Shanawany *et al.* 1999; Black 2001).

Farms visited in Oudtshoorn in the Klein Karoo, South Africa had comparable management practices but some marked differences were observed. Chicks from the hatchery were housed at night and released into an outside run during the day under fair weather conditions. Temperatures at night were maintained by the use of gas

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heaters and monitored by maximum/minimum thermometers but in some cases blankets were placed over cylindrical hard cardboard enclosures in which chicks were placed. Personal observation indicated that in general not much attention was paid to ventilation and air quality. Housing and provision of warmth at night offered protection from adverse environmental conditions such as sharp falls in night temperatures but lack of attention to ventilation could possibly expose chicks to poor quality air (i.e. ammonia build up from excreta).

The degree of cleaning and hygiene of the living quarters of ostrich chicks differed between the farms. The flooring for indoor housing ranged from iron grid to wood chippings to carpet and the surface of the outdoor run ranged from soil to cement to short cut lucerne pasture. Generally, the farm with the iron grid, indoor flooring cleaned and disinfected daily whilst those utilising wood chippings or carpet tended to clean and replace this substrate on a weekly basis. The soil outside run was swept of excess waste and droppings daily, the concrete run was more thoroughly cleaned with water and disinfectant and the lucerne pasture was left undisturbed. These observations reveal that no industry standard in terms of infrastructure and routine maintenance practices exists.

From their first day on the farm formulated feed was provided to the chicks and this was kept constant for the first three months of the chicks' life. Assuming that all chicks developed healthy feeding behaviour it can be established that the chicks received a balanced nutritional diet with no sudden changes that can predispose to enteric disease (Black 2001). It was observed that overcrowding was not a problem on the farms as chicks had ample feeding and watering space and room for exercise. Chlorine treated water to prevent microbial contamination was provided. Handling of the ostrich chicks was kept to a minimum and on a daily basis chicks were handled twice: in the morning to remove them from the house to the run and in the evening to return them to the house overnight.

Due to the artificial rearing of ostrich chicks it is assumed that the early development of the normal intestinal microflora is impaired and there is greater possibility for colonisation by pathogenic bacteria (Fuller 1989; Lopes *et al.* 2005). Probiotics are one of the treatments which can be used to help establish the normal intestinal microflora in chicks and promote good nutritional, growth and health status when administered soon after hatching (Fuller 1989). Probiotics have been adopted and administered to day old chicks as a preventative measure against enteritis on the

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farms in Oudtshoorn. This treatment would be beneficial but future stresses experienced by the animal in the rearing environment would upset the balance of the intestinal flora and make the individual more susceptible to disease yet again (Fuller 1989).

Similar to probiotics, other studies have utilised mannanoligosaccharides (as a prebiotic) and a competitive exclusion product comprising bacterial species derived from the intestinal tract of adult ostriches. The main findings from these studies were that mortality in treated ostrich chicks was reduced and growth performance was improved (Verwoerd *et al*. 1998; Lopes *et al*, 2005). The ostrich industry will have to decide based on future observations or studies what the most effective prophylactic treatment in this regard will be.

Biosecurity describes the area of management which focuses on preventing the introduction and spread of infectious disease on a farm (Huchzermeyer 1998). The biosecurity requirements should ideally be taken into account and implemented from inception and practiced consistently thereafter. Areas of concern for a comprehensive biosecurity protocol include the situation or location of the ostrich farming venture. The distance from other poultry or ostrich farms, the proximity to major roads, prevailing weather conditions and sources of feed and raw materials are to be considered (Huchzermeyer 1998).

Some pertinent measures are the control of movement (vehicles, workers, visitors) in and out of the farm, restriction of poultry and pet birds and the restriction of wild birds and rodents. This places management and infrastructure demands on the farm. Footbaths are to be provided at all access points and strategically for different sections or areas of the farm such as chick houses. Protective clothing should be provided to visitors and vehicle movement restricted from sensitive areas such as chick houses. Farm workers are to have clean protective clothing daily and they are to be knowledgeable and practise good hygiene and biosecurity measures (Huchzermeyer 1998).

Ostriches are susceptible to various diseases of birds (including poultry) and mammals. Thus it is recommended that ostrich farms are located well away from poultry farms. Wild birds and vermin (i.e. rodents) should be controlled but this is a major challenge because of feed lying around due to ad lib feeding (Huchzermeyer

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1998). Faecal excreta deposited around feeding areas can act as a source of infection, especially for such enteric diseases as salmonellosis (Shane *et al.* 1996).

Only one of the farms in Oudtshoorn was observed to utilise footbaths on a regular basis whereas all the other farms did not have this in place. The same farm was also the only one which observed that visitors had appropriate protective clothing when they entered the facilities. Farm access was not regulated on any of the farms and wild birds were seen in and around the feeding troughs at all farms. The majority of farms had rodent control measures in feed storage barns and in and around chick houses so rodents seemed to be well controlled.

Only one of the farms in the study appears to have taken biosecurity into account in their farming establishment but only partially. Overall the implementation of biosecurity in the ostrich farms was found to be significantly lacking. This lack of biosecurity represents many avenues for introduction of potentially harmful infectious agents including those for enteritis.

Escherichia coli was the most frequently isolated bacterium and it was found on the majority of farms in the study. This finding correlates with the observation that *E. coli* is the predominant enteric bacteria isolated from ostrich chicks suffering from enteritis (Verwoerd *et al.* 1998). *Escherichia coli* is known to form part of the normal intestinal flora and multiplex PCR for virulence factor genes was performed in order to determine whether any pathogenic strains were isolated.

Approximately 40% of isolates were determined to be enteropathogenic *E. coli* and this was established according to possession of the *paa* gene. Possession of the *eae* gene has been accepted to identify enteropathogenic *E. coli* but considering the finding that the *paa* gene is associated with the presence of the *eae* gene, the *paa* gene was used in this study (Batisson *et al.* 2003; Toma *et al.*, 2003). It is however acknowledged that ideally this should be confirmed by testing the isolates in this study for the *eae* gene to independently confirm this premise. Approximately 4% of isolates were found to be enterotoxigenic *E. coli* by possession of ST genes only and no enterohaemorrhagic *E. coli* were found as no Stx genes were present in the isolates. The 20% of isolates from which no virulence factor genes were identified can be considered to be non-pathogenic *E. coli*. Further studies may aim at identifying the presence of the *ipa*H gene to confirm any enteroinvasive *E. coli* and the HEp-2 cell culture assay to confirm any enteroaggregative *E. coli* or diffusely

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adherent *E. coli* (Sethabutr *et al.* 1993; Nataro *et al.* 1998). This raises the possibility that some of the non-pathogenic *E. coli* in this study may be of a pathogenic category that was not determined.

Studies that have investigated pathogenic *E. coli* in ostrich chicks with diarrhoea are very limited. One study identified enterotoxigenic *E. coli* possessing the LT gene only, from 4/24 (16.6%), three month old chicks with diarrhoea and no enterohaemorrhagic *E. coli* were found by PCR for the Stx gene (Nardi *et al.* 2005). Tests aimed at the other categories of diarrhoaegic *E. coli* were not pursued. The low prevalence of enterotoxigenic *E. coli* of 4% and the finding of no enterohaemorrhagic *E. coli* in this study is comparable to what was found by Nardi and co-workers (2005). The enterotoxigenic *E. coli* in the study of Nardi and co-workers (2005) however, differ in that they possessed the LT gene only whereas those in this study possessed the ST gene only. The reported study may have benefitted from investigating the presence of other categories of diarrhoeagenic *E. coli* as well.

Necrotic enteritis caused by *C. perfringens* is a disease that has been more comprehensively described and studied in broiler chickens (Engström *et al.* 2003). *Clostridium perfringens* type A is considered the principal cause of necrotic enteritis in poultry and type C less so (Keyburn *et al.* 2008). In this study, the majority of *C. perfingens* (93%) were type A and a few (7%) were type E. None of the other *C. perfringens* toxinotypes were isolated. The few published studies that mentioned necrotic enteritis of ostriches did not identify the toxinotype except for one study which mentioned the isolation of *C. perfringens* type A and D (Samson 1997; Huchzermeyer 1998; Huchzermeyer 2002; Kwon *et al.* 2004).

The *netB* toxin gene was identified from 16% of all the *C. perfringens* isolates. All the *C. perrfringens* type E isolates had the *netB* toxin gene and only 10% of the *C. perfringens* type A isolates had this gene. This is likely to be the first report of *C. perfringens* type E and of *netB* toxin gene from ostrich chicks and further the first to recognise the presence of *netB* toxin gene on a type E *C. perfringens* isolate. Sequencing of the *netB* toxin gene fragment amplified in this study indicated that it had 94% similarity to the *netB* toxin gene sequences on the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The nature of the product of this *netB* toxin gene and its pathogenicity may have to be determined as it may differ from the NetB toxin already described (Keyburn *et al.* 2008).

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There was no *C. difficile* isolated from all samples tested. *Clostridium difficile* is a fastidious organism that may require selective media for successful culture and it is possible that a positive case may have been missed due to this reason (Frazier *et al.* 1993). It is possible to determine the presence of *C. difficile* toxins A and B by ELISA but this approach was not pursued in this study.

Salmonella has been reported from cases of enteritis in ostrich chicks and different serotypes have been identified (Welsh *et al.* 1997; Huchzermeyer 1998; Verwoerd *et al*. 1998). Of the three different serotypes of *Salmonella* identified in this study, two of them, *S.* Hayindongo and *S.* Othmarschen do not appear in the lists (published elsewhere) of *Salmonella* serotypes that have been identified from ostriches (Welsh *et al*. 1997; Huchzermeyer 1998; Verwoerd *et al*. 1998).

In cases where *Salmonella* was isolated, a single serotype was identified. That serotype was associated with disease on a single farm at a particular time of disease occurrence. This can be illustrated in this study where on Farm A, *S.* Hayindongo was isolated in March 2011 and *S.* Muenchen was isolated in the following chick season in September 2011. This was also reported when *S.* Typhimurium was isolated from all the cases on a single farm from a study of 11 different farms (More 1996).

In the majority of cases in this study *Salmonella* was isolated together with other possible pathogenic bacteria (i.e. *E. coli* and *C. perfringens*). In the few cases that *Salmonella* was the sole bacteria isolated, it could be seen as the responsible pathogen but where it is found with other potential pathogenic bacteria this role becomes unclear.

No *Campylobacter* spp. and no *Pseudomonas* spp. were isolated from samples in this study. *Campylobacter* can be difficult to culture as it is a fastidious organism and an extended period from sample collection to processing (as was experienced in transporting samples from Oudtshoorn to the laboratory in Pretoria) would have a negative effect on isolation (Chaban *et al*. 2009). It may be that an ideal time period of 4 h from sampling to processing is required in order to isolate multiple *Campylobacter* species although some *Campylobacter* spp. can be retrieved from samples after three days (Koene *et al.* 2004). It is likely that if *Campylobacter* was present in any of the samples, it was not cultured because of its qualities as a fastidious and/or labile organism. *Pseudomonas* spp. on the other hand does not

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require any special growth media (Quinn *et al*. 2011) and its absence on culture from the samples can therefore be considered as an affirmative negative result.

The involvement of viruses in enteritis of ostrich chicks has been described as uncertain (Huchzermeyer 1998). The findings of this study confirm this observation as similarly to other studies a low incidence and a limited variety of viruses were identified (Els *et al.* 1998).

Electron microscopy was considered to be appropriate for the detection of viruses in this study because most viruses (e.g. rotaviruses and astroviruses) are shed in high concentrations of up to 10^{11} viral particles per ml which overcomes the limitation of low sensitivity of EM (Biel *et al*. 1999). No virus isolation attempts were made as most enteric viruses are not routinely isolated by the common cell culture techniques (McNulty *et al*. 1979).

It appears that coccidia, cryptosporidia and nematodes do not play a significant role in enteritis in ostrich chicks as is suggested by the findings from this study where none were found from the clinical samples. This correlates with other findings where no coccidia, no cryptosporidia and insignificant occurrence of nematodes (1 from 77 samples) have been reported (More 1996; Kwon *et al.* 2004).

An arbitrary characterisation of lesions was adopted based on the histopathological description. Bacteria were the predominant pathogens identified from this study and therefore the bacteria isolated in relation to the lesions observed from the samples were noted. Only a few lesions were categorised as necrotic enteritis but *C. perfringens* was associated with all of them. Experimentally induced lesions of poultry necrotic enteritis have been described as diffuse fibrinonecrotic enteritis with extensive erosions (Cooper *et al.* 2010). This is comparable to the lesions observed in one case in this study of severe, extensive, necrotic enteritis with a pseudomembrane. These lesions could possibly be attributed to *C. perfringens* although *E. coli* and in one instance *Enterococcus* spp. were also isolated.

If necrosis of intestinal tissue was not severe and extensive it was regarded as non-specific. Various bacteria including *E.coli*, *C. perfringens* and *Salmonella* spp. were associated with this type of lesion. Comparable lesions, associated with *Salmonella* Ituri, were described for necrosis of the mucosa with ulceration and an overlying fibrinocellular exudate (Welsh *et al*. 1997). The difference in this study is that the

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Salmonella serotypes involved were *S.* Muenchen and *S.* Othmarschen. It is feasible that *C. perfringens* which is known to cause necrotic lesions and certain pathogenic *E. coli* can be responsible for similar lesions. The relevance of such histopathological lesions which are not pathognomonic will depend on the isolation of a known pathogen and this can also be difficult where multiple bacteria are isolated as occurred with some samples in this study.

The sloughing lesion observed was similar to the "attaching-and-effacing" lesion which is a characteristic feature of enterohaemorrhagic *E. coli* and enteropathogenic *E. coli* infection. The epithelial effacement or sloughing was seen but the close attachment of bacteria to the epithelium was not always observed on histology (Moon *et al.* 1983; Oswald *et al*. 2000; Kaper *et al*. 2004). An electronmicrograph of one sample with enteropathogenic *E. coli* isolated showed attachment of the bacteria to the intestinal epithelium, the same sample had the sloughing lesion on histopathology. This observation lends some support to the pathogenic nature of this isolate, of attachment to the intestinal epithelium characteristic of enteropathogenic *E.coli*.

Characteristic lesions of *L. intracellularis* were not seen on histopathological examination (Prof Neil Duncan, Pathology Section, FVS, personal communication 2012). Proliferative enteropathy caused by *L. intracellularis* can be diagnosed by observing the typical enterocyte proliferation using microscopy of haematoxylin and eosin stained sections (Guedes *et al*. 2002). *Lawsonia intracellularis* is therefore unlikely to be involved in the cases of enteritis from this study.

Limitations of the study

There was often a delay between when the ostrich chicks died and when the postmortem was performed. As a result some autolytic changes were seen in some of the histopathological sections examined. The ideal would have been to collect tissues fresh (i.e. not more than an hour after death) to avoid autolysis. Refrigeration of carcasses at 4ºC after death would have preserved adequate histological integrity for 24-36 h post mortem (Canfield *et al.* 2000). However, these conditions were not practical or available and thus carcasses preserved in the shade as much as possible were used within eight hours after death. Considering that many samples could be interpreted histologically, this regime adequately served its purpose.

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The distance of the farms in Oudtshoorn to the laboratory in Pretoria meant that there was an extended period of time between collection and processing of samples. Cooled storage in transit should have helped to keep the microorganisms viable but labile organisms such as *Campylobacter* spp. and *C. difficile* may have been adversely affected.

The study focused on chicks from one day old to 12 weeks of age housed on the farm. These chicks were sourced from a hatchery and certain conditions related to the hatchery and/or transport to the farm could contribute to disease at a later stage. The poor survival rate of low birth weight chicks and degree of hygiene and biosecurity at the hatchery are some of the considerations (Black 2001). Infectious agents could be detected from ostrich chicks on the farm but an alternative study that investigated the hatchery conditions and quality of chicks could indicate whether hatchery conditions were a cause.

Not all the clinical cases observed had diarrhoea as a presenting sign and a significant number died without any preliminary signs or symptoms. It has been reported that diarrhoea may be absent if the enteritis is localised to the small intestine (Huchzermeyer 2002). This observation tends to confound a clear description of the clinical presentation of enteritis. Fading chick syndrome is a condition that has been known to afflict ostrich chicks, with signs of depression, anorexia and death. However, it is poorly understood as case definitions differ and it has been suggested that the syndrome may encompass a couple or more clinical conditions (More 1996). This ill definition may arise because enteritis and other related problems in ostrich chicks are not well characterised. An approach resembling that adopted for disease in turkeys could be useful, where poult enteritis complex is the overarching term for such related diseases of young turkeys (Barnes *et al*. 2000).

The use of antibiotics is common in cases of enteritis and it was applied on affected farms. One potential drawback of persistent antibiotic use is the development of drug resistant bacterial strains which may perpetuate disease (Shane *et al.* 1996; Huchzermeyer 1998).

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Conclusion

Certain criteria need to be taken into account when establishing disease causality. The contemporary approach is derived from the Koch-Henle postulates and one such method describes a process that determines congruence, consistency, cumulative dissonance and curtailment of the aetiological agent (Inglis 2007). Congruence is the induction of characteristic clinico-pathological signs and epidemiological patterns by the aetiological agent. Consistency is the predictable biological response of the host when the aetiological agent is introduced. Cumulative dissonance is the display of a typical disease process that progresses through all the biological systems that the aetiological agent is known to affect. Curtailment is the disruption of the disease process once intervention against the aetiological agent is applied (Inglis 2007).

The main observation from this study is that the cause of enteritis in ostrich chicks is bacterial involving enteropathogenic *E. coli* and to a lesser extent enterotoxigenic *E. coli; C. perfringens* type A and to a lesser extent type E (with the uncertain influence of *netB* toxin gene) and *S*. Muenchen and to a lesser extent *S*. Hayindongo and *S*. Othmarschen. Managerial factors increase susceptibility to disease by creating conditions that increase exposure to infection and also induce stress in the birds. Further studies should focus on confirming the role of these bacteria as aetiological agents of enteritis of ostrich chicks guided by the process of establishing congruence, consistency, cumulative dissonance and curtailment.

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CHAPTER 5

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APPENDICES

Appendix 1

Salmonella polyvalent antisera for determining the antigenic formula as indicated in the White-Kauffmann-Le Minor scheme (adopted from the Bio-rad product insert – Antiserum Salmonella).

Polyvalent antiserum	Group antisera	O somatic antigens
OMA	A, B, D, E, L	1, 2, 12 + 4, 5, 12 + 9, 12 + 9, 46 + 3, 10 + 3, 15 + 1, 3, 19 + 21
OMB	C, F, G, H	6, 7 + 6, 8 + 11 + 13, 22 + 13, 23 + 6, 14, 24 + 8, 20
OMC	I, J, K, M, N, O, P	16 + 17 + 18 + 28 + 30 + 35 + 38
OMD	Q, R, S, T, U, V, W	39 + 40 + 41 + 42 + 43 + 44 + 45
OME	X, Y, Z, 51-53	47 + 48 + 50 + 51 + 52 + 53 + 61
OMF	54 + 55 + 56 + 57 + 58 + 59	
OMG	60 + 62 + 63 + 65 + 66 + 67	

Polyvalent antiserum	H flagella antigens
HMA	a + b + c + d + i + z10 + z29
HMB	e, h + e, n, x + e, n, z15 + G
HMC	k + y + z + L + Z4 + r
HMD	z35 + z36 + z38 + z39 + z41 + z42 + z44 + z60
HMIII	z52 + z53 + z54 + z55 + z57 + z61
HE	e, h + e, n, x + e, n, z15
H1	1, 2 + 1, 5 + 1, 6 + 1, 7 + z6
HL	l, v + l, w + l, z13 + l, z28 + l, z40
HZ4	z4, z23 + z4, z24 + z4, z32
HG	f, g + g, p + g, m, s + g, m + m, t

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