Epidemiology of coccidiosis in calves and control of coccidiosis using toltazuril at the time of weaning.

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Abstract:

Two separate studies were conducted to investigate the impact of coccidiosis in young calves. In one study calves were reared to weaning (100kg liveweight) by feeding meal with or without monensin added. The oocyst counts were low in both groups up to weaning and there was no statistically significant ($p<0.05$) improvement in terms of body weight or a decline in oocyst counts in the monensin-treated group At weaning a single dose of toltrazuril (20mg/kg) was given to half the calves in both groups. A similar treatment regime was given in a second study where calves had been raised to weaning by commercial calf rearers. Half of these were treated with toltrazuril (20mg/kg) and half not. In both studies there was a statistically significant ($p<0.001$) reduction in oocyst counts in treated calves which remained very low for 4-5 weeks post treatment. The treatment also significantly increased ($p<0.001$) weight gains in treated calves by 3-5kgs at 5-6 weeks post treatment. The coccidial status of other calves on a variety of farms were also monitored including a group of organic beef farms. High oocyst counts were noted on occasions where calves were not on anti-coccidial treatment. Low oocyst counts were noted in adult cows where they were examined. The two most prevalent species overall were *Eimeria zuernii* (95%) and *E. bovis* (87%) followed by *E. auburnensis (62%), E. cylindrica (42%), E. canadensis (31%), E. wyomingensis (*23%), *E. bukidnonensis (*36%*), E. ellipsoidalis (24%) E. alabamensis (12%), E. brasiliensis (*12%**), and E. subspherica (*27%). The most predominant species, measured as the most numerous oocysts overall, were *E. bovis* (31%) *followed by E. zuernii* (27%), *E. auburnensis* (13%), *E. bukidnonensis* (7%), *E. cylindrica* (6%), *E. wyomingensis* (5.3%), *E. canadensis* (4.4%), *E. ellipsoidalis* (3.3%), *E. brasiliensis* (1.9%), *E. subspherica* (1.5%), *and E. alabamensis* (1%). The most prevalent species were also the most pathogenic species. On many occasions calves were infected with more than one species, sometimes as many as 5-6 *Eimeria* species. A redescription of the 11 species of *Eimeria* in cattle identified from New Zealand Farms was made.

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Abreviations

Sr- Square root

IFAT- Indirect fluorescent antibody Test

DAI- Days After Infection

LG - Low grade

HG - High grade

S.I. - small intestine

P.I. -Post infection

PP - pre patent

L.A - lasalocid

DEC -Decoquinate

MDBK -Madin-Darby Bovine Kidney

SDS - Sodium Dodecyl Sulphate

ELISA- Enzyme linked immunosorbant assay

PAGE- polyacrylamide gel electrophoresis

PVDF-Polyvinylidene Fluoride

FOC- Faecal oocyst count

PBL- Peripheral blood Leucocytes

PMN – Peripheral mononuclear Cells

NK – Natural killer cells

IEL- Intra Epethelial Lymphocytes

CMI- Cell mediated Immunity

IgG, IgA, IgM- Immunoglobulin G, A, M etc.

KDa- KiloDalton

Fc - Faecal Consistency

SE - Standard Error

VERO - African Monkey Kidney Cells

ANOVA – Analysis of variance

CO- Conventional

NC- No Chemical

IELs – Intraepithelial Lymphocytes

P- Probability

LSM- Least square mean

SRT- square root transformation

CHAPTER 1: Review of Literature.

1. Introduction:

1.1. Taxonomy:

Protozoa are unicellular organisms with a complex structure and are the most abundant of all living things. Protozoa can be found in the lumen of the intestine, blood plasma, blood cells and other tissues, and even in the nuclei of cells. Some protozoa cause disease. Protozoa form a subkingdom of the Kingdom Protista. There are about 65,000 named species, about half of which are fossils. In its latest classification 'The Society of Protozoologists' recognized seven phyla. Two are very small and so far relatively unimportant (Levine *et al.*, 1985).

The seven phyla are 1. Labryinthomorpha. 2. Aceptospora. 3. Microspora 4. Myxozoa. 5. Sarcomastigophora 6. Ciliophora. 7. Apicomplexa.

The protozoa of the Apicomplexa contain an apical complex at some stage of development and many of these are parasitic. The Phylum Apicomplexa is subdivided into two classes: Sporozoasida and Piroplasmasida. The class Sporozoasida produce oocysts or spores. They are further divided into two subclasses of Gregarinasina and Coccidiasina. The members of the Coccidiasina are intestinal parasites of vertebrates, marine annelids and are further categorized into four suborders. Out of four suborders, three suborders (Adeleorina, Haemospororina, and Piroplasmorina) are haemoparasites of vertebrates and the Suborder Eimeriorina contains mainly intestinal parasites, and has 9 families, one is Eimeriidae. Among 24 genera in the family Eimeriidae, two, *Eimeria* and *Isospora*, are commonly referred as the "coccidia". The Coccidia are generally highly host specific (Levine, 1985, Andrews, 1980) and cattle are only infected by species of *Eimeria.*

1.2. Life cycle of *Eimeria*:

The life cycle of *Eimeria* is monoxenous (one host) and each phase in the life cycle occurs in a particular site (stenoxenous). The general eimerian life cycle can be divided into 3 stages: Sporogony, schizogony (asexual development) and gametogony (sexual development). All stages of the organism are haploid except the zygote (Hammond, 1973). These parasites generally have a high

degree of site specificity. For example, the very large first generation schizonts of *E. bovis* are found in the posterior half of the small intestine, second generation schizonts are seen in the endothelial cells of the crypt epithelial cells of the large intestine and gametogony occurs in the surface epithelial cells of the large intestine, caecum and colon (Levine, 1985). Not all the life cycles of eimerian species infecting cattle are known. From experiments, the life cycles of *E. bovis, E. zuernii, E. ellipsoidalis, E. auburnensis and E. alabamensis* have been elucidated, but the life cycles of *E. cylindrica, E. illinoisensis, E. canadensis, E. pellita, and E. brasiliensis* remain unknown (Ernst and Benz 1986).

There are two endogenous phases of development (schizogony and gametogony) which occur mostly in the intestines, sometimes, in the liver and mesenteric lymph nodes (Lindsay and Dubey, 1990; Lima, 1979), whilst sporulation (sporogony) occurs outside the host (exogenous) (Fayer, 1980).

Oocysts are passed in the faeces and contain a single sporont. Sporulated oocysts contain four sporocysts and each sporocyst contains two sporozoites. Sporulation is strictly aerobic (Hammond, 1973) and takes 1 or more days depending on the species and temperatures. The fully sporulated oocyst is infective.

1.2.1. Asexual cycle:

When infective oocysts are ingested by ruminants, the sporozoites escape from the oocysts due to stimulation by carbon dioxide, trypsin and bile. This process is called "excystation" (Jackson, 1962; Hibbert, 1969; Landers Jr. 1959; Lotz and Leek, 1960). Individual sporozoites then penetrate into specific cells in specific locations. After entering the cell, the sporozoite becomes a round structure which is called a trophozoite. By multiple fission, a first generation schizont is formed in which numerous, often hundreds, of merozoites are developed which each contains one nucleus. The mature merozoites escape from the schizonts and penetrate another host cell and start another generation (second generation of schizogony). The *Eimeria* species of ruminants have two schizont stages, a giant first generation schizont and smaller second-generation

schizont. For example in *E. bovis*, there is a giant schizont (approximately 300µm diameter) containing 120,000 merozoites in the first generation and a smaller second generation schizonts of 8.9 x10 µ containing 30-36 merozoites (Hammond *et al.*, 1946 and 1963).

1.2.2. Sexual generation:

After the second generation of schizogony the merozoites enter new host cells and initiate gametogony, the sexual phase of the cycle. Most merozoites develop into macrogamonts - 'female' gametes and some into microgamonts- normally considered equivalent to 'male' gamonts (Levine, 1985; Ernst and Benz, 1986). Macrogamonts have a large central nucleus with a prominent nucleolus. In each microgamont a large number of dark blue staining peripherally arranged nuclei develop and these mature into hundreds of comma shaped microgametes. The flagellated male gamete migrates to and fertilizes the macrogamete and the fertilized macrogamete then forms an oocyst wall. The oocysts leave the host cell and enter the intestinal lumen and are shed in the faeces (Fayer, 1980; Ernst and Benz, 1986). The time from ingestion of sporulated oocysts to the appearance of oocysts in the faeces varies from about 1-3 weeks depending upon the species of *Eimeria*.

1.2.3. Sporulation:

The oocysts sporulate outside the body (exogenously) under aerobic conditions (Fayer, 1980). At the proper temperature and humidity the *Eimeria* oocyst cytoplasm divides to form four sporocysts, each with two sporozoites. This process is called sporogony (Ernst and Benz, 1985). The life cycle of different *Eimeria* species infecting cattle is reviewed and presented in Table 1.1.

1.2.4. Factors affecting the life cycle of *Eimeria* species:

The life cycle and endogenous development of eimerian parasites is not fixed. Sometimes the life cycle may be shorter when they have fewer generations of schizogony and smaller and faster maturing schizonts. Host factors such as genetic make-up, strain, and immunity are important in influencing the endogenous development of the parasite (Levine, 1985). Some anti-coccidial

drugs arrest the development of the sporozoites, resulting in abnormal sporulation of the oocysts (Levine, 1985).

Factors affecting the number of oocysts produced vary from the inherent potential of each species to reproduce in a non-immune host; immunity developed by the host; crowding factors; competition with other species; other concurrent infectious agents; nutrition of the host; and strain differences of the host and parasite. In addition, use of anti-coccidial drugs is also a factor for the number of oocysts produced (Fayer, 1980).

1.3. Pathogenicity:

Not all eimerian species are equally pathogenic. The most pathogenic species infecting cattle are *E. bovis and E. zuernii*, which are usually associated with clinical disease in cattle (Ernst and Benz, 1986). In contrast *E. wyomingensis and E. subspherica* are considered non-pathogenic. Others, though such as *E. alabamensis, E. auburnensis, and E. ellipsoidalis,* that are also considered non-pathogenic, may cause diarrhoea when large numbers of oocysts are given. For example, *E. alabamensis, E. wyomingensis, E. subspherica and E. auburnensis* required 17-140 million, 4 million, 1 million and 8000 sporulated oocysts to be given to calves to produce clinical infection (Davis *et al.*, 1955; Ernst and Benz, 1986; Oda and Nishida, 1990; Christensen et al., 1990). The pathogenicity of *E. pellita and E. brasiliensis* is not known. The pathophysiology of *Eimeria* species infecting cattle reported by several authors is shown in Table 1.2. The economical loss due to coccidiosis includes death of animals with the disease, weight loss in others, treatment expenses and impaired future performance of the herds (Quigley, 2001; Niilo, 1970).

1.4. Faecal consistency and oocyst numbers:

Little relationship has been noted between faecal consistency and the number of oocysts present in the sample. Many faecal samples with normal consistency had relatively large oocyst numbers and many diarrhoeic samples had low numbers (Ernst *et al.*, 1987; Oda and Nishida, 1990). However, these findings relate to mixed infections involving both pathogenic and non-pathogenic species.

Species	Infective dose	Asexual Phase	Sexual phase	Min PPP (days)	Patency (days)	Author
E. subspherica	$1x10^6$ - $1x\ 10^7$			10.6	1-5(2.9)	Oda and Nishida, 1991
E.bovis	1- 20 $x10^6$	Posterior part of the S.I, rarely in caecum and colon. As white structure in endothelial cells of central lacteal towards the tip. Size 281x203µm with 120,000 merozoites. 16-17days after infection	Caecum and colon. Epithelial cells of intestinal glands at the base and few in sub mucosa.			Hammond *et al.*, 1946
E.bovis	0.5-$1x10^6$ mero-zoites into caecum	Second generation schizonts after 6 to 36hrs. Size-8.9x10µm (30-36 merozoites)	Gametocytes at 18hrs on epithelial cells of the caecum and colon.			Hammond *et al.*, 1963
E. alabamensis	640,000	Schizonts on 12 days at 2,6,12,16,24,36 a feet anterior to the ileo-caecal valve. In the reticular connective tissue between the crypts of lieberkuhn mostly in the lamina propria close to the muscularis mucosa.	Micro gamete at 12 -19 days Size:79.5x125.5(mean size - 92x139.9µm)			Davis *et al.*, 1962

E. alabamensis	236x10^6	Excystation at 29 hrs Immature shcizonts seen on 4th day at 1\3 of SI, intranuclear scion in the apical cell in the tip of the villi size:at 6 days (9x.9 -1.44) But by the time they matured 4.4x1.4 with bent rounded tips in lumen to intestines at crypts of Lieberkuhn	Gametogony in lower SI, caecum and colon. Microgamete 12x9.1μm Macrogamete15.6 x 11.5 μm Lower ileum 6 days after infection	6-11 (8.6)	1-10 (21 days infection 1-13 (72 day infection) 5 -11 9 days	Davis, *et al.*, 1945 Davis, *et al.*, 1955 Smith and Davis, 1965)
E. alabamensis	10-400 million and challenge with 100 0r 400million				LG 1-10 (4.6) HG 1-3 (7.2)	Davis, *et al.*, 1955
E. alabamensis	1x10 ^{6} 80x10 ^{6} and challenge with 100x10^6					Soekardono, *et al.*, 1975
E. wyomingensis	0.2- 1x10 ^{6}	-		13-15 days (14.2)	1-7 days (3.6)	Ernst and Benz, 1980

E. wyomingensis	$1x10^6$		Seen on 12th day PI in the lamina propria of the villi in the distal 5m of SI. Infected cells clustered beneath - tips of the villi and distal 1\3 of the villi. Microgamete 52.8 x 43.0 Macrogamete 24.6 x19.3 µm numerous wall forming bodies (3.8µm)			Lindsay *et al.*, 1988
E.zuernii	500 oocysts	First generation schizont 3m of SI, greatest concentration at 2nd meter from ileo-caecal junction on 16 th day PI size: 225 -115x202µm-78µm In the lamina propria or close to muscularis mucosa. Mean no. of merozoites 32	Colon and caecum on 16 -22 days PI in the epithelial cells Size:114x13.5µm Gametogony in the caecum and colon on 16 -22 days PI with a mean size of 14.4 x12.94µm			Stockdale, 1977
E.zuernii	500,000 to $50x10^6$	Asexual stages seen as early as 2 days to 19 days PI. Mature schizonts seen upper, middle and lower S.I. and in the caecum and colon. Size-9.6x13.2 µm Merozoites size 5.6x12.2µm in S.I., caecum, colon, and rectum.	Seen in SI and large intestines (Caecum, colon, and rectum) at 12 to 19 days PI. Size-90.7 µm Macrogamete, size-10.6 seen at 15 to 19 days. And immature oocysts seen on 12 th day in colon, caecum, S.I.			Davis and Bowman, 1957

E.auburnensis	8,000			9	1-5days	Christensen, 1939
E.auburnensis	100,000-750,000		With a size of 85.61x66.5µm in the mesodermal cells, in the lamina propria of the villi in lower intestines. Macrogamete seen on 16 days PI with a size of 17.9 on 18 th days.	18	2-7	Hammond, 1961
E.auburnensis		Trophozoites and immature schizonts of the second generation seen on 10 to 12 days PI	Second generation schizonts and gametocytes seen in sub epithelial cells, mesodermal origin in the distal portion of the villi.			Hammond and Chobtar , 1969
E.auburnensis	640,000 oocysts.	Schizonts are seen in the middle and lower side of the small intestines. After 12 - 14 days PI. 92 x 39.9µm. in lamina propria.	Microgamete size 91 x 287.5µm.			Davis and Bowman, 1962

Table1.1: Life cycle of different bovine *Eimeria* species: Note: LG=low Grade, HG= High Grade, PP= Prepatent Period

1.5. Factors influencing the epidemiology:

A number of factors influence the epidemiology of coccidiosis in cattle.

1.5.1. Environmental factors:

1.5.1.1: Rate of sporulation and survival of oocyst:

Moisture, temperature and the availability of oxygen affect sporulation (Fayer 1980). The optimum temperature for sporulation is 25 to 27°C and for all bovine eimerian species temperature of >35°C causes permanent damage the unsporulated oocysts though sporulation is rapid at this temperature (Marquardt, 1960). At colder temperatures, the rate of sporulation is slow; however, it increases as the temperature increases (Fayer 1980). In general, unsporulated oocysts survive well at low temperatures. Storage at -30°C for 24hrs and -5°C for 60 days did not alter the viability of the oocysts (Landers, 1953) which is consistent with the observations that oocysts survived over winter in Wyoming (Landers, 1953). Relative humidity has been shown to affect survival and at a relative humidity of 90%, oocysts remained viable for 49 to 60 days, whereas at 61%, oocysts remained viable for only 32 days (Fayer, 1980). A small number of *E. zuernii* oocysts in calves could still sporulate in dry dusty conditions (Parker *et al.*, 1984).

1.5.1. 2. Hygiene:

Coccidiosis is a particular problem when groups of young calves are raised together. It is principally a factor of contamination of a small area then re-infection of each other. Poor hygiene in the calf rearing area provides a favorable microclimate that allows oocysts to sporulate and survive longer in the environment (Niilo, 1970b; Palvaseck, 1984; Chibunda *et al.*, 1996). Improved hygiene of calf pens reduced stocking density and prophylactic medication have been shown to contribute to reduced disease prevalence.

A lower prevalence of coccidiosis was seen with low stocking rates and less environmental contamination (Niilo, 1970b). For example when beef calves are widely spread on the pastures, they may not be exposed to a sufficient infective dose but keeping them in corrals and feed lots is reported to be a factor in the occurrence of severe winter coccidiosis in calves (Niilo, 1970b).

In one study, farms with a high stocking density, water contaminated with oocysts and animals not supplied with anticoccidials in feed, had prevalences of infection up to 78-82%. However, the use of cages, water troughs and lower stocking densities reduced the prevalence of infection to 49% (Matjila and Penzhorn, 2001).

1.5.1.3. Stress:

Stress factors such as a change in diet, climatic conditions (Fitzerald, 1961), weaning (Marsh, 1938), dry dusty conditions and challenge with other infectious agents (Parker, 1984) further contribute to infection, which is already present in the host. Stress due to harsh winter conditions was considered as one of the factors for winter coccidiosis in Canada (Niilo, 1970c; Radostits and Stockdale, 1980). Stress at weaning is acknowledged to reduce the limited immune response that has developed in calves by this age and this may result in the clinical disease outbreaks in calves (Fitzgerald, 1961; Niilo, 1970c)

1.5.2. Animal Factors:

1.5.2.1. Adult cattle serve as a source of infection:

Adult cows have been observed to shed low number of oocysts in several studies, (Svensson, 1993; Faber, 2002; Marquardt 1961; Balconi, 1963; Fitzgerald, 1961) and this is likely to produce the initial low level of contamination for calves. A peripartum rise in oocyst count especially for *E. bovis* has also been observed (Faber et al., 2002), which may further contribute to the initial infection in calves. The infection of the adult herd serves as the source of coccidiosis (Marsh, 1938; Boughton, 1944) and within 2-6 weeks, clinical coccidiosis may be seen in young animals and the severity in turn depends on the number of oocysts ingested (Boughton, 1944).

1.5.2.2. Age and immune status of calves:

Unexposed calves may develop severe disease after their first exposure and the severity of the disease depends on the level of infection. Previous exposure builds up resistance to the disease (Fitzgerald, 1967; Niilo, 1969). This resistance protects the calves for further infections (Niilo, 1969; Soekardono, 1975). Exposure of neonatal calves to the infection at a young age (3hrs -

40hrs) resulted in a poor immune response and these calves may then become a source of infection (Niilo, 1969). Further evidence of host immunity is the ability to modify this by using immunosuppressive drugs like dexamethazone (Niilo, 1970a). Host resistance to infection can be seen in terms of no clinical signs and discharging fewer oocysts following infection. A low dose infection with 100-500 oocysts/day for 11 days proved to be sufficient to stimulate immunity (Fitzgerald, 1967). The effect of low-level infection (premunisation) and treatment with a coccidiostat to control the disease was investigated in calves. Calves were infected with 2,000 *Eimeria bovis* oocysts/day for 5 days, whilst being treated with a coccidiostat and later challenged with 200,000 oocysts. They did not develop diarrhoea when they were on medication but when the drug was withdrawn the calves developed diarrhoea and had oocysts in their faeces. Premunisation without a coccidiostat could not prevent the disease when large numbers of *Eimeria bovis* oocysts were fed to calves (Foreyt, 1984).

1.6. Control of coccidiosis:

The control of coccidiosis depends on good hygiene practices such as clean dry stalls (wire or slatted floors), feeding from bunks (Schillhorn, 1986), treatment of clinically affected animals (Fox, 1985) and use of preventive anticoccidial drugs (Pritchard, 1983). It is difficult to treat the environment (pasture or feedlots) as the oocysts are ubiquitous and are resistant to many chemicals. Control can be achieved without drugs by taking precautions such as reducing stock densities, pasture rotation, avoidance of suspected contaminated pasture, feeding colostrum, using clean utensils and maintaining dry bedding.

Species	Dose (oocysts)	Clinical Signs	Lesions	Author
E. alabamensis	Up to 2 billion ($2x10^9$)	Deaths in 2 out of 5 calves given massive doses	Enteritis in the lower half of the small intestines, massive destruction of the epithelium, leucocytic infiltration and villous oedema, tufts of swollen villi which are macroscopically seen and sandy in texture.	Davis *et al.*, 1957.
E. alabamensis	10 - $400x10^6$	Slight diarrhoea in 2 month old bull calves and 10 calves developed watery diarrhoea, had poor appetite ,depression which affected the growth rates.	-	Hooshmandrad *et al.*, 1994
E. alabamensis	$17x10^6$ sporulated oocysts and twice this number oocysts of other species. $140x10^6$ sporulated oocysts in continuous days.	4 calves out of 5 developed clinical infection. clinical infection		Davis *et al.*, 1955
E. subspherica	$1x10^6$ to $1x10^7$ sporulated oocysts	No clinical disease but few oocysts were passed		Oda and Nishida, 1990.
E. auburnensis	640,000	Diarrhoea on 6th, 7th and 12th day after inoculation	Schizonts in the middle and lower third of small intestine and ranges from 75 to 250 µ in lamina propria near the muscularis mucosa.	Davis and Bowman, 1962
E. auburnensis	8000 sporulated oocysts	Clinical infection in a two week old calf which		Christensen *et al.*, 1990

		showed greenish water diarrhoea and discharged 319,000 oocysts on 24th day of infection.		
E. wyomingensis	0.2-1x10^6 sporulated oocysts/ sporocysts from 1 million sporulated oocysts and dexamethosone	Diarrhoea seen in all calves, Patent infection seen in 32 out of 50 calves. Calves excreted 100 - 3,300 oocysts for 2-4 days. No observable blood or sloughed mucosa passed. no relation between the oocyst number passed and oocyst no.given	No pathologic lesions	Ernst and Benz 1980
E. wyomingensis	1x10^6 sporulated oocysts	No clinical signs	Sexual stages in lamina propria of the villi in the terminal small intestines, Infected cells had nuclear and cytoplasmic hypertrophy	Lindsay *et al.*, 1988
E. wyomingensis	1-2 x 10^7 oocysts	Diarrhoea in 3 calves. Diarrhoea with flecks of blood in 2 calves Discharge of oocysts		Courtney *et al.*, 1976
E. zuernii	0.6x10^6 and 60mg dexamethazone	Consistent clinical infection		Niilo, 1976
E. zuernii	300,000 sporulated oocysts			Stockdale and Niilo, 1969

E. bovis	Sporulated oocysts at different levels from 10 to 15,000 for 62 days and challenge with 300 to 500,000 oocysts on day 47 or 49 day.	Produced bloody diarrhoea, 2 deaths and 5-25,000 oocysts in controls		
E. bovis	51 calves 1 to 20[6]	Diarrhoea when first oocysts are passed. Severe infections bloody diarrhoea with tenesmus.	Congestion, haemorrhage, destruction of the intestinal glands of mucosa and formation of membrane in caecum, colon. Symptoms and pathological changes are associated with sexual stages. Schizonts occupied lumens of endothelial ceils of central lacteals.	Hammond *et al.*, 1946

Table 1.2: A summary of some experiments investigating the pathophysiology of different bovine *Eimeria* species:

1.7. Anticoccidial Drugs:

Two terms are commonly used in relation to anticoccidial medication. **Coccidiostat:** coccidiostats cause stasis of infection/life cycle; they do not sterilise the infection but allow premunition and thus an immune response with subsequent infections. **Coccidiocide:** coccidiocidal drugs do result in a sterilising effect, killing some or all of the parasite stages and are suited for treatment of acute coccidiosis. A variety of different types of drugs, have been and are being, used for coccidiosis in cattle. The commonly used coccidiostats in cattle are amprolium, decoquinate, lasalocid, monensin, and sulphonamides.

1.7.1. Sulpha drugs:

During 1960 to 1970, various sulfonamide compounds such as sulfamethazine, sulfaquinoxilone and combinations of the various sulfonamides were used for treating cattle coccidiosis.

1.7. 2. Ionophores:

Ionophores are antibiotics used to control coccidia. The term ionophore is derived from the drug's ability to bind and transport ions and biogenic amines through biological membranes (Pressman, 1973). Ionophores selectively affect certain micro-organisms by altering the passage of cations through pores in their outer cell membrane. The ionophore antibiotics are produced by filamentous branching bacteria of the order Actinomycetales and commercially, different types of *Streptomyces* bacteria are used to produce them (Hall, 2000). Ionophores are hydrophobic molecules that selectively bind to a given metal ion and increase its cell permeability to cross the cell membrane. The inner part of the ionophore is made of polar groups forming a tetra or octahedral geometry that fits and encloses a specific ion. Ionophores change the primary ion concentration of the cells and can release sequestered intracellular ions thus altering intracellular pH and damaging cell organelles (Hall, 2000). In addition to activity on parasitic parasite protozoa, ionophores are also antibiotics that affect the rumen bacteria, protozoa and can suppress rumen fungi.

1.7.2.1. Monensin:

Monensin is an ionophore which is a fermentation product of *Streptomyces cinnamonensis* with a spectrum of activity which includes *Eimeria* infections in cattle and poultry; monensin is also used as growth promotant.

1.7.2.1. a. Mode of action:

Monensin forms soluble complexes with monovalent cations such as Na^+, K^+ in both host and parasite enabling them to cross plasma membranes which then draws excess water into the cell. The ion preference of monensin to different ions is $Na^+ > K^+$. Monensin is toxic for many species and each individual species differ in their tolerability for monensin. For example 2.5mg/kg is lethal to horses whereas trout can take up to 1,000mg /kg. Skeletal muscles are most prone to be affected by toxicity (Todd, 1984). Monensin is a weak antibiotic against gram-positive bacteria.

1.7.2.1.b. Monensin in a coccidiostatic role:

Monensin acts as a coccidiostat and reduces the clinical signs of coccidiosis in ruminants. Whether it can be regarded as a true coccidiostat or a coccidiocide is not clear. Some authors refer to it as a coccidiostat and others do not. Several experiments have been conducted on monensin treatment of calves and lambs infected with coccidia (Stromberg *et al.*, 1986; Genchi *et al.*, 1989; Fitzgerald, 1984; Stockdale, 1981) (see summary in Table 1.3). Treatment with monensin 2-3 days prior to infection and 31 days after infection not only prevented clinical disease but also reduced oocyst numbers by 60% (McDougald, 1978; Fitzgerald and Mansfield 1979; Stromberg et al., 1986) and in another study monensin feeding at 1mg/kg body weight reduced the incidence of clinical coccidiosis in calves (Stockdale, 1981).

1.7.2.1. c. Stages affected by Monensin:

Monensin treated sporozoites showed reduced penetration into cells and also inhibition of asexual stages in vitro (Smith, 1981). The damage is observed in terms of swelling, vacuolation and irregular cell surface. The damage to sporozoites was parallel to the dose of monensin administrated (Smith *et al.*, 1979, 1981).

Monensin increases the intracellular sodium and stimulates the activity of the sodium pump which is supported by amylopectin of sporozoites. The increased energy consumption by the sporozoites to counteract the effect of the monensin reduces the ability of the parasite to penetrate the host cell (Stockdale, 1981).

1.7.2.1. d. Dosage of Monensin:

Monensin has a dose related response to coccidia. When given in feed at 5ppm it was observed that lambs excreted excessive oocysts as the result of infection and suffered decreased weight gains. At 10 ppm, lambs passed fewer oocysts whilst at 20ppm the oocyst production in lambs was completely reduced (McDougald, 1978) over controls. Similar experiments with calves showed reductions in clinical signs at 1mg/kg weight (Fitzgerald, 1984; Stromberg *et al.*, 1986). The summary of several reports on the use of monensin as an anticoccidial treatment is detailed in Table1.3.

1.7.2.1. e. Effect of monensin on weight gains:

In previous studies, Monensin has had mixed effects on weight gains. In some trials monensin did increase daily weight gains (up to 8.1 -28.6%) and increased feed conversion efficiency (by 15%) in lambs (Fitzgerald 1978; Foreyt and Wescott, 1979; Calhoun *et al.*, 1979; McDougald, 1978) and calves (Wagner *et al.*, 1984; Rouquette *et al.*, 1980; Potter *et al.*, 1986). However, in other studies monensin reduced feed intake and improved feed conversion efficiency but had no effect on average daily gain (Potter *et al.*, 1985; Zinn, 1966; McDougald, 1978). Similarly, feeding of lambs 17 to 33 mg/kg of monensin for 35 days after inoculation with *Eimeria* had no effect on average daily weight gains (Horton and Stockdale, 1981; Gregory *et al.*, 1982).

1.7.2.1. f. Monensin regularly added to the feed as rumen modifier:

Monensin given in feed (Stockdale et al., 1982) or as an intra-ruminal bolus (Parker *et al.*, 1986) not only prevents coccidiosis but also acts as a rumen modifier and increases the efficiency of utilization of the feed in both grain and forage fed cattle. Monensin stimulates the production of propionic acid as a result of monensin selecting for certain bacteria, such as *Bacteroides* species and *Selemonaas ruminatium*. These bacteria convert decarboxylate succinate

to propionic acid thus increasing production of propionic acid in the rumen (Stockdale, 1981; Calhoun *et al.*, 1979; Hall, 2000; Oscar et al., 1987; Muntfering *et al.*, 1980). As a consequence, monensin increases the feed conversion efficiency in calves up to 15% (Stockdale, 1981, McDougald, 1978, Horton and Stockdale, 1981). In one trial, monensin-treated cattle grew 1.6% faster, consumed 6.4% less feed and required 7.5% less feed/100kg than controls (Goodrich, 1984). Monensin improved the utilization of feed energy by directly or indirectly influencing the metabolism of carbohydrates (Ritcher *et al.*, 1984). Monensin improves the digestibility of drymatter by 72 to 75%, (Goodrich *et al.*, 1983, 1984), reduces lactic acid and methane production by 26% (Wedegaertner *et al.*, 1983, Oscar *et al.*, 1987) and controls bloat in ruminants.

species	Infective dose Oocysts	Age at challenge (weeks)	Monensin dose rate	Clinical signs in treated	Clinical signs in controls	Authors
E. bovis+ E. zuernii and others	300,000	4	0.5mg/kg body weight, 1mg/kg, 3mg/kg, 3 pre and 30 days post infection.	No clinical signs in treated calves but 3mg was effective Improved weight gains	Deaths Loss of weight	Fitzgerald and Mansfield, 1979
E. bovis	100,000	calves	1mg/kg body weight given 10days after the infection	Few oocysts passed. 2 out of 6 treated passed many oocysts. Developed resistance to challenge. Better weight gains	Clinical disease Weight loss	Stockdale, 1981
E. zuernii	100,000	calves	1mg/kg body weight given 10 - 20days after infection	supressed the clinical signs prevented weight reduction		Stockdale, 1981
E. bovis or E. zuernii	250,000	7 (bulls)	10gms/ 20gms/ 30gms/in 906kgs feed	Fewer clinical signs Less no. of oocysts discharged Significant weight gains in 20 to 30gms received calves	Clinical disease with calves with increased number of oocysts passed in faeces	Fitzgerald and Mansfield, 1984
E. bovis or E. zuernii and/or both E. bovis + E. zuernii	500,000 20mg on 12, 15,16 PI dexa methasone 150,000 or 250,000 2nd time 500,000 + 300,000	4 (bulls)	Monensin 10,20,30g ton - 1-3days prior to inoculation and up to 30 days PI	The calves treated with 20 to 30g/ton reduced the oocyst numbers following the challenge	Calves inoculated with *E. bovis* and the calves had both species exhibited more severe signs compared to *E.zuernii* which was difficult to establish	Stromberg *et al.*, 1986
mixed	Natural infection	steers 252-255 kgs	monensin 14mg/ton feed	Day 1 counts were 1,104 but at 40 days were 50 opg, and 100	1day -495 opg 40 day -275opg	Berger *et al.*, 1981

			30g/ton feed	to 120 days they were 100 opg.With 30g, Day1-1603 opg and 40day to 120-0 opg. No significant weight gains and feed intake but feed efficiency increased by 8.2%	120 day -715 opg oocysts counts were high on day 1 -742, 40-19.7 120-12.7 Reduced feed intake seen	
		8months beef	25mg/kg 70mg/kg for 7days	Myocardial degeneration first and myocardial necrosis by 4 to 11 days		Ryley *et al.*, 1983
E. bovis	$5x10^5$ re infected after 35 days	4 (bull)	1mg/kg from 10 to 20 days PI	Peak oocyst production on 21st day, reduced oocyst numbers. Significant weight gains. Resisted second infection	Deaths, clinical coccidiosis, peak oocysts on day 25. Resisted second infection	Stockdale and Sheard , 1982
E. bovis+ E. zuernii	250,000	12-13	Monensin 33mg/kg body weight	Lower oocyst counts up 46 days No effect on weight gains	Clinical coccidiosis with high oocyst counts	Foreyt *et al.* , 1986
E. crandallis, E. ovinoidalis, E. ovina.	Natural infection	6 weeks age	Monensin 18mg/day before lambing for 6 weeks. Lambs. 0.3 to 0.6 mg/lamb for 10 weeks.	No periparturent peak in ewes and low diarrhoea during lambing. Oocyst output reduced in all animals < 2600/g Drier faeces and no significant weight gains	Diarrhoea around lambing and high oocyst out put	Gregory *et al.*, 1981-82.
E. ninakolinakimove E. ahsata.	Natural infection.	Lambs	5,10, 20 ppm	10 and 20 ppm had no clinical signs. Oocyst passage was not reduced. 5ppm lambs had no deaths but decreased weight gains and excessive oocyst counts up to $100x10^6$ per day. 20ppm no oocyst seen even after the withdrawal of the drug and 10ppm lambs had counts of less than $10x10^6$ on 15th	Anorexia diarrhoea 30%mortality, loss of weight, and oocyst counts were 169 to $298x10^6$.	McDougald, 1978.

				day of withdrawal of drug.		
E.ninakohlyakimove E. ahsata	Natural infection in lambs	Lambs	Monensin 20mg/kg body weight, or 31.9mg/day. From day 4 to 102 days.	No acute clinical signs Average oocyst count reduced to $1x10^{6}$ by 7th day. Feed consumption reduced by 7% and no weight gain seen and feed efficiency increased by 7%.		Horton *et al.*, 1981.
Eimeria poultry.	Natural infection. 53 days treatment	Poultry	120g/ton 80g/ton (70 days) 100g/ton (70 days)	Monensin reduced coccidiosis, reduced caecal lesions,reduced number of oocysts passed, and improved feed efficiency	34% of deaths, faecal scours were high, feed conversion was low, loss of weight.	Shumard and Callender, 1967.
5 Eimeria species	240,000 at 24 and 52 days.	Lambs	17 to 33mg/kg. 33 mg up to 24 days, 17mg from 25th day to 100th day.	Highly effective more than 99% in eliminating the oocysts. Lambs gained 6kgs more than the controls and consumed less feed of 0.05kg for each kg weight gained.	Had higher oocyst count 24,133 opg Blood tinged diarrohae 14 days after inoculation. Diarrhoea lasted for 6 to 10 days. Reduction in weight between 36 to 52 days.	Foreyt, 1979.
Bacteria.			Monensin 0.38, 0.75, 1.5, 3.0, 6.0, 9.0, 12.0, 24.0, 48.0mg/kg weight.	Inhibited lactate producing rumen bacteria Succinate production not inhibited. None of lactate fermenters were inhibited.		Dennis et al., 1981.

Table 1.3 : Summary of trials where monensin has been used to control coccidia

1.7.3. Toltrazuril:

1.7.3.1. Anticoccidial activity:

Toltrazuril is a symmetrical triazinone and chemically unrelated to other conventional anti-coccidial agents currently in the market. It has coccidiocidal activity which damages all the intracellular developmental stages during the schizogony cycles and of the gametogony phase (Haberkorn 1996; Froyman and Grief, 2002; Haberkorn and Stoltefuss 1987; Alaksandra, 2001). Toltrazuril is effective against all coccidial species of poultry (Haberkorn 1996) and all the coccidia of mammals studied until now. For example, ducks (Reynaud, 1999), Lambs (Alaksandra, 2001), puppies (Daugshies *et al.*, 2000), goats (McKenna, 1988) and rabbits (Peter and Geeroms, 1986).

1.7.3.2. Mode of action:

The exact mode of action is stull unclear. Toltrazuril directly affects the nucleus and mitochondria which in turn influence the ion exchange of the parasite. In the macrogamete it affects the wall forming bodies this in turn results in the vacuolation of intracellular development stages (Haberkorn, 1996).

1.7.3.3 Stages of life cycle affected by toltrazuril:

Toltrazuril does not affect the extra cellular stages such as sporozoites (Froyman and Grief, 2002). It does not affect the host tissue cells (Froyman and Grief, 2002) as seen on light, electron microscopic studies where all the microgametes, macrogametes and schizonts were damaged without causing any damage to the host cells (Haberkorn and Stoltefuss 1987). Treatment during early at the beginning of endogenous cycle completely eliminates the parasite (Reynaud *et al.*, 1999).

1.7.3.4. Single dose treatment of toltrazuril:

A dose of 10-20 mg/kg as a single dose or 10mg/kg on 2 separate days prevented coccidiosis in lambs (Gjerde and Helle 1986; Taylor and Kenny 1988; Stafford *et al.*, 1994; Alaksandra 1998), goats (McKenna, 1988), rabbits (Peters and Geeroms, 1986) and poultry (Haberkorn, 1996). Toltrazuril at the same dose is effective in treating clinical coccidiosis in calves (Emanuel *et al.*, 1988), cystoisoporosis in puppies (Daugschies *et al.*, 2000), neosporosis in mice and calves (Gottstein *et al.*, 2002; Kritzner *et al.*, , 2002). Faecal oocyst counts were

reduced for 2-3 weeks after treatment in lambs (Gjerde and Helle 1986, Taylor and Kenny, 1988) and puppies (Daugschies *et al.*, 2000).

In another study on rabbits, different *Eimeria* species required different dose levels of toltrazuril to have the same effect (Peters and Geeroms, 1986).
In poultry 5ppm was enough to reduce mortality, but a dose of 10-15ppm was more effective (Haberkorn and Stoltefuss, 1987). In ducks, treatment in the earlier days of infection prevented disease but given later it only had a curative effect (Reuynard *et al.*, 1999). In poultry, drinking water medication found to be more effective than per oral (Gottestein *et al.*, 2001).

1.7.3.5. Toltrazuril treatment and immunity:

It has been suggested that toltrazuril not only prevents the disease but may also help in the development of immunity in lambs (Gjerde and Helle 1986) and poultry (Grief, 2000, 2001). The anticoccidial drugs prevent the multiplication of parasite by acting on different stages of coccidia and these damaged stages stay a long time in the host cell and make the antigen available for the development of acquired immunity (Chapman, 1999).

1.7.3.6. Toltrazuril and weight gains:

Toltrazuril use has been shown to improve weight gains but it is variable in different species (lambs- Gjerde and Helle 1986; Stafford, 1994; Alaksandra 1998, 2001; Taylor and Kenny, 1988; Goats-McKenna, 1988).

1.8. Immunity to coccidia:

Immunity to coccidia is development of resistance to a challenge infection with a homologous *Eimeria* species. Immunity is measured in terms of reduced pathogenic effects, reduced size of visible lesions, decrease in the number of parasite stages and improved weight gains (Chapman, 1999).

1.8.1. Role of maternal antibodies:

Maternal antibodies transferred through colostrum protect calves during the first 3 weeks against many diseases. According to Faber (2002), antibody levels in the sera of cows and their corresponding colostrum were the same and

significant negative correlations were seen between oocyst excretion and serum antibody levels against *E. bovis* antigen.

1.8.2. Role of sexual stages in development of immunity:

The antibodies produced against the sexual stages of development potentially inhibit the development of the oocysts and provide a block in parasite development. This principle has been used by Wallach (1997) who isolated and characterised three major gametocyte antigens (230kDa, 82kDa and 56/54 kDa) of *Eimeria maxima* and used them them to immunise laying hens which could transfer transmission-blocking maternal antibodies to chicks via the yolk sac.

1.8.3. Immunity to *E. bovis*:

Several experiments have been done using *E. bovis* to study the development of immunity in calves. Table 1.4, summarises the experiments on immunity produced by *E. bovis.*

1.8.4. Dose of inoculum:

As can be seen in Table 1.4, it would appear there is an interaction between dose and magnitude of the immune response (Niilo 1969; Anderson *et al.*, 1965; Hammond *et al.*, 1963; Senger, 1959; Fitzgerald, 1967). Larger doses (500 sporulated oocysts and above) always elicit better immunity compared to lower doses, but multiple lower doses (110 oocysts) are effective in developing good immune responses that would protect the calves from severe infection (Fitzgerald, 1967). Multiple infections of *E. bovis* with a dose of 10,000 each on 5 consecutive days had no advantage over a single large dose at one time (Senger, 1959) and either was effective in promoting effective immunity. Immunity lasts for 2-3 months in young calves and 7 months in older cattle > 1 year if they are not exposed again (Senger, 1959). In rats and chickens a second inoculation increased antibodies but not a third inoculation (Rose and Mockett, 1983).

1.8.5. Immunity to other species: *E. zuernii*:

The immune response appears to be similar to that against *E. bovis*. Exposure to a large dose of 300,000 sporulated oocysts produced severe clinical disease

in calves but resulted in the development of excellent resistance to subsequent reinfection (Niilo, 1969).

1.8.5. a. Site of immune reaction:

The immune reaction occurs in both the small and large intestines of calves (Hammond, 1963). First generation merozoites inoculated into the caecum stimulated sufficient immunity to the extent that the calves could resist subsequent challenge (Hammond, 1964). This is probably due to the development of anti-merozoite antibodies (IgA, IgG and IgM) within the caecum.

1.8.5. b. Immune mechanism:

The immunity to *Eimeria* is very complex. *Eimeria* has different stages in the life cycle presenting several stage specific antigens that can be targeted by both humoral and cellular immunity components. Schizonts, gametocytes and oocyst components of poultry *Eimeria* spp. *(E. maxima, E. tenella)* have at least 2 immunogenic antigens (Rose, 1984; Rose and Hasketh, 1976; Davis *et al.*, 1978) but gametocytes of *Eimeria maxima* have 3 major antigens (230kda, 82kda, and 56/54kda) (Wallach, 1997). Sporozoites and merozoites not only have different antigens but also stimulate immunity of different duration in calves (Hughes, 1989). As seen in poultry, a primary infection with oocysts (2600) or with a combination of oocysts (50,000) and sulfa drugs developed sufficient immunity that birds could resist a second infection, but an infection with only merozoites, did not produce enough immunity to resist a second infection (Rose and Hasketh, 1976). Some authors have found that second-generation schizonts induce better immunity than first generation schizonts and the sexual stages are more susceptible to the immune response (Rose and Hasketh, 1976; Rose and Mockett, 1983).

Author	Dose of inoculum (oocysts)	Challenge	Immunity	Control
Hammond *et al.*, 1963	25 -60,000 oocysts of *E. bovis* 0.5 -1x 10^6 oocysts of *E. bovis*		Immune reactions both in small intestines and large intestines. Produced only 1,000 oocysts Fewer schizonts in small intestines on 14-16 days of inoculation and lower percentage of infected epithelium because of developed immunity	179,900 to 401,300 oocysts More schizonts Large infected epithelium
Hammond, 1964	0.4-0.9x10^7 first generation merozoites to caecal inoculum	Challenge of calves again with merozoites	No infection	Slight to moderate infection Mild infection
Senger *et al.*, 1959	10,000 50,000 100,000		No severe infection Similar in terms of immunity but 50,000 oocysts produced better immunity than 10,000 and 100,000 oocysts.	No severe infection Less severe coccidiosis More severe occidiosis and longer illness.
Senger *et al.*, 1959	10,000 50,000 100,000	Re-infection 500,000	Immunity developed rapidly within 14 days after the challenge. Immunity present at moderate degree up to 2-3 months after inoculation. Older animals (>1 year) developed high degree immunity, up to 7 months	Severe coccidiosis in all the previously un-inoculated control calves.
Senger *et al.*, 1959	50,000 as a single dose. 5 equal 10,000, on 5 consecutive days		Multiple infections have no advantage over the single inoculation.	
	100, 000 as a single dose or 5 equal doses of 20,000 on 5 consecutive		Multiple infection has no advantage over the single inoculation	

	days			
Fitzgerald , 1967	10 - 15,000 oocysts for 62 days 10, 100, 500,1000, 5000 up to 62 days 110 oocysts fed for 11 days	300,000-500,000 oocysts on 47th and 49th day.	No clinical signs Discharged fewer oocysts (1000-5000). Calves fed 500 oocysts had better immunity than 10-100,000 oocysts. Light infection but developed enough resistance to protect them.	Deaths, bloody diarrhoea with tissue.
Conlogue, 1984	Premunity (35 to 39 th day)with2000, *E.bovis*+ 52 days Lasalocid or DEC treatment		No diarrhoea	
	Premunity with(35 to 39 th day) 2000, *E.bovis*+ 52 days Lasalocid or DEC treatment	challenge with 200,000 on the 56th day	Diarrhoea 11 to 13 weeks after the challenge and the medicated drug with drawn	
	Same as above but 70 days		No diarrhoea	
	No treatment but premunised on 34 - 39 day with 2000 oocysts	challenge on 56 th day	Diarrhoea seen from 11 to 13 weeks.	
	Treated 70 days with out re-imunisation and challenged at 56 day		The diarrhoea is delayed by 1 week that is 12 week after the with drawl of the drug	

Table 1.4: Summary of various reports on the development of immunity to *E. bovis*.

1.8.6. Duration of immunity:

Antibodies are detectable within 5 -7days of infection (Davis *et al.*, 1978), reach a peak in about 2-3 weeks (Anderson, *et al.*, 1965; Hughes, 1989) and are present for up to 63 days in calves. There is an inverse relationship observed between levels of IgG1, IgG2 and oocyst production (Faber, 2002). One single larger dose of infection or inoculation raises antibody titres more quickly than two repeated doses and a second inoculum induces higher titres in calves and birds (Andersen *et al.*, 1965; Rose, 1984). This is more important than a third inoculation as the latter has no influence on titres in rats and chickens (Rose and Mockett, 1983). As mentioned earlier, sporozoite specific antibodies peak after 2-3 weeks and disappear by 40 days but merozoite specific antibodies were still detectable in calves for 63 days (Hughes *et al.*, 1989; Andersen *et al.*, 1965; Faber, 2002).

Mean titres are achieved in less time when the calves received larger doses of inoculum.

1.8.7. Cell mediated immunity:

Immunity to coccidia involves complex interactions of thymus-derived cell mediated immunity (CMI) and bursa-derived humoral immunity (Froyman, 2002). Athymic rats and bursectomised chickens excrete three times more oocysts than normal (Rose and Hesketh, 1970). In one study *E. bovis* antigen induced a delayed hypersensitivity reaction similar to *Mycobacterium* infection (Phillip *et al.*, 1977). It is claimed that CMI is more important than humoral immunity (Hughes et al., 1989).

Sub-populations of lymphocytes are cytotoxic and cytolytic and can kill and lyse-the parasite stages (Rose, 1974). As lymphocyte levels peak on 20^{th} day and remain elevated for up to 40 days indicates that cell mediated immunity (CMI) is also important (Hughes *et al.*, 1989). The CMI-associated factors like macrophages, natural killer cells (NK), lymphocytes and 2 types of T derived lymphocyte populations ($CD4^+$, $CD8^+$) are believed to play a role in immunity to *Eimeria* infections. Increased numbers of macrophage and NK and $CD4^+$ are seen during the primary infection and $CD8^+$ cells during the secondary infection. The lymph nodes draining the intestines and spleen were observed to be more actively secreting these cell types with infection (Hermosilla *et al.*, 1999).

Macrophages phagocytose the coccidial stages and the activity of macrophages seems to be increased in immunised chickens during 1-9 weeks after immunisation, with a peak at 5 weeks (Rose, 1974). The antibodies of the immunised chickens attach to the macrophages and enhance the activity of macrophages on sporozoites (Rose, 1974).
The intra-epithelial lymphocytes (IELS) of the gut play a role, not only in the development of the immunity by carrying the parasite from epithelial cells to the lamina propria and to the crypts, but they also seem to stop the sporozoites entering the enterocytes (McDonald, 1999).

1.8.8. Components of humoral immunity:

The earliest antibody detectable after primary infection is IgM which follow an initial large rise and then fall to lower levels that persist for a long period in rats. The second antibody which rises is IgG. Normally two fractions of IgG (IgGa and IgGb) are involved (Rose, 1984). The secondary infection recalled all the 3 fractions (IgM, IgGa, and IgGb) in rats and birds (Rose and Mockett, 1983). Probably this may be the reason the calves, which are exposed to primary infection, resist the second infection (Stockdale and Yates, 1978; Senger, 1959; Niilo, 1969).

1.8.9. Estimation of immunity:

Humoral immunity of *Eimeria* is estimated using a variety of different tests.

1.8.9. a. Neutralization and precipitation test:

Precipitating antibodies are detectable in birds within 7 days of primary infection by precipitation and neutralization tests by using tissue and caecal extracts of recovered birds (Davis *et al.*, 1978, Rose and Mockett, 1983).

1.8.9. b. Indirect Immuno-fluorescent Antibody test (IFAT):

Antibody titres against sporozoites and merozoites of *E. bovis* in calves were high during 10-20 days after infection (DAI) and reduced to basal level by 40 DAI (Hughes *et al.*, 1989). The IFAT tests using monoclonal antibodies detected antigens to *E. bovis* in the anterior half to two-thirds of merozoites (Haeber *et al.*, 1992).

1. 8.9. c. ELISA:

E.bovis antibody titres of cow and calf sera and colostrum can be estimated using an ELISA (Enzyme Linked Immunosorbent Assay) test (Faber *et al.*, 2002,).

1.8.9. d. Western blotting:

Stage specific differences in surface proteins in merozoites and sporozoites are commonly seen in coccidian parasites (Reduker and Speer, 1986). These can be selectively detected by antibodies using Western blotting. This technique has enabled researchers to identify antigenic proteins and together with immuno-blotting has been utilised to identify and isolate specific bands to study potential vaccine candidates for chickens (Wallach *et al.*, 1994; Mencher 1989; Smith *et al.*, 1994).

1.9. Western blotting using *E. bovis*:

Several experiments have been conducted to identify the immunogenic proteins in different stages of *Eimeria*. Experiments using SDS-PAGE gels identified protein bands of merozoites and sporozoite extracts of *E. bovis* ranging from 15,000 - 215,000KDa Molecular weight (Mr). Nitrocellulose immunoblots of immunised calves showed several binding proteins of Mr18,000 - 180,000KDa in merozoites and Mr28,000 to 118,000KDa in sporozoites. Though these two contained different bands, 4 antigens had the same migratory distance in both. They are Mr 58,000, 70,000, 83,000, and 98,000KDa. The auto radiographic analysis of radionated parasite identified surface proteins on merozoites between 15,000 and 18,000 and on sporozoites 28,000, 77,000 and 183,000Kda. Though several proteins were identified, only a few of these proteins, such as 183,000KDa proteins elicited an IgG antibody response (Redukar and Speer, 1986).

1.10. Tissue culture:

Parasites have been grown on tissue cultures to produce large quantities of parasites and in turn, antigens for molecular studies and for production of the vaccines. In earlier days the endogenous life cycles were studied by using cell lines (Hammond *et al.*, 1966, 1969, 1972). And *Eimeria* species seems to be well adapted to many mammalian cells bovine kidney, spleen, intestine and thymus.

1.11. Prevalence of *Eimeria* species in cattle:

1.11.1. Age prevalence:

Coccidiosis as a disease or infection is more prevalent in the younger animals than the older ones. Generally higher oocyst counts are seen in 3 week to 18 month old calves

(Hasche and Todd 1959, Wisconsin). There have been many studies on the change in prevalence with age. The highest prevalence is seen in the age group of 1 month to weaning calves i.e. 86.3% (USA), 56% (Tanzania), 29% (Tanzania) and 46% (Wisconsin). The adults show lowest prevalence varying from 3.8% (Kenya) to 30% (Tanzania).

The prevalence is more in diarrhoeic animals than in non-diarrhoeic animals. This difference is more obvious in calves than other aged animals. All types of calf diarrhoeas from 2 to 12 weeks of age and most of the diarrhoeas with blood are associated with coccidia (Andrews, 1954).

1.11.2. The effect of climate on the disease prevalence:

The seasonal distribution of bovine coccidiosis is not obvious (Hasbulla *et al.*, 1990) but oocyst counts seem to be high during the wet season in all age groups (Waruiru *et al.*, 2000; Munuya *et al.*, 1990), presumably as this is favourable for the sporulation and survival of oocysts.

1.11.3. World wide prevalence:

Table1.5. summarises various published papers on the prevalence and occurrence of coccidia in cattle. Coccidiosis is prevalent worldwide but there are geographical differences as to which species are present although in all countries at least 8-11 species are common. The most prevalent species are generally *E. bovis* and *E. zuernii*. These two species are not only the most prevalent but are also the most pathogenic. Regardless of which geographical region is considered coccidiosis as a disease or infection is more prevalent in younger animals than the older.

1.11.4. Prevalence in New Zealand:

There have been few studies on the prevalence of coccidiosis in cattle in New Zealand. Clinical disease is not uncommon with intensive dairying, weaning stress, poor hygiene of the premises. It is observed that 84% of blood scour cases and 25% non-blood scour cases among the <3months old calves and 6% among >3 months old calves were with coccidia when the calf scours were investigated on 81 different properties in New

Zealand. The most frequently found species are *E. zuernii* and *E. bovis* (Andrews *et al.*, 1954).

In New Zealand, half of all the recorded cases of coccidiosis occurred in three months of September, October and November among young and recently calves animals (Bailey, 1994). There was a small peak in the month of April, because of autumn born calves and this was distinct in the northern half of the North Island. The intensive agricultural regions like Waikato had more cases where higher stock densities are seen.

In New Zealand 11 species of *Eimeria* have been identified in cattle. In one study McKenna (1972) reported the prevalence of 10 of these as *E. bovis (4 4%), E. zuernii (19%), E. canadensis (14%), E. ellipsoidalis (14%), E. auburnensis (12%), E. alabamensis (7%), E. cylindrica (5%), E. brasiliensis (4%), E. wyomingensis (3%)* and *E. subspherica (2%)*. Most common and pathogenic species were *E. bovis* and *E. zuernii*. In a later report *E. bukidnonensis* was also recorded (McKenna, 1974). In a seperate study Arias, 1993; described seven *Eimeria* species with *E. canadensis* being most dominant species (37%) followed by *E. bovis* (16.6%), *E. auburnensis* (12.2), *E. ellipsoidalis* (6%), and *E. alabamensis* (6%).

1.12. Multiple species in one sample:

Most studies of coccidiosis in calves have shown the presence of multiple species in one animal and as many as 80 to 81% positive samples have 2 or more species (up to 9) (Ernst et, al 1984, 1987; Joyner, 1966; Kasim, 1985; McKenna, 1972; Munyua and Ngotho, 1990; Oda and Nishida, 1989; Hashe and Todd, 1959) with an average of 3.1.

Country	No. of cattle examined	Type and age	Any species	E.alabameninsis	E.auburnensis	E.bovis	E.braziliensis	E.bukidnonensis	E.canadensis	E.cylindrica	E. ellipsoidalis	E. pellita	E. subspherica	E. wyomingensis	E. zuernii	E.isospora/ Bareilea	Reporters
Austria	130	-	-	-	2.3	50.8	7.0	-	-	3.0	11.5	3.9	-	-	8.5	-	Supperer (1952)
Brazil	146	-	-	-	2.5	34.5	2.1	5.8	-	-	-	-	-	-	26.8	-	Torres & Ramos (1939)
Costa Rica	100	Adults	-	-	-	7	-	-	-	1	3	-	-	-	1	-	Ruiz (1959)
Costa Rica	100	Calves	-	20	1	31	-	-	-	-	4	-	3	-	2	-	Ruiz & Ortiz (1961)
Guatemala	100	Adults	69	43	43	45	3	6	39	19	47	9	12	-	25	-	Balconi (1963)
South West England	110	-	-	14.5	33.6	7.5	7.3	1.8	12.7	12.7	26.4	4.5	28.2	13.6	64.5	-	Joyner (1966)
Georgia, USA	534	Beef Calves	-	10.6	32.2	68.0	0.4	1.1	7	20.2	0.6	49.2	4.5	6.2	21.1	-	Ernst (1987)
South East US	2492	-	-	-	-	41	-	-	-	-	45	-	-	-	42	-	Boughton (1945)
Illinois	795	Beef calves	10.7	2.1	5.78	6.5	0.5	0.1	4.40	1.50	5.0	-	1.0	0.75	4.65	0.2	Szanto J (1964)
New Zealand	288	-	-	7.0	12.0	44.0	4.0	-	14.0	5.0	14.0	-	2.0	3.0	19.0	1	McKenna (1972)

Japan	201 9	<2-3 yr. old	-	10	18	26	3.9	5.4	15.0	1.3	8.1	0.2	1.5	1.9	7.0	-	Hasbulah et al., (1990)
Kenya	620	-	-	3.7	6.1	42.2	-	-	-	8.9	26.1	-	3.1	2.3	22.6	-	Munyua et al.,1989
India	88 /106	Buffalo calves	-	-	-	-	-	-	-	13.6	15.9	-	-	-	26.1	44.0	Bharkad et al., 2000
	24 /144	Cow calves	-	-	16.6	37.5	-	-	-	12.5	-	-	-	-	33.3	-	
Montana USA	486 +47 9	Calves+ Adults	-	0.2	22.8	46.5	0.1	6.7	4.3	1.0	8.4	_	_	_	5.8	_	Jacobson et al.,1969
Wisconsin	355	Dairy 1 week-18 month	23.9	11.8	12.7	11.3	1.7	1.4	12.7	5.7	12.1	-	3.1	-	7.3	-	Hasche & Todd, 1959

Table 1.5: summary of various reports on the Percent prevalence of bovine *Eimeria* species.

1.13. Species Descriptions:

The sizes and shapes of many oocysts overlap with other oocysts so there is recognized difficulty in identifying them (Joyner *et al.*, 1966). For example, *E. wyomingensis* is close in appearance to the oocyst of *E. auburnensis*, but differs in appearance and shape of the sporocyst. *E. pellita* described by (Supperer, 1952) is a dark coloured, thick-walled oocyst similar to *E. bukidnonensis* superficially but the oocyst wall of the latter and presence of a residuum allows them to be differentiated.

Sommer (1998) reported the importance of drawings to identify unknown *Eimeria* specimens and quantitative data as a reference set for identification. To classify cattle cocidia the quantitative data were employed in agglomerate clustering with an average linkage algorithm with equal weights assigned to size and shape. An inverse Fourier transform was used to reconstruct the oocyst outline, i.e., average shape and size. This method can be used to reconstruct and classify oocysts using quantitative data of any *Eimeria* species which vary in their sizes and shapes.

A summary of the morphology of bovine species of *Eimeria* as described by varous authors is given in Table 1.6. and Plate 1.1 shows the general morphological details of a sporulated oocyst.

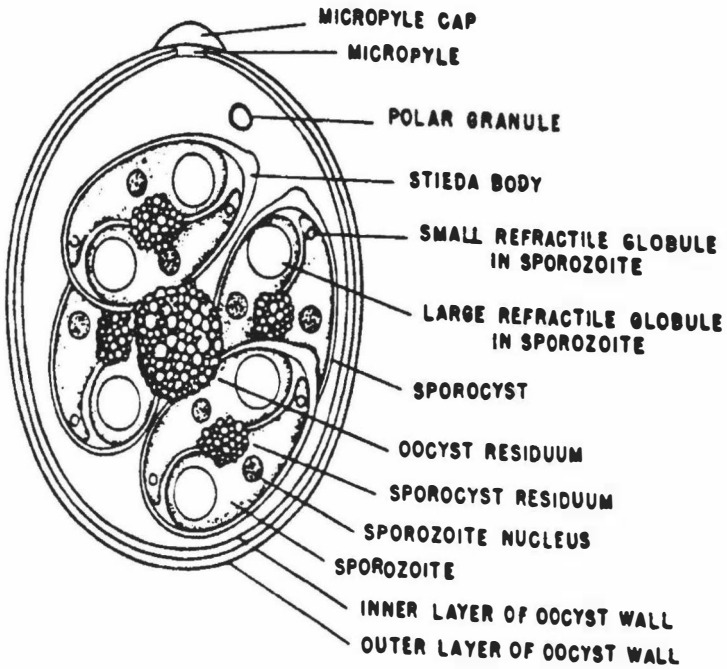


Plate. 1.1: A structure of sporulated *Eimeria oocyst* (from Levine, 1986).

1.13.1. *Eimeria* species without micropylar cap:

Ernst and Benz (1980), described *E.bovis, E. zuernii, E. ellipsoidalis*, and *E. auburnensis* as the most prevalent species whereas *E. cylindrica, E. alabamensis, E. illinoisensis, E. canadensis, E. wyomingensis* and *E. subspherica* as sporadic species and *E. bukidnonensis, E. pellita* and *E. brasiliensis* as the rarely occurring species.

1.13.1. a. *Eimeria subspherica*:

This species was first described by Christensen (1940), from calves in Alabama. They were described as the smallest oocysts observed with a transparent wall, subspherical to ellipsoidal shape. Later, in a study in Nigeria subspherical forms

were identified by Lee and Armour 1959. In other studies *E. subspherica* (Joyner et al., 1966 - South West England, Oda and Nishida, 1990 - Japan) was described similarly.

1.13.1. b. *Eimeria ellipsoidalis*:

The oocysts of *Eimeria ellipsoidalis* were first described from a Calf from Iowa, and were colourless, ellipsoidal (Becker and Frye, 1929). Then Christensen (1941) described the oocysts having lavender to yellowish coloured wall. Later, Lee (1959), found similar oocysts.

1.13.1. c. *Eimeria zuernii* (Rivolta 1878) Martin, 1909

Synonym: *Cytospermium zurneii*, Rivolta, 1878

These oocysts were described by Christensen (1941) from Alabama and also Lee (1959) from Nigeria and Levine and Ivens (1967) from Illinois. The oocysts were spherical to ellipsoidal, without a micropyle, with a transparent thin wall and contained one or more scattered polar granules. Sporocysts were elongate and ovoid each with a tiny stieda body. A sporocyst residuum was present either scattered or as a compact mass. Sporozoites were elongated, head to tail in sporocysts with a clear globule at the large end, and the nucleus in the center (Levine, 1985).

1.13.1. d. *Eimeria cylindrica* Wilson, 1961:

Christensen (1941) described the unsporulated oocysts as cylindrical shaped, non-micropylar with a homogenous transparent wall. In later descriptions it is reported that sporulated oocysts have no residuum and stieda body but had scattered polar granules. Sporozoites contained one or more clear globules.

1.13.1. e. *Eimeria alabamensis*:

Originally described from cattle, in Alabama, USA (Christensen, 1941). The unsporulated oocysts were typically pyriform, varying from sub-ellipsoidal to sub-cylindrical but the majority were tapered at one end. Oocysts contained a parachute shaped cap at each end of the sporocyst. No residual matter was present in either oocyst or sporocyst.

Levine and Ivens (1967) described these as ovoid with sides tapered towards the small end, with no micropyle, no sporocyst residuum and containing 2-3 clear globules in the sporozoite.

1.13.2. *Eimeria* species with micropylar cap:

1.13.2. a. *Eimeria bovis*:

Synonyms: *Coccidium bovis,* Zubliln, 1908.

Eimeria canadensis, Bruce, 1908.

Eimeria smithi, Yakimoff and Galiouzo, 1927.

Globidium fusiformis Hassan, 1935.

The unsporulated oocysts from Alabama calves were described as stout ovoid, blunt across narrow end sometimes, sub-ellipsoidal, asymmetrical and elongated. A micropyle was present. The oocysts were pale, cloudy, and greenish to yellowish – brown in colour under low magnification (Christensen, 1941). An oocyst residuum and polar granules were absent. Sporocysts had a stieda body and an oocyst residuum composed of pale granules of variable number.

1.13.2. b. *Eimeria canadensis*:

Synonym: *Eimeria zurnabadensis*: Yakimoff, 1931.

Christensen (1941) described this micropylar species from Alabama calves. The oocysts were regularly ellipsoidal in shape varying from cylindrical to stoutly ellipsoidal with a tapered end. Levine and Ivens (1967) described these as ovoid or ellipsoidal, with a smooth wall having no oocyst residuum but a number of splintered polar granules were seen in some oocysts. The sporocyst had a compact ball-like residuum and 2-3 clear globules were present in sporozoites.

1.13.2. c. *Eimeria auburnensis* (Christensen and Porter, 1939):

These oocysts were typically elongated, ovoid, varying between sub-ellipsoidal and markedly tapered. The micropyle was seen as a gap in the wall at the tapered end covered with thin, black line. The oocyst wall was typically smooth, homogeneous, transparent, and usually yellowish–brown in tint but varying in structure from a transparent homogenous type to a relatively semi-transparent

and heavily mammillated type. Smooth walled oocysts were present in more hosts than the rough walled. An oocyst residuum was absent. The sporocyst was ellipsoidal with a residuum as one or more compact masses. Sporozoites were comma-shaped with a large clear globule in the large end and 1 or 2 smaller globules (Levine and Ivens, 1967).

1.13.2. d. *Eimeria bukidnonensis*:

***Eimeria bukidnonensis* Tubangui, 1931**

This species was first described by Tubangui (1931) from the faeces of a bull from Bukidnon, Mindanao, Manila (Philippines). They were described as yellowish to darkish brown, uniformly pyriform shaped. The oocyst wall had radial striations and was about 2 microns thick except at the micropylar end where it was very thin. The micropyle was conspicuous, being about 4 microns wide. A definite residual body was absent in the oocyst or in the sporocysts. Later Christensen, 1941 described this species in the United States. His oocyst measurements agreed in all features with the description of Tubangui except the size was smaller. Similar descriptions were made by Levine and Ivens (1967) and Hiregaudar and Rao (1966) from India. The species were compared with other oocysts from the Philippines, Alabama and Nigeria and found to be generally similar.

1.13.2. e. *Eimeria wyomingensis* (Huizinga and Winger, 1942):

Synonyms: *bukidnonensis* Tubangui, 1931 of Christensen, 1938,
***E. bukidnonensis* Tubangui, 1931.**

This species was originally considered as *E.bukidnonensis* but later identified as a different species with minor differences from *E. bukidnonensis*. These differences include the oocyst being smaller in size with a smooth wall without striations. The other features were the same as for *E. bukidnonensis*. Levine and Ivens (1967) described the oocysts as ovoid with yellowish-brown to brownish-yellow walls, speckled and somewhat rough, composed of a single layer and lined by a membrane. The micropyle was 5µm in inside diameter at the small end of the oocyst. Oocyst residuum and polar granules were absent. Sporocysts were ellipsoidal with narrow end and a tiny stieda body at one end. Sporocyst residuum was generally absent but sometimes present in the form of

granules. Sporozoites were wider at one end with a large clear globule at the broader end.

1.13.2. f. *Eimeria brasiliensis:*

E. braziliensis was described as a new species by Torres and Romas (1939). A notable morphological feature was the presence of a polar cap measuring 8 -10 microns wide by 2-3 microns high. Lee and Armour (1954) found similar oocysts in Vom, Nigeria. The oocysts were yellowish - brown, the micropyle was like a dark line beneath the polar cap. The residual body was situated immediately behind the micropyle.

Oocyst Morphology					*Sporocyst Morphology*					
Eimeria species	**[range-LxW : mean: shape index (mean): Shape]**	**N**	**Micropyle**	**Oocyst wall**	**Polar granule**	**mean, range, shape index**	**Stieda body**	**Residuum shape**	**Globules**	**Author**
E. bukidnonensis	46.8-50.4 x 33.3-37.8: 48.40x35.6: 1.37, Pyriform.	25	Present	Thick radially striated.				Absent		Tabangui, 1931
E.bukidnonensis	38-48x24-34	82				18x9				Gill, 1968 (from Hiregaudar, 1966)
E.bukidnonensis	38-46x25-35	50				15-19 x8-11				Bhatia,*et al.*, 1968 (from Hiregaudar)
E.bukidnonensis	35.3-49.1 x 26.3-37.2, pyriform	153								Lee and Armour 1959.
E.bukidnonensis	32x21,	-				14.4x6.5				Patnaik, 1964 (from Hiregaudar, 1966)
E.bukidnonensis	44-50x 33.12-35.25: 47x34 :1.37:Pyriform		Present	Thick radially striated.						Hiregaudar and Rao, 1966.
E.bukidnonensis	32-41x24-30:	-								Christensen 1938 New York.
E. bukidnonensis	33-41x24-28: 37x26:1.37: Pyriform	80	Present							Christensen, 1941, Alabama.
E. khurodensis	40-44x28-30: 42x29:1.45: Ellipsoidal.		Present	Mammilated, thick						Rao and Hiregaudar. 1954
E. wyomingensis	37-45x26.4-	-	Present	-						Huizanga and

	30.8: 40.3x28 : 1.43:Ovoid to Elongate.									Winger, 1941
E. auburnensis	35 - 43 x 21-27, 41.3 x 2 4.5, Elongate ovoid.		Present	Smooth rarely rough.	Present					Levine and Ivens, 1967
E. auburnensis	32-45.5x20-26:38.4x23.1: 1.66: Elongated ovoid.		Present	Smooth homogenous						Hiregaudar and Rao, 1966
E. subspherica	13.8-27x 11.4-24.6									Majro and Dipole, 1981
E. subspherica	9.4 -13 x 8.7-12.2: 11.4x11: 1.06: Subspherical.	106	Absent	Thin, fragile transparent,						Lee and Armour, 1959
E. subspherica	10.13x9-12: 11x10.5: 1.06.									Ernst and Courtney, 1977
E. subspherica	9-13x 8-12: 11x10.4: 1.06, Sub spherical to ellipsoidal		Absent	Transparent	Absent					Christensen, 1941
E. subspherica	13-15.7x 10.5 - 4:14.5x12.3: 1.19: Spherical to ellipsoidal		Present	Smooth colorless wall						Oda and Nishida, 1990
E. ellipsoidalis	20-25x14-20: 23.1x16.1: ellipsoidal - ovoid			Smooth walled	Absent					Levine and Ivens, 1967
E. ellipsoidalis	13-24.9 x 14-	150		Smooth thin						Lee, 1959

	17.9: Ellipsoidal: sub-spherical, cylindrical			walled						
E. ellipsoidalis	12-27x10-18: 0.77: Ellipsoidal spherical to cylindrical	350	Absent	Smooth thin walled						Christensen, 1941
E. ellipsoidalis	20-26x13-17: 0.68 Ellipsoidal.		-							Becker and Frye, 1929
E. cylindrica.	17-28.4x11.8-16.6:0.60:									Lee and Armour, 1959.
E. cylindrica.	16-27x12-15:0.60									Christensen, 1941
E. cylindrica.	19.4-26.8 x 11.9-4.9:0.57:									Wilson, 1931
E. cylindrica	22-30x12-17: 25.3x14.8:1.7 :elongate ellipsoid, straight sides		Inconspi cu-ous	Smooth walled	Absent	12-16 x 4 - 6, 13.7 x 5.4, 2.5.		granule at one end	One or more	Levine and Ivens, 1967
E. canadensis	Ovoid or ellipsoidal		Present	Smooth wall	Absent			Present	2-3 clear	Levine and Ivens, 1967
E. alabamensis	19-24x14-16: 20.7x14.8: pyriform		Absent	Thin delicate, smooth	Absent			Absent scatter granule		Levine and Ivens, 1967
E. zuernii	13.5-20.5 x 12.3-18.0: 17x16.1:0.91:									Tabangui, 1931.
E. zuernii	14.7-20.7 x13.5-17.3: 0.91:17.3x15. 7:		Absent	Thin homogenous, transparent						Lee, 1959

	Subspherical Spherical or ellipsoidal.									
E. zuernii	15-22x1-18: 17.8x15.6: 0.88: 17.8x15.6									Christensen, 1941
E. zuernii	18-23x 16.1: 20.2x 16.1: ovoidal Subspherical,		absent	Smooth colorless	Present					Levine and Ivens1967
E. brasiliensis	34.20-42.75x27.05-24.2:29.x22,ovoid to regular			Smooth	Present					Torres& Romos, 1939
E. brasilensis	33.75-49.0x24.1-33.2,26.5, oval				Present					Supperer, 1952
E. brasiliensis	30.7-39.5 x 21.9-29.9: 35x 25.9:1.35: ellipsoidal				Present					Lee and Armour,1959
E. brasiliensis	32.0 - 40.0 x 23.x27.5: 36x25.1: ellipsoidal				Present					Marquardt, 1959
E. bovis	24-32.8x18.1-22.9:28.2x20.9:0.74	172								Lee and Armour, 1959
E. bovis	23-34x17-23: Stout ovoid, ellipsoidal	500		homogenous transparent.	Present					Christensen, 1941

	asymmetrical and elongated.									
E. bovis	24-34 x 19 - 22: 26.7 x 20.2: Ovoid			Smooth wall	present					Levine and Ivens,1967
E. canadensis	25.2-3.2x18-32.4: 34.1x25:0.73 : cylindrical, Two ends equally rounded.									Yakamov, 1933
E. canadensis	30.7-33.2x 24.9-26.5: Ellipsoidal.									Bruce,1921
E. canadensis	29-5.9x20.8-26.8:32.5 x 24.4:0.75: Ovoid or ellipsoidal.	107	Present	Smooth but sometimes rough.				Present	2 - 3 clear globule .	Lee and Armour, 1959.
E. canadensis	Ovoid or ellipsoidal		Present	Smooth wall rough wall	Absent	15 -22 x 6-9, 8.3 x7.9, 2.32		Small scattered granules	2-3 clear	Levine and Ivens,1967

Table 1.6: Morphological characteristics of oocysts of *Eimeria* species of cattle. Range- L=length, W=width range, mean of length x width, Shape index =ratio of length and width, N= Number of oocysts measured.

1.14. *Cryptosporidium:*

Tyzzer first described this protozoan parasite in 1907 in the gastric glands of the laboratory mouse. These parasites measured 6-7µm, and were named *Cryptosporidium muris*. Five years later, Tyzzer described a smaller form of the organism, 2- 5µm diameter in mice. This was named C*ryptosporidium parvum*.

C. parvum is responsible for calf diarrhoea in New Zealand. Bovine *Cryptosporidium* infection was first described in 1971 in an 8 old month Santa Gertrudis calf with diarrhoea. In New Zealand the Whangarei Animal Health Laboratory first reported *C. parvum* in 1980 (McSporran, 1983 and 1992). Calves are susceptible to infection for at least for the first 3 months of life. Exposed calves rapidly develop resistance to subsequent challenge. In an infected animal they are generally found in the gastrointestinal tract but may colonize epithelial cells of the trachea, bile duct, conjunctiva, nasal sinuses, salivary glands, small intestine and renal tubules of animals (McSporran, 1992). Young and immune-compromised animals are at greater risk. About 26-33% calves with neonatal diarrhoea are considered to be due to *Cryptosporidium (*McSporran, 1992). Cryptosporidiosis from cattle is zoonotic to human beings. The oocysts are very resistant to physical agents (McSporran, 1992). Cryptosporidiosis is prevalent in many countries and the summary of the recorded prevalence of *Crytosporidium* and *Giardia* is shown in Table 1.7

Giardia	*Cryptosporidium*	Age	Method	Authors	Country
14% animals	20-88% animals	3-13 days	Ziehl - Nielson	Bednarska *et a*l., 1998	Poland
45.7%	88.7%	Calves		Ruest *et al.*, 1998	Canada, Alaska, Monitab a.
25%	-	-	Ziehl - Nielson	Maldonado Camagoes, 1998	Mexico
73%	40-80%	New born -24 weeks	Immuno-fluorescent	Olson *et al.*, 1997	Alberta, British Columbi a
50%	17%	20 days	Immuno-fluorescent	Olson *et al.*, 1997	British Columbi a
-	50% (3 -days) 17% (4 day) 90-95% (8day)	4-10 days	Modified Ziehl Nielson	Naciri *et al.*, 1999	France
-	17.3%	6–14 month cows, calves.	Sucrose Flotation	Pena, *et al.*, 1997	Brazil
-	15.2%	Birth - 30days		Bandali *et al.*, 1999	France
-	52.6%	1-30 days	Modified Ziehl Nielson	de la Fuente *et al.*, 1999	Central Spain
-	43.85%	1- 7 days	Modified Ziehl Nielson	de la Fuente et al., 1999	Central Spain
	71.9%	8-14 days	Modified Ziehl Nielson	de la Fuente *et al.*, 1999	Central Spain
-	60.1%	15-21 days	Modified Ziehl Nielson	de la Fuente *et al.*, 1999	Central Spain
-	6.9%	22- 30 days	Modified Ziehl Nielson	de la Fuente *et al.*, 1999	Central Spain

Table 1. 7 : A summary of some of the recorded prevalence of *Cryptosporidium* and *Giardia* in cattle.

Chapter 2: Identification of *Eimeria* species in several studies and a redescription of their oocyst morphology

2.1. Introduction:

To date, 21 species of *Eimeria* have been described in cattle (Ernst, 1980) Geographical differences in prevalence of different species seems to be common as shown in Table 1.5. It is usual for multiple species to be observed in any one faecal sample, with an observed average of 3.5 and as many as 8 species present (Oda and Nishida, 1989, McKenna, 1972). Identification of species usually relies on identifying oocysts in faeces. The oocysts of each *Eimeria* species vary in size, shape and structure, with the combination being unique to a particular species. For some, the sizes and shapes overlap with other species. As described in Section 2.2., the *Eimeria* species of cattle are divided into 2 broad categories based on the presence or absence of a micropyle.

Two studies have been reported in detail on the species of *Eimeria* present in cattle in New Zealand. In one study, ten species were identified and their prevalence reported (McKenna, 1972). The species were *E. bovis* (44%), *E. zuernii* (19%), *E. canadensis* (14%), *E. ellipsoidalis* (14%), *E. auburnensis* (12%), *E. alabamensis* (7%), *E. cylindrica* (5%), *E. brasiliensis* (4%), *E. wyomingensis* (3%) and *E. subspherica* (2%). *E. bukidnonensis* was subsequently identified later by the same author (McKenna 1974). In the second New Zealand study, Arias (1993) described seven species. In this second study the species *E. canadensis* was the most common with a prevalence of 37% followed by *E. bovis* (16.6%), *E. zuernii* (15.7%), *E. auburnensis* (12.2%), *E. ellipsoidalis* (6%) and *E. alabamensis* (6%),

E. cylindrica, E. subspherica, E. bukidnonensis and *E. pellita* were not found in this second study.

The aim of the research reported in the current chapter was to re-describe the various species identified in all the various studies reported in other chapters and compare these descriptions with those previously published descriptions. Identification is based on a combination of various features and measurements and the aim was to determine how each species fitted previous descriptions. In

addition, the prevalence of each species was determined over the 3 main studies conducted during this research.

2.2. Materials and Methods:

Oocysts were recovered and sporulated from studies described in Chapters 3, 4, and 5. See Appendix 3.3. for a description of the technique used. Oocysts were generally identified using the keys provided by Christensen (1941) and Levine and Ivens (1967). For each species, at least 100 oocysts were measured and re-described, except for less common species where only a few oocysts were available for measurement. The parameters recorded were: presence or absence of a polar cap; micropyle shape; oocyst width; oocyst length; length: width ratio; presence and character of the oocyst residuum; a stieda body; presence and character of the sporocyst residuum.

A variety of terms are used to describe the general shape of oocysts and is in common usage without formal definitions of their meaning. These terms and a description of their meaning are as follows:

- Pyriform: pear shaped.
- Oval or ellipsoidal: oocyst shaped like a circle that is flattened, so that it is oval or an ellipse. The oocyst is not exactly circular in shape but is generally symmetrical but may taper at one end and be broader at the other end and slightly round in appearance.
- Ovoid: approaching being spherical or round but not tapered as for oval or ellipsoidal but more towards being round.
- Sub spherical: almost spherical but not an exact circle. Similar to ovoid but closer to being a true sphere.
- Spherical or Round: exactly circular in shape.

To determine the prevalence of species within a study at least 30 oocysts were identified from one faecal sample from each animal on each occasion. For an animal to be considered infected, the species was identified in that animal on atleast one occasion. Since many samples had few oocysts this was only estimated from those animals with a reasonably high oocyst count to make the

observation practically feasible. The overall prevalence of species was calculated by determining the mean prevalence over all 3 studies.

To obtain a measure of the predominant species the total number of oocysts of each species in a study that were identified was pooled and divided by the overall grand total of oocysts identified in that study to be expressed as a percentage. For all 3 studies the data from individual studies were pooled and the overall predominance of each species was expressed as a percentage.

2.3. Results:

2.3.1. Prevalence and predominance of species identified at Massey No.4 Farm (See Chapter 3):

Although this study continued for over 3 months, in total oocysts were recovered and identified in only 12 individual faecal samples from separate animals due to generally low oocyst counts throughout the study. The raw counts are shown in Appendix 2.4.1. Only 10 different species were identified. They were in order of decreasing prevalence as follows (with prevalence as %): *E. zuernii* (100%), *E. bovis* (83.3%), *E. auburnensis* (75%), *E. cylindrica* (66.6%), *E. bukidnonensis* (58.33%), *E. ellipsoidalis* (41.6%), *E. subspherica* (33.3%), *E. canadensis* (25%), *E. alabamensis* (25%), and *E. wyomingensis* (16.66%). Data is summarised in table 2.1.

2.3.2. Prevalence and predominance of *Eimeria* species identified at Tuapaka Farm (See Chapter 4):

In this study 81 young calves were faecal sampled at weaning and again at weekly intervals for 5 weeks with about half given a coccidiocide at weaning. Only 33 calves were used for estimation of prevalence where at least 30 oocysts could be recovered for identification. A total of 11 *Eimeria* species were identified on this farm. The raw data are shown in Appendix 2.4.2. and a summary in Table 2.1. In order of decreasing prevalence the species identified were *E. zuernii* (98%), *E. bovis* (90%), *E. auburnensis* (60.6%), *E. cylindrica* (33.3%), *E. canadensis* (18.18%), *E. wyomingensis* (15.15%), *E. bukidnonensis* (12.12%), *E. subspherica* (9.1%), *E. alabamensis* (9.1%), *E. brasiliensis* (9.1%) and *E. ellipsoidalis* (6.06%). Summary is shown in Table 2.1.

2.3.3. Prevalence and Predominance of Species identified in studies on other farms (see Chapter 5):

Oocysts were identified in 8 faecal samples from other calves in all remaining studies and a total of 10 species were identified. These 8 samples were obtained from 5 individual farms (Appendix 2.4.3). On some occasions several samples were pooled from the one group of animals because of low oocyst counts. In order of decreasing prevalence the species identified (and their prevalence) were: *E. bovis* (87.5%) followed by *E. zuernii* (87.5%), *E. auburnensis* (50%), *E. wyomingensis* (37.5%), *E. bukidnonensis* (37.5%), *E. canadensis* (37.5%), *E. subspherica* (37.5%), *E. brasiliensis* (25%), *E. cylindrica* (25%) and *E. ellipsoidalis* (25%). By far the two most predominant species were *E. bovis* and *E. zuernii.* Data is summarised in Table 2.1.

Farms on Study	Massey No.4	Tuapaka	Other Farms	Mean Over 3 Studies	Massey.No.4	Tuapaka	Other Farms	Mean Over 3 Studies
***Eimeria* Species**	**Prevalence %**				**Predominance %**			
E. zuernii	100.0	98.0	87.5	95.0	25.1	28.5	25.8	26.5
E. bovis	83.3	90.0	87.5	87.0	19.1	46.0	28.3	31.1
E. auburnensis	75.0	60.6	50.0	62.0	16.0	9.7	11.1	12.7
E. cylindrica	66.7	33.3	25.0	42.0	11.3	4.7	3.0	6.3
E. canadensis	25.0	18.1	50.0	31.0	5.9	3.2	4.0	4.4
E.wyomingensis	16.7	15.1	37.5	23.0	0.9	2.3	12.6	5.3
E. ellipsoidalis	41.6	6.1	25.0	12.0	0.0	1.2	4.5	1.9
E. subspherica	33.3	9.1	35.7	27.0	3.0	0.5	1.0	1.5
E. alabamensis	25.0	9.1	0.0	12.0	1.9	1.0	0.0	1.0
E. brasiliensis	0.0	9.1	25.0	12.0	0.0	1.2	4.5	1.9
E. bukidnonensis	58.3	12.1	37.5	36.0	10.8	0.9	8.6	6.8

Table 2. 1: Prevalence (%) and predominance (%) of *Eimeria* species from all three studies.

2.3.4. Prevalence and Predominance over the 3 studies.

The overall prevalence and predominance are summarized in Table 2.1.

The two most prevalent species were *E. zuernii* (95.2%) and *E. bovis* (87%) followed by *E. auburnensis* (62%), *E. cylindrica* (42%), *E. bukidnonensis* (36%), *E. canadensis* (31%), *E. subspherica* (27%), *E. ellipsoidalis* (24%), *E. wyomingensis* (23%), *E. alabamensis* (12%) and *E. brasiliensis* (12%).

The most predominant species was *E. bovis* (31.1%) followed by *E. zuernii* (26.5%), *E. auburnensis* (12.7%), *E. bukidnonensis* (6.8%), *E. cylindrica* (6.3%), *E. wyomingensis* (5.3%), *E. canadensis* (4.4%), *E. ellipsoidalis* (1.9%), *E. brasiliensis* (1.9%), *E. subspherica* (1.5%), and *E. alabamensis* (1%).

2.4. Species Description:

A summary of the basic morphological features of the oocysts of 11 bovine *Eimeria* species found in these studies is shown in Table 2.2. A sample of 100 oocysts was measured for 8 species and fewer for three species *(E. alabamensis, E. subspherica,* and *E. brasiliensis)* because only a few oocysts

were recovered for them. The sporulated oocysts of each species were then described in detail and comparison with previous descriptions is discussed.

Identification of individual oocysts is time consuming, as it includes scoring many parameters apart from size and shape. The shapes of many oocysts overlap with each other such as *E. zuernii* with *E. ellipsoidalis*, *E. ellipsoidalis* with *E. bovis*, *E. bovis* with *E. canadensis, E. wyomingensis* with *E. bukidnonensis* etc. Each oocyst has a wide range of length and width which is confusing and makes identification, based purely on size difficult. For example, the length and width of *E. canadensis* has a range which overlaps with *E. bovis* and *E. auburnensis*. Sporocyst structure is often difficult to determine. It differs with the angle of exposure under a microscope and overlapping of sporocysts makes it difficult to clearly measure the sporocyst and identify features such as the sporocyst residuum and large refractile globules in the sporocyst. Though there are many published papers with descriptions of species, the pictures given are often not very useful and are not the same all the time.

	Species	Size µm (L R x W R), Mean Length, Mean Width, mean L/W ratio	Shape	Cell wall	Stieda body	Polar granule	Oocyst Residuum	Colour	Refractile bodies	Micropyle
1	*E. zuernii*	14.0 - 25.2 x 10.3 - 22.7, 19.6 x 16.2 , 1.2	oval	thin	+ve distinct	+ve scattered	+ve	-	2-(large at base)	-ve
2	*E. ellipsoidalis*	20.0 - 29.60 x 10.96 - 25.0, 24.7 x 18.5, 1.4	oval or ellipsoidal	thin	+ve	+ve	+ve	-	1-2	-ve
3	*E. bovis*	20.0 -32.0 x 14.0 - 24.4,23.9 x 18.1, 1.3	oval flattened at one end	thin	+ve	-	+ve	-	2(one large at base)	+ve
4	*E. cylindrica*	15.5 - 30.0 x 11.8 - 27.0, 23.7 x 6.5, 1.5	cylindrical	thin	+ve distinct	+ve	+ve (centre)	-	2	-ve
5	*E. subspherica*	8.96 - 17.80 x 7.20 - 7.0,11.9 x 13.5, 1.2	spherical	thin	+ve	-	-	-	-	-ve
6	*E. canadensis*	14.4 -32.8 x 22.6 - 38.7, 21.9 x 29.1, 1.4	stout ovoid flattened end	moderate thick	+ve	+ve	+ve (centre)	yellowish	2-3	+ve
7	*E auburnensis*	24.8 - 48.8 x 15.5 - 32.9, 36.9 x 25.1, 1.5	oval , flattened end	thin	+ve distinct	-	+ve	yellowish	2	+ve
8	*E. bukidnonensis*	23.5 - 45.2 x 31.7 - 56.0, 31.9 x 47.0, 1.5	pyriform	thick	+ve distinct	-	-ve	dark	2	+ve
9	*E. wyomingensis*	30.0 - 49.6 x 21.4 - 34.4, 39.4 x 27.9, 1.4	pyriform	thick	+ve	-	-	yellowish	2	+ve
10	*E. brasiliensis*	27.2 - 48.0 X 20.6 - 41.4, 37.0 x 29.4, 1.3								+ve
11	*E. alabamensis*	15.1 - 26.0 X 11.4 - 23.2, 20.3 x 15.3, 1.4							2 to 3	-ve

Table 2. 2: A summary of the key morphological characteristics of sporulated oocysts of bovine *Eimeria* species. Note: L=Length, W=width R=Ratio of Length and width.

2.4.1. *E. alabamensis*, Christensen, 1941

Description: Oocysts (Plate.2.6) were ovoid but tapered at one end. Micropyle was absent. Oocyst wall was thin. The sporulated oocysts were 15.1-26.0 µm (L) by 11.4-23.2 µm (W) and with a mean of 20.3 µm (L) x15.3 µm (W). Their length: width ratios ranged from 1.0 - 1.6 with a mean of 1.2. Oocyst residuum was absent. Scattered polar granules were present. Sporocyst was elongated with a tiny stieda body. Sporocyst residuum was absent and sporozoites had 2 to 3 clear globules each.

Discussion: The oocysts looked similar to *E. bovis*, but were smaller in size and devoid of a micropyle. These descriptions are similar to those of Levine and Ivens (1967). The parachute shaped cap was not seen. As observed by Levine and Ivens (1967) oocyst and sporocyst residuum were not seen.

2.4.2. *E. auburnensis*, Christensen and Porter, 1939.

Description: Oocysts (Plate 2.1) were elongated and ovoid being flattened at the smaller end. A micropyle was present at the smaller end. Oocyst wall was generally smooth but sometimes rough and thin at the broad end. The micropylar end was even thinner. The oocysts were 24.8 - 48.8 µm (L), 15.5-24.8 µm (W) with mean 37.0 µm (L) x 25.1 µm (W). Their length: width ratios ranged from 1 - 2.2 with a mean of 1.5. Polar granules were present. Sporocyst elongated with one end smaller than the other. Stieda body was present. Sporocyst residuum was present as a compact mass or sometimes as scattered granules. Sporozoites possessed a large clear refractile globule and a small globule.

Discussion: The descriptions are equivalent to the descriptions of Levine and Ivens (1967). The rough oocyst wall appeared as if the wall is striated, but the presence of a sporocyst residuum, thick wall and shape of the oocyst made them distinct from *E. bukidnonensis*.

2.4.3. *E. bovis*: (Zublin, 1908), Fiebiger, 1912.

(Synonym*: Eimeria zurnabadensis*: Yakimoff, 1931)

Description: Oocysts (Plate 2.1) were ovoid with a flattened end. The oocyst wall was smooth. A micropyle was present at the smaller end. Sporulated

oocysts were 20.0 to 32.0 µm (L) x 14.0 to 24.4 µm (W) with a mean of 23.9 µm (L) x 18.1 µm (W). The length: width ratio ranged from 1 - 1.8 with a mean of 1.4. Oocyst residuum was present in some but a polar granule was absent. Sporocyst was elongated with a stieda body at the smaller end. A sporocyst residuum was present. Sporocysts had one large globule at the base and a small globule at the smaller end.

Discussion: Similar to the descriptions of Levine and Ivens (1967) and Christensen (1941). This species was typical of earlier descriptions. The oocyst residuum was visible in a few oocysts. Sometimes the indistinct micropyle made it difficult to distinguish from *E. ellipsoidalis*, but the sporocyst structure made it distinct from the latter.

2.4.4. *E. brasiliensis*: Torres and Ramos, 1969.

Description: Oocysts (Plate 2.4) ellipsoidal, smooth walled. Both a micropyle and micropylar cap were present. An oocyst residuum was absent but scattered polar granules were present. The sporulated oocysts were 27.2 to 48.0 µm (L) x 20.6 to 41.4 µm (W) with a mean of 37.6 µm (L) x 29.4 µm (W). Their length: width ratio ranged from 1.0 - 1.6 with a mean of 1.3. Sporocysts were elongated, ellipsoidal with a distinct stieda body. A sporocyst residuum was present as scattered granules. Sporozoites were elongated and contained one large refractile globule at each end. The sporocyst residuum was scattered in the middle.

Discussion: This species is considered a rare species, but some oocysts were seen in two faecal samples in this study. Morphologically they were similar to the earlier descriptions. Presence of a micropylar cap made them distinct from other species but sometimes if the micropylar cap was lost during the processing this oocyst resembled *E. canadensis*, which leads to some potential for confusion. However, their size was always comparatively larger than *E. canadensis*.

2.4.5. *E. bukidnonensis*, Tabangui, 1931.

Description: Oocysts (Plate 2.3) were pyriform. The oocyst wall was yellowish brown, radially striated, very thick, and wrinkled at the smaller end. A micropyle was present. The sporulated oocysts were 23.5-45.2 µm (L) x 31.7 - 56.0 µm

(W) with a mean 31.9 µm (L) x 47.0 µm (W) and their length: width ratios were1.1 - 2.0, with a mean of 1.5. Oocyst residuum and polar granules were present although in some, the polar granules were absent. Sporocysts were elongated with an indistinct stieda body. The sporocyst residuum was present as scattered small granules. Sporozoites were pointed at one end with large clear refractile globules at each end.

Discussion: The yellowish tinge of the oocyst and its large size, the shape of the sporocyst and the striations of the oocyst made it easy to identify this species. Sometimes higher magnifications were needed to see the striations. Many earlier authors reported an absence of a sporocyst residuum, but all the oocysts in the present study had a scattered granular sporocyst residuum as described by Levine and Ivens (1967). For a few oocysts it was difficult to appreciate the striations but the size was used as the criterion to identify them, as this species was the largest of all the species. The distinct pyriform shape, large size, dark yellowish brown colour and striations of *E. bukidnonensis* made it different from *E. wyomingensis*.

2.4.6. *E. canadensis*, Bruce, 1921.

Description: Oocysts (Plate 2.2) were slightly ovoid/ ellipsoidal to cylindrical with a flattened smooth thin wall, which was yellowish coloured. A micropyle was present but sometimes not very distinct. Sporulated oocysts were 14.4 - 32.8 µm (L) x 22.6 - 38.7 µm (W) with a mean of 29.1 µm (L) x 21.9 µm (W). The length: width ratio is 1.1 - 1.5 with a mean of 1.35. An oocyst residuum was absent, but scattered polar granules were present. The sporocyst was elongated and ovoid. A stieda body was present sometimes, but was not very distinct. A sporocyst residuum was present as a compact ball or scattered granules. Sporozoites each contained 2 to 3 clear refractile globules.

Discussion: The morphology is similar to the description of Christensen (1941) and Levine and Ivens (1967). The oocysts were slightly smaller than in earlier descriptions and the shapes varied from ellipsoidal to cylindrical which confused the identification of this species. On occasions these oocysts were similar in internal structure to *E. brasiliensis*, which is an oocyst with a micropylar cap. However, when these caps, which were very fragile, were lost during

processing, oocysts resemble *E. canadensis*. Smaller sized oocysts can be misidentified as *E. bovis* and larger as *E. brasiliensis*.

2.4.7. *E. cylindrica*, Wilson, 1961.

Description: Oocysts (Plate 2.5) were elongated, ellipsoidal to cylindrical, with relatively straight sides. They had a colourless oocyst wall, which was smooth and thin at the smaller end. Sporulated oocysts were 15.5-30.1 µm (L) x 11.8 - 27.1 µm (W) with a mean of 29.9 µm (L) x 21.1 µm in (W). Their length: width ratios ranged from 1.0-2.0 with a mean of 1.5. The oocyst polar granule was scattered as small fragments. The elongated sporocyst had a distinct stieda body. Sporocyst residuum was present as a compact ball or mass, and 2 to 3 clear refractile globules were present in the sporozoites.

Discussion: The descriptions of these oocysts are similar to those of Levine and Ivens (1967) and Christensen (1941). The typical shape of *E. cylindrica* and its size makes it relatively straight forward to identify, but it could be confused with *E. canadensis* and *E. ellipsoidalis* oocysts.

2.4.8. *E. ellipsoidalis,* Becker, Frye, 1929.

Description: Oocysts (Plate 2.5) were ellipsoidal in shape. The oocyst wall was smooth, colourless and the micropyle was absent. Sporulated oocysts were 20.0 - 29.6 µm (L) x 10.9- 25.0 µm (W) with a mean of 24.6 µm (L) x 18.5 µm (W). Their length: width ratios ranged from 1.0 - 2.5 with a mean of 1.35. The oocyst residuum was absent. Oocyst polar granules were present on most, but not all, occasions. The sporocyst was elongated, with an indistinct stieda body. Sporocyst residuum was present, either as a compact mass or as scattered granules. Sporozoites had one large and one small clear refractile globule.

Discussion: These oocysts are potentially confused with *E. bovis*, as they have a wide range of size and shape. These descriptions are similar to Lee (1954), Becker and Frye (1929) and Christensen (1941).

2.4.9. *E. subspherica,* Christensen, 1941.

Description: Oocysts (Plate 2.5) were spherical to sub-spherical. Oocyst wall was smooth and thin. Micropyle was absent. These were the smallest oocysts of cattle. Oocysts were 9.0 - 17.8 µm (L) x 7.2 – 17 µm (W), with a mean of 13.4

µm (L) x 11.8 µm (W). Their length: width ratio ranged from 1.0 - 1.2 with a mean of 1.1. Oocyst residuum and polar granules were absent. Sporocyst was elongated with a tiny stieda body. Sporocyst residuum was absent. Sporozoites had a clear globule at the larger end.

Discussion: These oocysts matched with the descriptions of Christensen (1941). The size (these were the smallest oocysts) seen and then the special structure of the oocysts, with a distinct stieda body made identification straight forward.

2.4.10. *E. wyomingensis*, Huizinga and Winger, 1942.

Description: Oocysts (Plate 2.2) were pyriform, had a thick oocyst wall and were yellowish-brown in colour. A micropyle was present at the smaller end of the oocyst. Sporulated oocysts were 30.0-49.6µm (L) x 21.4 – 34.4 µm (W) with a mean of 39.4 µm (L) x 27.9 µm (W). Their length: width ratios ranged from 1.2 - 2.0 with a mean of 1.4. Oocyst residuum and polar granules were absent. The sporocyst was elongated with an indistinct stieda body. The sporocyst residuum was present in the form of granules. Sporozoites had a large clear globule at their base.

Discussion: Many earlier reports described *E. bukidnonensis*, Tabangui, 1931 and this species as being the same, but later reports described both as separate species with slight differences in the oocyst wall. In this study, these oocysts had smooth walls, which separates them from the earlier species and the sporocyst residuum was present in the form of scattered granules.

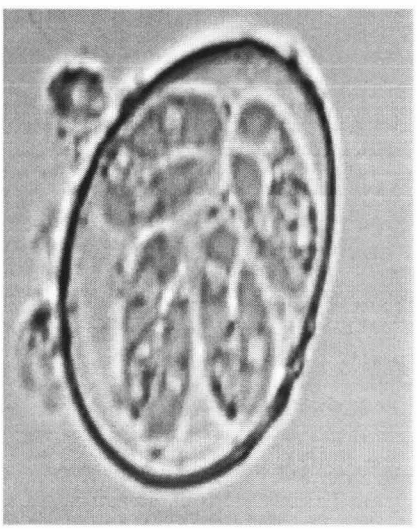
2.4.11. *E. zuernii*, (Rivolta, 1878) Martin, 1909.

Description: Oocysts (Plate 2.6) were spherical to ovoid in shape. The oocyst wall was smooth and colourless. A micropyle was absent. Sporulated oocysts were 14.1- 25.2 µm (L) x 10.3 - 22.7 µm (W), with a mean of 19.5 µm (L) x 16.2 µm (W). Their length: width ratios ranged from 1.0 -1.6 with a mean of 1.2. No oocyst residuum was seen and the polar granules were scattered. The sporocyst was ovoid with an indistinct stieda body. The sporocyst residuum was present as a compact mass or sometimes as fine granules. The sporozoite had a large clear globule at the base of its broad end.

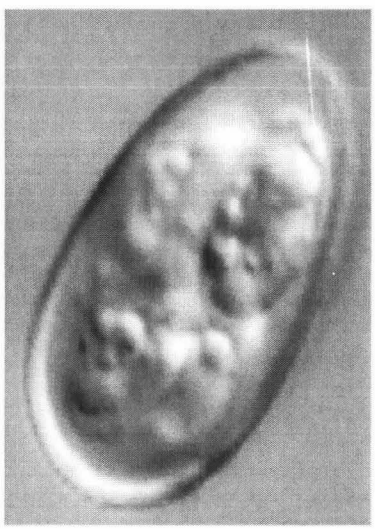
Discussion: The descriptions are similar to the earlier descriptions of Lee (1947), Tabangui (1931), Christensen (1941), and Levine and Ivens (1967). The oocyst descriptions were very clear, and as it was the most predominant species, *E. zuernii* was easily identified. However, there was overlap with oocysts of *E. ellipsoidalis*. The ovoid shape of *E. zuernii* sometimes resembles *E. ellipsoidalis,* but the number of 2 clear globules differentiates it from *E. zuernii*. The smaller sized, spherical oocysts resembled *E. subspherica*, but the latter was much smaller and lacked a sporocyst residuum.

The following Plates represent each species isolated in this study. They are all shown to the same scale.

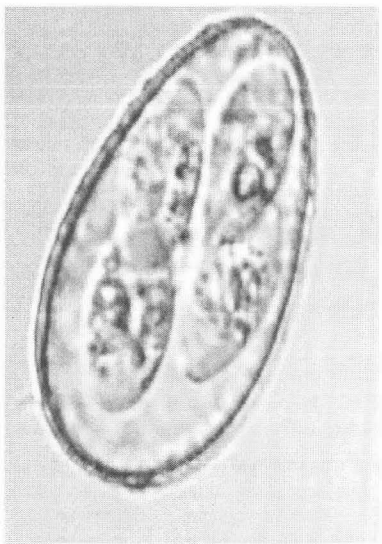
Plate 2.1. Species with Micropyle 1:



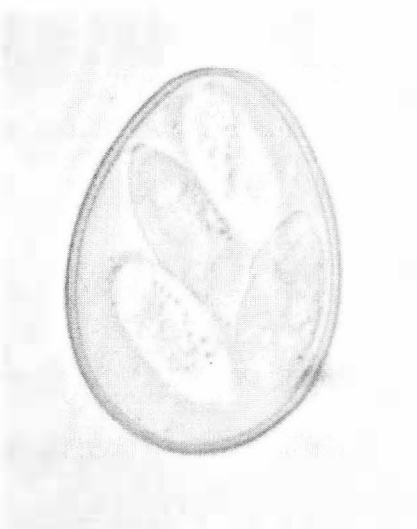
1. E. auburnensis



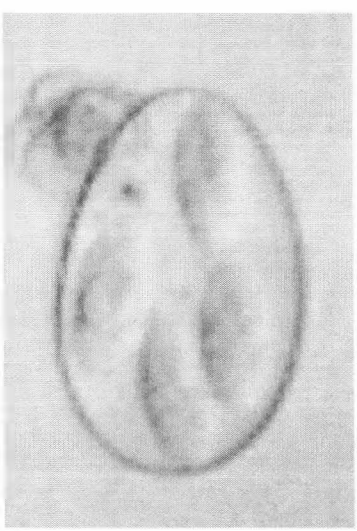
2 . E. auburnensis



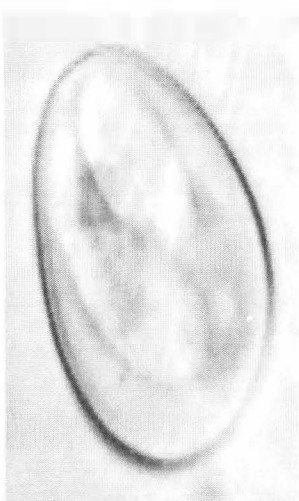
3. E. auburnensis



4. E. auburnensis

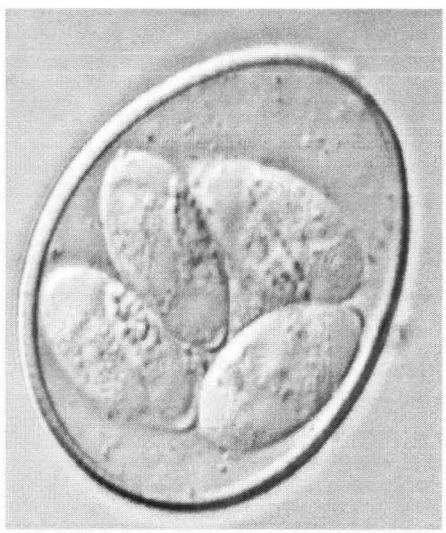


5. E. bovis



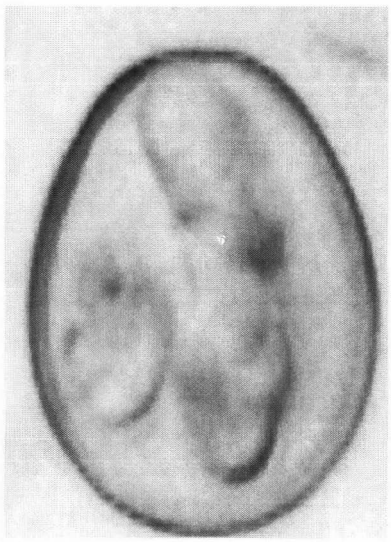
6. E. bovis

Plate 2. 2. Species with Micropyle 2:

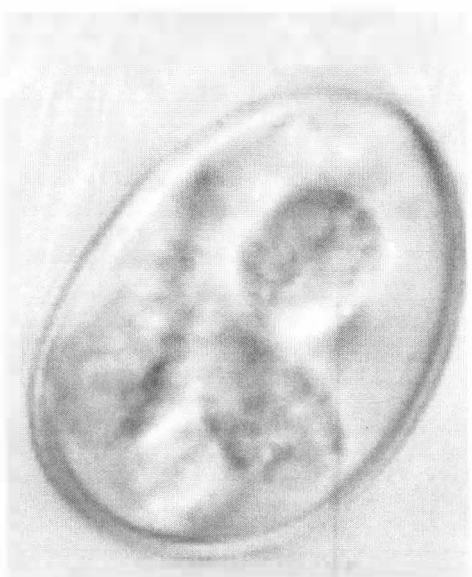


7. E. canadensis

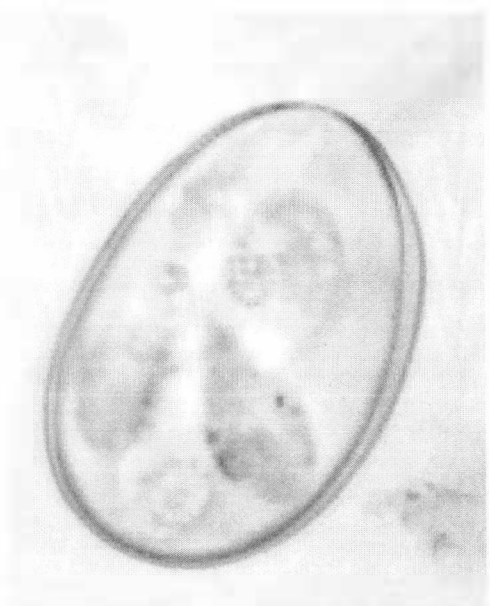
8. E. canadensis



9. E. canadensis



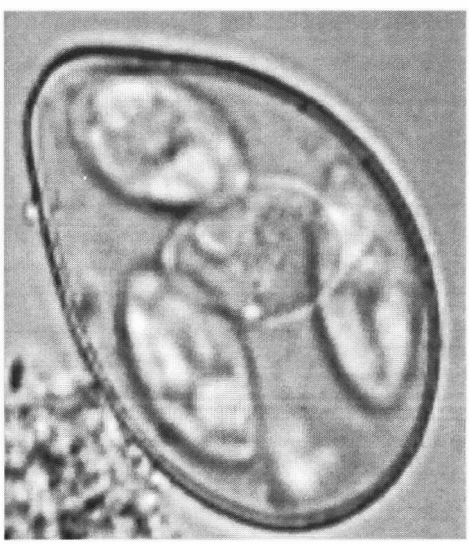
10. E. wyomingensis



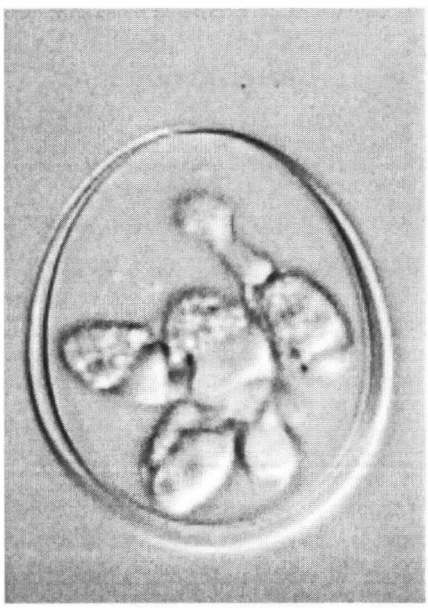
11. E. wyomingensis

*20*μ

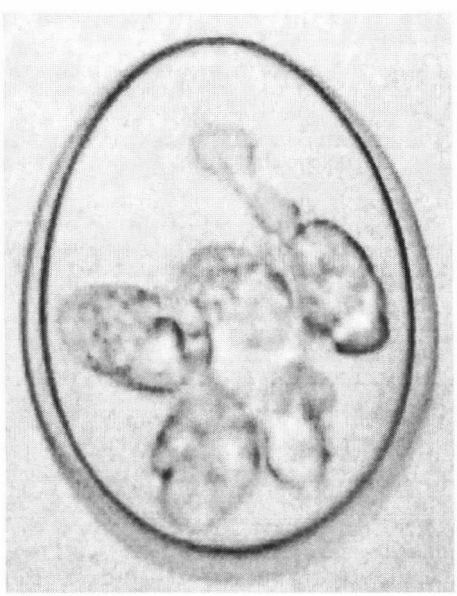
Plate 2.3. Species with Micropyle 3:



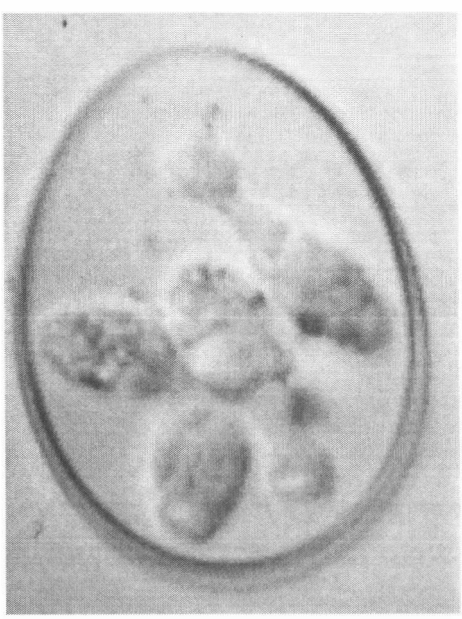
12. E. bukidnonensis (100x)



13. E. bukidnonensis

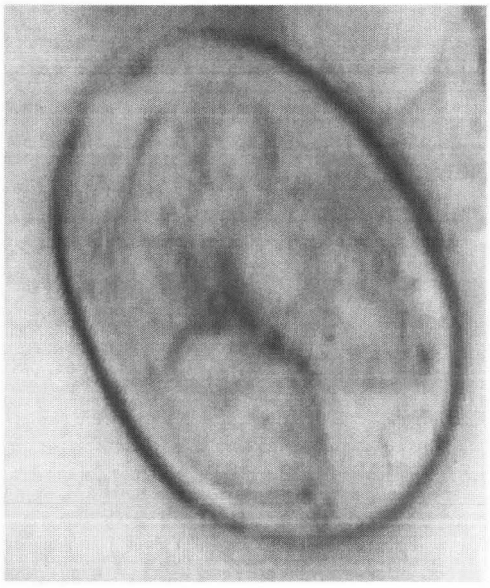


14. E. bukidnonensis

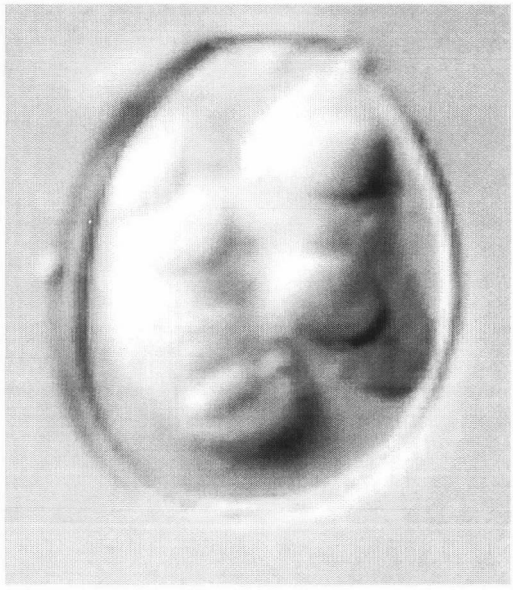


15. E. bukidnonensis

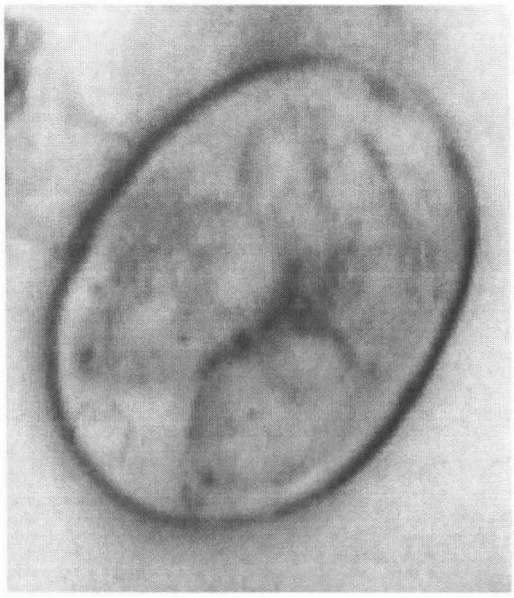
Plate 2.4. Species with Micropyle 4:



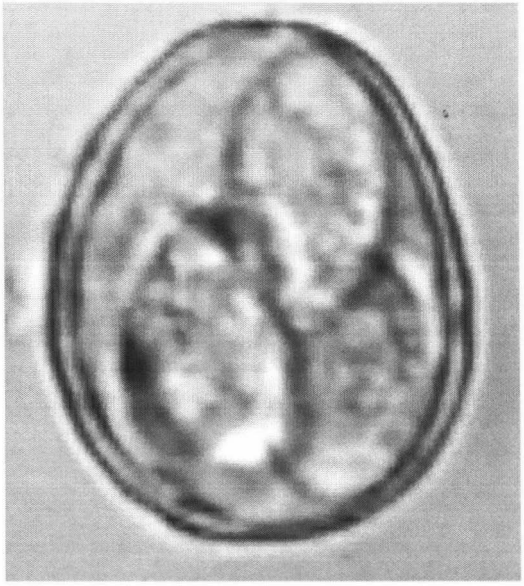
16. E. brasiliensis



17. E. brasiliensis



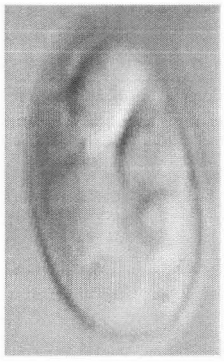
18. E. brasiliensis



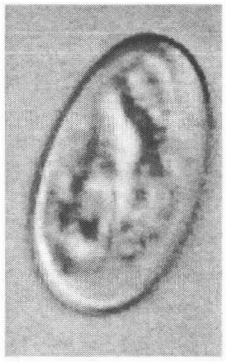
19. E. brasiliensis

20µ

Plate 2.5. Species without Micropyle 1:

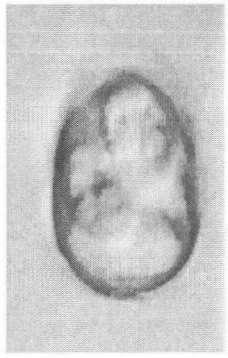


20. E. cylindrica

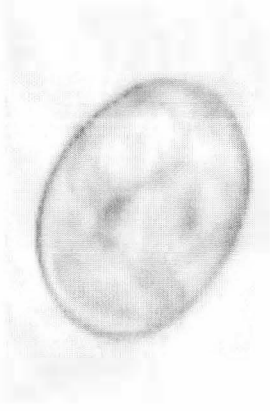


21. E. cylindrica

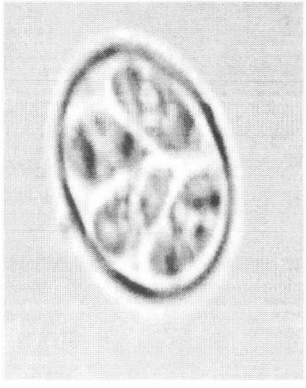
22. E. cylindrica



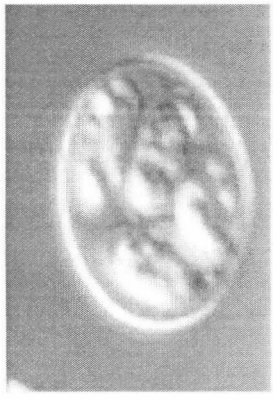
23. E.cylindrica



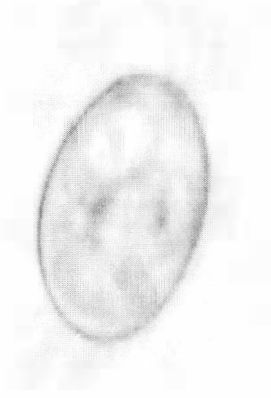
24. E. ellipsoidalis



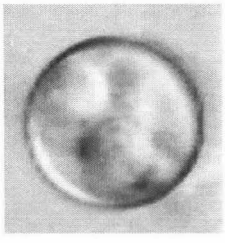
25. E. ellipsoidalis



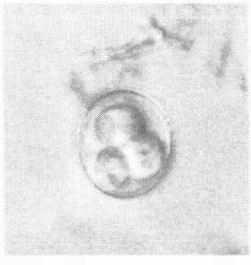
26. E. ellipsoidalis



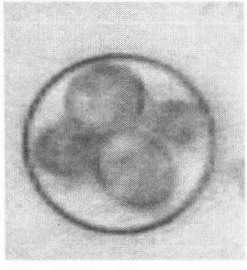
27.E. ellipsoidalis



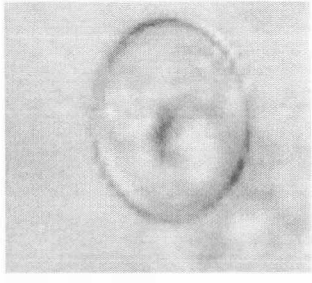
28. E. subspherica



29. E. subspherica

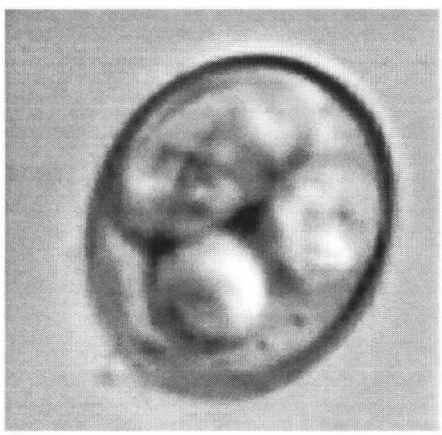


30.E. subspherica

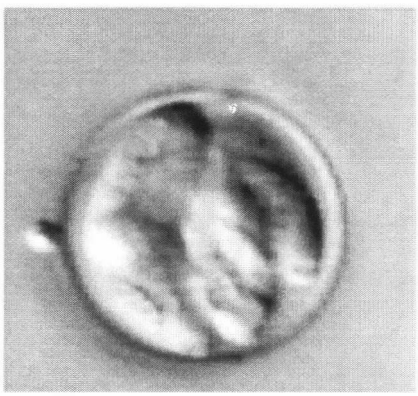


31.E. subspherica

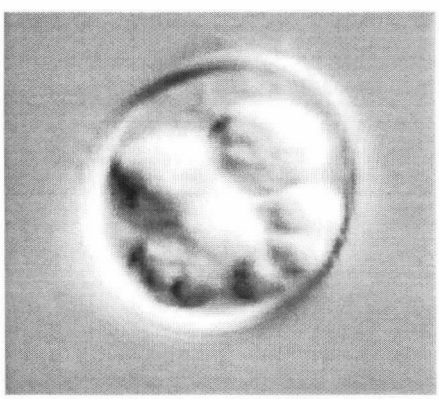
Plate 2.6. Species without Micropyle 2:



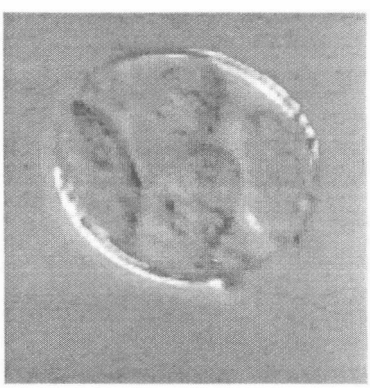
31 . E. zuernii



33. E. zuernii



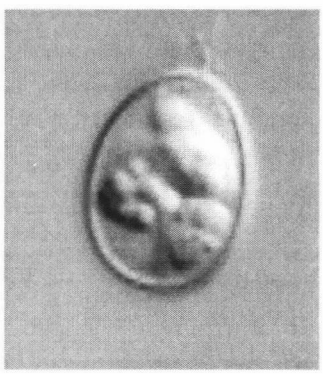
34. E. zuernii



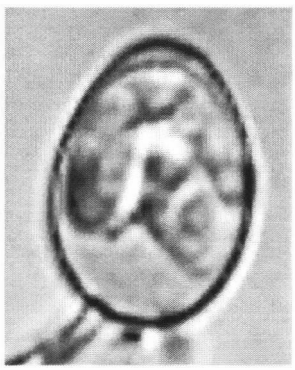
35. E. zuernii

20µ

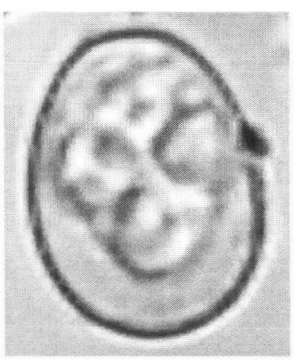
Plate 2.6. Species without Micropyle 2:



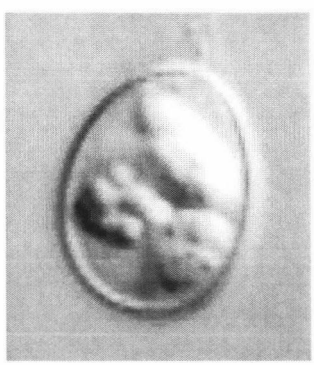
36. E. alabamensis



37. E. alabamensis



38 . E. alabamensis



39. E. alabamensis

20µ

bovis (87%) followed by *E. auburnensis* (62%), *E. cylindrica* (42%), *E. bukidnonensis* (36%), *E. canadensis* (31%), *E. subspherica* (27%), *E. ellipsoidalis* (24%), *E. wyomingensis* (23%), *E. alabamensis* (12%) *and E. brasiliensis* (12%).

The most predominant species was *E. bovis* (31.1%) followed by *E. zuernii* (26.5%), *E. auburnensis* (12.7%), *E. bukidnonensis* (6.8%), *E. cylindrica* (6.3%), *E. wyomingensis* (5.3%), *E. canadensis* (4.4%), *E. ellipsoidalis* (1.9%), *E. brasiliensis* (1.9%), *E. subspherica* (1.5%), and *E. alabamensis* (1%).

In earlier reports McKenna (1972 and 1974) also identified 11 species from New Zealand where the prevalence of different species vary slightly from this study but most predominant species were *E. bovis* (44%) and *E. zuernii* (19%) like this study. In a second study, Aria, 1993, isolated only 7 species and the predominant species was *E. canadensis* followed by *E. bovis*. The prevalence of *Eimeria* species differ globally (Table 1.5). This could be the reason for differences between the studies.

As this study included animals from several different farms is true representative of New Zealand than earlier studies.

Presence of multiple species in one animal is very common with *Eimeria* species. An average of 3.5 species in one animal was observed earlier (Oda and Nishida, 1989; McKenna, 1972) was also noted in this study with a range of 3-8 species in one animal. Most of the oocysts fitted with earlier descriptions.

Chapter 3. Study at Massey Number 4 Dairy Farm.

3.1. Introduction:

Coccidiosis is a disease of calves mainly seen in the age group of one month to a year old. Adults may be infected but almost never develop clinical signs. The infection is transmitted through ingestion of sporulated oocysts in contaminated feed, pasture, water and licking of contaminated surfaces. Disease is commonly linked to poor hygiene and higher stocking densities. The topography of the paddock, drainage facilities, placement of feed and water sources, pasture cover in the paddock, number of animals in the paddock and presence of adult cows grazing together with young stock will all influence the development of the disease. For dairy calves raised separately from their mothers, it is difficult to avoid infection. To further lower the risk, farmers add coccidiostats to the feed to keep the coccidial burdens low. Normally, monensin, a coccidiostat, is added to the calf milk replacer and calf meal. There are reports that, shortly after the feed is withdrawn at weaning disease seems to occur. Presumably weaning stress is an important factor in the development of the disease. The stress may also be due to transport, feed change, inclement weather, vaccination etc.

The objectives for this particular study were:

1. To investigate the effect of monensin on growth rate, production of oocysts and the development of immunity up until weaning by comparing calves fed meal containing monensin with those fed meal without monensin.
2. To determine the effect of the treatment of the calves with the coccidiocide-toltrazuril (20mg/kg-body weight) orally at the time of weaning on post weaning coccidiosis and productivity in terms of weight gains.

3.2. Materials and Methods:

3.2.1. Trial design:

This replicated study initially compared calves fed meal with or without monensin and comprised a total of 24 calves set-stocked as 6 groups of 4 calves in 6 areas subdivided from one paddock on Massey University's No 4 Dairy Farm. All were fed whole milk, supplemented with increasing quantities of

calf meal(See Appendix 3.2) Three of the groups (MG1, MG2 and MG3) were fed calf meal with the coccidiostat monensin included @1mg of meal and the other three (NMG1, NMG2 and NMG3) were fed calf meal without any coccidiostat included. The rearing practice in terms of rations fed was the same as for other non-experimental calves on this farm. The coccidiostat added was monensin ("Monensin premix") and the meal was a standard calf meal (See Appendix 3.1.a). The calves were weaned when the average weight was 100 kgs.

At weaning half of the calves (2) from each group were selected randomly and drenched with toltrazuril 20mg /kg body weight (Baycox Piglet Coccidiocide, Bayer Australia Ltd) (Refer to Appendix 3.1b). After weaning the calves were kept in a single mob in an area equivalent to two of the initial groups. The calves were kept in 2 cells as the grass was plentiful.

The trial commenced of 14th August 2002 (Day 1) with weaning occurring on 24th October (Day 72) and the trial ended on 10 December (Day 113). The calves were born between 10 July to 4 August and at the commencement of the study the average was 2.5 weeks. These calves were randomly allocated into 6 groups. The detailed feeding regime and other managerial activities are shown in the table in Appendix 3.2.

3.2. 2. Paddock preparation and sub division:

The paddock had been grazed by adult cows over winter and had been top-dressed with urea (23/7/02) to ensure adequate grass was available for set-stocking of calves. It had not been used in the past for calf rearing. This paddock was divided into 6 cells with electric fences. The calves were set-stocked at a rate of 4 calves in a grazing area of 0.68 ha, equivalent to about 6 calves/ha. A shed suitable to shelter 4 calves was placed in each cell. A set of portable yards was constructed adjacent to the paddock for the purpose of weighing and sampling animals. Each cell contained a feed and water trough. Water was available *ad lib* throughout the trial.

3.2.3. Husbandry practices:

The calves were moved to the experimental area at an average age of 2.5 weeks. They were initially fed on whole milk twice a day for the first week under experiment and then once a day until 75kg body weight. Initially they were on a

daily allowance of calf meal starting at 100g/calf/day in the second week and increasing by an extra 50g/day each week to a maximum of 1kg/day. Their initial milk allowance was 4litres/day increasing to 5litres/day on once a day feeding and then reducing by one litre per week until reaching 75kg bodyweight. From 75kg until weaning at 100kg they were fed meal only. The composition of the calf pellets is shown in Appendix 3.1.a.

The standard calf rearing practice of Massey University dairy farms was followed. Initially, the young calves were held at the No. 4 calf unit and established on twice daily whole milk feeds from one day of age.

On Day 43 of the experiment (25th Sept.) a group of 200 cows were kept overnight to reduce the pasture level as it was overgrown. This was repeated on Day 49 (11th Oct.) The calves were dis-budded on 16th October. On Day 111 (2nd Dec) a few calves were noted to be coughing so lungworm larval counts were assessed and most of the calves were shedding lungworm larvae so all calves were treated with anthelmintic for lungworm. The calves were maintained up to Day 113 (10th Dec) in this paddock under daily supervision. The feeding and other activities on the farm are shown in Appendix 3.2.

3. 2.4. Sample collection:

Each calf was faecal sampled twice per week for the first 3 weeks and then once a week until 7 weeks post-weaning. The technique was rectal stimulation of defaecation. In addition, once a week the calves were weighed on a Micropower 2000 (Donald Presses Ltd, Masterton New Zealand) electronic scales and a 10ml blood sample, from the jugular vein, was collected for recovery of serum and subsequent determination of anti-coccidial antibodies.

3.2.5. Examination of individual samples:

Faecal samples were screened for coccidia throughout the experiment and the positive samples with high counts were sporulated for species identification.

A faecal oocyst count (FOC) was carried out on a 2g sub sample of each sample collected. In addition, oocysts were recovered, if present, from a further sub-sample and sporulated, and the species present were described in a random sample of 100 oocysts.

In brief, oocysts were counted using a modified McMaster technique, using saturated NaCl as the flotation medium, where each oocyst counted represented 50 oocysts/g (see Appendix 3.3.1). Oocysts were recovered from positive samples as described in Appendix 3.3.2 and 3.3.3. In brief, a 5g sample of faeces was homogenized in water, subjected to flotation in saturated salt, oocysts were recovered from the supernatant, washed in water, centrifuged, recovered and sporulated at 27°C for 7 days in a shallow dish containing 2% H_2S0_4. Sporulated oocysts were recovered as per procedure in Appendix. 3.3.3.

To determine the prevalence of species within an animal, 30 oocysts were identified. This was only done from those animals with a reasonably high oocyst count to make the observation practically feasible. The percentage of individual species in this study was then calculated to find out the prevalence of individual species.

Faecal samples were also screened for *Cryptosporidium* and *Giardia* from Week 1 to 5 and *Giardia* on Week 1.

On the first occasion, a commercial direct fluorescence procedure (MERIFLUOR: Meridian Diagnostics) which detects *Cryptosporidium* and *Giardia* (see the Appendix 3.3.5) was used. Subsequently, faecal smears were stained with a modified Zeihl Neilson stain (see the Appendix 3.3.4)

3.3 Statistical analysis:

3.3.1. Faecal oocyst counts:

Arithmetic weekly mean oocyst counts were analyzed after square root transformation to normalise the data (Snedecor and Cochran, 1980). Data for live weight (LW) and transformed FOC were analysed using the MIXED procedure of SAS (2001). The linear model included the fixed effects of week, treatment either with "monensin" (pre-weaning or with toltrazuril post-weaning) and the interaction between week X treatment, and the random effect of replication. A replicate comprised a group of 4 animals for monensin up to weaning and 12 animals for toltrazuril after weaning. Using Akaike's information criterion, a compound symmetric error structure was determined as the most appropriate residual covariance structure for repeated measures over time within animals.

3.3.2. Live weight:

Live weight was analyzed using the MIXED procedure in SAS (2001). The model included the fixed effects of treatment with either monensin or toltrazuril (treated non-treated animals), time, their interaction and the random effect of animal within treatment. Using the Akaike's information criterion, a compound symmetry error structure was determined as the most appropriate residual covariance structure for repeated measures over time within animals. Least square means and their standard errors (SE) were obtained for the 11 weeks pre weaning and the 7 weeks post weaning.

3.3.3. Combined effect of two anti-coccidial treatments on oocyst counts up to weaning:

Statistical analysis was also carried out to see whether there was any effect of combined treatment with two anti-coccidials (monensin + toltrazuril) as some animals received both drugs. Data for transformed FOC were analyzed using the MIXED procedure of SAS (2001). The linear model included the fixed effects of week, treatment with monensin (pre-weaning) or with toltrazuril (post weaning), interaction between monensin and toltrazuril and the interaction between week X treatment (with toltrazuril + monensin). A replicate comprised a group of 6 animals. Using the Akaike's information criterion, a compound symmetric error structure was determined as the most appropriate residual covariance structure for repeated measures over time within animals.

3.4. Results:

3.4.1. Oocyst counts up to weaning:

The oocyst counts were very low in all the calves up to the 4^{th} collection or 2.5 weeks of treatment. Thereafter the oocyst counts rose to reach peak levels at 7^{th} collection or after 3.5 weeks of treatment. Then the oocyst counts started to decline to low levels, almost to their initial low levels, during collections 14 to 16 or 5 to 11 weeks on treatment. The monensin-treated groups (MG2, MG3) had higher oocyst counts from the 3^{rd} and 5^{th} week but thereafter the non-treated groups (NMG1, NMG2, NMG3) and the monensin-treated groups had similar oocyst counts (see Appendix 3.4.1 for raw data). The highest counts in their respective groups were for MG2 and NMG1.

The FOC of all the samples were pooled to calculate the group mean (Groupwise) oocyst counts and treatment mean (Treatmentwise) oocyst counts. Least square mean (LSM) oocyst counts were determined for both Treatmentwise and Groupwise and the summary of ANOVA is shown in Table 3.1. The Groupwise and Treatmentwise arithmetic mean oocyst counts and the least square mean oocyst counts (Groupwise and Treatmentwise) in calves up to weaning are shown in Figs: 3.1, 3.2, 3.3 and 3.4.

As shown in Table 3.1, there was no significant difference between monensin-treated and non-treated calves ($p=0.6$) and the LSM oocyst counts of both treated and non-treated calves showed similar patterns all through the experiment. The effect of week was significant ($p<0.0001$) indicating the oocyst counts varied over time. Full details of this statistical analysis are shown in Appendices 3.4.3 and 3.4.4.

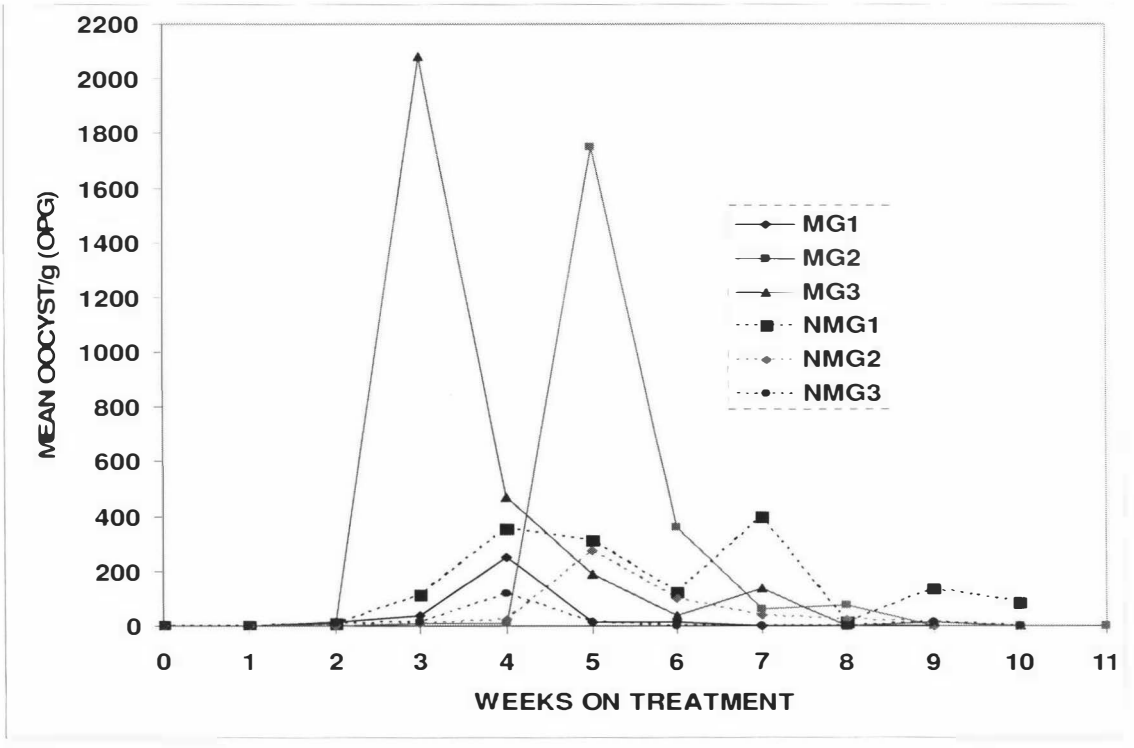


Figure 3. 1: Comparison of arithmetic mean oocyst counts of groups of calves up to weaning fed meal containing monensin (M) and calves fed meal without monensin (NM). Each group (G1-3) comprising 4 calves.

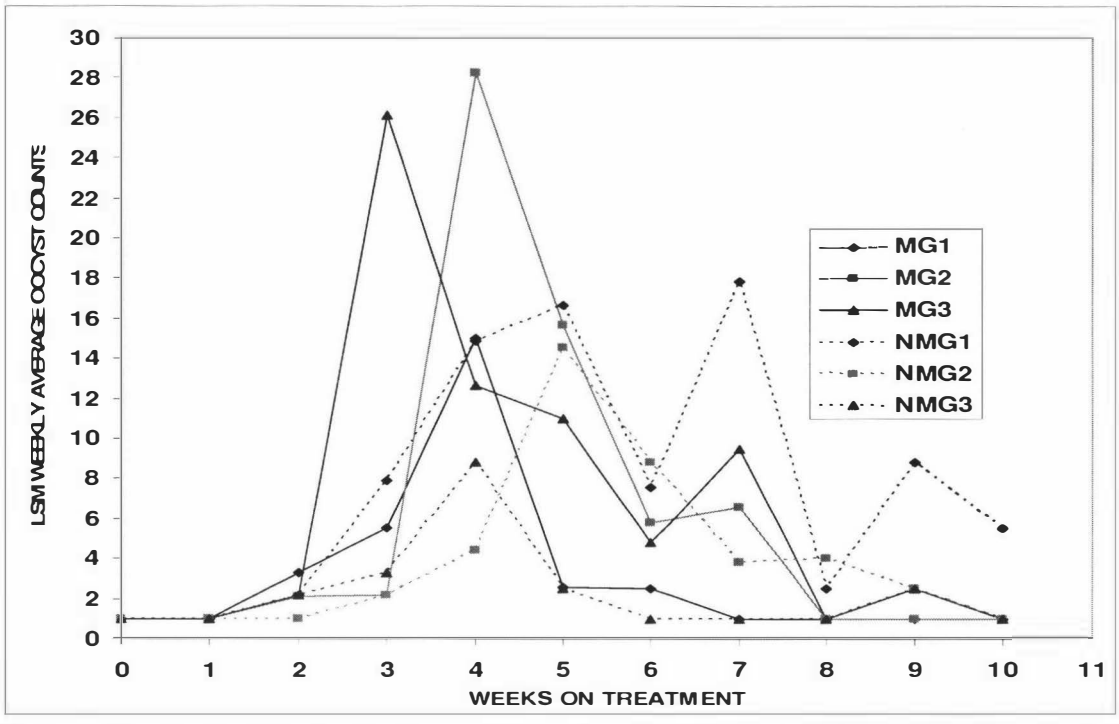


Figure 3. 2: Comparison of least square mean oocyst counts of groups of calves up to weaning fed meal containing monensin (M) and calves fed meal without monensin (NM). Each group (G1-3) comprising 4 calves.

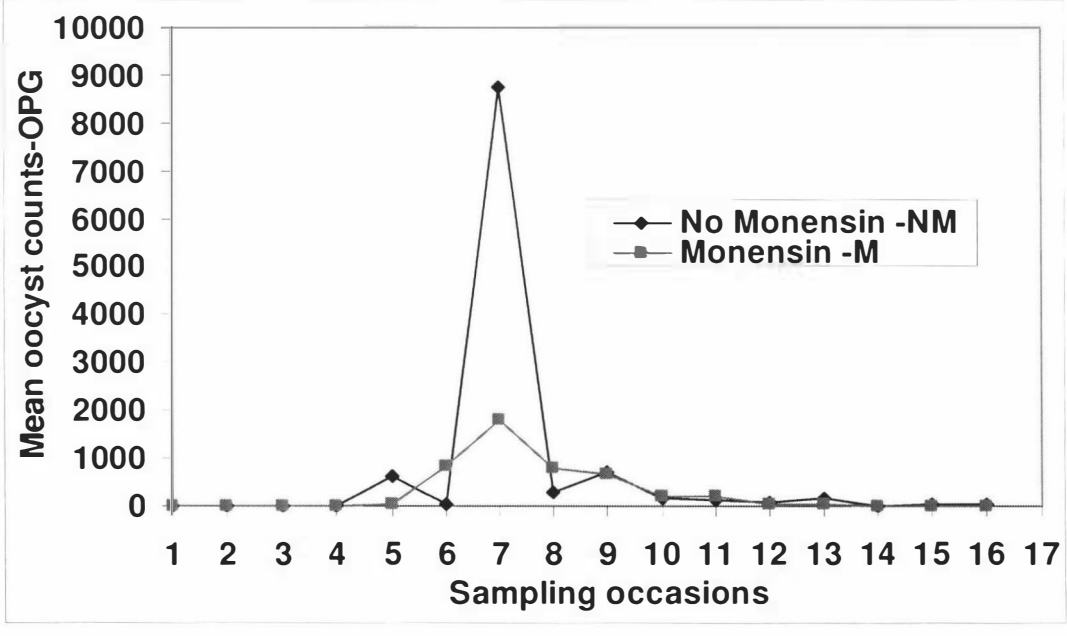


Figure 3. 3: Comparison of arithmetic mean oocyst counts of calves up to weaning fed meal containing monensin (M) and calves fed meal without monensin (NM) by sampling occasion. Calves were sampled twice a week for the first 3 weeks and then once a week. Each group consisted of 12 animals.

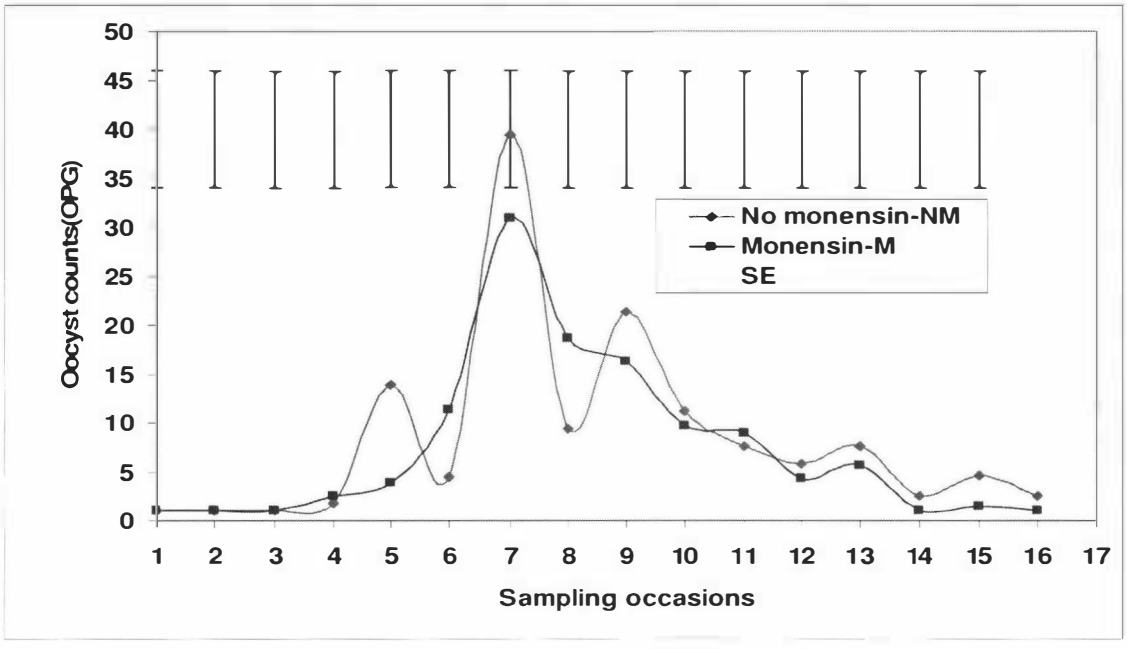


Figure 3. 4: Comparison of least square mean oocyst counts of calves up to weaning either fed meal containing monensin (M) or fed meal without monensin (NM). Each group consisted of 12 animals. The error bars represent the standard error.

Effect	Numerator	Denominator	F Value	Pr>F
	DF	DF		
Week	10	178	5.90	<0.0001
Treatment	1	20	0.26	0.6169
Rep	2	20	0.31	0.7364
RepXTreatXWeek	52	178	1.28	0.1209

Table 3. 1: Summary of the statistical results for a repeated ANOVA of oocyst counts up to weaning

As shown in Fig 3.5 the calves being fed meal which included monensin received less than the required amount of monensin in the feed for the initial 4

weeks. But from the 5th week onwards the calves received the recommended dose, which is 1mg/kg body weight. (See Appendix 3.7 for full details).

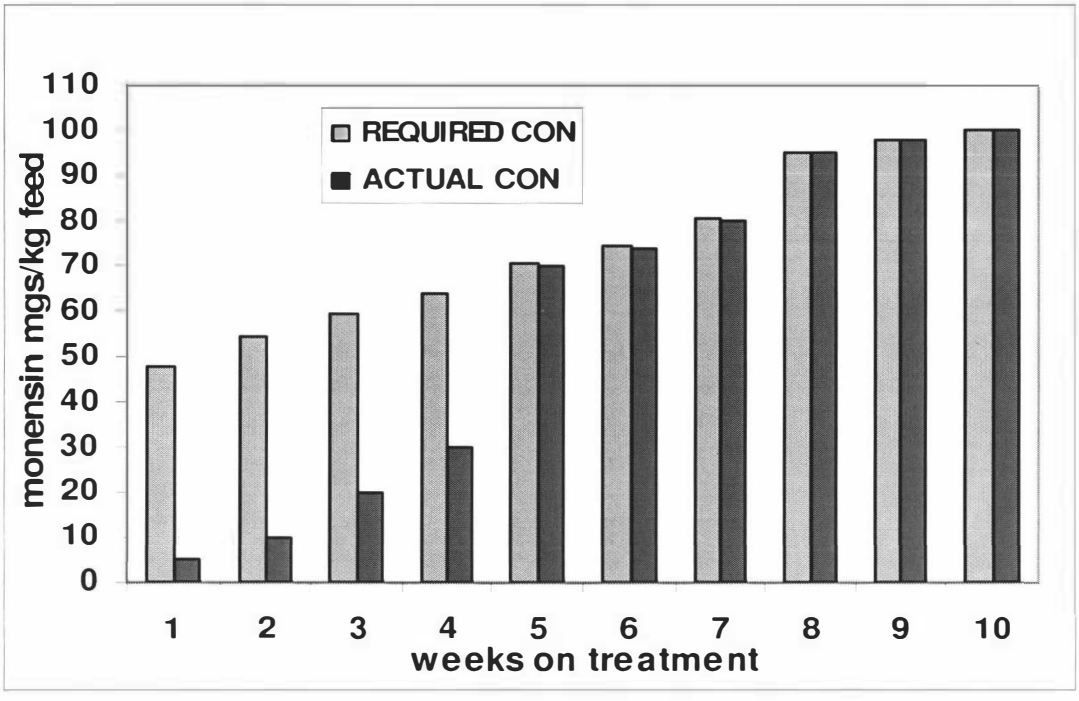


Figure 3. 5: Monensin consumption (mg/kg feed) compared to required consumption by calves preweaning based on their weekly average live weights.

3.4.2. Oocyst counts after weaning:

Fig 3.6 and Fig 3.7 show the mean and least square mean oocyst counts respectively for groups treated with toltrazuril or not treated with toltrazuril at weaning.

As shown in Fig. 3.6 and Fig 3.7 the oocyst counts of the calves treated with toltrazuril remained low, whereas the oocyst counts of the untreated calves increased from the first week to reach a maximum level during the third week. Thereafter, their counts declined to reach their lowest level similar to the initial level by the 6th week post treatment. Interestingly, the counts of the treated and non-treated calves were almost similar from the 4th week.

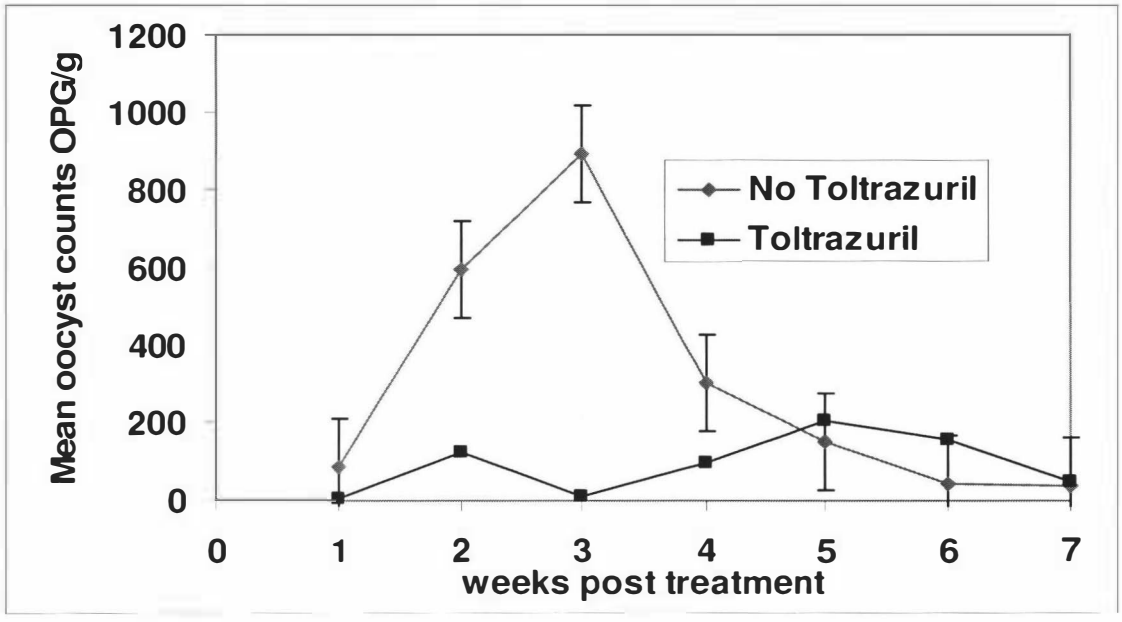


Figure 3. 6: Arithmetic mean of oocyst counts for calves after weaning either treated with toltrazuril 20 mg/kg body weight or not treated with toltrazuril, each group consisting 12 animals. Treatment on Week Zero.

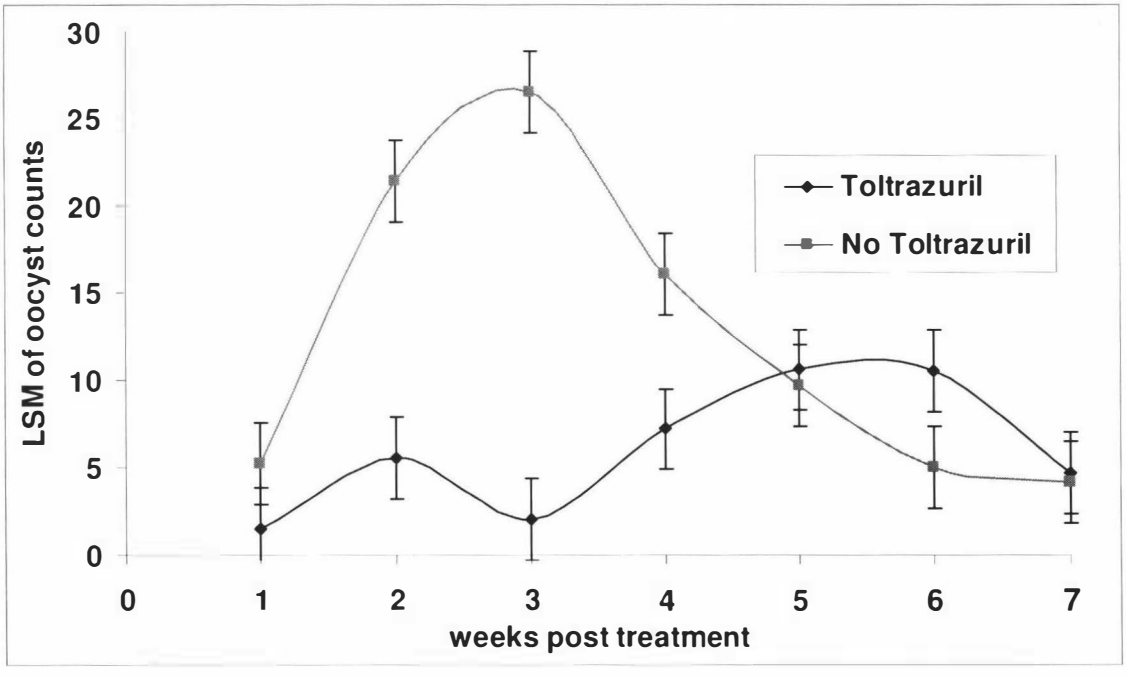


Figure 3. 7: Least square mean oocyst counts for calves after weaning, either treated with toltrazuril or not treated with toltrazuril, each group consisting of 12 animals. Note : Treatment given at 0 Week.

Effect	Numerator DF	Denominator DF	F Value	Pr>F
Week	5	100	5.97	<0.001
Monensin	1	20	0.19	0.6696
Toltrazuril	1	20	22.24	0.0001
ToltXMon	1	20	0.33	0.5740
ToltXMonXWeek	15	100	4.62	<0.0001

Table 3. 2: Analysis Of Variance for oocyst count after weaning including prior treatment with monensin. (Key: Tolt = toltrazuril, Mon= monensin).

The summary results for the initial model used for analysis is shown in Table 3.2. The effect of the week was significant ($p<0.001$), indicating that the oocyst counts varied significantly over time through the experiment. The effect of the monensin and the combined effect of toltrazuril and monensin were non significant, but the effect of the toltrazuril was highly significant ($p<0.0001$). But, the interaction of MonensinXtoltrazurilXweek was significant. This indicates that monensin had no effect in reducing the oocyst numbers in calves after weaning but toltrazuril given at weaning had significant effect on oocyst counts. The combined effect of two treatments was significant as the calves treated with both drugs had the lowest counts when compared to calves treated with either of two drugs separately and calves that were not treated with anticoccidials. Since prior treatment with monensin was not significant, a further analysis was undertaken excluding monensin (see Appendix 3.5.4).

Monensin	Toltrazuril	29 Oct	5 Nov	11 Nov	18 Nov	26 Nov	3 Dec	10 Dec
M	T	0	8	8	100	150	167	33
NM	T	8	242	8	92	258	142	67
M	NT	0	500	1250	433	150	17	33
NM	NT	175	692	533	175	150	67	42

Table 3. 3: Arithmetic mean oocyst counts (oocysts/g) of calves treated either with monensin (M) or toltrazuril (T) and treated with both (M, T) or not treated with either (NM+NT), each group consisting of 6 calves.

Effect	Numerator DF	Denominator DF	F Value	Pr>F
Week	6	132	7.13	<0.0001
Toltrazuril	1	22	20.90	0.0001
ToltrazurilX Week	6	132	10.96	<0.0001

Table 3. 4: Type 3 Tests of Fixed Effects. Repeated measures analysis of variance for oocyst counts after weaning.

Table 3.4 shows the summary of the repeated measures ANOVA (see Appendix 3.5.3 for full report) when monensin treatment is removed from the model. The effects of the week, treatment and the interaction between the treatment (Toltrazuril) were all highly significant (p>0.0001), indicating that the treatment reduced the oocyst counts significantly in calves. There was an overall change in oocyst count with time consistent with an increase then a decline and the significant interaction is consistent with this being different between the two treatments.

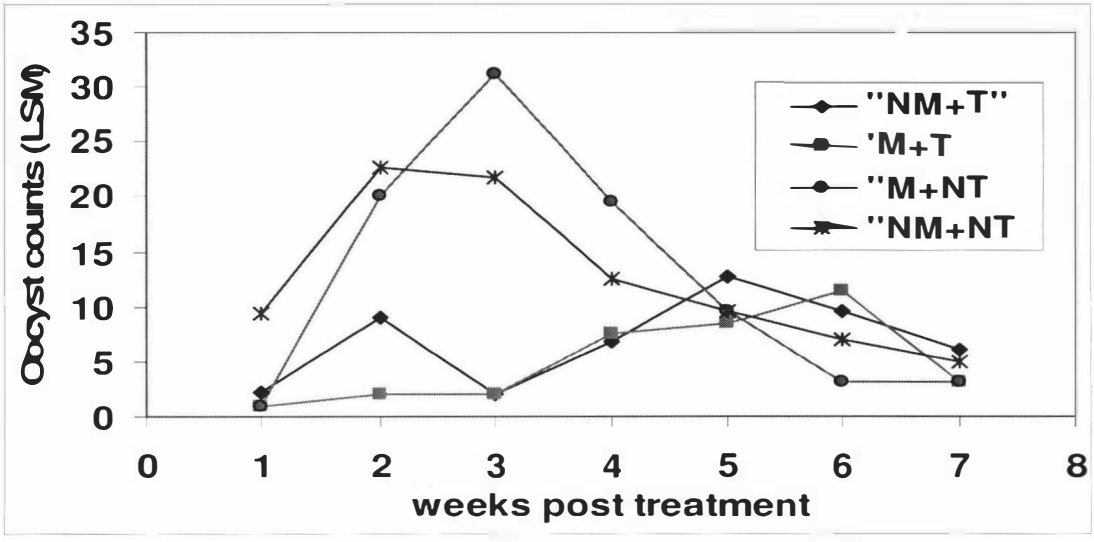


Figure 3. 8: Comparison of the arithmetic mean oocyst counts of 4 groups treated with both anticoccidials (toltrazuril+ monensin = T+M), not treated with either drugs (NM +NT), treated with only one anticoccidial at least (NM+T) = treated with toltrazuril only; treated with monensin only(M+NT).

3.4.3. Live weight of the calves up to weaning:

The arithmetic mean live weights by group up to weaning are shown in Fig. 3.9 and by treatment in Fig. 3.10. At the beginning of the experiment, all 3 groups of monensin-treated calves (n=12) had an average weight of 47.7kg. This was slightly higher than the average weight (47.1kg) of calves (n=12) which did not receive any monensin in their feed, with a difference of 0.6 kg between treated and non-treated. At the end of the experiment, the treated groups weighed an average of 100.5kg and the group not treated had an average of 100kg, with a difference of 0.5kg.

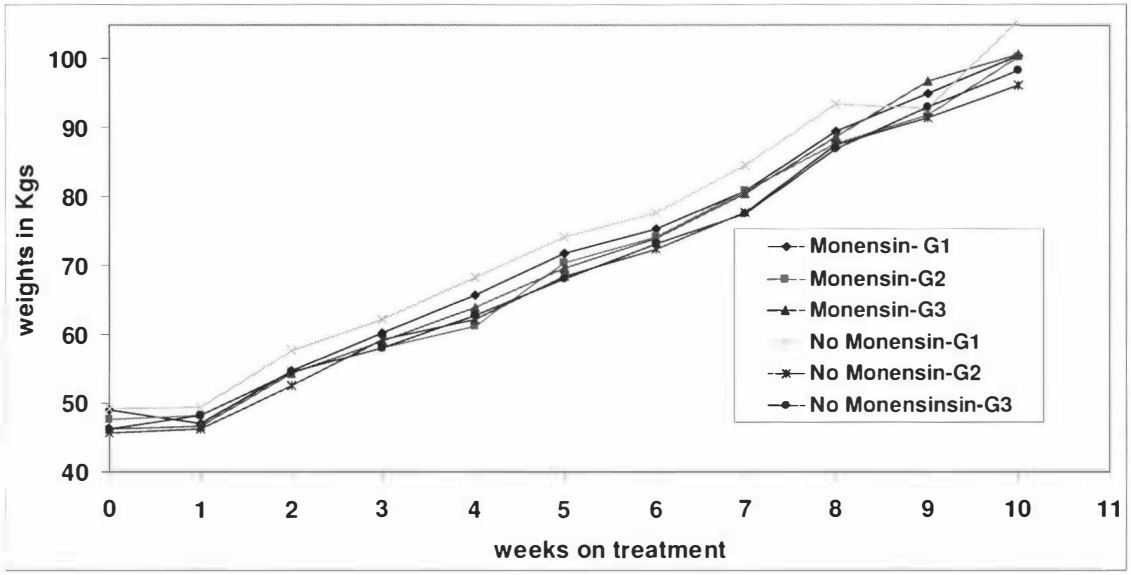


Figure 3. 9: Comparison of arithmetic mean live weights of calves fed pellets containing monensin (MG1, MG2, and MG3) and calves fed pellets without monensin (NMG1, NMG2, NMG3). Each group comprised 4 calves.

Effect	Numerator DF	Denominator DF	F Value	Pr>F
Week	10	178	1217.2	<0.0001
Monensin	1	20	0	0.96
Rep	2	20	1.03	0.37
RepXMonensinXWeek	52	178	0.90	0.66

Table 3. 5: Repeated measures Analysis of variance for live weights for calves up to weaning either fed pellets containing monensin or without monensin.

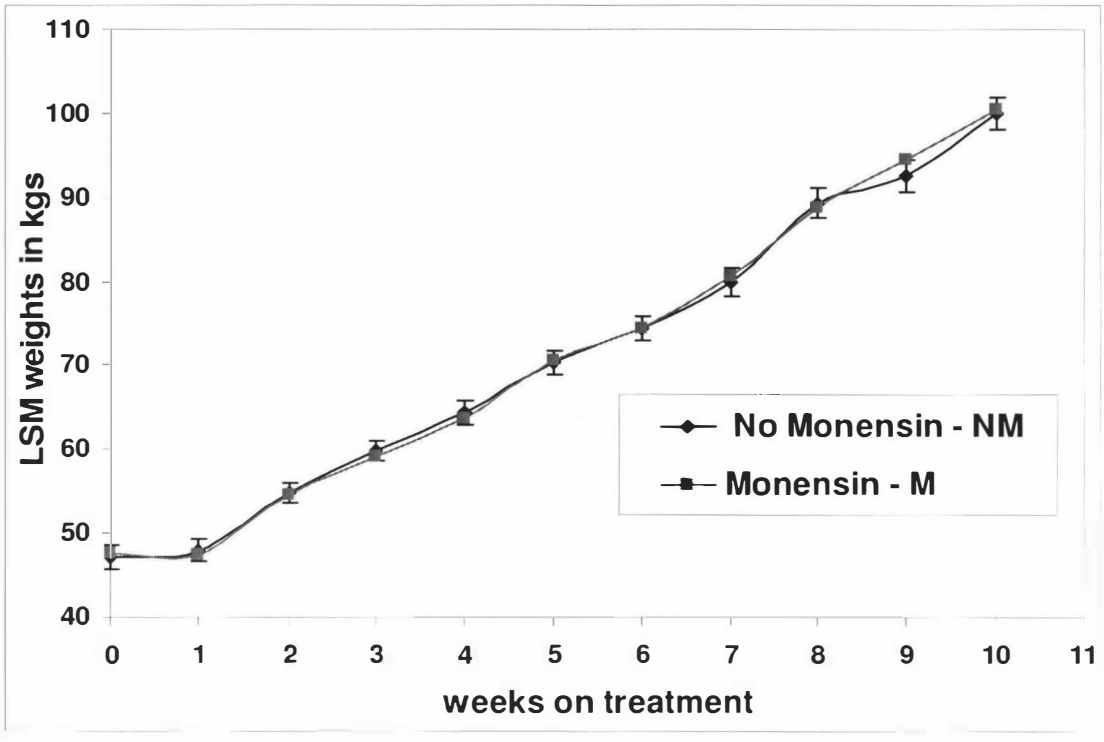


Figure 3. 10: Comparison of Least square mean live weights of calves (Error bars represent SE) either fed pellets containing monensin (M) or fed pellets without (NM). Each treatment consisting of 12 animals

As shown in Table 3.5, there was no significant effect ($p>0.05$) of group, treatment or the interaction of treatment x group x week. But the effect of the week was significant at ($p>0.001$). This indicates that the calves increased in their weight during the experiment but an effect of monensin was not significant statistically, as seen in the Fig 3.9 and Fig 3.10 as the treated and non treated calves did not differ much in their live weight gains over 11 weeks of observation (see Appendix.3.6.1 and Appendix 3.6.2. for raw data). The statistical analysis of monensin treatment on weight gain is shown in Appendix.3.6.4.

3.4.4. Live weights after weaning:

The arithmetic mean live weights by toltrazuril treatment are shown in Fig. 3.11 and those corrected for initial live weights in Fig. 3.12. The analysis showing correction for initial live weight is shown in Table. 3.6. There was a difference of > 5kg in weight gain between the treated and non-treated calves by 7 weeks post toltrazuril treatment. The arithmetic mean difference between toltrazuril treated (133.25kgs) and untreated (129.42kgs) was 3.82kgs and the corrected

LSM difference between treated (133.91kgs) and un-treated (128.76kgs) was 5.12kg (see details in Appendix.3.6.5 and 3.6.6).

Effect	Numerator DF	Denominator DF	F Value	Pr>F
Monensin	1	20	0.97	0.3356
Toltrazuril	1	20	5.65	0.0275
Week	4	79	374.28	<0.0001
Tolt X Week	4	79	2.81	0.0608
Mon XTolt XWeek	9	79	0.33	0.9628
Liveweight Week1	*1*	*20*	*132.90*	<0.001

Table 3. 6: ANOVA of Liveweights after weaning corrected for initial liveweight at weaning.

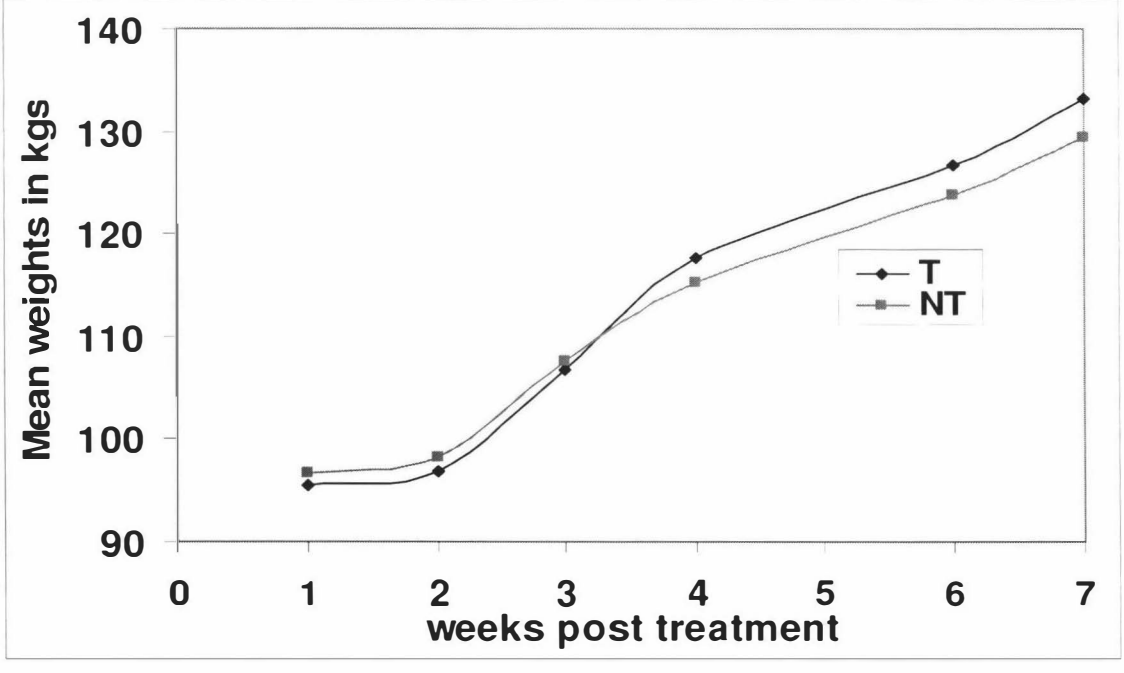


Figure 3. 11: Arithmetic mean live weights of calves after weaning either treated with toltrazuril 20mg /kg at the time of weaning (T) or not treated (NT). Each group consisted of 12 animals.

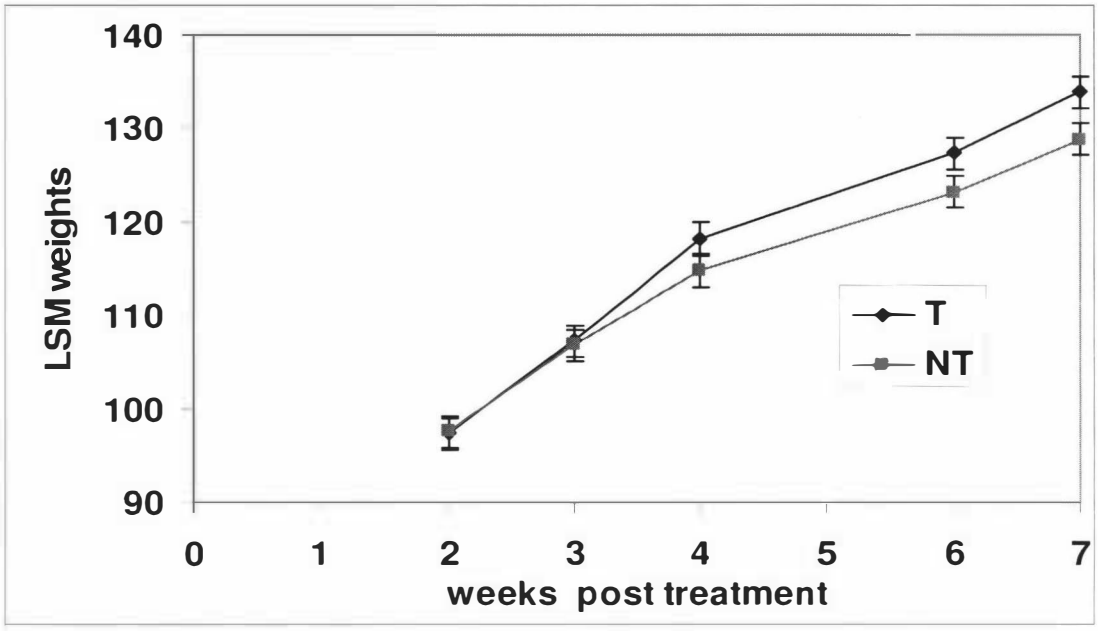


Figure 3. 12: Least square mean weights for a ccomparison of 2 groups of calves either treated at weaning with toltrazuril (T) or not treated with toltrazuril (NT) with live weights adjusted for initial weights. Each group consisted of 12 animals.

As shown in Table 3.6, the effect of week was significant ($p<0.001$) reflecting calves getting heavier with time. The effect of the toltrazuril treatment on liveweight was also significant ($p<0.05$) and the interaction between toltrazuril treatment and week was approaching being significant ($p=0.06$). This interaction suggests that the divergence in live weights between the two groups was becoming greater over time. Prior treatment with monensin had no significant effect ($p>0.05$).

3.4.5. *Cryptosporidium* and *Giardia* results:

The prevalence of *Cryptosporidium* in these calves using either technique is shown in Fig. 3.13. The prevalence of *Cryptosporidium* and *Giardia* in Week 1 is shown in Table 3.7. It is notable that 14 of the calves were infected with *Cryptosporidium* and this declined to no calves infected 4 weeks later. Also, 14 of the calves were infected with *Giardia* in Week 1.

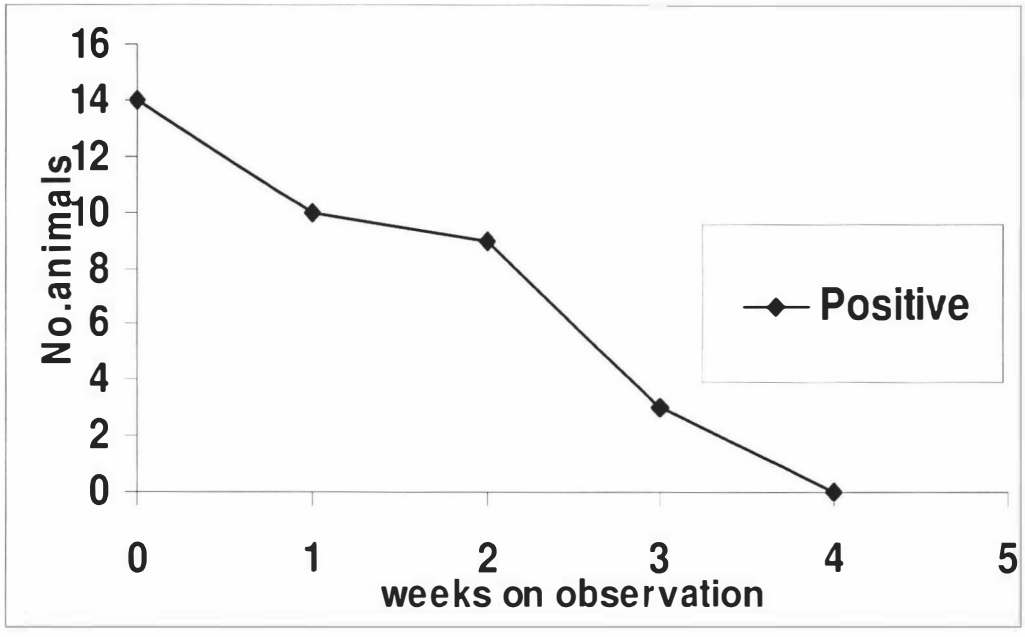


Figure 3. 13: Status of *Cryptosporidium* infections in calves up to 5 weeks of observation.

As shown in the Table 3.7 more than half of the animals were detected positive for both *Cryptosporidium* (58.33%) and *Giardia* (54.16%) at the beginning of the study when the calves were about 2.5 weeks of age. *Giardia* levels were recorded only on this occasion.

Animal no	*Cryptosporidium*	*Giardia*
43	-	-
17	-	-
116	-	-
118	+	-
25	-	-
57	-	+
39	+	+
113	-	+
6	+	+
16	+	+
15	-	-
117	+	-
3	+	+
33	+	+
18	+	-
56	+	-
32	+	+
28	+	+
9	-	+
29	-	+
31	+	+
20	+	-
14	-	-
47	+	+

Table 3. 7: *Cryptosporidium* and *Giardia* status of the calves by Meriflour test kit in Week 1.

3.5. Discussion:

A significant feature of this study was the generally low oocyst count throughout the experiment. At the commencement of the study this was not that surprising as the mean age of calves was only 2.5 weeks. The prepatent period for cattle *Eimeria* species varies from 8-21 days. This group of calves represented the very earliest calves born on this farm this year. So contamination in the calf rearing area was likely to have been low. At the calf unit, calves were initially housed in pens and then moved to paddocks adjacent to the pens that had been used by calves in successive years. This occurred a few days before movement to the experimental area. Oocyst counts were nil in this study for up to 4 collections, i.e. after 2 weeks on the pasture, which is presumably due to non-exposure to the infection whilst in the calf pens. Earlier researchers reported at least eight days after turning out onto pasture, for development of the infection with peak excretion of oocysts between 9-18 days after infection (Svensson *et al*, 1993). The rise in oocyst counts after 2 weeks, to reach the peak oocyst counts in 3 - 3.5 weeks of time on the experimental paddocks may coincide with the maturation of infection resulting from the calf-rearing at the Massey Calf Rearing facility immediately before being transferred to the experimental paddocks. After this time, oocyst counts were low, possibly because of immunity developing, as has been observed previously (Niilo, 1969, 1970; Senger, 1959; Rose, 1987). An alternative reason is that *Eimeria* proceeded through their life cycles and developed into oocysts, but in the absence of further infection on the experimental paddocks oocyst counts subsequently dropped to low levels. The relatively large size of the grazing cells meant that little reinfection probably occurred over the course of the experiment because the initial contamination by these calves was widely dispersed and pasture growth was good, further diluting any oocysts. In addition, the experimental area was not previously used for calf rearing and had only been grazed by adult cattle which would have had generally low oocyst counts and hence the area would have only had a low level of contamination with *Eimeria* oocysts.

Monensin should contribute to lower oocyst counts in treated animals (Stockdale 1973 &1981; McDougald, 1978; Fitzgerald 1984). The monensin-added meal-fed animals had comparatively lower counts but untreated animals also had low numbers, possibly because of good managerial practices in the paddock. It was reported that monensin has a dose relevant effect (Stromberg *et al.,* 1986). Therefore, if oocyst numbers had been high then a higher dosage of monensin might be required to obtain the same effect. Monensin treatment had no significant effect on weight gains.

As shown in Fig 3.5, the calves were not eating enough monensin for the first 4 weeks, as the whole fresh milk had no coccidiostats and the amount of meal eaten contributed less than the recommended 1mg/kg body weight (McDougald, 1978; Stromberg *et al.*, 1986). This occurred despite sufficient meal being offered to achieve this dose but the meal was not adequately consumed. It is not surprising that both treated and non-treated animals showed similar oocyst counts up to 4 weeks of time. After 4 weeks the oocyst counts were generally low in all groups, so no obvious effect of monensin could be determined. Whether there was a developing immune response is also hard to determine. The immunity to the parasite depends on the previous exposure and the level of exposure (Stockdale and Yates, 1978; Senger, 1959; Niilo, 1969), which in this experiment appeared to be low. A large dose of infection provides good immunity and the immunity lasts for at least 40 days (Hughes *et al*, 1989; Ferron *et al.,* 1965; Faber, 2002).

As shown in Table 3.6, the effect of week on liveweight was significant ($p<0.001$), indicating calves were getting heavier with time. The effect of the toltrazuril treatment on liveweight was significant ($p<0.05$) and the interaction between toltrazuril treatment and week was approaching being significant ($p=0.06$). This indicates that toltrazuril did have a postive effect on liveweight and the divergence in live weights between the two groups tended to be larger over time. Prior treatment with monensin had no significant effect ($p>0.05$).

The other notable feature was the generally low oocyst count in relation to those associated with clinical disease. There are published reports that counts of more than 5000 opg, are associated with clinical disease in cattle (Horton-Smith, 1958; Oda and Nishida, 1990).

The impact of monensin treatment on liveweight gain up to weaning was not significant ($p<0.05$), which is not surprising given the level of FOC that was noted. This may not be a fair reflection of monensin given the low challenge in the untreated calves. In most of the experiments that have been reported monensin improved the live weight gain in lambs and calves (Foreyt and Wescott, 1984; Fitzgerald 1984; MacDougald, 1978; Goodrich, 1984; Stockdale, 1981; McDougald, 1978). But in some other studies monensin did not improve weight gains in lambs with naturally acquired infections (Horton and Stockdale, 1981; McDougald, 1978). In this present study monensin had no significant effect on weight gains. This may be because monensin consumption was not enough during initial period of 4 weeks as shown in Fig 3.5. This allowed calves to have access to more oocysts as the effect of monensin is dose responsive (McDougald, 1978) and the treated calves shed almost equal number of oocysts as untreated animals. Probably this initial infection in animals had some effect on weight gain, as the disease causes weight loss in animals (Quigley, 2001).

After weaning, there was a rise in the oocyst counts of the animals which were not treated with the toltrazuril whereas those that were treated maintained low FOC. The rise in the untreated calves might have been because of the stress of weaning or else because of the withdrawal of monensin allowing inhibited stages to mature and calves picking up new infection. These two factors might both operate independently or together, but this study was not able to determine which was the case. These animals were subjected to several stressful conditions at the time of weaning such as vaccination, change of diet (no supplemental diet), bleeding, weighing, drenching (coccidiocide) and removal of shelter (pens removed from the paddock). None of the animals developed clinical disease. Indeed the FOC remained low even though there was a difference between the toltrazuril-treated and not-treated calves.

A single treatment of the calves with toltrazuril significantly reduced the oocyst counts up to 4 weeks post treatment and the oocyst counts of the calves which were not treated were higher over this period. Toltrazuril has been shown to be an effective coccidiocide and is effective in killing all stages of the life cycle except the extracellular stages and is effective against all species of *Eimeria* species (Froyman and Grief, 2002). However, there is no persistent activity.

Hence, any effect on FOC is only likely to persist until a new infection becomes patent. There have been several studies with various ruminants. Toltrazuril treatment of lambs (20mg/kg) reduced their oocyst counts to low levels and weekly treatment of the same lambs for 10 weeks kept FOC low (Stafford et al, 1994). In 3 different trials with goats, a single treatment with toltrazuril resulted in rapid and significant reduction of oocyst counts in treated goats. In one of these the control animals had 21,546 OPG count but the treated animals had only 360 opg count by the 4th day after treatment and this further reduced to 163 opg on the 10th day (McKenna, 1988). A single treatment to lambs with toltrazuril (20mg/kg) on Day 10 on pasture reduced the oocyst counts for up to 34 days. Lambs treated with 20mg/kg toltrazuril had better results over the lambs treated with 10 mg/kg (Gjerde and Helle, 1986).

The improvement in the weight gain (corrected LSM) in the 6 week period after weaning was significant with > 5kgs difference between treated and untreated animals. The lower weight gains in the controls may be because of the oocyst burdens these calves had during the immediate post weaning time. The effect seen here is particularly surprising given the generally low oocyst counts seen after weaning in the calves not given toltrazuril treatment. There have been few studies, investigating the effect of coccidiosis in calves after weaning under similar type of grazing conditions and apparently no studies on weight gain when using toltrazuril in calves. Toltrazuril treatment of lambs naturally infected with *Eimeria* improved the weight gains when treated with 15–20mg/kg toltrazuril and the weight gains were significant in the period from 14 to 35 days after treatment (Gjerde and Helle, 1986). As noted in Chapter 2, the dominant species in these calves were *E. bovis* and *E. zuernii* which are generally acknowledged as the two most pathogenic species in cattle. Even so, it seems surprising that there was a significant effect on weight gain given the generally low FOC, even with these two species dominating.

***Cryptosporidium* status:**

At the beginning of the experiment at least 58.3% of calves were positive to *Cryptosporidium* and 54.2% of calves for *Giardia.* The average age of the calves in this experiment was 2.5 weeks at which age the infection seems to be most prevalent in calves (Maldonado *et al*, 1998, Atwill *et al*, 1998).This was the maximum level of shedding recorded in this study with counts declining each week to become nil in the fifth week of observation when the calves were 6.5 weeks old. It has been observed that the risk of infections decreases with increasing age (Mohammed *et al,* 1999).

Chapter 4: Experiment at Tuapaka:

4.1. Introduction:

The study reported in the previous chapter examined coccidiosis both before and after weaning. Coccidiosis has been reported immediately after weaning because of the stress calves undergo at the time of weaning in terms of transport, feed change and vaccination (Parker, 1984; Fitzgerald 1961; Niilo, 1970; Marsh 1938). It has been previously shown that administration of anti-coccidial medication early during the stress period can decrease the clinical and sub-clinical disease seen in feed lots (Pritchard, 1993). A single dose of toltrazuril at 15 - 20 mg/kg reduced oocyst counts and they remained low for 3 - 4 weeks post treatment in previous studies (McKenna, 1998; Gjerde and Helle, 1986; Taylor and Kenny, 1988).

This present study was run in conjunction with the study reported in Chapter 3 at Massey Number 4 Dairy Farm and was designed to investigate the effect of toltrazuril treatment at weaning in young Holstein-Friesian bull calves reared by commercial rearers under commercial conditions where the probability of calves being exposed to infection with coccidia was likely to be high. Traditional rearing of dairy-breed bull calves involves a commercial calf rearer raising the calves from 3 days of age until weaning at about 100kg liveweight and 12 weeks of age. These animals are then sold to farmers who keep these bulls until they are slaughtered. It is also a time when anticoccidial treatment ceases as calf meal is no longer fed.

4.2 Materials and Methods:

4.2.1. Farm and Animals

"Tuapaka" is a Massey University farm that regularly rears calves from weaning to 18-24 months of age. The area of the farm where the study bulls were grazed has only been used for the rearing of dairy bull beef since the early 1980s. It is current policy for calves to be sourced from Massey University dairy farms, raised under contract by commercial calf rearers until 100kg body weight and then returned to Tuapaka to be grazed until sold at 18-24 months of age. These commercial calf rearers would generally follow the same rearing practice as for those calves studied in Chapter 3, except they would use milk replacer

rather than whole milk. For this present study calves from two different calf rearers were used with some from a third rearer arriving 1 week after the commencement of the study and being monitored as a form of additional control group. To ensure that this current experiment started with both groups of calves treated synchronously it was necessary to hold some calves after arrival at Tuapaka but in this case all calves were maintained on calf meal with coccidiostat as would have occurred on the property of the calf rearer. No calves were monitored whilst with the calf rearer.

4.2.2. Experimental Schedule:

Two groups (Group A and B) of 30 calves each were selected, one from each of the two calf rearers. Half (n=15) of both groups were treated once with 20mg/kg body weight toltrazuril (Baycox Piglet Coccidiocide containing toltrazuril 50g/L; Batch. No.1848A2005; Expiry Date, June 2003). All treatments were given orally with a 20ml syringe. The animals were randomly selected in the order of their arrival in the race at the time of the first sampling. All the calves were faecal sampled, weighed and bled. From the second week onwards the third group (Group C) of 21 calves arrived on the farm and were sampled as for Groups A and B but none of this group was treated with toltrazuril. Each group of calves were grazed together as a group but each group was grazed in a different paddock. Calves remained in these same paddocks for the duration of the experiment.

Each calf was faecal sampled and weighed once a week for 5 weeks post-treatment. The technique for faecal collection was rectal stimulation of defaecation. In addition, a 10ml blood sample from the jugular vein was collected on the first and last week of the experiment from half of the Groups A and B for recovery of serum and subsequent determination of anti-coccidial antibodies (see Chapter 6).

The experiment commenced on the 7th November 2002 (day of treatment) and was completed 5 weeks later on 12th December (Week 5 post treatment). On arrival all calves were treated with an anthelmintic to remove the complication of nematodes affecting liveweight gain.

4.2.3 Oocyst counts:

A total oocyst count was carried out on a 2g sub-sample of faeces collected as previously described (see Appendix 3.3.1). In addition, oocysts were recovered from a further sub-sample for those animals with high oocyst counts and sporulated as previously described (Appendix 3.3.2 and 3.3.3). Each oocyst counted represented 50 oocysts per gram. A total of 516 faecal samples were screened for coccidia throughout the experiment and all the positive samples with high counts were sporulated for species identification.

4.2.4. Species prevalence:

To determine the prevalence of species within an animal 30 oocysts were identified. This was only estimated from those animals with a reasonably high oocyst count to make the observation practically feasible. These results have been reported in Chapter 2.

4.2.5. Faecal consistency:

A total of 128 faecal samples on two occasions (3rd and 4^{th} week post treatment – 26^{th} Nov and 3^{rd} Dec) were categorised based on their consistency of faeces as solid (1), semi-solid (2), and liquid (3) and the faecal oocyst counts were compared to see whether there was any relationship between the faecal consistency and the oocyst counts.

4.2.6. Statistical analysis of faecal oocyst counts:

Faecal oocyst counts (FOC) were analysed after square root transformation to normalise the data (Snedecor and Cochran, 1980). Data were analysed using the MIXED procedure of SAS (2001). The linear model included the fixed effects of week, treatment with toltrazuril, the interaction between week X treatment, and the random effect of replication. A replicate comprised a group of 15 animals that were treated with toltrazuril and 15 untreated animals.

Using the Akaike's information criterion (SAS 2001), a compound symmetric error structure was determined as the most appropriate residual covariance structure for repeated measures over time within animals (Appendix. 4.1.2).

4.2.7. Statistical analysis of Live Weights:

Live weight was analysed using the MIXED procedure in SAS (2001). The model included the fixed effects of week, treatment with toltrazuril, the interaction between week X treatment and the random effect of animal within treatment. It also included liveweight at Week 1 to standardise initial differences between groups. Using the Akaike's information criterion, a compound symmetry error structure was determined as the most appropriate residual covariance structure for repeated measures over time within animals. Least square means and their standard errors (SE) were obtained for each treatment.

4.2.8. Statistical analysis of faecal consistency:

The correlation between faecal consistency and oocyst counts was determined using SAS (2001) after transforming the FOC by their square root.

4. 3. Results

4.3.1 Faecal oocyst counts:

Arithmetic mean oocyst counts for Groups A, B and C are shown in Figs 4.1, 4.2 and 4.3 respectively. The Least Square Mean (LSM) oocyst counts for Groups A and B are shown in Fig. 4.4 and Fig. 4.5 respectively. The raw oocyst counts are recorded in Appendix 4.4.1. The oocyst counts of Group A calves which were treated with toltrazuril were dramatically reduced by the first week from a mean of 4120 oocysts/g to 17 oocysts/g and the counts of these treated calves remained low for the duration of the experiment (Fig. 4.1). Oocyst counts remained lower than the untreated calves for 5 weeks post treatment. The counts for calves which were not treated with toltrazuril remained as high in Week 2 as pre-treatment, then increased a little in Week 3 and reduced thereafter to be similar to treated calves by the end of the experiment. A similar pattern was seen with calves in Group B. However, by chance the oocyst counts of toltrazuril-treated calves were initially lower than the non-treated calves. Regardless, they remained low for the duration of the experiment increasing very slightly in Week 5. The untreated calves in Group B averaged 337 oocysts/g at the start of experiment in Week 0, increasing to 1196 oocysts/g in Week 2 post-treatment and then reducing to be similar to the

treated calves by Week 5 post-treatment. The mean counts for Group C calves which were not treated with toltrazuril also increased slightly after weaning from a mean of 140 oocysts/g on arrival in Week 1 to 460 oocysts/g in Week 3 and then reducing to low levels by Week 5.

The statistical analysis of the oocyst variation of Group A and B is shown in Table 4.1. The effect of toltrazuril treatment was highly significant ($p<0.0001$) indicating that toltrazuril was effective in reducing the oocyst counts. The effect of Group was also significant ($p<0.05$) indicating a difference between calves from different rearers. Week was not significant ($p>0.05$) indicating oocyst counts overall did not vary over time. The interaction of "GroupxTreatXWeek" was also significant ($p<0.001$) indicating that there was a variation between Group and Treatment over time which is consistent with the decline in oocyst counts after treatment.

Effect	**Num DF**	**Den DF**	**F Value**	**Pr >F**
Week	5	278	1.90	0.09
Treatment	1	5	17.88	0.0001
Group	1	57	5.26	0.025
Group*Treatment*Week	16	278	2.56	0.001

Table 4. 1: Type 3 tests of fixed effects of time series analysis for oocyst counts for calves from Group A and B treated or not treated at weaning with toltrazuril. Week = week of experiment, Treat = toltrazuril treatment and Group = Group A or B.

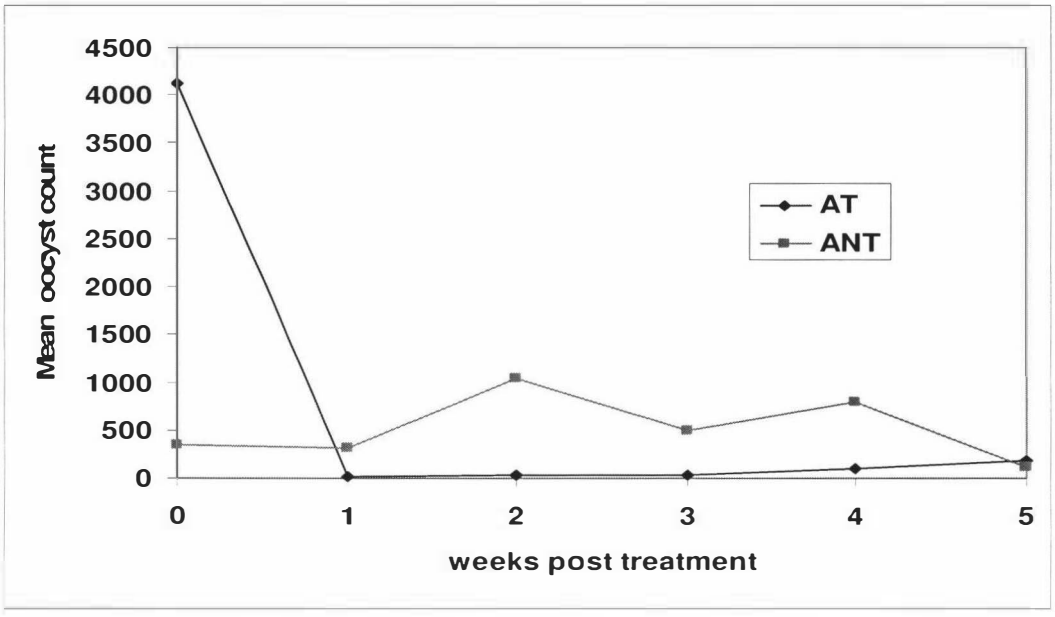


Figure 4. 1: Comparison of arithmetic mean oocyst counts of Group A calves treated at weaning (Week 0) with toltrazuril 20mg/kg body weight (AT) or not treated with toltrazuril (ANT). Each group comprised 15 calves.

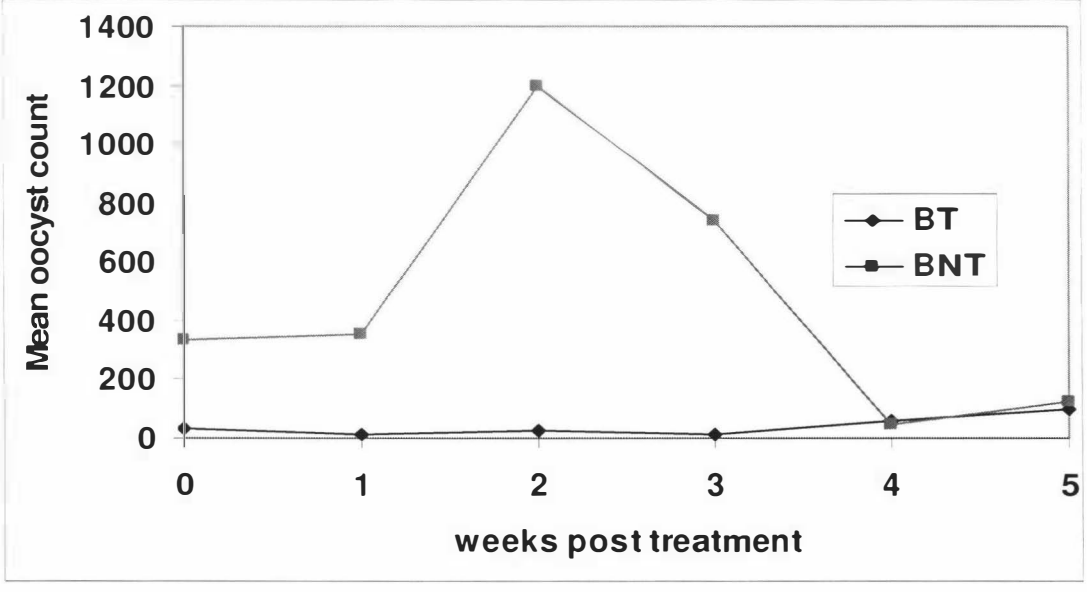


Figure 4. 2: Comparison of arithmetic mean oocyst counts of Group B calves treated at weaning (week 0) with toltrazuril (BT) or not treated with toltrazuril (BNT). Each group comprised15 calves.

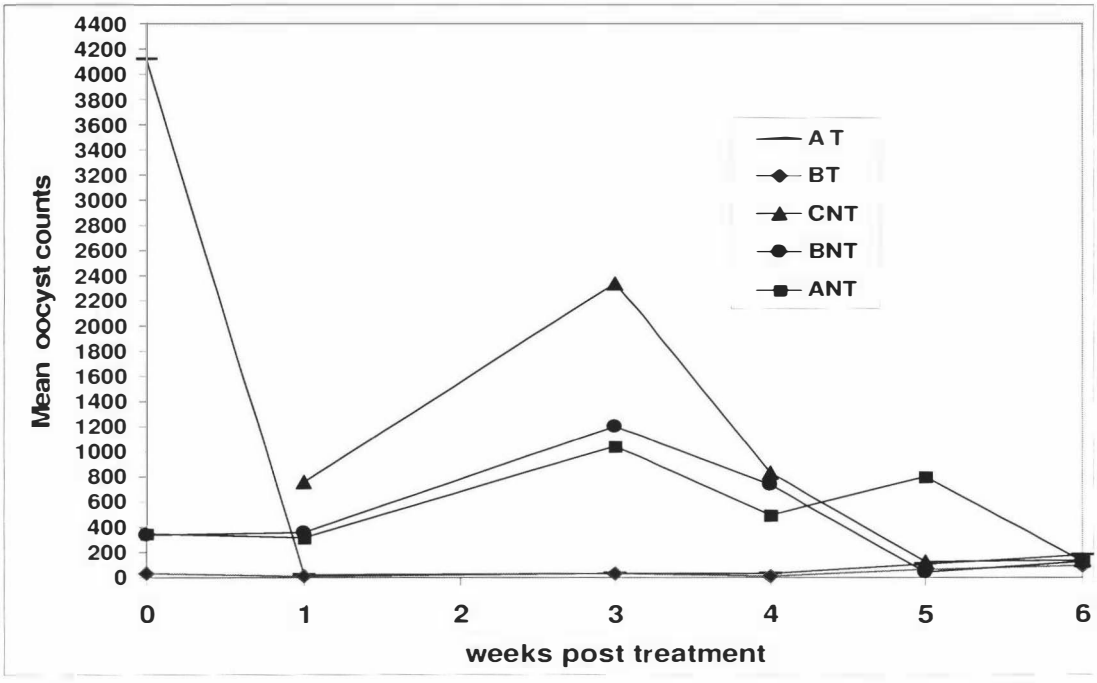


Figure 4. 3: Comparison of Means of Group A treated with toltrazuril (AT), Group A not treated with toltrazuril (ANT), Group B treated with toltrazuril (BT) or Group B not treated with toltrazuril (BNT) and Group C not treated with toltrazuril (CNT) Each group comprised 15 animals except group C of 21 calves.

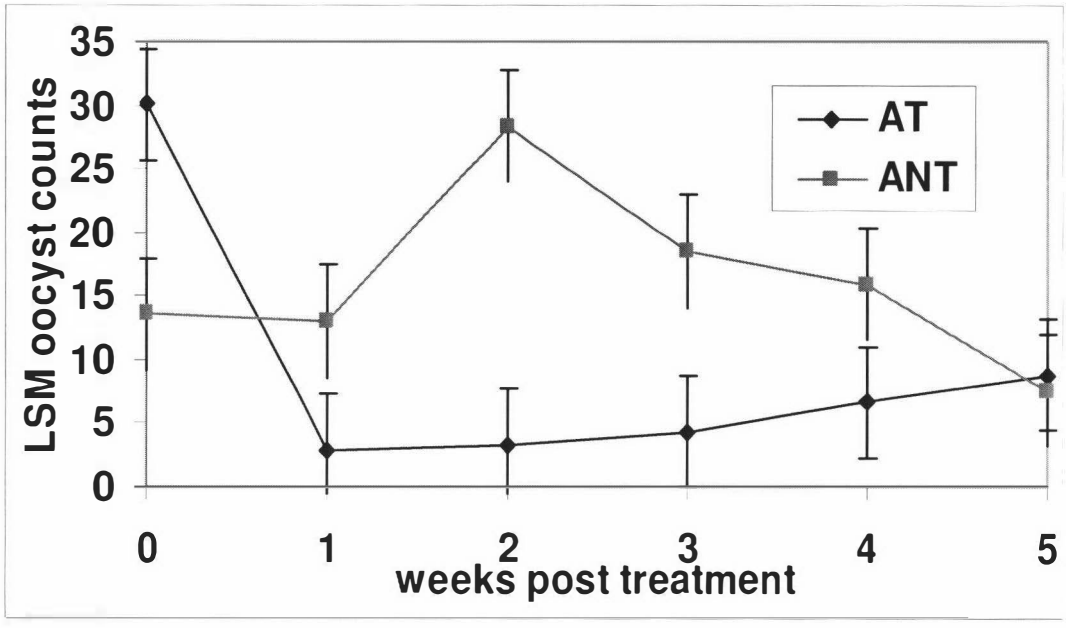


Figure 4. 4: Comparison of Least Square Mean oocyst count (+/-) of Group A treated with toltrazuril (AT), Group A not treated with toltrazuril (ANT). Each group comprised 15 animals. Error bars represent Standard Error.

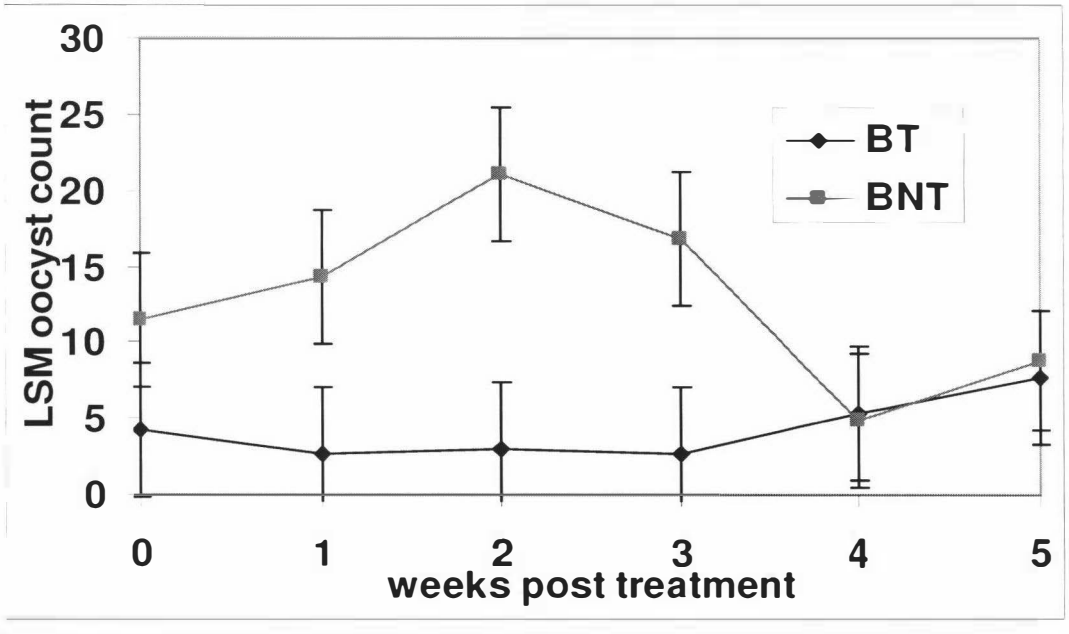


Figure 4. 5: Comparison of Least Square Mean oocyst count (+/-) of Group B treated with toltrazuril (BT) or Group B not treated with toltrazuril (BNT). Each group comprised 15 animals. Error bars represent Standard Error.

4.3.2. Liveweights of calves:

Arithmetic mean liveweights for Group A are shown in Fig 4.6, for Group B in Fig 4.7 and the Least Square Means for both Groups in Fig 4.8 and Fig 4.9 respectively. The summary of the statistical comparison of weight gains is shown in Table 4.2. By 5 weeks post weaning there was >5-8kgs difference in weight gain between animals treated with toltrazuril or not. As shown in Table 4.2 the effect of week was significant at ($p<0.0001$) reflecting the liveweight increase over time. Treatment with toltrazuril was not significant but the interaction of GroupXTreatmentXWeek was significant ($p<0.01$) indicating the relationship was not constant over the experiment but consistent with one group weighing more than the other at the end of the experiment.

Effect	Numerator DF	Denominator DF	F Value	Pr>F
Group	1	56	0.20	0.6583
Treat	1	56	1.13	0.2932
Week	5	279	315.55	0.0001
Group*Treatment*Week	16	279	2.62	0.0008
Liveweight week 0	0	56	407.92	0.0001

Table 4. 2: The repeated analysis variance of live weights of calves adjusted for initial liveweight (liveweight week 0) showing the effect of treatment with toltrazuril ('treat"), "group" (Group A and Group B), time by week post treatment ("week") and the interaction of group, treatment and week ("group*treat*week) on the live weight.

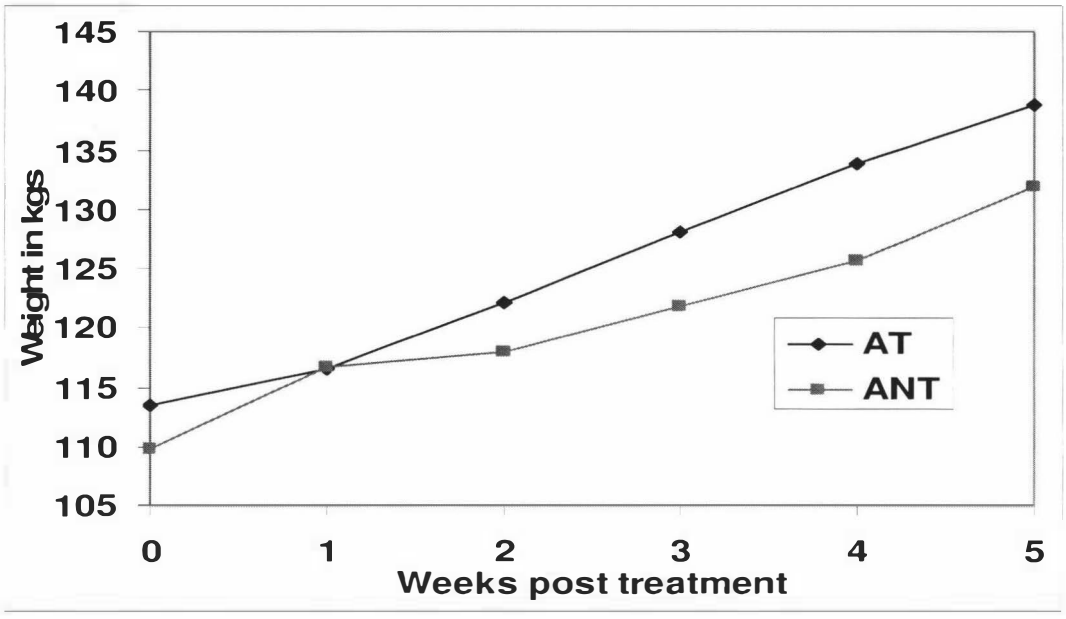


Figure 4. 6: Comparison of arithmetic mean live weights of Group A calves either treated with toltrazuril at weaning (AT) or not treated with toltrazuril (ANT). Each group comprised 15 animals.

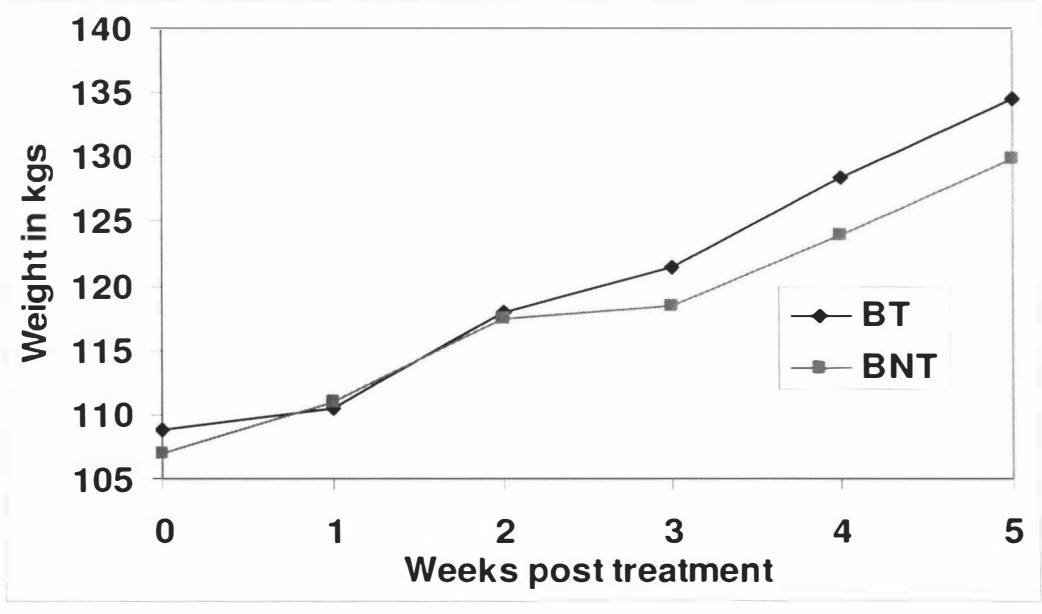


Figure 4. 7: Comparison of live weights of group B calves treated with toltrazuril (BT) or not treated with toltrazuril (BNT). Each group comprised of 15 animals.

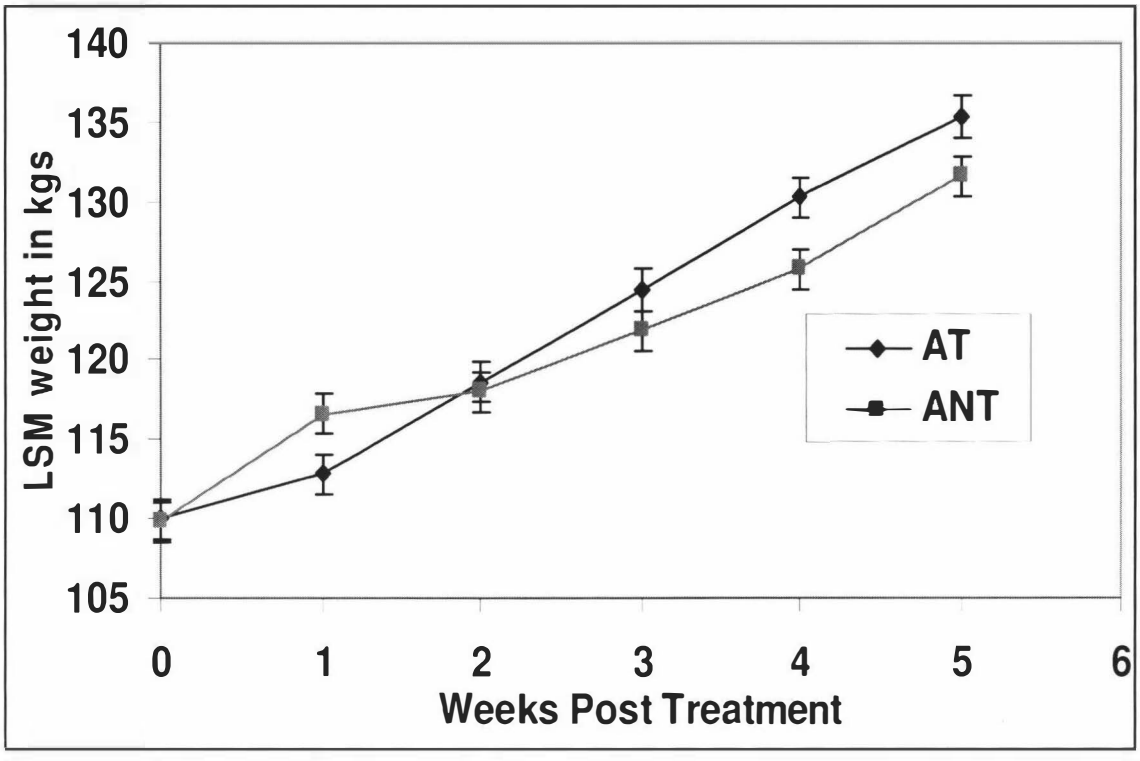


Figure 4. 8: Comparison of LSM live weights, of Group A calves treated with toltrazuril (AT) and not treated with toltrazuril (ANT). Each group comprised 15 animals

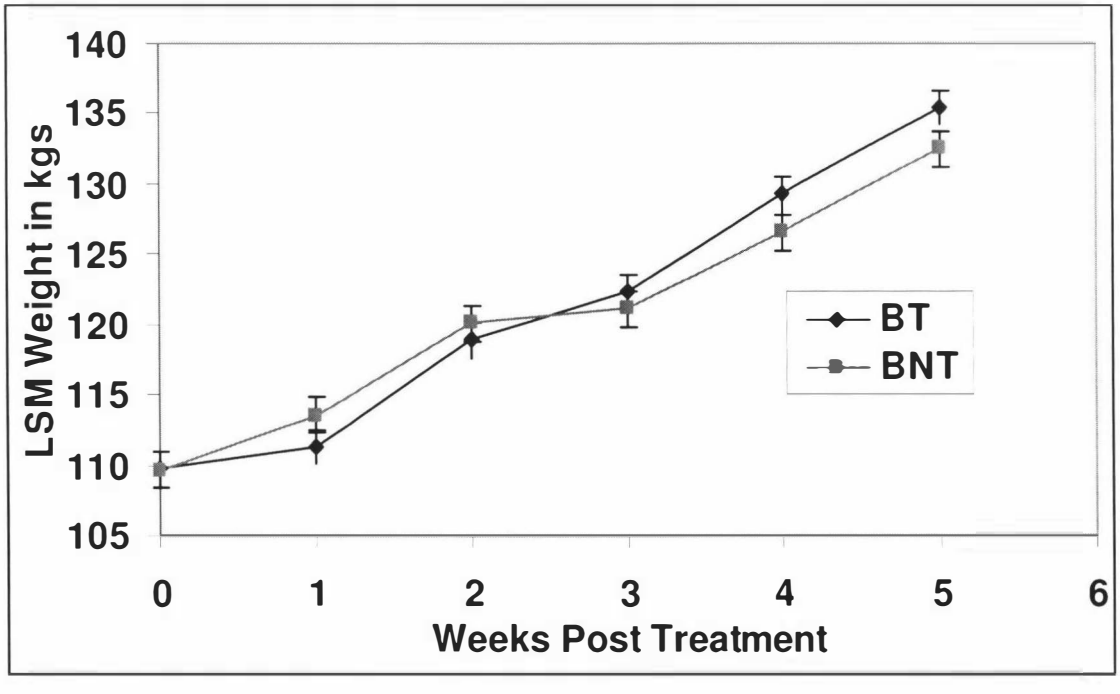


Figure 4. 9: Comparison of LSM live weights, of Group B calves not treated with toltrazuril (BNT) and treated with toltrazuril (BT). Each group comprised 15 animals. Error bars represent SE.

4.3.3. Faecal consistency and oocyst counts:

A total of 128 faecal samples were categorised based on their consistency of faeces as solid (1), semi solid (2), and fluid or liquid (3) and the faecal oocyst counts were compared to see whether there was any relationship between the faecal consistency and the oocyst counts as shown in Table 4.3 (see Appendix 4.3. for raw data).

Consistency of faeces	No. samples	Faecal counts
1	69	0-4550 (286)
2	47	0-1350 (236.)
3	12	0-750 (175)

Table 4. 3: Faecal consistency and the oocyst counts of the calves.

As shown in Table 4.4 and Fig. 4.10, there was no relationship observed between the faecal consistency and the oocyst counts in the faeces as all types (faecal consistency solid, semi-solid and liquid) had minimum counts as low as zero as minimum count and solid faeces had highest counts up to 4,550. The average counts of liquid, semi - solid and solid were 175, 235.5 and 286.6.

Pearson Correlation Coefficients			
	Fc	**Foc**	**Sr_Foc**
Fc **P**	1.00000 0.4909	-0.06193 0.9336	-0.00750
Foc **P**	-0.06193 0.4009	1.00000 <0.0001	0.90842
	Fc	**Foc**	**Sr_Foc**

Table 4. 4: Correlation coefficients of faecal oocyst counts. Note: Fc- faecal consistency, Foc=Faecal oocyst counts, Sr = Square root, P=Probability

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Fc	2	123	0.49	0.6141

Table 4. 5: Comparison of faecal consistency and faecal oocyst counts.

As shown is Table 4.4. and 4.5., there was no significant relationship between faecal consistency and faecal oocyst counts as the correlation coefficient before square root transformation (SRT) of oocyst counts was -0.06 and after SRT was -0.007. Analysis of variance of the fixed effect was also not significant at (p=0.614) which means that faecal consistency is not a true measure for the level of infection as the most liquid sample can have low oocyst counts and solid samples with high counts may mislead the level of infection of animal.

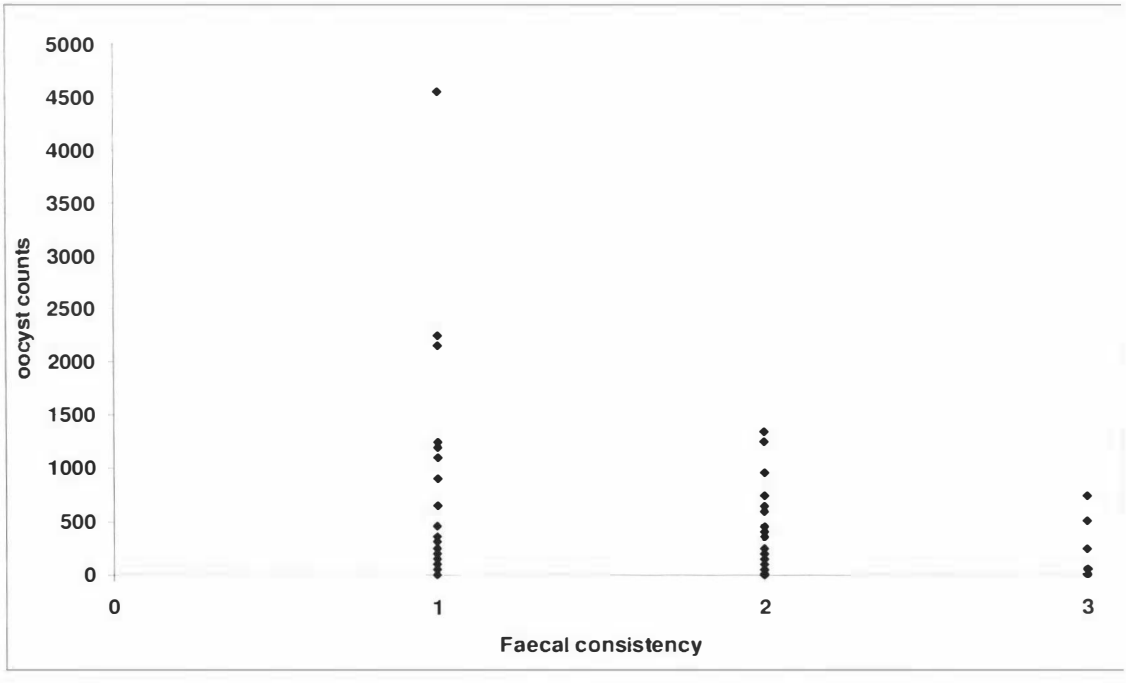


Figure 4. 10: Comparison of faecal consistency solid (1), semi solid (2) and fluid (3) and oocyst counts of 127 calves.

4.4. Discussion:

The development of coccidiosis is affected by several factors of host and parasite (Levine, 1986) such as host age, nutritional status and immune status of an animal. Adult animals are generally resistant to the disease compared to calves this could be because of previous exposure (Stockdale and Yates; Senger, 1959; Niilo, 1969) and severe infection results in the development of good immunity that can last for at least 40 days (Hughes *et al*, 1989; Ferron *et al.,* 1965; Faber, 2002).

Management conditions and climatic conditions will influence numbers of infective oocysts available to the calves (Matjila & Penzhoru, 2001; Hasbulla *et al*., 1990; Chibundu *et al*., 1996; Salisbury *et al*., 1955; Niilo, 1970). In this present study, bull calves from three different rearers had different levels of infection before treatment although none would be considered to be very high, suggesting that management on the farms had an influence on the oocyst counts. Group A calves had the highest mean oocyst counts (343 to 4120 ocyst/g) whereas Group B were lower means (33 to 336 oocysts/g) whilst Group C had mean counts of 0 to 760 oocysts/g.

All the groups were subjected to a similar degree of stress at the time of weaning. The animals were transported to the Tuapaka farm, faecal sampled, weighed and half of the animals were bled and drenched with Toltrazuril orally (as described). Toltrazuril-treated animals showed a reduction in the oocyst counts after one week of treatment, consistent with toltrazuril effectively killing the existing infection. However, although toltrazuril reduced the counts after treatment there were still some oocysts were passed in treated calves. This study is in agreement with previous studies in goats where the oocyst counts were reduced almost to zero levels by toltrazuril treatment and then kept very low for 30 days post treatment (McKenna, 1988; Taylor and Kenny, 1988). In the present experiment this reduction was seen up to 4 weeks post treatment.

The oocyst counts of untreated calves of all the groups rose slightly after the start of the experiment with the peak being seen during the second week. The

mean counts remained relatively high until the 4th week post treatment. Both treated and untreated calves had similar counts around Week 5 post treatment.

Overall there was no significant effect of treatment on liveweights ($p>0.05$) although the liveweights of treated animals were improved after treatment with toltrazuril. Treated calves in Group A gained >8kgs live weight in the 5 weeks after the treatment over the untreated calves. The increased live weight gain of treated animals started to be apparent after 3 weeks post treatment (Fig. 4.8) giving an impression that the oocyst count reduction during these 3 weeks might have helped the animals to gain weight. Treated calves in Group B also had an improved weight gain of >4kgs over untreated calves. In all untreated groups the live weights were lower (Group A, B, C) when compared to treated animals, and this may be due to sub-clinical disease process going on in the animals. Although, the effect of treatment was not significant, the significant interaction of liveweight with time and treatment ($p<0.01$) is indicative of a divergence in liveweight gain between treated and untreated animals.

In this study the faecal samples were graded as solid, semi solid and runny based on the consistency and the oocysts counts of the same animals were tallied with consistency to investigate whether there is any relationship between diarrhoea and oocyst counts. No relationship between faecal consistency and oocyst counts was however observed. All types of faecal consistencies ("Solid", "Semisolid" and" Runny") had counts as low as zero as a minimum count and high counts up to > 4,000 oocysts/g. The average counts of "liquid category" had the highest counts of 758 oocysts/g when compared to Semi solid (237 oocysts/g) and Solid (287oocysts/g). This is similar to the results of other studies (Oda and Nishida, 1990; Ernst, 1985) that also failed to show any relationship between counts and faecal consistency.

Chapter 5: Study of coccidiosis and oocyst shedding on various beef farms

5.1. Introduction:

The previous two chapters detail experiments on coccidiosis pre- and post-weaning and the effect of toltrazuril treatment at weaning. Studies in this chapter detail a series of investigations designed to be complementary to these by monitoring the shedding of coccidial oocysts in beef cows and their calves, calves raised by conventional dairy calf rearers and cows with their calves on organic farms. The aim was to expand our limited knowledge of the epidemiology of bovine coccidiosis in New Zealand. In particular, to see how different rearing practices influence the coccidial burdens in calves.

5.2. Materials and methods:

Cattle were sampled on several different farms as detailed below. Samples were either collected per rectum or fresh faeces were collected from the pasture after observing animals, defaecating. Oocysts counts were estimated with the McMaster oocyst count technique as previously described (Appendix. 3.3.1). If counts using this technique were all zero then a faecal float was usually performed as previously described (Appendix 3.3.1) and the results were recorded as either positive or negative.

Some of the positive samples were processed for oocyst sporulation as previously described (Appendix 3.3.2) and the sporulated oocysts were speciated by measuring their sizes and shapes as described in Chapter 2 (Section 2.2).

5.2.1 Massey University calves:

(a) Conventional calves raised on No.4. Dairy Farm that were the cohorts of the calves used in the experiment reported in Chapter 3, were sampled on two occasions in October. These were two separate groups of calves. They were initially fed on whole milk and then meal containing monensin as for the calves used in the experiment in Chapter 3. In addition a group of 10 adult cows was sampled from this same herd on one occasion in October.

(b) A group of 10 organically-reared calves were screened four times prior to weaning and once after weaning by collecting faeces per rectum and estimating faecal oocyst counts. The organically-reared calves were reared on the Massey University Dairy Research Unit and were part of the organic dairy research programme. They were raised without any coccidiostat in the meal that was fed to them. A group of 10 calves from the conventionally-reared control group from this research programme were also screened 4 times prior to weaning.

5.2.2 Organic Beef Farms

Calves from two farms were monitored.

(a) Farm B was a fully certified commercial organic farm located in the Rangitikei region of New Zealand. It had a mixture of beef cows suckling calves and organic dairy beef. Faecal samples were collected from adult cows, calves and autumn born bull calves on several occasions as detailed in Table 5.4.

(b) Ballantrae Hill Country Research Station: This farm is owned by AgResearch and the animals sampled were from the organic farm research block. This organic farm compares cattle and sheep raised under organic farming criteria with other raised conventionally. The animals sampled were from two organic (No Chemical; referred to as NC1 and NC2) and two conventional (non-organic) farmlets (referred to as CO1 and CO2). Faecal samples were collected from the same cows and calves on 3 occasions. Neither the conventional or organic calves had access to coccidiostats.

5.2.3 Commercial Calf Rearers and Farmers:

Calves from three different commercial calf rearers were sampled.

(a) Apiti commercial calf rearer:

This farm is located near Apiti and rears 500 to 600 calves to weaning per year. Calves are initially fed in sheds with wooden floors and fed milk replacer and increasing quantities of calf pellets containing monensin until weaned off milk and moved to pasture at 3 weeks of age. The shed contained 20 pens and each pen had 12 animals each. Ten faecal samples were collected randomly from several of these inside pens, another 10 samples from calves just weaned and moved

outside and a 3rd group of 10 samples from autumn born calves that were about 5-6 months old but which were still being fed about 0.5kg meal containing monensin per day.

(b) Stafford commercial farm:

This farm reared a small number of dairy bull calves. A sample of 10 calves was screened on one occasion to determine the coccidial status of the calves. Samples were collected a week after weaning.

(c) Alley Commercial farm:

This is a hill country farm in the Manawatu. Both sheep and beef cattle were farmed. The calves (n=10) were sampled once and were still suckling their mothers. They varied in age from about 3 to 6 weeks old.

5.2.4: Statistical Analysis:

Ballantrae Farm data were analysed by ANOVA (SAS 2001), where the faecal oocyst counts were log transformed by using the following formula:

LnFoc = Ln (Foc+1).

The model included the effect of treatment, month, interaction between treatment and month, group nested within the treatment, the animal and procedural error (See Details in Appendix. 5.3).

5.2.5. Prevalence and species identification:

To determine the prevalence of species within an animal at least 30 oocysts were identified. This was only estimated from those animals with a reasonably high oocyst count to make the observation practically feasible. The percentage of individual species in each study was then calculated to find out the prevalence of individual species in that study. The overall prevalence of species was calculated by pooling all the studies to come up with an overall prevalence. These results were discussed in Chapter 2.

5.3. Results:

5.3.1 Massey University Farm:

The oocyst counts of calves described in 5.2.1(a) and (b) are shown in Tables 5.1a and 5.1b and 5.2.

S.No.	Aug	Sep	Oct	Jan
1	0	50	0	POSITIVE
2	0	0	0	POSITIVE
3	0	0	0	POSITIVE
4	0	0	0	POSITIVE
5	0	0	0	POSITIVE
6	0	0	0	POSITIVE
7	0	0	0	POSITIVE
8	0	0	0	POSITIVE
9	0	0	0	POSITIVE
10	0	0	0	POSITIVE

Table 5. 1a: Counts (oocysts/g) of conventional calves (n=10) on Massey University Dairy Research Unit.

Adult Cows		Calves Paddock A	Calves Paddock B
1	0	positive	positive
2	50	Positive	Positive
3	0	Positive	positive
4	0	Positive	Negative
5	0	Positive	Negative
6	0	Positive	Negative
7	0	Positive	Positive
8	0	Positive	Positive
9	0	Positive	Negative
10	0	Positive	Negative

Table 5. 1b: Oocyst counts (oocysts/g) of conventional calves (n=10) and cows (n=10) on Massey No. 4 .Dairy Farm.

As shown in Table 5.1b the oocyst counts in the adult cows were low and the conventionally reared calves on Massey University No.4 Dairy Farm were also low as oocysts were only detected by floatation and thus reported as positive (oocysts seen) or negative (no oocysts seen). The counts of the organically reared calves are shown in Table 5.2. Initially these were low in September but showed a modest increase in the month of October to a mean count of 529 oocysts/g before declining to relatively low counts in November, December and January. All the conventional control calves were negative to coccidia in August but during September one calf was positive with a count of 50 oocysts/g then during October counts were nil but all the calves were positive in the month of January on faecal floatation. The data is shown in table 5.1a.

Tag	**Sep**	**0ct**	**Nov**	**Dec**	**Jan**
20	50	0	250	300	POSITIVE
10	0	1400	100	150	POSITIVE
15	50	150	250	800	POSITIVE
2	0	50	150	100	POSITIVE
13	0	3800	0	200	POSITIVE
4	100	0	500	150	POSITIVE
16	50	0	700	250	POSITIVE
7	50	0	0	100	POSITIVE
19	50	50	250	0	POSITIVE
3	0	150	0	100	POSITIVE
12	0	750	0	50	POSITIVE
14	0	0	50	150	POSITIVE
36	-	-	-	400	-
65	-	-	-	250	-
69	-	-	-	0	-
66	-	-	-	0	-
Average	29.16	529.16	187.5	140.62	-

Table 5.2: Oocyst counts of calves (n=16) raised organically at Massey University Dairy Research Unit.

5.3.2. Study of Organic Beef Farms:

(a) Farm B: Oocyst counts for young calves on Farm B are shown in Table 5.3. They were generally low with only 6 samples in both age groups being over 1000 oocysts/g and all were <5000 oocysts/g. Some cows were shedding oocysts but many had zero counts. The autumn-born calves had the highest counts amongst the 3 groups, which was a mean of 1567 oocysts/g in the month of November.

ID	19 Nov 02	20 Dec 02	28 Mar 03
Calves born 3rd week of October			
Calf 59	1500	650	200
Calf 54	0	800	-
Calf 45	50	250	50
Calf 53	150	300	-
Calf 46	50	-	-
Calf 60	0	-	500
Mean	292	500	250
Adult Cows			
Cow 36	0	0	-
Cow NT1	0	0	-
Cow 20	100	-	-
Autumn born			
6	1150		
5	450		
3	900		
2	700		
4	4850		
7	1350		
Mean	**1567**		

Table 5. 3 : Oocyst counts oocysts/g of calves, their dams and autumn born bull calves estimated on up to 3 occasions on Farm B. Note: '-' means no sample is obtained.

ID	19 Nov 02	20 Dec 02	28 Mar 03
Calves born 1st week of September			
Calf 32	2050	500	200
Calf 15	950	0	300
Calf 21	550	500	150
Calf25	300	0	600
Calf 27	50	1350	350
Calf 26	50	200	2750
Calf12	350	200	-
Calf 11	1500	200	-
Calf 30	250	-	150
Calf 29	2250	-	600
Mean	**830**	**200**	**1167**
Cow 25	0	0	600
Little heifer	50	50	-
Cow Twinkle	50	0	-
NT2	0	-	-
cow 4	0	-	-

Table 5. 3 : Oocyst counts oocysts/g of calves, their dams and autumn born bull calves estimated on up to 3 occasions on Farm B. Note: '-' means no sample is obtained.

(b) Ballantrae Hill Country Research Station:

	Sep	Dec	Mar
NC-C	106	35	638
CO-C	128	35	255
NC-D	25	10	10
COD	5	32	25

Table 5.4: Arithmetic Mean oocyst counts (ocysts/g)of No Chemical organic calves(NC-C), their dams (NC-D) and Conventional calves (CO-C), and their dams (CO-D) from farmlets at Ballantrae Hill Country Research Station.

As shown in the Table 5.4 the oocyst counts of all the calves from Ballantrae Organic Unit were low men ranged (100 – 154) oocyst/g in September lower at the second sampling occasion in December (35 - 50) oocysts/g and rose slightly to (638) oocysts/g in the month of March. See Appendix 5.1. Fig. 5.1 and Fig. 5.2 show the details of pooled status of the oocyst counts of the conventional and No-Chemical calf and dam groups. The No-Chemical organic groups had slightly higher counts at the last collection but this was not so in the first collection as the Conventional 1 group had higher counts over the No-Chemical animals.

DF	Num DF	Den DF	F Value	Pr>F
Treatment	1	23	3.01	0.0964
Month	2	50	18.60	0.0001
Treatment x month	2	50	2.70	0.0771
Group(Treatment)	2	23	0.24	0.7862

Table 5.5: Type 3 tests of fixed effects of time series analysis for oocyst counts of Calves on Ballantrae Farm. Treatment is Conventional or No-Chemical.

Effect	Num DF	Den DF	F Value	Pr>F
Treatment	1	15	1.34	0.2659
Month	2	34	0.88	0.4260
Treatment x month	2	34	1.61	0.2155
Group(Treatment)	2	15	3.15	0.0719

Table 5.6: Type 3 tests of fixed effects of time series analysis for oocyst counts of cows on Ballantrae farm.

As shown in the Table 5.5 there was no significant effect of treatment, and interaction of month and treatment in calves at ($p>0.01$) and no significant effect of group nested for treatment but a significant effect of the month (>0.0001) was seen. That means the oocyst counts were different in different months but the there was no significant difference in between oocyst counts of No-Chemical groups and the conventional groups.

Similarly, in cows (Dams) as shown in Table 5.6 there was no effect of treatment, month, interaction between treatment and the month and group at ($p>0.01$) that means the there was no difference between in oocyst counts of the cows of both groups.

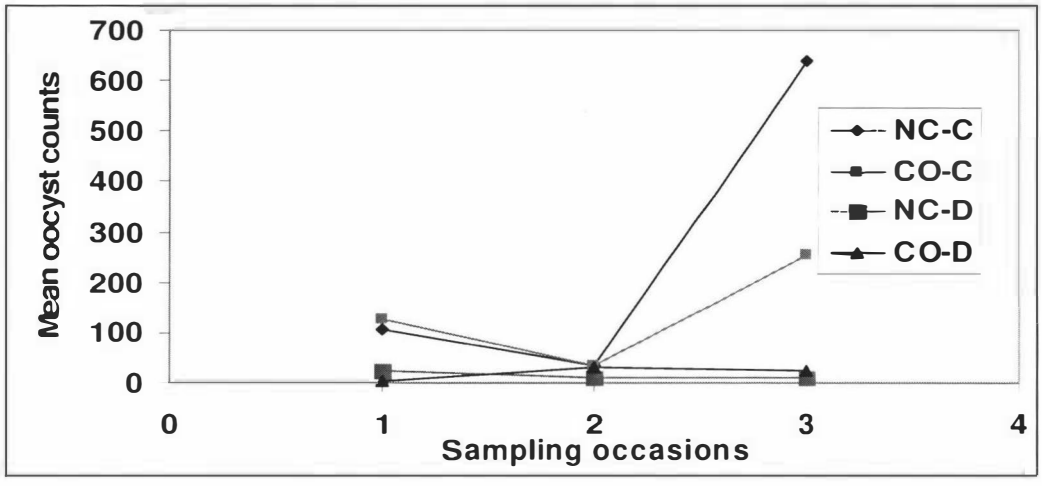


Figure 5. 1: Comparison of Mean oocyst counts of No Chemical organic calves (NC-C) , their dams(NC-D) and Conventional calves(CO-C) , their dams(CO-D) on 3 sampling occasions (1-Sep, 2-Dec, 3-Mar).

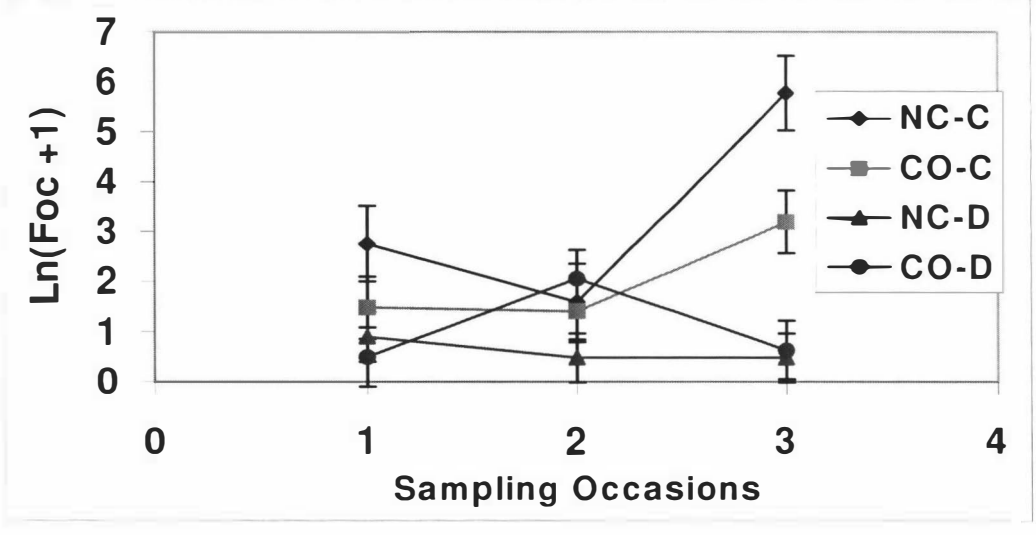


Figure 5. 2 :Comparison of log-transformed oocyst counts of No Chemical organic calves (NC-C) , their dams (NC-D) and Conventional calves (CO-C) , their dams (CO-D) on 3 sampling occasions (1-Sep, 2-Dec, 3-Mar). Note:Foc=Faecal oocyst count, Ln =log.

5.3.3. Commercial Calf Rearers and Farmers:

(a) Apiti commercial calf rearer:

The young calves (n=10) in the calf-rearing shed that were being fed meal containing monensin and some milk replacer were all negative for oocysts in their faeces except 1 calf being positive on a float. The second group (n=10), which were grazing and just weaned were negative on counts. The autumn-born calves had 3 out of 7 calves positive for coccidia on a float.

(b) Stafford commercial farm:

Young calves were sampled on one occasion (24th December) and these results are shown in Table 5.7

Animal ID	Oocysts/g
1	200
2	550
3	450
4	2150
5	3370
6	400
7	150
8	1400
9	950
10	50
Average	967

Table 5.7: Oocyst counts (oocysts/g) of calves on Stafford commercial farm

(c) Alley commercial farm:

A total of 8 calves of mixed ages of 3-8 weeks old were sampled and the oocyst counts are shown in Table 5.8. Calves were with their mothers. Oocyst counts were generally low. In addition, 3 cows examined were negative for oocysts.

Calf no.	Oocyst counts
1	450
2	1300
3	200
4	300
5	50
6	700
7	Negative
8	2350

Table 5.8: Oocyst counts (oocysts/g) of calves from Alley commercial farm.

5.4. Prevalence Species identified on other farms:

A total of 10 species were identified in this study; details are shown in Table 5.9. The most prevalent species was *E. bovis (28.9%)* followed by *E. zuernii (25.8%), E. wyomingensis (11.6%), E. auburnensis (11.1%), E. bukidnonensis (8.6%), E. brasiliensis (4.5%), E.canadensis (4.0%), E. cylindrica (3.0%), E. ellipsoidalis (2.0%) and E. subspherica (1.0%).*

I.D.	*E. zuernii*	*E. bovis*	*E. canadensis*	*E. cylindrica*	*E. ellipsoidalis*	*E. brasiliensis*	*E. auburnensis*	*E. subspherica*	*E. bukidnonensis*	*E. alabamensis*	*E. wyomingensis*
07B	4	17	0	0	0	0	3	0	1	0	11
04Ball	4	17	0	0	0	0	3	0	1	0	11
06Ball	12	0	0	2	0	1	0	0	15	0	0
011Ball	1	1	0	0	0	8	0	0	0	0	0
059Ball	17	14	5	0	0	0	0	0	0	0	0
032Ball	11	3	0	0	0	0	15	0	0	0	0
Apiti 6	2	3	2	4	4	0	0	1	0	0	1
Massey organic	0	1	1	0	0	0	1	1	0	0	0
Total	51	56	8	6	4	9	22	2	17	0	23
Percentage	**2.8**	**28.9**	**4.0**	**3.0**	**2.0**	**4.5**	**11.1**	**1.0**	**8.6**	**0**	**11.6**

Table 5.9: Total Counts of species identified from different animals on different farms. Note: B=Farm B, Ball=Ballantrae

5.5. Discussion:

Cows on all farms showed mild infections as observed in earlier studies (Svensson, 1981; Faber, 2002; Marquardt 1961; Balconi, 1963; Fitzgerald, 1961). This mild infection in the dams could be the source of infection to calves in all farms.

Massey No.4:

The organic calves had higher counts than the conventional calves in the Massey study, which is likely to be due to the use of monensin in the feed of conventional calves even though they were grazing in separate areas. Several experiments have been conducted on calves and lambs infected with coccidia to test the efficacy of monensin in controlling coccidiosis (Stromberg *et al*, 1986, Genchi *et al.*, 1989, Fitzgerald and Stockdale, 1981). Oocyst counts of conventional calves were lower than the organic calves as the coccidiostat suppresses parasite growth when it is present in the system, when it is withdrawn, disease can recur (Chapman 1999).

Farm B:

In Farm B, different age group animals had different levels of infection. One month old calves had a mean oocyst count of 292 oocysts/g which rose to 500 when they were 2 months old and counts were only 250 when the calves were 6 months old. In their dams, the mean count was 33 oocysts / g, but only once did any of the cows shed the parasite. The oocyst counts in the autumn-born calves which were about 6months old were quite low. This is probably consistent with the development of immunity in these calves.

The calves born in the first week of September which were two months old when sampled were shedding a mean of 830 oocysts/g. By the time they were 3 months they were shedding only 200 oocysts/g; but at the age of 6 months they were shedding the highest count of 1166 oocysts/g. This series suggests some changes over time but even at the highest mean count was not particularly high. Their dams shed oocysts on all occasions but the average counts were only 50, 50 and 600 oocysts/g. Such low oocyst counts are expected in adult cattle.

Commercial Farms:

Apiti farm had three calf groups of which two groups were still being fed with meal containing monensin so the counts were predictably very low in these two groups although they were still shedding oocysts (detectable on float). One group (autumn-born) showed moderate infection which was higher than that noted for Farm B. Stafford Farm had meal added with monensin until they were weaned. The animals were sampled 1 week after weaning and had relatively high counts ranging from 50-3370 oocysts/g. This is consistent with results from the two Massey studies reported in Chapters 2 and 3 where there was an increase in oocyst counts immediately after weaning.

Several studies have showed different percentages of prevalence in different age groups. In a study in Wisconsin, the calves of < three months, 3 - 6 month old and 7-12 months had 40-51%, 46-27% and 11-16% prevalence (Harsche *et al.*, 1959). Calves of 1 month old and up to weaning had the highest prevalence of 86.3% in USA (Ernst *et al.*, 1985). The calves <3 weeks of age had lowest counts of oocysts in New Zealand reflecting the prepatent period of the parasites (Osborne and Ensor,1952) and in Wisconsin 40% prevalence was seen in 2 week to one month old calves.

Several conditions on farms such as contamination of paddocks, stocking density, rearing with adult cows, use of coccidiostats and climate had influenced the intensity of the disease in previous studies (Matjila and Penzhorn, 2001; Niilo, 1970; Pavlaseck, 1984; Chibunda *et al.*, 1996) and will have influenced the results in the present study.

The number of oocysts produced also depends on other factors as the inherent potential of each species to reproduce in a non-immune host, immunity developed by the host, crowding factor, competition with other species, other infectious agents, nutrition of the host, strain differences of the host and parasite, stress factors and anticoccidial drugs (Fayer, 1980).

Ballantrae Farm: There are two different management systems compared on this farm. One is raised using conventional farming approach whilst the other is farmed without the use of chemicals. There are two separate farmlets of each. On the No-chemical farmlets there is alternative grazing of sheep and cattle to keep nematode parasite burdens low as only a few parasites of sheep cross infect cattle. So neither the No-Chemical group nor Conventional groups have any access to coccidiostats which is consistent with usual management of beef cattle. Though these animals were not given any coccidiostat the coccidia burden was comparatively very low. There was a slight difference between NC and CO groups both in calves and cows, but the differences were not significant statistically. The cows of both groups were shedding coccidia on all occasions but counts were very low.

5.6. Conclusions:

This study revealed that all conventionally reared calves which were treated with monensin up to weaning had low coccidial burdens when compared to organically reared calves. The infection seems to recur when the calves were weaned and monensin feeding was withdrawn. Adult cows were also infected most of the time and the infection was very mild. The different conditions between farms are likely to have a role on the status of infection. There were about 10 *Eimeria* species isolated from this study. The most prevalent species was *E. bovis* (28.9%) followed by *E. zuernii* (25.8%). Of note is the fact that these are considered the two most pathogenic species. The next two most prevalent species were *E. wyomingensis (11.6%)* and *E. auburnensis* (11.1) which are generally considered to be the next in order of pathogenicity. The least pathogenic species were generally not very prevalent and included *E. bukidnonensis* (8.6%), *E. brasiliensis* (4.5%), *E. canadensis* (4.0%), *E. cylindrica* (3.0%), *E. ellipsoidalis* (2.0%) and *E. subspherica* (1.0%).

Chapter 6: Western blotting:

6.1. Introduction:

In the studies reported in Chapters 3 and 4 calves were regularly bled to monitor the development of immune response and there antibody levels. Although it is well accepted that cattle develop an immune response to *Eimeria* there have been few attempts to measure it. This chapter will report an attempt to measure this immunity using the technique of Western blotting. This technique is reviewed in Section 1.8.9.d. Western blotting has been used in poultry to identify the antigenic and immunogenic proteins in *Eimeria maxima* as part of a project to produce a vaccine in chicken (Wallach *et al.*, 1993, 1989; Smith *et al.*, 1994). The stage specific differences in surface antigens produce different bands and the intensity of the bands reflect the degree of protection (Wallach, 1994).

Similar trials with *E. bovis* identified protein bands from merozoites and sporozoites ranging from 15,000-215,000 kDa and the immuno-blots on nitrocellulose with immunized calves had binding proteins of 18, 000 to 180,000kDa for merozoites and 28,000 to 118,000kDa for sporozoites. Both sporozoites and merozoites had common bands at 58,000, 70,000, 83,000 and 98,000kda but only the 183,000kda protein elicited an IgG antibody response (Reduker and Speer, 1986). Antibodies peak in 2 to 3 weeks after infection and remain detectable for about 40 days in calves (Hughes *et al.*, 1989; Ferron *et al.*, 1965; Faber, 2002).

The aim of this study was to develop a western blotting technique for use with cattle and use this to measure the immunity of calves to *Eimeria* species in the studies reported in Chapters 3 and 4.

6.2. Materials and methods:

Calves of Massey No.4 study (Chap 3) were bled every week for 18 weeks. The sera were extracted and kept at -20ºC until used for testing. Similarly, sera were collected from calves of the Tuapaka study (Chapter 4) at the time of toltrazuril treatment and once at 5 weeks post-treatment with toltrazuril and also

stored at -20°C until used. These sera were used as a primary antibody in the Western blotting technique described below.

6.2.1. Parasites:

Oocysts of *Eimeria* were collected from the faeces of calves from all studies when an individual sample had a reasonably high oocyst count. These were sporulated and then cleaned as described in Appendix 6.1.

6.2.2. Production of parasite antigens:

Several different procedures were tried to isolate parasite proteins from oocysts. After each procedure samples were examined for proteins by SDS-Page. See Appendix.6.3. These included the following:

6.2.2.1 French press:

A total of 60,650 cleaned oocysts in 2ml of PBS were passed through a French Cell press (French Press is a cell press which uses pressure to fracture the oocysts) (7,000psi) three times in an attempt to fracture the oocysts. The whole volume of fluid which passed through the French Press was used as a source of protein on the gels.

6.2.2.2. Vortexing with glass beads:

Vortexing with glass beads was performed as described in Appendix 6.2. In brief, clean oocysts in a concentrated pellet were mixed with an equal volume of glass beads (0.5mm diameter) in 2ml hard plastic cryo-tubes. These were subject to vortexing in a bench vortex machine for 5-10 second intervals of 5 cycles. After each vortex the suspension was examined under the microscope to see whether the oocysts were fractured or not.

6.2.2.3. Freezing and thawing:

This method involves quick freezing of oocysts in freeze resistant tubes in liquid nitrogen and then thawing the oocysts in boiling water. This was repeated for 12 freeze-thaw cycles. Samples were examined to see how many oocysts had fractured and then this whole volume was used on gels for detecting protein.

6.2.2.4 Combination of vortexing with glass beads, freezing and thawing, and sonication:

As earlier methods were unsuccessful to produce enough proteins on gels, a combination of all 3 above described methods together with sonication (2 cycles) was tried to get as many oocysts to fracture and release proteins as possible. Sonication was performed in 2ml Ependorf tubes using a [Sonics- Vibra m Cell –Ultrasonic Processor- Code-SN VCX 500] at 2 cycles of 25 seconds. Tubes were kept on ice while sonication was performed. The whole volume of fluid was used as a protein source for gels.

6.2.2.5 Tissue culturing:

The oocyst pellet was subjected to combination of all 3 procedures as detailed in 6.2.2.4 above (vortexing with glass beads, freezing with liquid nitrogen and 2 cycles of sonication). An attempt was made to establish a cell culture line using MDBK cells to produce more parasites and hence more antigen for use in gels. MDBK cells were established in a monolayer with $4x10^4$ cells in 10 ml of medium as the initial seeding volume. Prepared oocysts were added when the number of cells had increased to $5x10^5$ cells/ml. The cells were grown using 1x Minimum Essential Medium (Gibco, Invitrogen Corp., Cat.NO.12492-013) with 1% glutamax (GIBCO, Invitrogen Corp.Cat.NO.350350-061), non-essential amino acids (Gibco, Invitrogen Cat.No.11140-050 -5ml), a combination of 10,000iu penicillin and 10,000µg streptomycin (Penicillin-Streptomycin Gibco, Invitrogen Corp.No.15140-122) per 1ml and 25µg/ml photericin (Fungizone-Gibco, Invitrogen Corp.Cat.No.15290-018), 2% sodium bicarbonate (sodium bicarbonate 7.5% Gibco-Invitrogen-cat.No.25050-094) and 10% foetal calf serum (Foetal Bovine Serum- Gibco, Invitrogen Corp. Cat. No.100093-144). After the initial inoculation, this medium was changed to a maintenance media which was similar except that, only 2% foetal calf serum was included. The media were changed every 3rd day.

The cell cultures were monitored daily for at least two weeks to spot the first motile merozoites. Motile structures resembling merozoites were seen at the end of the second week and counted. After 2 weeks the whole culture was

harvested by disrupting the cell monolayer manually and the medium was centrifuged to concentrate any parasite stages present together with cell debris. Because sufficient numbers were not obtained for the use in Western blotting after 2 weeks, the original culture was split and put onto a new monolayer to increase the numbers.

The merozoites were centrifuged, counted with cell counter and treated with 2% sodium dodecyl sulphate (SDS, BDH. Code No. 108073), 10% glycerol, $6.25x10^{-2}$ M tris (Hydroxymethyl) aminomethane (Invitrogen. Code. No. 75504-020), 4% mercaptoethanol (BDH - Code.No.75504-020) in a boiling water bath for 10 minutes at a ratio of $6x10^{6}$ merozoites per 10 µl of solution as described by Reduker and Speer (1986).

6.2.3. Western Blotting:

The proteins obtained by the different procedures were quantified by optical density to estimate protein concentration using a spectrophotometer [Helios-UNICAM-UV-VIS Spectrometer-Sl.No.UVA070707, 1999]. Prior to using the spectrophotometer a protein estimation kit (Bio-rad) which binds to protein was added to the fractured oocyst preparation.

The proteins obtained by the above procedure were run every time on 10% SDS PAGE [Sodium Dodecyl Sulphate – Polyacrylamide Gel electrophoresis). See Appendix 6.3 for the detailed Western blotting procedure. On each occasion to confirm some proteins were present the gels were stained with 0.1% Coomassie blue stain and/or silver staining (Bio-Rad, Catalog No. 161-0443). If SDS PAGE showed any bands then the blotting procedure continued. PDF [Bio-Rad- Ready Gel Blotting sandwiches-Immuno-Blot PVDF filter Papers] membrane or nitrocellulose membrane was then placed on the gel to transfer the separated proteins. After transfer, the membrane was then incubated in 1% skim milk powder solution for 1 hour to block non-specific proteins.

The membranes were then probed with the test sera from the calves at several concentrations including 1:10, 1:100 and 1:1000 dilutions. Any bound antibody was detected with rabbit anti-bovine peroxidase - labelled IgG as the secondary

antibody to identify any proteins specific to *Eimeria*. Several dilutions of secondary antibody were used from 1:1000 to 1:80,000. Westfemto was then added to the PDF which will react with the peroxidase and indicate the presence of bands. The PDF was then radiographed to demonstrate the presence of bands.

A positive control of *Neospora* antigen was used for detection of protein using Coomassie Blue and silver staining and also as a positive control for the Western Blotting procedure.

6.3. Results:

All attempts to recover protein were frustrated by the small number of oocysts available.

6.3.1. French press cell:

The washed (cleaned) oocysts were passed through the French cell (7,000psi) and only 700 un-fractured oocysts were seen in the recovered 2ml volume suggesting most oocysts had been fractured. However, no proteins were seen on SDS gels after Coomassie Blue staining or silver staining.

6.3.2. Vortexing with glass beads:

This method fractured few oocysts and separated of fractured oocysts from the beads was difficult. This method did not yield sufficient protein to see on a SDS gel.

6.3.3. Freeze -thaw.

The original oocyst pellet had 49,150 oocysts and each 0.5µl had 273 oocysts before freezing and thawing. The SDS gels showed no protein band on the gel.

6.3.4. Combination of Vortexing, freeze- thawing, and sonication.

All remaining oocysts were subject to this procedure. No protein bands were seen.

6.3.5. Tissue culturing:

When no bands were seen, the remaining volume containing motile sporozoites was used for inoculating the cell culture monolayer. After 2 weeks of culture merozoites were harvested and counted and proteins were separated as detailed processed as described in 6.2.2.5

The Figure 6.3 shows the details of different proteins separated on the nitrocellulose membrane. Bands were visualised by staining with Ponceau S staining. As shown in the Figure 6.3, tissue culture grown *Eimeria* contained many bands on gels of different molecular weights. There were faint bands above the level of 11kDa and in between 17 and 24 there were two bands and another at the level of 24kDa. In between 33 to 72kda 2 faint and 1 prominent bands were noted. In between 100 to 170kDa, a few faint bands were seen.

When compared to uninfected MDBK cells there are a small number of bands that were present in the infected cells compared to the uninfected as seen in Fig 6.1 and 6.2. The gels showed several bands 1-2 below 20 kda , 3 bands in between 20-30 one prominent band around 35 kda, 45 kDa and 55kDa and bands were also seen at 70, 80 and above 120kDa. The quality of the gels is poor as the availability of material to do repeats to improve the quality was low.

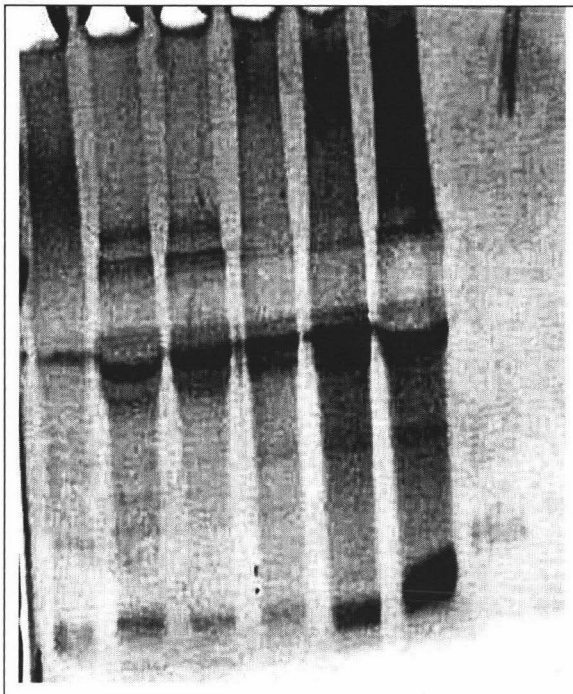


Figure 6.1

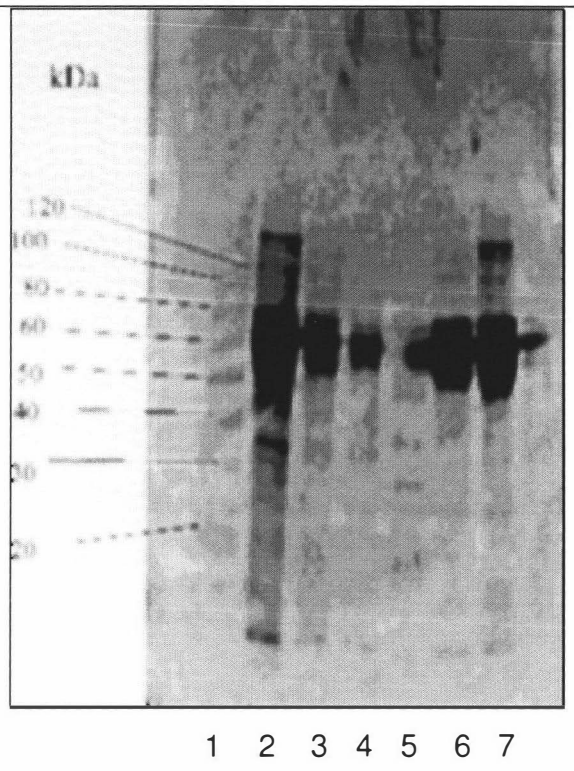


Figure 6.2

Figure 6. 1: Silver stained SDS-PAGE gels. Lanes 1-3 contain proteins from MDBK culture containing *Eimeria* proteins loaded at volumes of 5µl, 10µl and 20µl per lane. Lanes 4-6 contain proteins from uninfected MDBK cultures at the same respective volumes

Figure 6. 2: 0.1% Coomassie stained SDS-PAGE gels. Lanes 2-4 contain proteins from MDBK culture containing *Eimeria* proteins loaded at volumes of 20µl, 10µl and 5µl per lane. Lanes 5-7 contain proteins from uninfected MDBK cultures at the same respective volumes.

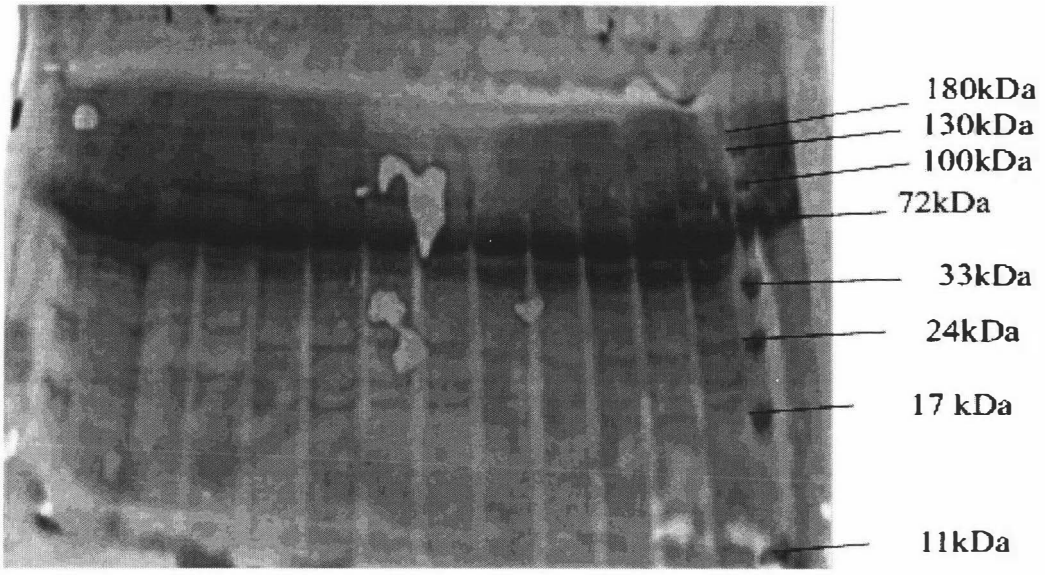


Figure 6. 3: Different antigenic proteins of *Eimeria* on Nitrocellulose membrane from cell culture grown parasites after transfer from SDS-PAGE gels, stained with 0.2% Ponceau S.

6.4. Discussion:

Tissue culture has been used to grow *Eimeria* and study the micro- structure of *E. bovis (*Hammond *et al*, 1966 and 1969, Fayer and Hammond, 1967).

In this study, this procedure has been adopted to multiply the numbers *in vitro* as the parasite numbers obtained otherwise were few to carry out the work. The oocysts were very tough and many procedures tried in this study further wasted the few oocysts obtained. All the methods were unsuitable for cracking the oocysts and each procedure had its own limitations. Immuno-blotting was used in chicken to estimate the antibody titers and isolate the immunogenic proteins which lead to the development of vaccine to chicken *Eimeria*. Immuno-blotting to SDS-PAGE separated sporozoite antigen with sera of the birds treated with toltrazuril detected strong antibody response to three antigens of 14 to 20kDa molecular weight which titre was also corresponded to the reduction of oocyst excretion and lesions (Grief, 2000).

In cattle only a few studies have been conducted to identify the immunogenic proteins. In such earlier studies proteins were identified from *E. bovis* merozoites and sporozoites (Reduker and Speer, 1986). The merozoites and sporozoites had proteins which ranged from 47 to 125kDa.and cell lysate proteins. Merozoite specific proteins were at 15, 17, 39, 152 and 180kDa.
In this present study there were faint bands above the level of 11kDa (probably at 15 kDa) and in between the 17 and 24 there were two bands and one at the level of 24kDa. In between 33 to 72kDa 2 faint and 1 prominent band were noted. In between 100 to 170kDa there were also a few faint bands seen. These are similar to the proteins identified earlier from merozoites (Reduker and Speer, 1986).

Smaller sized 14–20 kDa proteins were recorded in *Eimeria* of chicken (Grief, 2000) and cattle (Redukar and Speer, 1986) which are immuno-dominant are also seen in this study.

The principle reason for failure to utilise the western blotting technique for assessing serological status of the calves was the limited number of available oocysts from which to recover antigen. No attempt was made to probe the gels with calf sera.

Conclusions: The object of this study was to standerdise the Western blotting technique and estmate the immunity using it. The protein extraction from available oocysts using procedures like French press, freezing and thawing, vortexing with glass beads was unsuccessful. So tissue culture technique was used to multiply the parasite numbers. Though tissue culturing technique was time consuming did it produce enough parasites for protein estimation. Several parasite specific protein bands of different sizes (one at 15kda, two between 17-24, one at 24kda, two faint and one prominent bands between 33-72kDa and few bands between 100 -170kDa) were identified on nitrocellulose gels
but probing with calf sera was not successful.

Chapter 7: Final Discussion:

7.1. Pattern of oocyst shedding and monensin treatment:

The research in this project has involved studying oocyst shedding by calves in a number of different situations. For the study on Massey No.4 dairy farm the earliest born calves on the farm were used and as a result had only a limited exposure to oocysts that were present in the calf paddocks for prior to calving before arriving on the experiment. Nevertheless oocysts were seen 2 weeks after arrival with a peak at 3-3.5 weeks indicating they did not get infected.The relatively large size of the grazing cells meant that little re-infection probably occurred over the course of the experiment because the initial contamination by diluting any oocysts. In addition, the experimental area was not previously used for calf rearing and had only been grazed by adult cattle which would have had generally low oocyst counts and hence the area would have only had a low level of contamination with *Eimeria* oocysts. Consequently it is not surprising that oocyst counts remained low in this experiment. By contrast the calves at Tuapaka were raised under standard commercial conditions and oocyst counts were higher.

Monensin should contribute to lower oocyst counts in treated animals (Stockdale 1973 &1981; McDougald, 1978; Fitzgerald 1984). The monensin-added meal-fed animals had comparatively lower counts but untreated animals also had low numbers, possibly because of good managerial practices in the paddock. Commercial farms Apiti and Stafford farm calves had oocyst counts very low when they were on monensin added feed but a week after the monensin withdrawal, the oocyst count increased (200-3370 oocyst/g) as observed by others (Chapman, 1999).

The organic farms had higher oocyst counts than the conventional calves in the Massey study, which is likely due to the monensin in the feed of conventional calves suppressing the parasite growth (Chapman, 1999).

Several studies have showed different percentages of prevalence in different age groups. In a study in Wisconsin, the calves of >3months , 3-6 months old and 7-12 months had 40-51%, 46-67% and 11-16% prevalence (Harsche *et al.*,1959).

Calves of 1 month old and up to weaning had the highest prevalence of 86.3% in USA (Ernst *et al.,* 1985). The calves >3weeks of age had lowest counts of oocysts in New Zealand reflecting the prepatent period of the parasite(Osborne and Ensor, 1952) and in Wisconsin 40% prevalence was seen in 2 week to one month old calves.

Similarly, in this study on Farm B, different age groups showed differ level of infection. In two different groups from this farm the oocyst counts of 1 month old calves were 292oocyst/g, two month old had 500 to 800 oocysts/g and six month old had oocyst counts of 250-1166 oocysts/g.

Cows on all farms showed mild infections as observed in earlier studies (Svensson, 1981; Faber, 2002; Marquardt, 1961; Fitzgerald, 1961). This mild infection in the dams could be the source of infection to calves in all farms.

7.2. Weight up to Weaning:

In most of the experiments that have been reported monensin improved the live weight gain in lambs and calves (Foreyt and Wescott, 1984; Fitzgerald, 1984; MacDougald, 1978; Goodrich, 1984; Stockdale, 1981). But in some studies monensin did not improve weight gains in lambs with naturally acquired infections (Horton and Stockdale, 1981; MacDougald, 1978). In this present study monensin had no significant ($p>0.05$) effect on weight gains over untreated calves. This may be because monensin consumption was not enough during initial period of 4 weeks as the amount of meal eaten supplied less than the required 1mg/kg monensin (McDougald, 1978; Stromberg *et al.,* 1986) and the milk had no coccidiostats included. It could also reflect the low oocyst counts in the group without monensin in their feed, especially after 4 weeks.

When there was a developing immune response is also hard to determine. The immunity to the parasite depends on the previous exposure and the level of exposure (Stockdale and Yates,1978; Senger, 1959; Niilo, 1979), which in this experiment appeared to be low. A large dose of infection provides good immunity and the immunity lasts for at least 40 days (Hughes *et al.,* 1989; Ferron *et al.,* 1965; Faber, 2002).

7.3. Single oral treatment at weaning and oocyst shedding after treatment:

A single treatment of the calves with toltrazuril significantly reduced the oocyst counts for up to 4 weeks post treatment in both Massey No.4 and Tuapaka) and the oocyst counts of the calves which were not treated were higher over this period. Toltrazuril-treated calves showed a reduction in the oocyst counts within one week of treatment consistent with toltrazuril effectively killing all the intracellular stages of life cycle and all the species of *Eimeria* (Froyman and Grief, 2002). This study is in agreement with previous studies in goats where the oocyst counts were reduced almost to zero levels by toltrazuril treatment and then kept very low for 30 days post treatment (McKenna, 1988, Taylor and Kenny, 1988).

However, although toltrazuril reduced the counts after treatment there were still some oocysts were passed in treated calves. Hence, any effect on FOC is only likely to persist until a new infection becomes patent. There have been several studies with various ruminants. In lambs weekly toltrazuril treatment (20 mg/kg) reduced their oocyst counts to low levels for the 10 weeks of study and this kept FOC low (Stafford et al, 1994). In 3 different trials with goats, a single treatment with toltrazuril resulted in rapid and significant reduction of oocyst counts in treated goats (McKenna, 1988). A single treatment to lambs with toltrazuril (20 mg/kg) reduced oocyst counts for up to 34 days.

7.4. Weight gain with toltrazuril:

The improvement in the weight gain in the 6 week period after weaning was significant with > 5kgs difference between treated and untreated animals in Massey No.4 study. Similarly, in Tuapaka study, treated calves in Group A gained >8kgs live weight in the 5 weeks after the treatment over the untreated calves. The increased live weight gain of treated animals started to be apparent 3 weeks after treatment giving an impression that the oocyst count reduction during these three weeks might have helped the animals to gain weight. Treated calves in Group B also had an improved weight gain over 4kgs over untreated calves. Overall there was no significant effect of treatment on live weights ($p>0.05$) although the live weights of treated animals were improved after treatment with toltrazuril. The significant interaction of liveweight with time

and treatment ($p<0.01$) is indicative of a divergence in live weight gain between treated and untreated animals.

These growth rates occurred despite low counts in control calves during the immediate post weaning time. However, although the oocyst counts were low in these calves, the species predominating in these calves were *E. bovis and E. zuernii* which are the most pathogenic species in cattle *Eimeria* (Ernst and Benz, 1986).

7.5. Stress around weaning:

There was a rise in the oocyst counts after weaning, of the animals which were not treated with the toltrazuril until the 4th week post treatment. Both treated and untreated had similar oocyst counts around Week 5 post treatment.

The rise in the untreated calves might have been because of the stress of weaning or else because of the withdrawal of monensin allowing inhibited stages to mature. These two factors might both operate independently or together, but this study was not able to determine which the case was. These animals were subjected to several stressful conditions at the time of weaning such as vaccination, change of diet (no supplemental diet), bleeding, weighing, drenching (coccidiocide) and removal of shelter, (pens removed from the paddock).

The actual magnitude of the increase in oocyst count was not very great in the two Massey studies where it was followed none of the animals developed clinical disease even though there was a difference between the toltrazuril-treated and untreated calves.

Several conditions on the farm such as contamination of paddocks, stocking density, rearing with adult cows, use of coccidiostats and climate had influenced the intensity of the disease in previous studies (Matjila and Penzhorn, 201; Niilo, 1970; Pavlaseck, 1984; Chibunda *et al.,* 1996) and will have influenced the results in the present study.

The number of oocysts produced also depends on other factors studied so far as the inherent potential of each species to reproduce in a non-immune host, immunity developed by the host, crowding factor, competition with other

species, other infectious agents, nutrition of the host, strain differences of the host and parasite, stress factors and anti-coccidial drugs (Fayer, 1980).

7.6. Management on farm:

Management conditions and climate conditions will influence numbers of infective oocysts available to the calves (Matjila and Penzhorn, 2001; Hasbullah *et al.*, 1990; Chibunda *et al.,* 1996; Salisbury *et al.,* 1955; Niilo, 1970). In the Tuapaka study, bull calves from three different rearers were on coccidiostats up to weaning and had different levels of infection. This suggests that management on the farms had an influence on the oocyst counts. Group A calves had the highest mean oocyst counts (343 t0 4120 oocysts/g) where as Group B were lower means (33 to 336 oocysts/g) whilst Group C had mean counts of 0 to 760 oocysts/g.

On Ballantrae farm there are two different management systems compared. One is raised using conventional farming approach whilst the other is farmed without the use of chemicals. There are two separate farmlets of each. On the No-chemical farmlets there is alternative grazing of sheep and cattle which is not usually the case on the conventional farmlets. Neither have any access to coccidiostats which is consistent with usual management of beef cattle. Despite this the coccidia burden was comparatively very low in both treatments. There was a slight difference between NC and CO groups both in calves and cows, but the differences were not significant statistically. The cows of both groups were shedding coccidian on all occasions but counts were very low. This will reflect the more extensive nature of beef cattle farms.

7.7. Prevalence and Predominance of *Eimeria* species:

In cattle 21 *Eimeria* species have been described (Ernst, 1980) and the prevalence's of the species differed globally. In New Zealand 2 studies described 11 *Eimeria* species (McKenna, 1972 and 1974; Arias, 1993). In this present study, 11 *Eimeria* species were isolated and de-described. The two most prevalent species over all farms were *E. zuernii* (95.2%) and *E. bovis* (87%) followed by *E. auburnensis* (62%), *E. cylindrica* (42%), *E. bukidnonensis* (36%), *E. canadensis* (31%), *E. subspherica* (27%), *E. elipsoidalis* (24%), *E. wyomingensis* (23%), *E. alabamemsis* (12%) and *E. brasiliensis* (12%).

The most predominant species was *E. bovis* (31.1%) followed by *E. zuernii* (26.5%), *E. auburnensis* (12.7%), *E. bukidnonensis* (6.8%), *E. cylindrica* (6.3%), *E. wyomingensis* (5.3%), *E. canadensis (4.4%), E. ellipsoidalis* (1.9%), *E. brasiliensis* (1.9%), *E. subspherica* (1.5%), and *E. alabamensis* (1%). The most prevalent and predominant species in New Zealand (Andrew, 1954), *E. bovis* and *E. zuernii* were reported to be the most pathogenic (Ernst and Benz, 1986).

It is usual for multiple species to be observed in any one faecal sample, with an observed average of 3.5 and as many as 8 species present (Oda and Nishida, 1989; McKenna, 1972) and a similar pattern was observed in this study.

7.8. Western Blotting:

Disruption of oocyst cell wall using procedures like the French press, Freezing and thawing, Overtaxing with glass beads was found to be unsuccessful. However, the combined of Vortexing, grinding after each Freeze-and –thaw cycle in liquid nitrogen (5-7 cycles), and Sonication was successfully used to crack many oocysts.

This study identified tissue culturing technique as the best way to multiply the parasite numbers to produce enough parasites for protein estimation. In this study, several parasite protein bands of different sizes (one at 15kda, two between 17-24, one at 24kda, two faint and one prominent bands between 33-72kda and few between 100-170kda) were identified on nitrocellulose gels which were similar to previous studies (Reduker and Speer, 1986).
Unfortunately it was not possible to pursue with this procedure to analyse collected serum.

Appendix. 2.1. Oocyst counts:

A total oocyst count was carried out on a 2g-sub-sample of sample collected. In addition, oocysts were recovered from a further sub-sample and sporulated, and the species present identified in a random sample of 100 oocysts. The details of the procedures are shown in appendix 3.3. 1.

In brief, oocysts were counted using a modified McMaster technique, using salt saturated as the flotation media and where each oocyst counted represented 50 oocysts per gram. Oocysts were recovered from positive samples as described in Appendix 3.3.2 In brief a 5g sample of faeces was homogenized in water, subjected to flotation in saturated salt and sporulated at 27°C for 7 days in 2% H_2SO_4.

Appendix. 2.1.1. Method of identification of species:

Most samples contained mixed infections. Identification of unsporulated oocysts is difficult. Many species have specific structural characteristics that can only be seen clearly in fully sporulated oocysts. In the present study, therefore, species were only identified after sporulation.

Species were identified according to descriptions given by the following authors. Levine (1985) and Levine & Ivens (1986).

Species were identified on the characteristics considered in the following order:

1. Presence and absence of micropylar cap and its characteristics.
2. The oocyst size and shape.
3. Characteristics of the micropyle if present (distinct or indistinct).
4. Number of polar granules.
5. Size and shape of sporocysts.
6. Presence or absence of sporocyst residuum and its characteristics, if present.
7. Presence or absence of stieda body.
8. Position of sporozoites in the sporocyst.
9. Number and size of refractile globule in each sporozoite.

To determine oocyst and sporocyst dimensions for comparative purposes and statistical analysis, 100 oocysts of each species were measured using an Olympus BH2 microscope with apochromatic objectives and a digital micrometer. The oocysts measured for each species were from samples collected from different farms on different days.

Appendix. 2.2. Table showing multiple species present in calves: Note: Numbers followed by T are animal no.s

	Total				Total
0T					
E.zuernii	7				
E.canadensis	13				
E.bovis	4				
E.cylindrica	4				
E.ellipsoidalis	2				
11T					
21-Nov		**14-Nov**		**29-Nov**	
E.zuernii	3	E.zuernii		E.zuernii	5
E.bovis	21	E.bovis		E.bovis	9
E.brasiliensis	6	E.brasiliensis		E.cylindrica	7
E.auburnensis	3			E.auburnensis	4
56					
24-Sep		**24-Oct**		**29-Oct**	
E.bovis	8	Un sporulated		E.zuernii	15
E.zuernii	1			E.subspherica	4
E.auburnensis	1			E.cylindrica	9
4T					
18-Nov		**26-Nov**			
E.brasiliensis	3	E.zuernii	17		
E.auburnensis	8	E.bovis	13		
25					
25-Nov		**18-Nov**		**26-Nov**	
		E.bukidnonensis	9	E.alabamensis	2
E.bovis	6	E.bovis	5	E.zuernii	20
E.zuernii	17	E.auburnensis	16	E.bovis	6
E.subspherica	8				

E.wyomingensis 1

29

18-Nov

E.bukidnonensis 12
E.bovis 3

E.canadensis 16
E.auburnensis 8

5-Nov

E.zuernii 6
E.alabamensis 9

E.subspherica 3
E.cylindrica 3

11-Nov

E.auburnensis 27
E.bovis 3

14-Nov

E.zuernii 13
E.bovis 17

18

26-Nov

E.alabamensis

E.zuernii
E.bovis

18-Nov

E.auburnensis
E.bukidnonensis
E.bovis

5-Nov

Unsporulated

12-Dec

E.bovis

57

26-Nov

E.bovis 1

E.cylindrica 4
E.canadensis 3

E.wyomingensis 6

5-Nov

E.canadensis 9

E.cylindrica 19
E.zuernii 3

E.subspherica 2

24-Sep

E.zuernii 4

E.canadensis 5
E.bovis 3

e.cylindrica 1
E. subspherica 2

18-Nov

E.canadensis 11

E.cylindrica

29-Oct

E.aburnensis 13

E.bovis 2
E.zuernii 5

5-Nov

E.canadensis 30

E.cylindrica
E.zuernii
E.subspherica

11-Nov

E.zuernii 4
E.bukidnonensis 1
19

E.canadensis 6

15

29-Nov

E.bukidnonensis 23
E.zuernii 3

E.ellipsoidalis 7

24-Sep

E.zuernii
E.canadensis
E.bukidnonensis

Dec-05

E.canadensis 1
E.zuernii 1

E.bovis 2
E.cylindrica 2

24-Sep

E.canadensis 10
E.bovis 8

21-Oct

E.canadensis 2

32

24-Sep

E.canadensis
E.zuernii

26-Nov

E.zuernii 9
E.bovis 11

24-Sep

E.bovis 1
E.auburnensis 6

E.subspherica
E.bovis 1
E.ellipsoidalis

61T
29-Nov
E.zuernii 13
E.cylindrica 17
E.wyomingensis

67T
29-Nov
E.zuernii 11
E.bovis 10
E.ellipsoidalis 9

8T
3-Nov
E.zuernii 12
E.bovis 18

16
18-Nov
E.auburnensis 40
E.zuernii 3

E.cylindrica 1

117
18-Nov
E.zuernii 5
E.wyomingensis 25
39
6-Nov
E.bovis 9

E.ellipsoidalis 10

E.bukidnonen
sis 5+3
E.auburnensis 2

68T
5-Dec
E.auburnensis 3
E.zuernii
E.subspherica 9
E.bovis 12
67T
21-Nov
E.bovis 3
E.zuernii 2
E.ellipsoidalis 5

4-Nov
E.bovis 15
E.zuernii 14
E.auburnensis 1

12-Sep
E.zuernii
E.canadensis

17

E.zuernii 3
E.cylindrica 3

29-Oct
E.zuernii 3
E.bukidnonen
sis 1

14-Nov
E.ellipsoidalis 10
E.bovis 2
E.zuernii 12
E.auburnensis 6
66T
5-Dec
E.zuernii 8
E.bovis 13
E.aburnensis 9

5-Nov
E.zuernii
E.cylindrica

E.ellipsoidalis
E.canadensis
E.auburnensis
118

Un sporulated

11-Nov
E.bovis 11

E.zuernii 13

12-Dec
E.bovis 12
E.ellipsoidalis 5
E.zuernii 6
E.aburnensis 8

11-Nov
E.zuernii 10
E.ellipsoidalis 8
E.bukidnonen
sis 18

20
29-Oct
E.zuernii 2

E.zuernii	6
E.cylindrica	3

116
5-Nov

E.zuernii	11
E.cylindrica	9
E.ellipsoidalis	8
E.aburnensis	2

38T
14-Nov

E.zuernii	14
E.bovis	1
E.canadensis	9
E.bukidnonensis	2

18T
14-Nov

E.bovis	25
E.zuernii	5

29T
14-Nov

E.zuernii	
E.bovis	

20T
7-Nov

E.zuernii	1
E.canadensis	5
E.bovis	7

12T
7-Nov

E.aburnensis	1

9
24-Sep

E.zuernii	5
E.auburnensis	2
E.bovis	24
E.bukidnonensis	1

21-Nov

E.zuernii	3
E.bovis	13
E.wyomingensis	8
E.auburnensis	6

12-Dec

E.bovis	7
E.auburnensis	1
E.alabamensis	2

E.aburnensis	2
E.alabamensis	2

12-Dec

E.zuernii	4
E.bovis	3
E.wyomingensis	17

E.zuernii
E.bovis
E.cylindrica
13T

7-Nov		**21-Nov**	
E.zuernii	10	E.zuernii	21
			13-Jan
E.bovis	13	E.bovis	
E.auburnensis	8	E.auburnensis	1
E.subspherica	2		

71T

7-Nov	
E.bovis	21
E.zuernii	9

22T

3-Nov		**5-Dec**	
E.zuernii	9	E.bovis	25
E.bovis	21	E.zuernii	5
E.canadensis	1		

38T

E.zuernii	3
E.bovis	27

10T

7-Nov	
E.bovis	27
E.zuernii	3

31T

21-Nov	
E.zuernii	6
E.canadensis	7
E.wyomingensis	5
E.auburnensis	7
E.bovis	5

19T

27-Nov

E.zuernii	
E.bovis	20
E.canadensis	6
E.bukidnonensis	?1

57T

21-Nov

E.zuernii	25
E.bukidnonensis	5

50T

29-Nov

E.zuernii	5
E.auburnensis	1
E.bovis	1

51T

21-Nov

E.bovis	
E.zuernii	14
E.wyomingensis	7
E.alabamensis	3
E.auburnensis	6

39T

21-Nov

E.zuernii	2
E.aburnensis	28

72T

21-Nov

E.zuernii	6
E.bovis	21
E.wyomingensis	4

15T

12-Dec

E.bovis	12
E.cylindrica	9
E.alabamensis	5
E.auburnensis	5
E.zuernii	3

21-Nov

E.bovis	28
E.zuernii	5

29-Nov

E.zuernii	3
un sporulated	

72T

29-Nov

E.bovis	2
E.zuernii	20
E.cylindrica	8

51T

E.bovis	24
E.zuernii	7
E.wyomingensis	1

5-Dec

E.bovis	12
E.brasiliensis	5
E.auburnensis	2
E.zuernii	18

29-Nov

E.bovis	18
E.zuernii	7
E.auburnensis	7

6T

29-Nov		**5-Dec**	
E.zuernii	13	E.bovis	19
E.bovis	17	E.zuernii	9
		E.bukidn	5

29-Nov- 44 T

E.bovis	21
E.zuernii	5
E.wyomingensis	1

74T

29-Nov		**5-Dec**	
E.bovis	15	E.bovis	23
E.cylindrica	6	E.zuernii	8
E.subspherica	2	E.auburnensis	3
E.zuernii	7		

63T

12-Dec		**5-Dec**	
E.auburnensis	2	E.bovis	7
E.bovis	8	E.zuernii	2
E.zuernii	1		

59T

29-Nov		**5-Dec**	
E.zuernii	11	E.auburnensis	3
E.bovis	13	E.bovis	18
E.cylindrica	6	E.zuernii	8
		E.cylindrica	2

O7 DB

19-Nov

E.bovis	17
E.zuernii	4
E.auburnensis	3
E.bukidnonensi	1

s
E.wyomingensis 11
O4B
19-Nov
E.bovis 26
E.zuernii 4
E.brasiliensis 16
E.cylindrica 1
O6B

19-Nov
E.bukidnonensi
s 15
E.zuernii 12
E.cylindrica 2
E.brasiliensis 1
011B
19-Nov

E.brasiliensis 8

E.zuernii 1
E.bovis 1

059B

19-Nov
E.zuernii 11
E.bovis 14
E.auburnensis 15
E.zuernii 17

E.bovis 3

27T
E.cylindrica 5
E.auburnensis 23
E.auburnensis 1

032B

19-Nov

E.aburnensis 15

E.canadensis 11

E. cylindrica 2

	E.bovis 2
3 OT	
1 2-Dec	
E.bovis	2
E.auburnensis	1
E.zuernii	1
43T	
E.zuernii	8
E.alabamensis	10
E.bovis	10
E.auburnensis	2

Appendix. 2. 3. Species measurements : Note: L=Length, W=width, R= Ratio of L /w

no	***E. zuerni***			***E.subspericа***			***E.auburnensis***			***E.bukidnonensis***			***E.canadensis***			***E. bovis***			***E.cylindrica***			***E.ellipsoidal***			***E.wyomingensis***			***E.brasilliensis***			***E.alabamensis***		
No	L	W	R	L	W	ratio	L	W	R	L	W	R	L	W	R	L	W	R	L	W	R	L	W	R	L	W	R	L	W	R	L	W	R
1.0	19.5	15.1	1.3	14.3	9.0	1.6	28.4	22.0	1.3	36.2	48.0	1.3	21.8	26.0	1.2	25.6	17.4	1.5	28.4	15.6	1.8	24.8	17.7	1.4	41.5	28.7	1.5	37.1	32.0	1.2	18.4	12.2	1.5
2.0	21.4	16.2	1.3	14.3	9.0	1.6	33.2	20.8	1.6	30.0	44.3	1.5	18.9	25.7	1.4	28.9	16.5	1.8	20.5	15.0	1.4	24.9	20.4	1.2	36.2	27.8	1.3	39.4	32.0	1.2	17.4	11.4	1.5
3.0	22.0	16.0	1.4	9.0	8.2	1.1	32.0	21.0	1.5	45.2	52.2	1.2	21.0	27.4	1.3	28.5	17.0	1.7	28.7	16.0	1.8	25.8	19.6	1.3	37.5	26.1	1.4	33.2	32.5	1.0	18.9	14.6	1.3
4.0	20.7	14.7	1.4	9.0	8.6	1.1	28.7	28.7	1.0	32.0	44.0	1.4	21.6	28.3	1.3	20.0	17.8	1.1	20.1	13.0	1.6	24.2	20.2	1.2	39.4	31.1	1.3	31.0	26.4	1.2	19.2	12.6	1.5
5.0	20.9	18.2	1.1	9.7	8.6	1.1	48.0	28.0	1.7	29.4	48.0	1.6	19.2	28.5	1.5	24.5	20.0	1.2	21.3	13.4	1.6	25.5	23.9	1.1	37.6	29.6	1.3	27.2	23.1	1.2	18.6	13.3	1.4
6.0	19.3	15.9	1.2	11.7	11.3	1.0	43.2	30.6	1.4	30.6	35.4	1.2	26.6	28.3	1.1	29.3	18.2	1.6	18.8	27.0	0.7	27.2	20.8	1.3	32.0	22.4	1.4	28.8	20.6	1.4	19.7	12.2	1.6
7.0	16.6	14.6	1.1	14.4	11.8	1.2	43.5	29.9	1.5	32.4	41.8	1.3	24.0	24.9	1.0	25.6	17.4	1.5	24.9	17.4	1.4	26.4	19.2	1.4	31.0	21.4	1.5	28.6	25.7	1.1	16.8	11.5	1.5
8.0	17.3	15.3	1.1	14.5	11.2	1.3	48.0	25.0	1.9	41.6	53.8	1.3	20.6	24.2	1.2	23.9	14.0	1.7	24.9	20.9	1.2	24.4	20.7	1.2	32.5	24.6	1.3	45.1	29.5	1.5	20.2	14.4	1.4
9.0	20.3	14.4	1.4	14.7	14.2	1.0	46.6	31.2	1.5	32.7	45.5	1.4	28.2	26.2	0.9	22.0	17.3	1.3	22.5	16.1	1.4	26.8	18.6	1.4	46.4	25.2	1.8	42.2	32.6	1.3	21.8	16.2	1.3
10.0	22.2	18.4	1.2	14.9	14.0	1.1	45.4	26.3	1.7	37.2	51.0	1.4	30.6	35.4	1.2	23.4	16.0	1.5	27.5	18.9	1.5	26.4	20.4	1.3	47.7	31.8	1.5	36.0	30.6	1.2	18.7	15.9	1.2
11.0	17.6	16.0	1.1	14.0	13.0	1.1	45.6	32.8	1.4	33.0	50.8	1.5	17.9	25.0	1.4	23.0	16.4	1.4	16.4	14.1	1.2	27.2	20.8	1.3	39.2	26.9	1.5	38.6	30.2	1.3	21.0	14.4	1.5
12.0	18.2	15.9	1.2	12.8	12.8	1.0	48.0	27.2	1.8	32.2	45.1	1.4	16.0	28.8	1.8	27.2	20.0	1.4	23.8	19.5	1.2	27.7	11.0	2.5	46.9	26.2	1.8	39.5	30.2	1.3	23.5	15.4	1.5
13.0	19.4	15.0	1.3	13.4	12.2	1.1	48.8	26.7	1.8	36.4	55.0	1.5	24.0	27.8	1.2	24.7	16.3	1.5	20.5	17.1	1.2	25.5	17.2	1.5	34.0	27.0	1.3	35.0	24.3	1.4	20.8	13.5	1.5
14.0	21.4	16.0	1.3	13.4	10.3	1.3	40.0	31.0	1.3	35.4	50.0	1.4	21.6	29.5	1.4	25.0	19.1	1.3	25.2	18.8	1.3	25.9	19.2	1.4	34.4	29.1	1.2	40.8	24.9	1.6	15.1	12.2	1.2
15.0	20.8	14.8	1.4	13.0	10.7	1.2	32.8	17.6	1.9	31.6	48.6	1.5	18.8	29.1	1.6	29.2	17.8	1.6	23.7	17.6	1.4	21.5	18.7	1.2	46.1	29.8	1.5	40.8	28.7	1.4	18.3	17.2	1.1

no	***E. zuerni***	***E.subspericа***	***E.auburnensis***	***E.bukidnonensis***	***E.canadensis***	***E. bovis***	***E.cylindrica***	***E.ellipsoidal***	***E.wyomingensis***	***E.brasilliensis***	***E.alabamensis***

16.0	21.2	16.2	1.3	12.6	12.0	1.1	37.6	27.8	1.4	30.8	50.1	1.6	26.2	35.0	1.3	27.0	18.8	1.4	21.2	16.8	1.3	28.8	20.0	1.4	48.2	24.0	2.0	48.0	41.4	1.2	24.0	15.2	1.6
17.0	21.2	17.4	1.2	13.4	11.7	1.1	45.6	28.7	1.6	30.0	48.2	1.6	24.0	36.0	1.5	27.2	19.2	1.4	25.6	19.7	1.3	28.2	17.4	1.6	47.3	27.2	1.7	37.6	30.2	1.2	19.8	17.6	1.1
18	19	17 4	1.1	14	13	1.1	40	24	1.6	31.	51	1.6	21.	29	1.4	23	20	1.1	24	17.	1.4	27	22.	1.2	49	27	1.8	40	28	1.4	24.	19	1.3
19.0	21.3	16.2	1.3	13.0	13.0	1.0	48.0	29.4	1.6	32.6	50.2	1.5	32.8	35.0	1.1	25.1	21.0	1.2	25.9	17.2	1.5	24.7	18.6	1.3	41.2	29.6	1.4	38.2	28.6	1.3	21.8	17.6	1.2
20.0	19.7	14.5	1.4	14.1	13.8	1.0	32.0	22.7	1.4	30.4	48.6	1.6	26.3	33.7	1.3	23.0	19.6	1.2	28.3	17.2	1.7	25.6	18.0	1.4	46.3	27.6	1.7	37.8	27.5	1.4	26.0	23.2	1.1
21.0	21.0	13.8	1.5	14.2	13.2	1.1	30.4	30.4	1.0	29.4	43.0	1.5	20.3	24.6	1.2	24.2	17.0	1.4	24.7	17.3	1.4	23.9	20.5	1.2	34.2	24.0	1.4	41.1	33.4	1.2	17.0	15.0	1.1
22.0	19.9	18.9	1.1	14.4	11.6	1.2	43.3	28.0	1.6	30.5	49.8	1.6	14.4	25.0	1.7	23.4	19.8	1.2	24.7	17.2	1.4	27.9	16.8	1.7	48.7	28.8	1.7	37.1	32.0	1.2	20.2	15.6	1.3
23.0	19.0	15.2	1.3	11.7	11.1	1.1	37.3	25.2	1.5	31.3	46.3	1.5	21.0	29.5	1.4	27.4	18.5	1.5	17.8	25.0	0.7	20.3	17.0	1.2	46.4	25.2	1.8	39.5	32.0	1.2	19.2	16.2	1.2
24.0	16.3	15.2	1.1	13.6	12.0	1.1	43.0	28.2	1.5	36.0	49.6	1.4	21.4	30.1	1.4	23.0	17.0	1.4	26.8	18.6	1.4	28.8	21.0	1.4	47.7	31.8	1.5	33.2	32.5	1.0	18.6	18.5	1.0
25.0	19.2	13.2	1.5	10.2	8.9	1.1	34.6	25.6	1.4	33.4	49.4	1.5	19.2	30.0	1.6	22.3	16.3	1.4	28.2	20.0	1.4	23.8	18.4	1.3	46.9	26.2	1.8	31.0	26.4	1.2	19.9	15.9	1.3
26.0	18.4	14.4	1.3	10.0	10.0	1.0	31.9	19.6	1.6	28.8	33.4	1.2	27.8	38.7	1.4	24.6	19.2	1.3	27.1	20.0	1.4	27.0	17.6	1.5	39.2	26.9	1.5				24.0	15.8	1.5
27.0	20.0	19.9	1.0	15.2	7.2	2.1	43.0	28.1	1.5	32.9	50.9	1.6	25.3	30.3	1.2	24.4	24.4	1.0	20.9	16.2	1.3	26.3	20.8	1.3	34.0	27.0	1.3				21.2	13.0	1.6
28.0	19.0	16.0	1.2	15.1	13.5	1.1	31.0	25.0	1.2	38.9	53.7	1.4	22.2	25.6	1.2	22.4	17.0	1.3	23.4	18.2	1.3	25.0	19.0	1.3	34.4	29.1	1.2				21.6	14.8	1.5
29.0	21.0	17.0	1.2	14.4	13.6	1.1	28.6	19.5	1.5	36.9	51.7	1.4	24.8	28.0	1.1	23.1	18.4	1.3	23.1	15.4	1.5	27.0	18.0	1.5	36.2	27.8	1.3				21.0	16.4	1.3
30.0	15.0	14.4	1.0	14.4	12.0	1.2	29.9	26.8	1.1	33.8	49.4	1.5	20.6	33.7	1.6	23.5	17.4	1.4	24.4	18.0	1.4	25.2	18.7	1.4	41.5	28.7	1.5				22.4	15.8	1.4
31.0	14.1	10.3	1.4	9.8	9.4	1.0	28.5	16.0	1.8	37.8	51.6	1.4	22.2	24.6	1.1	24.6	20.3	1.2	21.8	13.8	1.6	25.2	18.7	1.4	37.5	26.2	1.4				21.7	17.6	1.2
32.0	20.2	14.2	1.4	15.0	14.4	1.0	47.3	26.8	1.8	34.1	41.6	1.2	26.6	29.1	1.1	26.7	16.0	1.7	19.2	12.9	1.5	22.0	18.0	1.2	39.4	31.1	1.3				21.0	16.0	1.3
33.0	21.0	13.0	1.6	14.4	12.4	1.2	32.0	24.0	1.3	27.2	47.3	1.7	21.6	31.4	1.5	25.6	15.6	1.6	23.0	16.0	1.4	20.2	18.9	1.1	46.2	34.4	1.3				18.0	13.0	1.4
34.0	16.0	13.9	1.2	9.8	9.4	1.0	31.6	31.0	1.0	33.4	48.2	1.4	22.4	29.4	1.3	28.0	16.2	1.7	27.4	13.6	2.0	24.8	15.0	1.7	36.2	29.3	1.2						
35.0	17.4	12.8	1.4	15.0	14.4	1.0	33.6	22.4	1.5	27.2	47.3	1.7	22.8	30.0	1.3	23.0	16.9	1.4	22.4	12.7	1.8	22.8	16.0	1.4	42.6	30.2	1.4						

no			*E. zuerni*			*E.subspericа*			*E.auburnensis*			*E.bukidnonensis*			*E.canadensis*			*E. bovis*			*E.cylindricа*			*E.ellipsoidal*			*E.wyomingensis*			*E.brasilliensis*			*E.alabamensis*		
36.0	15.6	11.8	1.3	17.8	17.0	1.1	39.4	24.2	1.6	34.8	55.2	1.6	21.0	28.8	1.4	24.8	16.2	1.5	18.5	14.5	1.3	22.9	25.0	0.9	36.5	26.0	1.4									
37.0	18.6	13.4	1.4	16.0	15.0	1.1	34.8	32.0	1.1	31.2	46.2	1.5	21.2	29.9	1.4	25.1	17.2	1.5	23.2	12.8	1.8	26.0	17.6	1.5	34.8	28.0	1.2									
38.0	22.7	22.7	1.0	16.0	14.4	1.1	29.9	23.4	1.3	28.8	48.7	1.7	20.5	27.1	1.3	25.5	18.9	1.4	19.2	14.9	1.3	23.0	18.8	1.2	30.6	24.6	1.3									
39.0	21.0	13.3	1.6	14.9	13.2	1.1	32.3	23.0	1.4	24.0	34.2	1.4	19.0	27.7	1.5	24.6	16.9	1.5	19.7	12.2	1.6	26.4	18.6	1.4	37.9	30.9	1.2									
40.0	18.6	17.6	1.1	10.3	10.2	1.0	34.7	23.3	1.5	27.6	46.3	1.7	20.6	31.4	1.5	32.0	19.2	1.7	18.9	14.9	1.3	24.2	14.4	1.7	36.4	29.1	1.3									
41.0	24.0	20.9	1.2	16.0	15.2	1.1	31.9	21.6	1.5	37.8	53.4	1.4	21.8	26.0	1.2	22.2	22.2	1.0	22.6	16.5	1.4	24.8	18.3	1.4	44.4	29.0	1.5									
42.0	19.5	17.5	1.1	14.0	8.7	1.6	31.3	24.5	1.3	37.0	48.6	1.3	21.4	30.6	1.4	23.6	17.2	1.4	19.3	12.7	1.5	25.1	19.7	1.3	37.0	30.0	1.2									
43.0	19.4	17.8	1.1	15.0	11.8	1.3	32.9	22.2	1.5	37.6	48.4	1.3	19.0	26.0	1.4	23.1	18.9	1.2	15.5	11.8	1.3	24.2	19.2	1.3	34.9	27.1	1.3									
44.0	18.2	18.7	1.0	16.0	13.5	1.2	34.4	24.9	1.4	32.5	45.6	1.4	26.3	27.2	1.0	22.7	17.9	1.3	27.8	19.8	1.4	24.2	14.4	1.7	36.0	25.0	1.4									
45.0	21.3	17.3	1.2	10.2	9.0	1.1	33.0	26.0	1.3	31.2	42.2	1.4	23.0	32.0	1.4	24.3	16.9	1.4	24.0	12.2	2.0	29.6	14.8	2.0	35.4	27.6	1.3									
46.0	22.8	18.6	1.2	16.0	14.2	1.1	32.9	21.6	1.5	26.8	46.4	1.7	21.3	26.6	1.3	23.5	18.7	1.3	20.0	16.0	1.3	22.9	16.3	1.4	32.0	25.0	1.3									
47.0	19.4	19.4	1.0	13.5	11.8	1.2	31.7	20.0	1.6	27.2	46.8	1.7	20.4	30.5	1.5	22.4	19.0	1.2	28.8	20.8	1.4	22.1	13.9	1.6	34.0	23.1	1.5									
48.0	20.8	17.0	1.2				32.0	24.8	1.3	25.0	41.9	1.7	20.0	31.5	1.6	23.8	19.8	1.2	25.0	16.0	1.6	26.1	19.2	1.4	30.0	23.1	1.3									
49.0	20.8	16.2	1.3				30.4	20.4	1.5	23.5	39.6	1.7	26.8	34.3	1.3	23.8	20.2	1.2	20.5	13.9	1.5	23.7	18.0	1.3	33.6	26.0	1.3									
50.0	20.2	16.0	1.3				33.2	20.8	1.6	25.0	37.2	1.5	17.0	27.4	1.6	24.2	20.7	1.2	18.0	14.9	1.2	28.0	18.0	1.6	46.4	25.5	1.8									
51.0	21.6	16.3	1.3				32.5	24.0	1.4	24.2	31.8	1.3	26.6	38.4	1.4	25.7	21.8	1.2	23.8	13.0	1.8	25.2	19.4	1.3	37.2	28.2	1.3									
52.0	20.0	16.2	1.2				31.8	21.0	1.5	24.8	37.9	1.5	26.3	35.2	1.3	27.2	22.4	1.2	30.1	15.8	1.9	28.6	18.3	1.6	40.2	30.9	1.3									
53.0	18.8	13.7	1.4				31.7	22.2	1.4	30.3	42.2	1.4	22.5	35.2	1.6	26.4	18.6	1.4	25.2	14.6	1.7	22.8	18.4	1.2	35.7	24.3	1.5									

no			*E. zuerni*			*E.subspericaca*			*E.auburnensis*			*E.bukidnonensis*			*E.canadensis*			*E. bovis*			*E.cylindrica*			*E.ellipsoidal*			*E.wyomingensis*			*E.brasilliensis*			*E.alabamensis*		
54.0	19.7	15.4	1.3				45.4	26.3	1.7	35.2	48.2	1.4	17.6	28.2	1.6	22.0	16.2	1.4	23.8	17.0	1.4	24.8	18.0	1.4	37.8	26.0	1.5									
55.0	20.8	16.8	1.2				45.6	32.8	1.4	26.5	45.4	1.7	18.3	24.8	1.4	24.3	16.0	1.5	22.9	14.1	1.6	23.5	18.0	1.3	32.6	27.0	1.2									
56.0	21.3	16.2	1.3				43.5	28.7	1.5	28.4	40.8	1.4	18.6	26.4	1.4	26.0	20.6	1.3	24.8	16.6	1.5	24.4	17.7	1.4	38.0	28.0	1.4									
57.0	21.8	15.6	1.4				48.0	28.0	1.7	24.2	40.4	1.7	19.2	25.1	1.3	23.2	15.2	1.5	26.0	14.4	1.8	21.8	15.9	1.4	35.8	27.0	1.3									
58.0	22.0	18.6	1.2				43.5	29.9	1.5	25.0	48.0	1.9	22.2	30.1	1.4	24.0	18.8	1.3	23.6	14.6	1.6	24.0	21.9	1.1	43.4	28.4	1.5									
59.0	19.3	12.7	1.5				48.0	24.7	1.9	35.4	52.6	1.5	23.9	31.1	1.3	22.4	18.4	1.2	25.4	13.9	1.8	26.5	17.2	1.5	39.4	31.1	1.3									
60.0	16.3	14.0	1.2				46.0	31.2	1.5	36.0	49.8	1.4	25.9	30.6	1.2	21.0	16.8	1.3	17.8	13.5	1.3	28.0	20.2	1.4	41.5	28.7	1.5									
61.0	15.5	15.5	1.0				42.0	22.4	0.5	31.8	56.0	1.8	22.0	31.0	1.4	22.4	16.4	1.4	21.4	20.0	1.1	26.6	20.2	1.3	37.5	26.2	1.4									
62.0	17.0	15.9	1.1				29.9	20.8	1.4	32.6	44.6	1.4	20.0	27.4	1.4	22.2	16.0	1.4	25.4	25.4	1.0	25.3	21.0	1.2	37.0	28.0	1.3									
63.0	15.2	13.8	1.1				45.4	26.3	1.7	33.3	47.6	1.4	20.6	27.8	1.4	22.7	17.1	1.3	27.0	14.3	1.9	27.4	19.2	1.4	38.0	30.0	1.3									
64.0	17.0	17.0	1.0				45.6	32.8	1.4	28.0	42.2	1.5	16.6	22.6	1.4	25.4	17.1	1.5	18.4	18.4	1.0	21.1	18.3	1.2	41.8	28.0	1.5									
65.0	14.9	14.3	1.0				43.5	28.7	1.5	24.3	45.8	1.9	22.6	25.6	1.1	22.0	22.0	1.0	27.0	15.4	1.8	27.0	19.2	1.4	38.0	26.3	1.4									
66.0	15.3	13.4	1.1				48.0	28.0	1.7	33.8	49.0	1.5	20.3	30.9	1.5	24.7	19.2	1.3	20.0	15.0	1.3	21.1	18.3	1.2	35.6	26.7	1.3									
67.0	16.9	13.7	1.2				43.2	30.6	1.4	28.6	48.3	1.7	21.8	26.0	1.2	22.9	17.8	1.3	22.4	15.0	1.5	27.0	19.2	1.4	40.2	32.0	1.3									
68.0	19.4	18.4	1.1				24.8	22.3	1.1	33.5	46.6	1.4	18.9	25.7	1.4	22.0	17.8	1.2	25.0	17.0	1.5	23.0	18.9	1.2	40.0	30.1	1.3									
69.0	18.8	17.6	1.1				43.5	29.9	1.5	31.4	47.6	1.5	21.0	27.2	1.3	21.6	16.0	1.4	25.0	17.0	1.5	24.6	19.6	1.3	37.5	27.0	1.4									
70.0	21.6	15.0	1.4				48.0	25.0	1.9	31.2	50.2	1.6	21.6	28.3	1.3	22.0	16.8	1.3	24.5	15.0	1.6	21.1	18.3	1.2	40.0	31.6	1.3									
71.0	24.6	18.9	1.3				46.6	31.2	1.5	35.0	52.2	1.5	19.2	28.5	1.5	22.2	18.4	1.2	25.9	17.4	1.5	22.0	16.0	1.4	39.2	30.1	1.3									

no			*E. zuerni*			*E.subspericaa*			*E.auburnensis*			*E.bukidnonensis*			*E.canadensis*			*E. bovis*			*E.cylindrica*			*E.ellipsoidal*			*E.wyomingensis*			*E.brasilliensis*			*E.alabamensis*		
72.0	19.0	19.0	1.0				32.0	21.0	1.5	29.6	45.6	1.5	21.9	34.0	1.6	24.0	16.0	1.5	28.4	15.4	1.9	22.6	20.0	1.1	33.0	28.0	1.2									
73.0	16.1	11.1	1.5				34.0	24.0	1.4	24.0	48.0	2.0	23.0	30.3	1.3	23.0	16.0	1.4	18.8	15.9	1.2	20.5	17.6	1.2	39.4	31.7	1.2									
74.0	17.0	10.9	1.6				40.4	24.2	1.7	34.2	49.6	1.5	19.2	32.3	1.7	23.4	17.8	1.3	25.0	14.4	1.7	24.3	16.9	1.4	41.5	28.7	1.5									
75.0	20.9	13.4	1.6				48.0	25.0	1.9	33.4	53.6	1.6	19.2	27.6	1.4	24.0	16.2	1.5	28.4	15.4	1.9	23.5	18.5	1.3	37.5	26.2	1.4									
75.0	20.9	13.4	1.6				48.0	25.0	1.9	33.4	53.6	1.6	19.2	27.6	1.4	24.0	16.2	1.5	28.4	15.4	1.9	23.5	18.5	1.3	37.5	26.2	1.4									
76.0	18.2	14.8	1.2				31.7	21.0	1.5	34.9	50.6	1.5	23.1	30.9	1.3	23.0	18.0	1.3	23.6	15.8	1.5	23.8	19.8	1.2	36.2	27.8	1.3									
77.0	16.8	16.8	1.0				32.0	24.0	1.3	36.4	51.0	1.4	22.1	30.6	1.4	23.0	17.3	1.3	23.0	14.0	1.6	22.4	20.2	1.1	45.8	24.3	1.9									
78.0	22.4	21.9	1.0				34.2	15.5	2.2	31.4	47.0	1.5	19.2	32.3	1.7	24.7	16.2	1.5	27.7	16.0	1.7	26.2	17.7	1.5	47.2	32.0	1.5									
79.0	20.0	17.8	1.1				33.0	20.8	1.6	31.6	45.1	1.4	24.0	26.0	1.1	22.2	16.0	1.4	27.4	17.6	1.6	23.1	18.9	1.2	49.6	33.8	1.5									
80.0	21.8	15.0	1.5				33.3	24.3	1.4	29.4	48.6	1.7	22.6	35.2	1.6	21.9	16.2	1.4	25.1	19.7	1.3	24.3	22.7	1.1	48.3	28.6	1.7									
81.0	15.0	15.0	1.0				39.4	24.2	1.6	32.8	49.0	1.5	17.6	28.2	1.6	20.0	16.2	1.2	24.2	19.2	1.3	25.0	17.9	1.4	42.2	27.5	1.5									
82.0	21.0	19.2	1.1				34.8	23.2	1.5	34.0	50.9	1.5	25.2	30.1	1.2	21.8	16.0	1.4	24.2	14.4	1.7	22.6	16.0	1.4	45.4	27.4	1.7									
83.0	18.9	15.8	1.2				29.9	23.4	1.3	31.0	37.6	1.2	23.9	31.1	1.3	24.0	16.6	1.5	29.6	14.8	2.0	22.7	18.0	1.3	48.0	29.6	1.6									
84.0	17.1	15.4	1.1				32.3	23.0	1.4	29.2	36.5	1.3	24.0	31.8	1.3	24.2	21.8	1.1	22.9	16.3	1.4	20.2	15.2	1.3	45.6	29.6	1.5									
85.0	20.5	17.6	1.2				34.7	23.3	1.5	29.5	38.4	1.3	25.9	30.6	1.2	24.0	16.6	1.5	22.1	13.9	1.6	26.0	16.4	1.6	48.6	29.9	1.6									
86.0	22.6	20.0	1.1				31.9	23.3	1.4	35.8	48.8	1.4	21.8	26.0	1.2	23.9	17.7	1.4	26.4	18.6	1.4	23.1	19.5	1.2	42.2	28.0	1.5									
87.0	19.9	16.5	1.2				30.9	21.6	1.4	35.4	40.0	1.1	18.9	25.7	1.4	22.0	17.5	1.3	24.8	24.8	1.0	24.6	19.2	1.3	36.8	29.9	1.2									
88.0	17.8	16.4	1.1				31.3	32.9	1.0	31.6	48.6	1.5	21.0	27.2	1.3	23.9	18.9	1.3	27.0	15.7	1.7	23.0	17.6	1.3	38.9	29.5	1.3									

no			*E. zuerni*				*E.subspericа*			*E.auburnensis*			*E.bukidnonensis*			*E.canadensis*			*E. bovis*			*E.cylindricа*			*E.ellipsoidal*			*E.wyomingensis*			*E.brasilliensis*			*E.alabamensis*		
89.0	18.4	18.4	1.0				31.3	24.5	1.3	27.0	50.0	1.9	21.6	28.3	1.3	22.9	17.7	1.3	24.0	13.6	1.8	23.4	19.8	1.2	36.5	29.2	1.3									
90.0	18.0	16.3	1.1				32.8	22.2	1.5	36.4	55.0	1.5	19.2	28.5	1.5	20.2	16.6	1.2	25.6	17.6	1.5	27.4	18.8	1.5	37.6	30.9	1.2									
91.0	20.3	20.0	1.0				34.4	25.0	1.4	35.0	51.6	1.5	26.6	28.3	1.1	23.0	20.5	1.1	24.0	17.6	1.4	22.6	17.2	1.3	42.4	29.5	1.4									
92.0	25.2	18.7	1.4				31.6	21.7	1.5	32.7	45.5	1.4	24.0	24.9	1.0	21.0	17.0	1.2	25.1	16.3	1.5	23.2	18.2	1.3	37.5	31.1	1.2									
93.0	20.2	18.1	1.1				38.2	30.8	1.2	41.6	53.8	1.3	20.6	24.7	1.2	25.6	19.0	1.3	28.0	16.4	1.7	25.1	17.4	1.5	39.4	31.1	1.3									
94.0	25.2	18.7	1.4				36.0	28.2	1.3	34.8	55.2	1.6	26.2	28.2	1.1	24.0	19.4	1.2	19.2	15.2	1.3	20.0	16.0	1.3	33.2	26.6	1.3									
95.0	22.0	18.0	1.2				35.0	25.6	1.4	33.4	48.2	1.4	30.6	35.4	1.2	23.6	19.8	1.2	21.9	15.5	1.4	21.6	19.8	1.1	32.0	21.6	1.5									
96.0	21.0	17.5	1.2				32.6	20.5	1.6	34.1	41.6	1.2	17.9	25.0	1.4	20.0	19.1	1.1	24.7	18.6	1.3	22.6	17.2	1.3	32.7	22.9	1.4									
97.0	19.5	18.9	1.0				32.0	20.0	1.6	30.5	49.8	1.6	24.0	27.8	1.2	23.5	19.5	1.2	22.0	21.2	1.0	22.9	18.7	1.2	36.6	28.0	1.3									
98.0	20.2	14.2	1.4				32.0	25.0	1.3	31.3	46.3	1.5	16.0	28.8	1.8	24.9	21.8	1.1	23.6	15.8	1.5	25.3	17.0	1.5	32.3	27.6	1.2									
99.0	19.4	17.8	1.1				32.4	23.4	1.4	36.0	49.6	1.4	17.4	23.7	1.4	23.6	19.2	1.2	27.4	17.8	1.5	26.6	18.7	1.4	32.0	25.6	1.3									
100.0	18.7	18.2	1.0				31.1	25.0	1.3	33.3	49.4	1.5	17.6	32.8	1.9	22.9	17.9	1.3	25.1	19.7	1.3	25.8	19.0	1.4	46.4	25.2	1.8									
Max	25.2	22.7	1.6	17.8	17.0	1.0	48.8	32.9	2.2	45.2	56.0	2.0	32.8	38.7	1.2	32.0	24.4	1.8	30.1	27.0	2.0	29.6	25.0	2.5	49.6	34.4	2.0	48.0	41.4	1.6	26.0	23.2	1.6
Min	14.1	10.3	1.0	9.0	7.2	1.2	24.8	15.5	0.5	23.5	31.8	1.1	14.4	22.6	1.6	20.0	14.0	1.0	15.5	11.8	0.7	20.0	11.0	0.9	30.0	21.4	1.2	27.2	20.6	1.0	15.1	11.4	1.0
Total	1951.2	1619.3	121.9	632.7	555.6	54.4	3695.8	2513.8	147.0	3195.9	4706.9	149.0	2193.2	2912.9	135.0	2398.1	1808.1	134.0	2373.0	1651.8	147.0	2463.0	1848.5	135.0	3936.7	2786.8	142.0	964.1	764.8	33.0	710.6	534.1	47.2

Sl. no.	*E. zuernii*	*Esubspericа*	*E.auburnensis*	*E.bukidnonensis*	*E.canadensis*	*E. bovis*	*E .cylindricа*	*E.ellipsoidal*	*E.wyomingensis*	*E.brasilliensis*	*E.alabamensis*

Appendix 2.4.1. Oocysts identified to species from the study on Massey University No. 4 Dairy Farm.

NO.	Id/Date	E.zuernii	E.bovis	E.canadensis	E.cylindrica	E.ellipsoidalis	E.brasiliensis	Auburnensis	E.subspherica	E.bukidnonensis	E.wyomingensis	E.alabamensis
1	58											
		16	8	0	9	0	0	1	4	0	0	0
2	25											
	Nov 7	15	0	0	10	0	0	0	5	0	0	0
	Nov 18	0	5	0	0	0	0	16	0	9	0	0
	Nov 25	17	6	0	0	0	0	0	8	0	1	
3	29											
	NOv5	6	0	0	3	0	0	0	3	0	0	9
	Nov 11	0	3	0	0	0	0	23	0	0	0	0
	Nov 14	13	17	0	0	0	0	0	0	0	0	0
4	18											
	Nov 18	0	7	0	0	0	0	7	0	16	0	2
	Nov 26	18	8	0	0	0	0	0	0	0	0	0
	Dec 12	0	6	0	0	0	0	0	0	0	0	0
5	57											
	Oct 29	5	2	0	0	0	0	13	0	1	0	0
	Nov 5	3	0	9	19	0	0	0	2	0	0	
	Nov 11	4	0	6	19	0	0	0	0	0	0	0
	Nov 26	0	1	3	4	0	0	0	0	0	6	0
6	15											
	Sep 24	2	8	10	0	0	0	0	0	0	0	0
	Nov 26	3	0	0	0	7	0	0	0	23	0	0
	Dec 5	1	2	1	2	0	0	0	0	0	0	
	32											
	Sep 24	1	1	13	0	0	0	7	0	0	0	0
	Nov 26	9	11	0	0	0	0	2	0	8	0	0
7	16											
		13	0	0	1	8	0	40	0	18	0	0
8	39											
	Oct	3	0	0	0	0	0	1	0	1	0	0
	Nov 6	6	9	0	3	10	0	0	0	0	0	0
	Nov 11	13	11	0	0	0	0	3	0	1	0	2
9	116											
	Nov 5	11	0	0	9	8	0	3	0	0	0	0
10	117											
11	Nov 18	5	25	0	0	0	0	0	0	0	0	0
12	113											
	Nov 26	16	7	0	0	10	0	0	0	0	0	0
	No.12	180	137	42	79	43	0	116	22	77	7	13
		12	10/12	3/12	8/12	5/12	0	9/12	4/12	7/12	2/12	3/25
	preval ence%	100	83.3	25	66.6	41.6	0	75	33.33	58.33	16.66	25

Appendix 2.4.2. Oocyst identification from calves from Tuapaka Farm. In total 23 faecal samples were examined

NO.	Id/Date	E.zuernii	E.bovis	E.canadensis	E.cylindrica	E.ellipsoidalis	E.brasiliensis	E.auburnensis	E.subspherica	E.bukidnonensis	E.wyomingensis	E.alabamensis
1	**40T**											
	Nov 21	7	4	13	4	2	0	0	0	0	0	0
2	**11T**											
	Nov 14	3	21	0	0	0	6	3	0	0	0	0
	Nov 21	13	9	0	0	0	4	5	0	0	0	0
	Nov 29	5	9	0	7	0	0	4	0	0	0	0
3	**4T**											
	Nov 7	15	0	0	9	0	0	0	4	0	0	0
	Nov 26	17	13	0	0	0	0	0	0	0	0	0
4	**61T**											
	Nov 29	13	0	0	17	0	0	0	0	0	0	0
5	**67T**											
	Nov14	12	2	0	0	10	0	6	0	0	0	0
	Nov 21	2	3	0	0	5	0	0	0	0	0	0
	Nov 29	11	10	0	0	9	0	0	0	0	0	0
	Dec 12	6	12	0	0	5	0	0	0	0	0	0
6	**8T**											
		12	18	0	0	0	0	11	0	0	0	0
7	**66T**											
	Nov 4	14	15	0	0	0	0	1	0	0	0	0
	Dec 12	8	13	0	0	0	0	9	0	0	0	0
8	**38T**											
	Nov 7	3	27	0	0	0	0	0	0	0	0	0
	Nov 11	5	25	0	0	0	0	0	0	0	0	0
	Nov 14	14	1	9	4	0	0	0	0	2	0	0
9	**18T**											
	Nov14	5	25	0	0	0	0	0	0	0	0	0
10	**29T**											
	Nov 14	13	17	0	0	0	0	0	0	0	0	0
	Nov 21	3	13	0	0	0	0	6	0	0	8	0
	Dec 12	4	3	0	0	0	0	0	0	0	17	0
11	**20T**											
	Nov 7	1	7	5	0	0	0	0	0	0	0	0
	Dec 12	0	7	0	0	0	0	1	0	0	0	2
12	**12T**											

	Nov 7	9	19	0	2	0	0	0	0	0	0	0
13	**8T**											
	Nov 11	12	18	0	0	0	0	0	0	0	0	0
	Nov 14	14	15	0	0	0	0	1	0	0	0	0
14	**13T**											
	Nov 7	10	13	0	0	0	0	8	2	0	0	0
	Nov21	21	13	0	0	0	0	1	0	0	0	0
15	**71T**											
	Nov7	9	21	0	0	0	0	0	0	0	0	0
16	**22T**											
	Nov 3	9	21	1	0	0	0	0	0	0	0	0
	Dec 5	5	25	0	0	0	0	0	0	0	0	0
17	**10T**											
	Nov 7	3	27	0	0	0	0	0	0	0	0	0
18	**31T**											
	Nov 21	6	5	7	0	0	0	7	0	0	5	0
19	**19T**											
	Nov 27	3	20	6	0	0	0	0	0	2	0	0
	Dec 12	3	12	0	9	0	5	0	0	0	0	5
20	**57T**											
	Nov 21	25	0	0	0	0	0	0	0	5	0	0
21	**50T**											
	Nov 21	2	28	0	0	0	0	0	0	0	0	0
	Nov 29	5	1	0	0	0	0	1	0	0	0	0
22	**51T**											
	Nov 21	7	21	0	0	0	0	0	0	0	2	0
	Nov 29	6	24	0	0	0	0	1	0	0	1	0
23	**39T**											
	Nov 21	2	0	0	0	0	0	28	0	0	0	0
24	**72T**											
	Nov 21	6	21	0	0	0	0	0	0	0	4	0
	Nov 29	20	2	0	8	0	0	0	0	0	0	0
	Dec 5	18	12				5	2	0	0	0	0
25	**15T**											
	Nov 29	7	18	0	0	0	0	7	0	0	0	0
26	**6T**											
	Nov 29	13	17	0	0	0	0	0	0	0	0	0
	Dec 5	9	19	0	0	0	0	0	0	5	0	0
27	**44T**											
	Nov 29	5	21	0	0	0	0	0	0	0	1	0
28	**74T**											
	Nov 29	7	15	0	6	0	0	0	2	0	0	0

	Dec 5	8	23	0	0	0	0	3	0	0	0	0
29	**63T**											
	Dec 5	2	7	0	0	0	0	0	0	0	0	0
	Dec 12	1	8	0	0	0	0	1	0	0	0	0
30	**59T**											
	Nov 29	11	13	0	6	0	0	0	0	0	0	0
	Dec 5	8	18	0	2	0	0	3	0	0	0	0
31	**27T**											
	Nov 29	0	2	11	2	0	0	15	0	0	0	0
	Dec 12	0	0	0	0	0	0	30	0	0	0	0
32	**30T**											
	Dec 12	1	2	0	0	0	0	1	0	0	0	0
33	**43T**											
	Nov 11	8	10	0	0	0	0	2	0	0	0	10
total =33	**+ve/total**	**32/33**	**30/33**	**6/33**	**11/33**	**2/33**	**3/33**	**20/33**	**3/33**	**4/33**	**5/33**	**2/33**
prevalence	**No.33**	**96.97**	**90.0**	**18.18**	**33.33**	**6.06**	**9.09**	**60.6**	**9.09**	**12.12**	**15.15**	**9.09**

Appendix 2.4.3: Identification of oocysts in faeces of calves from other Farms.

N0.	Id/Date	E.zuernii	E.bovis	E.canadensis	E.cylindrica	E.ellipsoidalis	E.brasiliensis	E.auburnensis	E.subspherica	E.bukidnonensis	E.wyomingensis	E. alabamensis
1	**Blakies 07 DB**											
	Nov 19 59	4	17	0	0	0	0	3	0	1	11	0
	Nov 19	17	14	5	0	0	0	0	0	0	0	0
2	**011 DB**											
	Nov 19	1	1	0	0	0	8	0	0	0	0	0
3	**032DB**											
	Nov 19	11	3	0	0	0	0	15	0	0	0	0
4	**Ballentrae**											
	04B(NC1)											
	Nov 19	4	17	0	0	0	0	3	0	1	11	0
5	06B(NC2)											
	Nov 19	12	0	0	2	0	1	0	0	15	0	0
6	**Apiti 6**											
	Sep 23	2	3	2	4	4	0	0	1	0	1	0
7	**massey organic(pooled)**											
		0	1	1	0	0	0	1	1	0	0	0
8	**Maurice Farm**	**0**	**1**	**1**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**
	No.8	**51**	**56**	**8**	**6**	**4**	**9**	**22**	**2**	**17**	**23**	**0**
	Prevalence %	**87.5**	**87.5**	**50**	**25**	**12.5**	**25**	**50**	**35.5**	**37.5**	**0**	**0**

Appendix 3.1. Calf treatment:

Appendix 3.1.a. Calf performance 20% pellets

Supplied by Denver Stock Feeds

Barley meal	60%
Soya bean meal	20%
Copra Meal	10%
Molasses	2.5%
Limestone	2%
Salt	2%
Soya bean oil	0.5%
Calf premix	0.25%
Total	100.15%
Monensin	0.10%

Batch No. 0211400011 Supplied by: Denver Stock Feeds
Expiry date: April 2004.
Final monensin concentration of feed was 100mg/kg of calf meal

Appendix 3.1. b. Baycox (Toltrazuril) specifications:

Trade name: Baycox
Concentration: Baycox (piglet coccidiocide - toltrazuril 50g/L)
Batch No: 1848A2005
Expiry Date: June 2003

Toltrazuril 200ml contained active constituent of Toltrazuril 50g/l. batch. No.1848A2005. Expiry Date: June 2003 and the animals were dosed using 20ml syringe.

Appendix 3.2. Feeding Schedule of the calves.

Day	Date	Activity.	Milk liter		Meal Fed		Dose of monensin per day	Required monensin dose to achieve 100mg/kg feed (mean calf weight)
			Am	Pm	Am	Pm		
1	14/8/ 02	F/S, B/S and Weighed.	2.5	2.5	50g	-	5mg	
2	15 /8/02	Calves had coats on.	2.5	2.5	50g	-	5mg	
3	16/8/02	-	2.5	2.5	50g	-	5mg	
4	17/8/02		2.5	2.5	50g	-	5mg	
5	18/8/02	-	2.5	2.5	50g	-	5mg	
6	19/8/02	-	3.5	1.5	50g	-	5mg	
7	20/8/02	F/S, B/S, Weighed	3.5	1.5	50g	-	5mg	47.66mg/ 47.66kg
8	21/8/02	-	3.5	1.5	50g	-	5mg	
9	22/8/02	-	4.0	1.0	50g	Pellets, Hay	5mg	
10	23/8/02	-	4.0	1.0	50g	-	5mg	
11	24/8/02	-	5.0		100g	-	10mg	
12	25/8/02	-	5.0	-	100g	-	10mg	
13	26/8/02	-	5.0	-	100 G	-	10mg	
14	27/8/02	F/S, B/S, Weighed.	5.0	-	100g	-	10mg	547mg/ 54.70kg
15	28/8/02	-	5.0	-	100g	-	10mg	
16	29/8/02	-	5.0	-	100g		10mg	
17	30/8/02	-	5.0	-	100g	100g	20mg	
18	31/8/02	-	5.0	-	100g	100g,Hay.	20mg	
19	1/9/02	-	5.0	-	100g	100g	20mg	
20	2/9/02	-	5.0	-	100g	100g	20mg	
21	3/9/02	-	5.0	-	100g	100g	20mg	59.5mg/ 59.45kg
22	4/9/02	-	5.0	-	100g	150g	20mg	
23	5/9/02	-	5.0	-	100g	150g	25mg	
24	6/9/02	-	5.0	-	100g	150g	25mg	
25	7/9/02	-	5.0	-	100g	150g	25mg	
26	8/9/02	-	5.0	-	150g	150g	30mg	
27	9/9/02	-	5.0	-	150g	150g	30mg	
28	10/9/02	F/S, B/S, Weighed	5.0	-	150g	150g	30mg	64.0mg/ 64.04kg
29	11/9/02	-	5.0	-	150g	200g	35mg	
30	12/9/02	-	5.0	-	200g	200g	40mg	
31	13/9/02	-	5.0	-	200g	200g	40mg	
32	14/9/02	-	5.0	-	200g	200g	40mg	

33	15/9/02	-	5.0	-	200g	200g	40mg	
34	16/9/02	-	5.0	-	200g	200g	40mg	
35	17/9/02	F/S,B/S, Weighed	5.0	-	200g	200g	40mg	70mg/ 70.45kg
36	18/9/02	-	5.0	-	200g	250g	45mg	
37	19/9/02	-	5.0	-	250g	250g	50mg	
38	20/9/02	-	5.0	-	250g	300g	55mg	
39	21/9/02	-	5.0	-	300g	300g	60mg	
40	22/9/02	-	5.0	-	300g	300g	60mg	
41	23/9/02	200 cows were allowed to graze the grass for one day.	5.0	-	350g	350g	70mg	
42	24/9/02	F/S, B/S, Weighed.	-	-	350g	350g	70mg	74.5mg/ 74.5kg
43	25/9/02	Cows were allowed to graze overnight.	4.0	-	350g	400g	75mg	
44	26/9/02	-	4.0	-	400g	400g	80mg	
45	27/9/02	-	3.0	-	400g	450g	85mg	
46	28/9/02	-	3.0	-	450g	450g	90mg	
47	29/9/02	-	2.0	-	500g	500g	100mg	
48	30/9/02	-	2.0	-	500g	500g	100mg	
49	1/10/02	F/S, B/S, Weighed.	2.0	-	500g	500g	100mg	80.2mg/ 80.29kg
50	2/10/02	-	2.0	-	500g	500g	100mg	
51	3/10/02	-	-	-	500g	500g	100mg	
52	4/10/02	-	-	-	500g	500g	100mg	
53	5/10/02	-	-	-	500g	500g	100mg	
54	6/10/02	-	-	-	500g	500g	100mg	
55	7/10/02	-	-	-	500g	500g	100mg	
56	8/10/02	F/S, B/S, Weighed.	-	-	500g	500g	100mg	
57	9/10/02	-	-	-	500g	500g	100mg	
58	10/10/02	-	-	-	500g	500g	100mg	95.0 mg/ 95kg
59	11/10/02	Grazing paddock with cows	-	-	500g	500g	100mg	
60	12/10/02	-	-	-	500g	500g	100mg	
61	13/10/02	-	-	-	500g	500g	100mg	
62	14/10/02	-	-	-	500g	500g	100mg	
63	15/10/02	F/S, B/S, Weighed.	-	-	500g	500g	100mg	
64	16/10/02	Debudding 33calves bleed.	-	-	500g	500g	100mg	
65	17/10/02	-	-	-	500g	500g	100mg	
66	18/10/02	-	-	-	500g	500g	100mg	
67	19/10/02	-	-	-	500g	500g	100mg	

68	20/10/02	-	-	-	500g	500g	100mg	
69	21/10/02	-	-	-	500g	500g	100mg	
70	22/10/02	F/S, B/S, Weighed.	-	-	500g	500g	100mg	1022mg/ 102.25kg
71	23/10/02	-	-	-	500g	500g	100mg	
72	24/10/02	Weaned. F/S,B/S, Drenched with Toltrazuril, Vaccinated with 7 in one.	-	-				
73	25/10/02	-	-	-			-	
74	26/10/02	-	-	-			-	
75	27/10/02	-	-	-			-	
76	28/10/02	-	-	-			-	
77	29/10/02	-	-	-			-	96.0mg/ 96.00kg
78	30/10/02	-	-	-			-	
79	31/10/02	-	-	-			-	
80-82	1/11/02 - 3/11/02	-	-	-				
83	4/11/02	Gate open between last twopaddocks and calves were allowed all together in the first two paddocks						
84	5/11/02	F/S, B/S, Weighed.	-	-			-	97.5kg
85-90	6/11/02- 11/11/02	-	-	-			-	106.8kg
91	12/11/02	F/S, B/S, Weighed.	-	-			-	
92-95	13/11/02 to 16/11/02							
96	17/11/02	-	-	-			-	116.87kg
97	18/11/02	F/S, B/S, Weighed.	-	-			-	
98	19/11/02	-	-	-			-	
99-104	20/11/02- 25/11/02	-	-	-			-	
105	26/11/02	F/S, B/S,	-	-			-	

		Weighed.						
106-109	27-30 /11/02	-	-	-			-	
110	1/12/02	-	-	-	-	-	-	
111	2/12/02	14 calves were coughing	-	-	-	-	-	
112	3/12/02	F/S, B/S, Weighed.	-	-	-	-	-	125.29kg
113	4/12/02-9/12/02	End of the work.						

Appendix 3.3. SOPs:

Appendix 3.3.1.Oocyst counting:

a. Equipment used:

1. McMaster chamber.
2. Filter (Coffee Strainer).
3. Steel bowl ans spoons.
4. Cover slips.
5. Pipettes.
6. Universal Glass bottle (28ml).
7. Centrifuge Tubes.

b.Solutions:

1. Saturated salt solution. (Specific gravity 1.2)

c.Technique used

All the samples were collected directly from the rectum and stored at 4 °C until used. Oocysts were counted using a modified McMaster technique as follows:

1. Two gram faeces were weighed and 28ml saturated NaCl solution (specific gravity 1.2) measured out. The feces were homogenized in some of the salt solution in a bowl. The suspension was then poured through a 500um-aperture sieve and the remaining salt solution was used to wash the bowl and the material retained on the sieve.
1. Samples were withdrawn using a Pasteur pipette and run into two counting chambers. The total number of oocysts counted, multiplied by 50, and represented the number of oocysts contained in one gram of faeces (OPG).

Appendix 3.3.2. Separation of oocysts for sporulation for samples > 100 Opg:

a. Equipment:

1. Steel bowls
2. 2. Filter (tea strainer)
3. 100 Sieve and water jet.
4. Centrifuge tubes (50ml and 20ml).
5. 'J' pipette and Pasteur pipette.
6. Suction pump and a jar.
7. Test tube stands.
8. Centrifuge.
9. Petridishes.

b. Solutions:

1. Saturated salt solution
2. 2% $H^2 So^4$

C. Technique:

1. 5g faeces from each sample were homogenized with 50ml tap water and filtered through a 500um-sieve.The residue in each sieve was washed with a jet of tap water from a wash- bottle.
2. Approximately 50mls filtrate was centrifuged at 800g for 6 minutes.
3. Two thirds of the supernatant was discarded. The sediment was re suspended in tap water and re- centrifuged as in step2.
4. The resultant sediment was re-suspended in NaCl solution (specific gravity1.2) and allow to stand for 10 minutes to allow coarse material to sink with little chance of trapping oocysts. The suspension was then centrifuged at 400g for 6 minutes.
5. The tube was removed gently from the centrifuge and allowed to stand for a further 10 minutes in order to compensate for any disturbance of the oocyst band at the top.
6. Approximately 5mls was sucked from the top using a "J" tip pipette attached to a suction pump and collected in a 50ml centrifuge tube.
7. The oocysts were washed free of salt solution by suspension and centrifugation in distilled water twice.
8. The washed sediment was transferred to a 15ml graduated conical centrifuge tube and centrifuged at 150g for 6 minutes.
9. The supernatant (appoximately 12mls) was discarded and the sediment re-suspended in 2% $H_2 So_4$ solution. The total volume was not > 5ml. The suspension was the placed in a 35mm petridish.
10. The petridish was placed in a 27°C room for 7 days. It was checked regularly to avoid drying out. Further 2% of H_2SO_4 was added as needed.

Appendix 3.3.3. Recovery of sporulated oocysts:

Petridishes were removed from the 27°C room and the oocysts were recovered as follows:

1. The oocyst suspension was stirred thoroughly in order to free the oocysts, which usually adhere to the bottom.
2. The suspension was washed into micro tubes and stored at 4°C until used for species identification.

Appendix 3.3.4. Staining of *Cryptosporidium* oocysts by a modified Zeihl Neilson technique:

The following procedure was used

1. Dry smear at room temperature
2. Fix in 96% methanol for 2 to 5 minutes

3. Dry at room temperature
4. Fix briefly in flame
5. Stain with concentrated Carbol fuchsin 20 to 30 minutes without heating
6. Rinse in tap water
7. Differentiate with H_2SO_4 for 20 to 60 minutes (concentrations 5 - 10%)
8. Rinse in tap water
9. Counter stain with 5% malachite green for 5 minutes
10. Rinse in tap water
11. Dry at room temperature
12. Mount in eukitt

In smears stained by this technique *Cryptosporidium* appear as 3 to 6 µm in diameter, densely stained red bodies clearly distinguishable against a green background. Some of the parasites however are rather stained, these are probably oocysts or their precursors. The cryptosporidium contain a varying number of darker blue or brownish internal bodies. The parasites are easily detected at magnifications of 200 to 400 x.

The properties of the carbol fuchsin dye may vary, and with certain preparations step 5 in the staining procedure may have to be extended to 1hour even overnight. For differentiation (step 7) H_2SO_4, 10% may be used in concentrations from 0.25% to 10%.The most appropriate concentration of the acid and length of the differentiation procedure would seem to depend on the properties of the carbol fuchsin dye. So before adopting the technique, it is advisable to make a few experiments with positive control smears, in order to adjust steps 5 and 7 to the dye preparation available.

In smears from fecal samples or ileal mucosa scrapings, yeasts were found to be stained by the Giemsa method, but not by the Zeihl Neilsen method.

Appendix 3.3.5. MERIFLUOR (Meridian diagnostics) C*ryptosporidium/ Giardia*, direct immunofluorescent detection procedure for the simultaneous detection of *Cryptosporidium* oocysts and *Giardia* cysts in faecal material.

1. Use a transfer loop to transfer a drop of fecal sample to a treated slide well. Spread the specimen over the entire well. Do not scratch the treated surface of the slide.
2. Use a new transfer loop to transfer a drop of positive control to a separate treated slide well. Spread the positive control over the entire well. Do not scratch the treated surface of the well.
3. Use a new transfer loop to transfer a drop of negative control to a separated treated side well.
4. Allow the slides to air dry completely at room temperature (usually requires 30 minutes).
5. Place one drop of detection reagent in each well.
6. Place one drop of counterstain in each well.
7. Mix the reagents with an applicator stick and spread over the entire well. Do not scratch the treated surface of the well.
8. Incubate the slides in a humidified chamber for 30 minutes at room temperature. Note: Protect from the light.
9. Use a wash bottle to rinse the slides with a gentle stream of 1x wash buffer until excess detection reagent and counterstain is removed.
Note: Do not submerge the slides during rinsing. Avoid disturbing the specimen or causing cross contamination of the specimens.
10. Remove excess buffer by tapping the long edge of the slide on a clean paper towel. Note: Do not allow the slide to dry.
11. Add one drop of mounting medium to each well and apply a cover slip.
13. Scan each well thoroughly using 100-200X magnification. The presence of oocysts should be confirmed at higher magnification.

Appendix 3.4.1. Oocyst counts up to weaning:

			14 Aug	20 Aug	23 Aug	27 Aug	30 Aug	3 Sep	6 Sep	10 Sep	13 Sep	17 Sep	20 Sep	24 Sep	1st oct	8th oct	15th oct	22 Oct
No.	Treat	rep	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
43	R	1	0	0	0	0	0	30	1700	200	2650	0	100	0	0	0	0	0
17	R	1	0	0	0	0	0	30	1850	450	750	0	100	50	0	0	0	0
116	R	1	0	0	0	30	50	0	0	300	0	50	0	0	0	0	0	0
118	R	1	0	0	0	30	100	100	50	50	400	0	0	0	0	0	0	0
Mean		**1**	**0**	**0**	**0**	**15**	**37.5**	**40**	**900**	**250**	**950**	**12.5**	**50**	**12.5**	**0**	**0**	**0**	**0**
6	R	2	0	0	0	0	30	0	0	50	0	200	450	50	100	0	0	0
16	R	2	0	0	0	0	30	0	300	650	50	0	0	0	0	0	0	0
15	R	2	0	0	0	0	0	30	1050	6300	2200	1000	1250	200	0	0	0	0
117	R	2	0	0	0	30	0	0	8250	0	650	250	150	0	200	0	0	0
Mean		**2**	**0**	**0**	**0**	**7.5**	**15**	**7.5**	**2400**	**1750**	**725**	**362.5**	**462.5**	**62.5**	**75**	**0**	**0**	**0**
32	R	3	0	0	0	0	0	0	0	30	0	0	0	100	0	0	50	0
28	R	3	0	0	0	0	30	8240	4900	0	0	550	0	0	100	0	0	0
9	R	3	0	0	0	30	50	50	0	1850	150	50	50	0	50	0	0	0
29	R	3	0	0	0	0	0	30	4200	0	0	150	50	50	400	0	0	0
			0	**0**	**0**	**7.5**	**20**	**2080**	**2275**	**470**	**37.5**	**187.5**	**25**	**37.5**	**137.5**	**0**	**12.5**	**0**
25	NR	1	0	0	0	0	30	0	0	0	800	650	100	0	250	50	250	0
57	NR	1	0	0	0	0	0	0	0	30	550	150	950	450	100	0	300	350
39	NR	1	0	0	0	0	150	150	650	650	2250	100	0	50	150	0	0	0
113	NR	1	0	0	0	30	7550	300	450	750	2700	350	150	0	1100	0	0	0
Mean			**0**	**0**	**0**	**7.5**	**1933**	**113**	**275**	**358**	**1575**	**313**	**300**	**125**	**400**	**13**	**138**	**88**
3	NR	2	0	0	0	0	0	0	1100	30	2850	0	0	0	0	50	0	0
33	NR	2	0	0	0	0	0	0	0	30	750	500	100	100	0	0	50	0
18	NR	2	0	0	0	0	50	30	350	0	150	300	50	100	150	50	0	0
56	NR	2	0	0	0	0	0	0	250	30	0	300	250	200	0	0	0	0
Mean			**0**	**0**	**0**	**0**	**12.5**	**7.5**	**425**	**22.5**	**937.5**	**275**	**100**	**100**	**37.5**	**25**	**12.5**	**0**
31	NR	3	0	0	0	0	850	30	200	350	0	0	0	0	0	0	0	0
20	NR	3	0	0	0	0	350	30	115500	100	0	50	0	0	0	0	0	0
14	NR	3	0	0	0	30	0	0	0	0	50	0	0	0	0	0	0	0
47	NR	3	0	0	0	0	0	0	0	30	0	0	0	0	0	0	50	0
			0	0	0	7.5	300	15	28925	120	12.5	12.5	0	0	0	0	12.5	0
Mean			**0**	**0**	**0**	**6.2**	**647**	**166**	**7393**	**277**	**753**	**199**	**125**	**72**	**163.2**	**11.02**	**48**	26

The second column of the above table represents the "treatment " R means monensin added to pellets and NR means monensin is not added to pellets. Rep represents 3 different groups (1 to 3) under each group that is Monensin treated and non-treated groups. Rest of the columns are different occasions of sample collections.

Appendix 3.4. 2. Weekly average oocyst counts of calves up to weaning (Group wise):

T	Group	1	2	3	4	5	6	7	8	9	10	11
R	1	0	0	15.0	40.0	250.0	12.5	12.5	0.0	0.0	0.0	0.0
R	2	0	0	7.5	7.5	1750.0	362.5	62.5	75.0	0.0	0.0	0.0
R	3	0	0	7.5	2080.0	470.0	187.5	37.5	137.5	0.0	12.5	87.5
NR	1	0	0	7.5	112.5	357.5	312.5	125.0	400.0	12.5	137.5	0.0
NR	2	0	0	0.0	7.5	22.5	275.0	100.0	37.5	25.0	12.5	0.0
NR	3	0	0	7.5	15.0	120.0	12.5	0.0	0.0	0.0	12.50	0.0

The first column of the above table represents the "treatment " R means monensin added to pellets and NR means monensin is not added to pellets. Group represents 3 different groups (1 to 3) under each group that is Monensin treated and non-treated groups. Rest of the columns are, different weeks on Monensin treatment.

Appendix 3.4.3. Statistical analysis for group wise oocyst counts up to weaning:

Type 3 Tests of Fixed Effects

Effect		DF	DF	F Value	Pr > F
Week		10	78.00	5.90	<.0001
Treat	1	20	0.26	0.67	
Rep	2	20	0.31	0.73	
Rep*treat*week		52	178.00	1.28	0.1209

Least mean square means

Effect	rep	week	Estimate	Standard Error	DF	t Value	Pr > |t|
Treat	NR		4.7104	0.7506	20	6.28	<.0001
Treat	R		5.2499	0.7506	20	6.99	<.0001
Week		1	1.0000	1.7786	178	0.56	0.5747
Week		2	1.0000	1.7786	178	0.56	0.5747
Week		3	2.1419	1.7786	178	1.20	0.2301
Week		4	7.8576	1.7786	178	4.42	<.0001
Week		5	14.0031	1.7786	178	7.87	<.0001
Week		6	10.4798	1.7786	178	5.89	<.0001
Week		7	5.0961	1.7786	178	2.87	0.0047
Week		8	6.6289	1.7786	178	3.73	0.0003
Week		9	1.7677	1.7786	178	0.99	0.3216
Week		10	3.0674	1.7786	178	1.72	0.0863
Week		11	1.7390	1.7786	178	0.98	0.3295

Effect	rep	week	Estimate	Standard Error	DF	t Value	Pr > |t|
rep*treat*week	NR	1	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	2	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	3	2.1419	4.3566	178	0.49	0.6236
rep*treat*week	NR	4	7.9094	4.3566	178	1.82	0.0711
rep*treat*week	NR	5	14.8717	4.3566	178	3.41	0.0008
rep*treat*week	NR	6	16.6469	4.3566	178	3.82	0.0002
rep*treat*week	NR	7	7.5945	4.3566	178	1.74	0.0830
rep*treat*week	NR	8	17.8406	4.3566	178	4.10	<.0001
rep*treat*week	NR	9	2.5354	4.3566	178	0.58	0.5613
rep*treat*week	NR	10	8.7981	4.3566	178	2.02	0.0449
rep*treat*week	NR	11	5.4337	4.3566	178	1.25	0.2139
rep*treat*week	R	1	1.0000	4.3566	178	23	0.8187
rep*treat*week	R	2	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	3	3.2839	4.3566	178	0.75	0.4520
rep*treat*week	R	4	5.5464	4.3566	178	1.27	0.2046
rep*treat*week	R	5	14.9762	4.3566	178	3.44	0.0007
rep*treat*week	R	6	2.5354	4.3566	178	0.58	0.5613
rep*treat*week	R	7	2.5354	4.3566	178	0.58	0.5613
rep*treat*week	R	8	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	9	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	10	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	11	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	1	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	2	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	3	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	4	2.1419	4.3566	178	0.49	0.6236
rep*treat*week	NR	5	4.4258	4.3566	178	1.02	0.3111

rep*treat*week	NR	2	6	14.5204	4.3566	178	3.33	0.0010
rep*treat*week	NR	2	7	8.8193	4.3566	178	2.02	0.0444
rep*treat*week	NR	2	8	3.8221	4.3566	178	0.88	0.3815
rep*treat*week	NR	2	9	4.0707	4.3566	178	0.93	0.3514
rep*treat*week	NR	2	10	2.5354	4.3566	178	0.58	0.5613
rep*treat*week	NR	2	11	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	2	1	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	2	2	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	2	3	2.1419	4.3566	178	0.49	0.6236
rep*treat*week	R	2	4	2.1419	4.3566	178	0.49	0.6236
rep*treat*week	R	2	5	28.2587	4.3566	178	6.49	<.0001
rep*treat*week	R	2	6	15.6648	4.3566	178	3.60	0.0004
rep*treat*week	R	2	7	5.8297	4.3566	178	1.34	0.1826
rep*treat*week	R	2	8	6.5568	4.3566	178	1.51	0.1341
rep*treat*week	R	2	9	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	2	10	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	2	11	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	3	1	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	3	2	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	3	3	2.1419	4.3566	178	0.49	0.6236
rep*treat*week	NR	3	4	3.2839	4.3566	178	0.75	0.4520
rep*treat*week	NR	3	5	8.8382	4.3566	178	2.03	0.0440
rep*treat*week	NR	3	6	2.5354	4.3566	178	0.58	0.5613
rep*treat*week	NR	3	7	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	3	8	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	3	9	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	NR	3	10	2.5354	4.3566	178	0.58	0.5613
rep*treat*week	NR	3	11	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	3	1	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	3	2	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	3	3	2.1419	4.3566	178	0.49	0.6236
rep*treat*week	R	3	4	26.1223	4.3566	178	6.00	<.0001
rep*treat*week	R	3	5	12.6478	4.3566	178	2.90	0.0042
rep*treat*week	R	3	6	10.9758	4.3566	178	2.52	0.0126
rep*treat*week	R	3	7	4.7978	4.3566	178	1.10	0.2723
rep*treat*week	R	3	8	9.5541	4.3566	178	2.19	0.0296
rep*treat*week	R	3	9	1.0000	4.3566	178	0.23	0.8187
rep*treat*week	R	3	10	2.5354	4.3566	178	0.58	0.5613
rep*treat*week	R	3	11	1.0000	4.3566	178	0.23	0.8187

Appendix 3.4.4. Oocyst counts up to weaning (Treatment wise):

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
week	15	330	4.61	<.0001
treat	1	22	0.20	0.6578
treat*week	15	330	0.33	0.9927

Least Squares Means

Effect	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|
treat	NR		8.4414	1.5898	22	5.31	<.0001
treat	R		7.4318	1.5898	22	4.67	0.0001
week		1	1.0000	4.1734	330	0.24	0.8108
week		2	1.0000	4.1734	330	0.24	0.8108
week		3	1.0000	4.1734	330	0.24	0.8108
week		4	2.1419	4.1734	330	0.51	0.6081
week		5	8.8682	4.1734	330	2.12	0.0343
week		6	7.8576	4.1734	330	1.88	0.0606
week		7	35.1823	4.1734	330	8.43	<.0001
week		8	14.0031	4.1734	330	3.36	0.0009
week		9	18.7998	4.1734	330	4.50	<.0001
week		10	10.4798	4.1734	330	2.51	0.0125
week		11	8.3537	4.1734	330	2.00	0.0461
week		12	5.0961	4.1734	330	1.22	0.2229
week		13	6.6289	4.1734	330	1.59	0.1132
week		14	1.7677	4.1734	330	0.42	0.6722
week		15	3.0674	4.1734	330	0.73	0.4629
week		16	1.7390	4.1734	330	0.42	0.6772
treat*week	NR	1	1.0000	5.9021	330	0.17	0.8656
treat*week	NR	2	1.0000	5.9021	330	0.17	0.8656
treat*week	NR	3	1.0000	5.9021	330	0.17	0.8656
treat*week	NR	4	1.7613	5.9021	330	0.30	0.7656
treat*week	NR	5	13.8167	5.9021	330	2.34	0.0198
treat*week	NR	6	4.4451	5.9021	330	0.75	0.4519
treat*week	NR	7	39.4619	5.9021	330	6.69	<.0001
treat*week	NR	8	9.3786	5.9021	330	1.59	0.1130
treat*week	NR	9	21.2850	5.9021	330	3.61	0.0004
treat*week	NR	10	11.2342	5.9021	330	1.90	0.0579
treat*week	NR	11	7.6842	5.9021	330	1.30	0.1938
treat*week	NR	12	5.8046	5.9021	330	0.98	0.3261
treat*week	NR	13	7.5542	5.9021	330	1.28	0.2015
treat*week	NR	14	2.5354	5.9021	330	0.43	0.6678
treat*week	NR	15	4.6229	5.9021	330	0.78	0.4340
treat*week	NR	16	2.4779	5.9021	330	0.42	0.6749
treat*week	R	1	1.0000	5.9021	330	0.17	0.8656
treat*week	R	2	1.0000	5.9021	330	0.17	0.8656
treat*week	R	3	1.0000	5.9021	330	0.17	0.8656
treat*week	R	4	2.5226	5.9021	330	0.43	0.6694
treat*week	R	5	3.9197	5.9021	330	0.66	0.5071
treat*week	R	6	11.2702	5.9021	330	1.91	0.0571
treat*week	R	7	30.9028	5.9021	330	5.24	<.0001
treat*week	R	8	18.6276	5.9021	330	3.16	0.0017
treat*week	R	9	16.3147	5.9021	330	2.76	0.0060
treat*week	R	10	9.7253	5.9021	330	1.65	0.1004
treat*week	R	11	9.0231	5.9021	330	1.53	0.1273
treat*week	R	12	4.3876	5.9021	330	0.74	0.4578
treat*week	R	13	5.7036	5.9021	330	0.97	0.3346
treat*week	R	14	1.0000	5.9021	330	0.17	0.8656
treat*week	R	15	1.5118	5.902	330	0.26	0.7980
treat*week	R	16	1.0000	5.901	330	0.17	0.8656

Appendix 3.5.1. Oocyst counts after weaning showing the status of Two (anti-coccidials treatment):

No.	Treatment		29th Oct	5th Nov	11th Nov	18th Nov	26th Nov	3rd Dec
43	R	Tol	0	0	0	100	0	150
116	R	Tol	0	0	0	50	150	100
6	R	Tol	0	0	0	0	0	500
15	R	Tol	0	50	0	100	650	0
28	R	Tol	0	0	0	250	0	150
9	R	Tol	0	0	50		100	100
		Average	0	8.33	8.33	100	150	166.67
14	NR	BC	0	0	0	0	100	250
31	NR	BC	0	0	0	0	0	0
56	NR	BC	50	0	0	150	200	100
33	NR	BC	0	50	0	0	0	400
113	NR	BC	0	50	50	350	650	100
25	NR	BC	0	1350	0	50	600	0
		Average	8.33	241.67	8.33	91.67	258.33	141.67
17	R	NBC	0	300	600	350	200	0
118	R	NBC	0	400	800	350	0	50
117	R	NBC	0	0	250	950	50	0
16	R	NBC	0	600	4550	350	50	0
32	R	NBC	0	150	750	50	450	50
29	R	NBC	0	550	550	550	0	0
		Average	0	500	1250	433.33	150	16.67
39	NR	NBC	50	650	500	150	100	100
57	NR	NBC	800	2100	1200	100	400	0
3	NR	NBC	150	550	300	250	0	50
18	NR	NBC	0	600	700	400	350	0
47	NR	NBC	0	0	100	100	0	150
20	NR	NBC	50	250	400	50	50	100
		Average	175	691.67	533.33	175	150	66.67

Note: NR= meal with out monensin, R= meal with monensin , BC= treated with toltrazuril and NBC= not treated with toltrazuril.

Appendix 3. 5. 2. Weekly average weights after weaning

Week	1	2	3	4	5	6
Treated	**95.41**	**96.75**	**106.66**	**117.58**	**126.75**	**133.25**
Untreated	**96.58**	**98.25**	**107.5**	**115.33**	**123.83**	**129.42**

Appendix 3.5.3: Statistical analysis of oocyst counts after weaning:

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
week	6	132	7.13	<.0001
bc	1	22	20.90	0.0001
bc*week	6	132	10.96	<.0001

Least Squares Means

Effect	bc	week	Estimate	Standard Error	DF	t Value	Pr > |t|
week		1	3.3756	1.6463	132	2.05	0.0423
week		2	13.4439	1.6463	132	8.17	<.0001
week		3	14.2592	1.6463	132	8.66	<.0001
week		4	11.6377	1.6463	132	7.07	<.0001
week		5	10.1208	1.6463	132	6.15	<.0001
week		6	7.7433	1.6463	132	4.70	<.0001
week		7	4.383	1.6463	132	2.66	0.0087
bc	BC		5.993	1.0156	22	5.91	<.0001
bc	NBC		12.5638	1.0156	22	12.37	<.0001
bc*week	BC	1	1.5118	2.3283	132	0.65	0.5173
bc*week	BC	2	5.5150	2.3283	132	2.37	0.0193
bc*week	BC	3	2.0236	2.3283	132	0.87	0.3864
bc*week	BC	4	7.1874	2.3283	132	3.09	0.0025
bc*week	BC	5	10.5925	2.3283	132	4.55	<.0001
bc*week	BC	6	10.5022	2.3283	132	4.51	<.0001
bc*week	BC	7	4.6487	2.3283	132	2.00	0.0479
bc*week	NBC	1	5.2394	2.3283	132	2.25	0.0261
bc*week	NBC	2	21.3727	2.3283	132	9.18	<.0001
bc*week	NBC	3	26.4948	2.3283	132	11.38	<.0001
bc*week	NBC	4	16.0880	2.3283	132	6.91	<.0001
bc*week	NBC	5	9.6491	2.3283	132	4.14	<.0001
bc*week	NBC	6	4.9844	2.3283	132	2.14	0.0341
bc*week	NBC	7	4.1182	2.3283	132	1.77	0.0792

Appendix 3.5.4. Statistical analysis of oocyst counts with two anti - coccidials:

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Week	5	100	5.97	<.0001
rum	1	20	0.19	0.6696
bc	1	20	22.24	0.0001
rum*bc	1	20	0.33	0.5740
rum*bc*week	15	100	4.62	<.0001

Least Squares Means

Effect	rum	bc	week	Estimate	Standard Error	DF	t Value	Pr > |t|
week			1	3.3756	1.7181	100	1.96	0.0522
week			2	13.4439	1.7181	100	7.82	<.0001
week			3	14.2592	1.7181	100	8.30	<.0001
week			4	11.6377	1.7181	100	6.77	<.0001
week			5	10.1208	1.7181	100	5.89	<.0001
week			6	7.7433	1.7181	100	4.51	<.0001
rum	NR			10.4526	1.1620	20	9.00	<.0001
rum	R			9.7409	1.1620	20	8.38	<.0001
bc	BC			6.2221	1.1620	20	5.35	<.0001
bc	NBC			13.9714	1.1620	20	12.02	<.0001
rum*bc	NR	BC		7.0475	1.6433	20	4.29	0.0004
rum*bc	NR	NBC		13.8576	1.6433	20	8.43	<.0001
rum*bc	R	BC		5.3966	1.6433	20	3.28	0.0037
rum*bc	R	NBC		14.0852	1.6433	20	8.57	<.0001

Effect	rum	bc	week	Estimate	Standard Error	DF	t Value	Pr > |t|
rum*bc*week	NR	BC	1	2.0236	3.4362	100	0.59	0.5573
rum*bc*week	NR	BC	2	9.0065	3.4362	100	2.62	0.0101
rum*bc*week	NR	BC	3	2.0236	3.4362	100	0.59	0.5573
rum*bc*week	NR	BC	4	6.8608	3.4362	100	2.00	0.0486
rum*bc*week	NR	BC	5	12.7096	3.4362	100	3.70	0.0004
rum*bc*week	NR	BC	6	9.6613	3.4362	100	2.81	0.0059
rum*bc*week	NR	NBC	1	9.4788	3.4362	100	2.76	0.0069
rum*bc*week	NR	NBC	2	22.6972	3.4362	100	6.61	<.0001
rum*bc*week	NR	NBC	3	21.8232	3.4362	100	6.35	<.0001
rum*bc*week	NR	NBC	4	12.5662	3.4362	100	3.66	0.0004
rum*bc*week	NR	NBC	5	9.6585	3.4362	100	2.81	0.0059
rum*bc*week	NR	NBC	6	6.9216	3.4362	100	2.01	0.0467
rum*bc*week	R	BC	1	1.0000	3.4362	100	0.29	0.7716
rum*bc*week	R	BC	2	2.0236	3.4362	100	0.59	0.5573
rum*bc*week	R	BC	3	2.0236	3.4362	100	0.59	0.5573
rum*bc*week	R	BC	4	7.5140	3.4362	100	2.19	0.0311
rum*bc*week	R	BC	5	8.4755	3.4362	100	2.47	0.0153
rum*bc*week	R	BC	6	11.3432	3.4362	100	3.30	0.0013
rum*bc*week	R	NBC	1	1.0000	3.4362	100	0.29	0.7716
rum*bc*week	R	NBC	2	20.0482	3.4362	100	5.83	<.0001
rum*bc*week	R	NBC	3	31.1665	3.4362	100	9.07	<.0001
rum*bc*week	R	NBC	4	19.6097	3.4362	100	5.71	<.0001
rum*bc*week	R	NBC	5	9.6397	3.4362	100	2.81	0.0060
rum*bc*week	R	NBC	6	3.0471	3.4362	100	0.89	0.3773

Differences of Least Squares Means

Effect	rum	bc	week	_rum	_bc	_week	Estimate	Standard Error	DF	t Value	Pr > |t|

Week			1		2	-10.0683	2.3375	100	-4.31	<.0001
Week			1		3	-10.8836	2.3375	100	-4.66	<.0001
Week			1		4	-8.2621	2.3375	100	-3.53	0.0006
Week			1		5	-6.7452	2.3375	100	-2.89	0.0048
Week			1		6	-4.3677	2.3375	100	-1.87	0.0646
Week			2		3	-0.8153	2.3375	100	-0.35	0.7280
Week			2		4	1.8062	2.3375	100	0.77	0.4415
Week			2		5	3.3230	2.3375	100	1.42	0.1583
Week			2		6	5.7006	2.3375	100	2.44	0.0165
Week			3		4	2.6215	2.3375	100	1.12	0.2648
Week			3		5	4.1384	2.3375	100	1.77	0.0797
Week			3		6	6.5159	2.3375	100	2.79	0.0064
Week			4		5	1.5168	2.3375	100	0.65	0.5179
Week			4		6	3.8944	2.3375	100	1.67	0.0988
Week			5		6	2.3775	2.3375	100	1.02	0.3116
rum	NR			R		0.7116	1.6433	20	0.43	0.6696
bc	BC		NBC			-7.7493	1.6433	20	-4.72	0.0001
rum*bc	NR	BC	NR	NBC		-6.8101	2.3240	20	-2.93	0.0083
rum*bc	NR	BC	R	BC		1.6509	2.3240	20	0.71	0.4857
rum*bc	NR	BC	R	NBC		7.0377	2.3240	20	-3.03	0.0066
rum*bc	NR	NBC	R	BC		8.4609	2.3240	20	3.64	0.0016
rum*bc	NR	NBC	R	NBC		-0.2276	2.3240	20	-0.10	0.9229
rum*bc	R	BC	R	NBC		-8.6886	2.3240	20	-3.74	0.0013

Appendix 3.6.1. Live weights of individual calves up to weaning (Group wise):

Animal	Treat	Rep	14th Aug	20th Aug	27th Aug	3rd Sep	10th Sep	17th Sep	24th Sep	1st Oct	8th Oct	15th Oct	22nd Oct
43	R	1	54	52	59	65	70	76	80	85	92	97	100
17	R	1	49	46	54	60	65	70	73	78	87	90	98
116	R	1	45	44	52	57	64	70	74	78	89	96	101
118	R	1	48	46	54	59	64	71	74	82	90	97	103
Average			**49**	**47**	**54.75**	**60.25**	**65.75**	**71.75**	**75.25**	**80.75**	**89.5**	**95**	**100.50**
6	R	2	52	52	59	65	59	78	82	89	98	104	112
16	R	2	49	49	55	54	66	72	75	85	88	96	101
15	R	2	43	43	49	54	57	63	67	69	76	83	89
117	R	2	47	49	55	59	63	69	73	80	89	84	99
			47.75	**48.25**	**54.5**	**58**	**61.25**	**70.50**	**74.25**	**80.75**	**87.75**	**91.75**	**100.25**
32	R	3	52	52	58	63	69	73	78	84	93	99	105
28	R	3	48	45	54	59	62	69	74	72	87	97	101
9	R	3	46	49	56	60	65	71	75	82	88	96	98
29	R	3	39	41	49	54	60	66	69	84	87	95	99
Average			**46.25**	**46.75**	**54.25**	**59**	**64**	**69.75**	**74**	**80.5**	**88.75**	**96.75**	**100.75**
25	NR	1	52	50	57	61	67	72	76	82	92	97	100
57	NR	1	49	47	56	62	67	74	77	84	93	93	106
39	NR	1	42	49	61	61	67	73	75	83	91	95	105
113	NR	1	54	52	57	65	72	78	83	89	98	87	111
Average			**49.25**	**49.50**	**57.75**	**62.25**	**68.25**	**74.25**	**77.75**	**84.5**	**93.50**	**92.83**	**105.5**
3	NR	2	52	51	57	62	66	72	75	81	90	99	97
33	NR	2	49	49	55	60	66	73	77	81	94	96	101
18	NR	2	46	47	54	59	63	70	74	79	88	95	99
56	NR	2	36	38	44	56	54	59	64	70	78	76	88
Average			**45.75**	**46.25**	**52.5**	**59.25**	**62.25**	**68.5**	**72.5**	**77.75**	**87.50**	**91.52**	**96.25**
31	NR	3	50	53	59	63	66	71	76	80	91	94	102
20	NR	3	48	51	57	61	67	71	77	80	91	97	102
14	NR	3	48	49	55	59	65	71	77	82	92	99	104
47	NR	3	39	40	47	49	53	59	63	68	74	82	85
average			**46.25**	**48.25**	**54.50**	**58.00**	**62.75**	**68.00**	**73.25**	**77.50**	**87.00**	**93.03**	**98.25**

Note: NR= fed with meal not added with monensin, R= fed with monensin added meal

Appendix 3.6.2. Group mean weekly live weights up to weaning:

Treat-ment	Rep	14th Aug	20th Aug	27th Aug	3rd Sep	10th Sep	17th Sep	24th Sep	1st Oct	8th Oct	15th Oct	22nd Oct
R	1	49.00	47.00	54.75	60.25	65.75	71.75	75.25	80.75	89.50	95.00	100.50
R	2	47.75	48.25	54.50	58.00	61.25	70.50	74.25	80.75	87.75	91.75	100.25
R	3	46.25	46.75	54.25	59.00	64.00	69.75	74.00	80.50	88.75	96.75	100.75
NR	1	49.25	49.50	57.75	62.25	68.25	74.25	77.75	84.50	93.50	92.83	105.5
NR	2	45.75	46.25	52.50	59.25	62.25	68.50	72.50	77.75	87.50	91.56	96.25
NR	3	46.25	48.25	54.50	58.00	62.75	68.00	73.25	77.50	87.00	93.03	98.25

Appendix 3.6.3.Statistical analysis of live weights up to weaning (treatment wise):

Note= treat=treatment NR= No Monensin feed, R= Monensin added feed

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
week	10	220	276.31	<.0001
treat	1	22	0.02	0.8884
treat*week	10	220	0.14	0.9990

Least Squares Means

Effect	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|
treat	NR		70.9773	0.4530	22	156.68	<.0001
treat	R		71.0682	0.4530	22	156.88	<.0001
week		1	47.3750	0.9901	220	47.85	<.0001
week		2	47.6667	0.8535	220	55.85	<.0001
week		3	54.7083	0.8460	220	64.67	<.0001
week		4	59.4583	0.8172	220	72.76	<.0001
week		5	64.0417	0.9669	220	66.24	<.0001
week		6	70.4583	0.9990	220	70.53	<.0001
week		7	74.5000	1.0075	220	73.94	<.0001
week		8	80.2917	1.1674	220	68.78	<.0001
week		9	89.0000	1.2211	220	72.88	<.0001
week		10	93.5000	1.3512	220	69.20	<.0001
week		11	100.25	1.3020	220	77.00	<.0001

Effect	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|
treat*week	NR	1	47.0833	1.4002	220	33.63	<.0001
treat*week	NR	2	48.0000	1.2071	220	39.76	<.0001
treat*week	NR	3	54.9167	1.1965	220	45.90	<.0001
treat*week	NR	4	59.8333	1.1557	220	51.77	<.0001
treat*week	NR	5	64.4167	1.3673	220	47.11	<.0001
treat*week	NR	6	70.2500	1.4128	220	49.73	<.0001
treat*week	NR	7	74.5000	1.4249	220	52.28	<.0001
treat*week	NR	8	79.9167	1.6510	220	48.41	<.0001
treat*week	NR	9	89.3333	1.7269	220	51.73	<.0001
treat*week	NR	10	92.5000	1.9109	220	48.41	<.0001
treat*week	NR	11	100.00	1.8412	220	54.31	<.0001
treat*week	R	1	47.6667	1.4002	220	34.04	<.0001
treat*week	R	2	47.3333	1.2071	220	39.21	<.0001
treat*week	R	3	54.5000	1.1965	220	45.55	<.0001
treat*week	R	4	59.0833	1.1557	220	51.13	<.0001
treat*week	R	5	63.6667	1.3673	220	46.56	<.0001
treat*week	R	6	70.6667	1.4128	220	50.02	<.0001
treat*week	R	7	74.5000	1.4249	220	52.28	<.0001
treat*week	R	8	80.6667	1.6510	220	48.86	<.0001
treat*week	R	9	88.6667	1.7269	220	51.34	<.0001
treat*week	R	10	94.5000	1.9109	220	49.45	<.0001
treat*week	R	11	100.50	1.8412	220	54.58	<.0001

Appendix 3.6.4. Statistical analysis of weight group wise up to weaning:
treat= Treatment, rep=replicate,
Note: NR= fed with meal not added with monensin, R= fed with monensin added meal,

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
week	10	178	1217.32	<.0001
treat	1	20	0.00	0.9628
rep	2	20	1.03	0.3737
rep*treat*week	52	178	0.90	0.6693

Least Squares Means

Effect	treat	rep	week	Estimate	Standard Error	DF	t Value	Pr > |t|
treat	NR			70.9773	1.3623	20	52.10	<.0001
treat	R			71.0682	1.3623	20	52.17	<.0001
week			1	47.3750	1.0848	178	43.67	<.0001
week			2	47.6667	1.0848	178	43.94	<.0001
week			3	54.7083	1.0848	178	50.43	<.0001
week			4	59.4583	1.0848	178	54.81	<.0001
week			5	64.0417	1.0848	178	59.03	<.0001
week			6	70.4583	1.0848	178	64.95	<.0001
week			7	74.5000	1.0848	178	68.67	<.0001
week			8	80.2917	1.0848	178	74.01	<.0001
week			9	89.0000	1.0848	178	82.04	<.0001
week			10	93.5000	1.0848	178	86.19	<.0001
week			11	100.25	1.0848	178	92.41	<.0001

Effect	treat	rep	week	Estimate	Standard Error	DF	t Value	Pr > |t|
rep*treat*week	NR	1	1	49.2500	2.6573	178	18.53	<.0001
rep*treat*week	NR	1	2	49.5000	2.6573	178	18.63	<.0001
rep*treat*week	NR	1	3	57.7500	2.6573	178	21.73	<.0001
rep*treat*week	NR	1	4	62.2500	2.6573	178	23.43	<.0001
rep*treat*week	NR	1	5	68.2500	2.6573	178	25.68	<.0001
rep*treat*week	NR	1	6	74.2500	2.6573	178	27.94	<.0001
rep*treat*week	NR	1	7	77.7500	2.6573	178	29.26	<.0001
rep*treat*week	NR	1	8	84.5000	2.6573	178	31.80	<.0001
rep*treat*week	NR	1	9	93.5000	2.6573	178	35.19	<.0001
rep*treat*week	NR	1	10	93.0000	2.6573	178	35.00	<.0001
rep*treat*week	NR	1	11	105.50	2.6573	178	39.70	<.0001
rep*treat*week	R	1	1	49.0000	2.6573	178	18.44	<.0001
rep*treat*week	R	1	2	47.0000	2.6573	178	17.69	<.0001
rep*treat*week	R	1	3	54.7500	2.6573	178	20.60	<.0001
rep*treat*week	R	1	4	60.2500	2.6573	178	22.67	<.0001
rep*treat*week	R	1	5	65.7500	2.6573	178	24.74	<.0001
rep*treat*week	R	1	6	71.7500	2.6573	178	27.00	<.0001
rep*treat*week	R	1	7	75.2500	2.6573	178	28.32	<.0001
rep*treat*week	R	1	8	80.7500	2.6573	178	30.39	<.0001
rep*treat*week	R	1	9	89.5000	2.6573	178	33.68	<.0001
rep*treat*week	R	1	10	95.0000	2.6573	178	35.75	<.0001
rep*treat*week	R	1	11	100.50	2.6573	178	37.82	<.0001

Effect	treat	rep	week	Estimate	Standard Error	DF	t Value	Pr > |t|
rep*treat*week	NR	2	1	45.7500	2.6573	178	17.22	<.0001

rep*treat*week	NR	2	2	46.2500	2.6573	178	17.40	<.0001
rep*treat*week	NR	2	3	52.5000	2.6573	178	19.76	<.0001
rep*treat*week	NR	2	4	59.2500	2.6573	178	22.30	<.0001
rep*treat*week	NR	2	5	62.2500	2.6573	178	23.43	<.0001
rep*treat*week	NR	2	6	68.5000	2.6573	178	25.78	<.0001
rep*treat*week	NR	2	7	72.5000	2.6573	178	27.28	<.0001
rep*treat*week	NR	2	8	77.7500	2.6573	178	29.26	<.0001
rep*treat*week	NR	2	9	87.5000	2.6573	178	32.93	<.0001
rep*treat*week	NR	2	10	91.5000	2.6573	178	34.43	<.0001
rep*treat*week	NR	2	11	96.2500	2.6573	178	36.22	<.0001
rep*treat*week	R	2	1	47.7500	2.6573	178	17.97	<.0001
rep*treat*week	R	2	2	48.2500	2.6573	178	18.16	<.0001
rep*treat*week	R	2	3	54.5000	2.6573	178	20.51	<.0001
rep*treat*week	R	2	4	58.0000	2.6573	178	21.83	<.0001
rep*treat*week	R	2	5	61.2500	2.6573	178	23.05	<.0001
rep*treat*week	R	2	6	70.5000	2.6573	178	26.53	<.0001
rep*treat*week	R	2	7	74.2500	2.6573	178	27.94	<.0001
rep*treat*week	R	2	8	80.7500	2.6573	178	30.39	<.0001
rep*treat*week	R	2	9	87.7500	2.6573	178	33.02	<.0001
rep*treat*week	R	2	10	91.7500	2.6573	178	34.53	<.0001
rep*treat*week	R	2	11	100.25	2.6573	178	37.73	<.0001
rep*treat*week	NR	3	1	46.2500	2.6573	178	17.40	<.0001
rep*treat*week	NR	3	2	48.2500	2.6573	178	18.16	<.0001
rep*treat*week	NR	3	3	54.5000	2.6573	178	20.51	<.0001
rep*treat*week	NR	3	4	58.0000	2.6573	178	21.83	<.0001
rep*treat*week	NR	3	5	62.7500	2.6573	178	23.61	<.0001
rep*treat*week	NR	3	6	68.0000	2.6573	178	25.59	<.0001
rep*treat*week	NR	3	7	73.2500	2.6573	178	27.57	<.0001
rep*treat*week	NR	3	8	77.5000	2.6573	178	29.16	<.0001
rep*treat*week	NR	3	9	87.0000	2.6573	178	32.74	<.0001
rep*treat*week	NR	3	10	93.0000	2.6573	178	35.00	<.0001
rep*treat*week	NR	3	11	98.2500	2.6573	178	36.97	<.0001
rep*treat*week	R	3	1	46.2500	2.6573	178	17.40	<.0001
rep*treat*week	R	3	2	46.7500	2.6573	178	17.59	<.0001
rep*treat*week	R	3	3	54.2500	2.6573	178	20.42	<.0001
rep*treat*week	R	3	4	59.0000	2.6573	178	22.20	<.0001
rep*treat*week	R	3	5	64.0000	2.6573	178	24.08	<.0001
rep*treat*week	R	3	6	69.7500	2.6573	178	26.25	<.0001
rep*treat*week	R	3	7	74.0000	2.6573	178	27.85	<.0001
rep*treat*week	R	3	8	80.5000	2.6573	178	30.29	<.0001
rep*treat*week	R	3	9	88.7500	2.6573	178	33.40	<.0001
rep*treat*week	R	3	10	96.7500	2.6573	178	36.41	<.0001
rep*treat*week	R	3	11	100.75	2.6573	178	37.91	<.0001

Appendix 3.6.7.Statistical analysis adjusted live weights:

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
rum	1	20	0.97	0.3356
bc	1	20	5.65	0.0275
week	4	79	374.28	<.0001
bc*week	4	79	2.81	0.0308
rum*bc*week	9	79	0.33	0.9628
lw1	1	20	132.90	<.0001

Least Squares Means

Effect	rum	bc	week	Estimate	Standard Error	DF	t Value	Pr > |t|
rum NR				116.08	0.7840	20	148.06	<.0001
rum	R			114.99	0.7840	20	146.66	<.0001
bc	BC			116.86	0.7850	20	148.86	<.0001
bc	NBC			114.21	0.7850	20	145.49	<.0001
week			2	97.50	0.8386	79	116.27	<.0001
week			3	107.08	0.8386	79	127.70	<.0001
week			4	116.46	0.8386	79	138.88	<.0001
week			6	125.29	0.8386	79	149.41	<.0001
week			7	131.33	0.8386	79	156.62	<.0001
bc*week		BC	2	97.40	1.1873	79	82.04	<.0001
bc*week		BC	3	107.32	1.1873	79	90.40	<.0001
bc*week		BC	4	118.24	1.1873	79	99.59	<.0001
bc*week		BC	6	127.41	1.1873	79	107.31	<.0001
bc*week		BC	7	133.91	1.1873	79	112.79	<.0001
bc*week		NBC	2	97.59	1.1873	79	82.20	<.0001
bc*week		NBC	3	106.84	1.1873	79	89.99	<.0001
bc*week		NBC	4	114.68	1.1873	79	96.59	<.0001
bc*week		NBC	6	123.18	1.1873	79	103.75	<.0001
bc*week		NBC	7	128.76	1.1873	79	108.45	<.0001
rum*bc*week	NR	BC	2	97.33	1.6821	79	57.86	<.0001
rum*bc*week	NR	BC	3	106.67	1.6821	79	63.41	<.0001
rum*bc*week	NR	BC	4	118.83	1.6821	79	70.64	<.0001
rum*bc*week	NR	BC	6	127.83	1.6821	79	75.99	<.0001
rum*bc*week	NR	BC	7	134.17	1.6821	79	79.76	<.0001
rum*bc*week	NR	NBC	2	98.72	1.6778	79	58.84	<.0001
rum*bc*week	NR	NBC	3	107.40	1.6778	79	64.01	<.0001
rum*bc*week	NR	NBC	4	114.90	1.6778	79	68.48	<.0001
rum*bc*week	NR	NBC	6	124.56	1.6778	79	74.24	<.0001
rum*bc*week	NR	NBC	7	130.40	1.6778	79	77.72	<.0001
rum*bc*week	R	BC	2	97.48	1.6948	79	57.52	<.0001
rum*bc*week	R	BC	3	107.98	1.6948	79	63.71	<.0001
rum*bc*week	R	BC	4	117.65	1.6948	79	69.42	<.0001
rum*bc*week	R	BC	6	126.98	1.6948	79	74.92	<.0001
rum*bc*week	R	BC	7	133.65	1.6948	79	78.86	<.0001
rum*bc*week	R	NBC	2	96.45	1.6850	79	57.25	<.0001
rum*bc*week	R	NBC	3	106.29	1.6850	79	63.08	<.0001
rum*bc*week	R	NBC	4	114.46	1.6850	79	67.93	<.0001
rum*bc*week	R	NBC	6	121.79	1.6850	79	72.28	<.0001
rum*bc*week	R	NBC	7	127.12	1.6850	79	75.45	<.0001

Note: NR = fed with meal not added with monensin, R or Rum= fed with monensin added mea treated with toltrazuril and NBC= not treated with toltrazuril.

Appendix 3.6.5. Weight of calves after weaning:

Weights after treating with toltrazuril @ 20mg/kg weight

Animal No:	Treatment		29th Oct	5th Nov	11th Nov	18th Nov	3rd Dec	10th Dec
43	R	BC	91	92	101	109	122	128
116	R	BC	92	97	108	116	131	139
6	R	BC	105	107	117	125	137	145
15	R	BC	84	85	94	112	107	111
28	R	BC	95	93	104	112	122	130
9	R	BC	94	94	107	115	126	132
113	NR	BC	105	106	117	131	141	151
25	NR	BC	99	100	106	117	131	138
56	NR	BC	87	86	96	114	112	116
33	NR	BC	95	97	108	120	131	136
14	NR	BC	101	107	114	124	135	143
31	NR	BC	97	97	108	116	126	130
Average			**95.42**	**96.75**	**106.67**	**117.58**	**126.75**	**133.25**
17	R	NBC	92	94	101	109	115	123
118	R	NBC	98	101	105	113	122	125
117	R	NBC	98	96	109	117	116	118
16	R	NBC	100	102	111	119	131	138
32	R	NBC	103	104	115	124	129	133
29	R	NBC	95	93	108	116	129	137
39	NR	NBC	102	103	111	119	131	137
57	NR	NBC	99	103	110	118	130	137
3	NR	NBC	95	95	106	114	129	135
18	NR	NBC	98	98	110	118	125	134
47	NR	NBC	81	88	93	98	107	109
20	NR	NBC	98	102	111	119	122	127
Average			**96.58**	**98.25**	**107.50**	**115.33**	**123.83**	**129.42**

Note: NR= fed with meal not added with monensin, R = fed with monensin added meal, BC= treated with toltrazuril and NBC= not treated with toltrazuril.

Appendix 3.6. 6. Weekly average weights after weaning

Week	1	2	3	4	5	6
Treated	95.41	96.75	106.66	117.58	126.75	133.25
untreated	96.58	98.25	107.5	115.33	123.83	129.42

Appendix 3.7. Comparison of Monensin concentration of feed actually recquired (100/kg feed) and supplied based on the weekly average weights of the animals.

Weeks treatment	Required Dose on average weight mg	Actual Received Dose mg
1	47.66	5
2	54.7	10
3	59.45	20
4	64.04	30
5	70.45	70
6	74.5	74
7	80.29	80
8	95	95
9	98	98
10	100	100

Appendix 4. 1. 1: Oocysts counts of calves: BC means treated with toltrazuril and NBC means not treated with toltrazuril

Group A. No	Treatment	7th Nov	14th Nov	21st Nov	26thNov	3rd Dec	12th Dec
75	BC	0	0	150	0	100	200
19	BC	4550	0	0	100	150	150
17	BC	0	0	0	0	0	0
64	BC	50	0	0	50	100	0
22	BC	53000	50	0	0	350	50
16	BC	150	50	50	0	0	0
20	BC	700	0	200	0	0	1150
7	BC	500	0	0	0	0	0
62	BC	0	50	0	0	0	500
77	BC	0	0	0	50	0	0
3	BC	200	0	0	0	0	0
2	BC	250	0	0	100	0	0
60	BC	0	100	0	200	0	0
66	BC	0	0	0	50	650	150
12	BC	2400	0	0	0	200	500
	Average	4120	16.66	26.66	36.66	103.33	180
18	NBC	0	1000	150	350	0	0
65	NBC	0	50	550	200	250	0
8	NBC	800	1050	1450	200	0	0
13	NBC	500	700	3450	50	0	0
10	NBC	850	50	3350	0	0	100
63	NBC	0	50	1200	1350	800	650
5	NBC	50	450	1500	350	200	150
4	NBC	600	0	850	950	250	50
21	NBC	50	250	300	0	0	0
68	NBC	250	0	0	100	650	0
59	NBC	50	0	900	700	500	0
74	NBC	50	0	1000	2150	9150	200
71	NBC	900	0	100	100	0	500
69	NBC	50	1000	400	600	200	200
76	NBC	0	100	500	300	0	0
	Average	343.33	313.33	1046.66	493.33	800	123.33
Group B							
36	BC	0	0	0	0	0	300
34	BC	0	0	0	0	0	50
52	BC	0	0	0	50	50	250
40	BC	0	0	0	0	0	0
42	BC	50	50	0	0	50	0
54	BC	0	0	250	50	150	0
48	BC	50	0	100	0	0	0
55	BC	0	0	0	0	0	150
53	BC	0	0	0	50	150	0
32	BC	0	0	0	0	0	0
45	BC	0	50	50	0	0	200
35	BC	150	0	0	0	0	50
28	BC	0	50	0	0	0	50

46	BC	100	0	0	0	250	150
25	BC	50	50	0	50	250	250
	Average	33.33	13.33	26.66	13.33	60	96.66
58	NBC	0	0	1 00	15.2381	100	50
47	NBC	50	0	100	150	100	250
50	NBC	0	50	13100	450	0	50
23	NBC	100	150	450	0	0	50
37	NBC	0	750	0	900	100	0
56	NBC	100	300	0	0	0	150
26	NBC	100	200	200	0	150	800
51	NBC	0	0	2000	450	0	150
43	NBC	100	50	50	0	0	50
38	NBC	250	1700	200	0	0	0
57	NBC	50	50	200	400	0	0
61	NBC	50	50	600	7750	200	100
49	NBC	0	950	0	500	50	150
33	NBC	100	50	50	150	0	0
39	NBC	150	1000	900	100	0	50
	Average	337	353	1197	724	47	123
Group C							
73	NBC	No Sampling	0	0	0	0	0
11	NBC		2950	3500	1100	200	250
1	NBC		0	300	250	0	0
6	NBC		700	200	4550	400	350
67	NBC		1150	3800	1250	300	100
14	NBC		2150	250	2250	0	0
15	NBC		200	1900	950	0	150
71	NBC		0	50	100	0	500
70	NBC		0	1450	0	0	250
9	NBC		50	0	100	50	50
72	NBC		0	4750	1350	750	0
57	NBC		50	200	400	0	0
39	NBC		1000	900	100	0	50
30	NBC		550	3300	250	100	400
31	NBC		2950	400	0	0	100
50	NBC		50	13100	450	0	50
61	NBC		50	600	1150	200	100
44	NBC		950	50	1200	100	100
24	NBC		350	100	200	0	0
41	NBC		150	250	250	200	250
29	NBC		1900	1700	650	150	100
Average	**NBC**		**760**	**2340**	**827.5**	**122.5**	**140**

Appendix 4.1.2. Statistical analysis of oocyst counts:
not considering group C
The Mixed Procedure

Model Information

Dependent Variable	tfec
Covariance Structure	Compound Symmetry
Subject Effect	anim(group*treat)
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
anim	60	2 3 4 5 7 8 10 12 13 16 17 18 19 20 21 22 23 25 26 28 32 33 34 35 36 37 38 39 40 42 43 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 68 69 71 74 75 76 77
group	**2**	**A B**
treat	**2**	**bc nbc**
week	**6**	**1 2 3 4 5 6**

Animal –calves, Treatment-BC= treated with toltrazuril, NBC= not treated with toltrazuril

Dimensions

Covariance Parameters	2
Columns in X	35
Columns in Z	0
Subjects	60
Max Obs Per Subject	6
Observations Used	359
Observations Not Used	1
Total Observations	360

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	2916.02578481	
1	2	2915.99265897	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
CS	anim(group*treat)	1.8434
Residual		289.03

Fit Statistics

-2 Res Log Likelihood	2916.0
AIC (smaller is better)	2920.0
AICC (smaller is better)	2920.0
BIC (smaller is better)	2924.2

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	0.03	0.8556

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
week	5	278	1.90	0.0937
treat	1	57	17.88	<.0001
group	1	57	5.26	0.0256
group*treat*week	16	278	2.56	0.0010

Least Squares Means

Effect	group	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|
week			1	14.8298	2.2018	278	6.74	<.0001
week			2	8.1902	2.2018	278	3.72	0.0002
week			3	13.8848	2.2213	278	6.25	<.0001
week			4	10.5710	2.2018	278	4.80	<.0001
week			5	8.1836	2.2018	278	3.72	0.0002
week			6	8.2050	2.2018	278	3.73	0.0002
treat		bc		6.777	1.2949	57	5.23	<.0001
treat		nbc		14.5106	1.2912	57	11.24	<.0001
group	A			12.7404	1.2949	57	9.84	<.0001
group	B			8.547	1.2912	57	6.62	<.0001

Effect	group	treat	week	Estimate	Error	DF	t Value	Pr > |t|
group*treat*week	A	bc	1	30.0660	4.4036	278	6.83	<.0001
group*treat*week	A	bc	2	2.8316	4.4036	278	0.64	0.5207
group*treat*week	A	bc	3	3.1773	4.5581	278	0.70	0.4863
group*treat*week	A	bc	4	4.3134	4.4036	278	0.98	0.3282
group*treat*week	A	bc	5	6.6543	4.4036	278	1.51	0.1319
group*treat*week	A	bc	6	8.8392	4.4036	278	2.01	0.0457
group*treat*week	A	nbc	1	13.5860	4.4036	278	3.09	0.0022
group*treat*week	A	nbc	2	13.0484	4.4036	278	2.96	0.0033
group*treat*week	A	nbc	3	28.3679	4.4036	278	6.44	<.0001
group*treat*week	A	nbc	4	18.4920	4.4036	278	4.20	<.0001
group*treat*week	A	nbc	5	15.9268	4.4036	278	3.62	0.0004
group*treat*week	A	nbc	6	7.582	4.4036	278	1.72	0.0862
group*treat*week	B	bc	1	4.1875	4.4036	278	0.95	0.3425
group*treat*week	B	bc	2	2.6377	4.4036	278	0.60	0.5497
group*treat*week	B	bc	3	3.0023	4.4036	278	0.68	0.4959
group*treat*week	B	bc	4	2.6377	4.4036	278	0.60	0.5497
group*treat*week	B	bc	5	5.3030	4.4036	278	1.20	0.2295
group*treat*week	B	bc	6	7.6809	4.4036	278	1.74	0.0822
group*treat*week	B	nbc	1	11.4796	4.4036	278	2.61	0.0096
group*treat*week	B	nbc	2	14.2431	4.4036	278	3.23	0.0014
group*treat*week	B	nbc	3	20.9917	4.4036	278	4.77	<.0001
group*treat*week	B	nbc	4	16.8409	4.4036	278	3.82	0.0002
group*treat*week	B	nbc	5	4.8504	4.4036	278	1.10	0.2716
group*treat*week	B	nbc	6	8.7178	4.4036	278	1.98	0.0487

Appendix 4.2. 1. Live weights of calves:

Group A: BC calves treated with toltrazuril and NBC calves not treated with toltrazuril

NO.	Treatment	7th Nov	14th Nov	21st Nov	28th Nov	3rd Dec	12th Dec
75	BC	102	113	109	116	123	120
19	BC	130	134	141	146	154	157
17	BC	111	109	117	124	129	135
64	BC	103	110	114	118	121	140
22	BC	113	117	129	133	140	145
16	BC	124	107	136	141	151	156
20	BC	121	124	122	128	136	145
7	BC	120	128	129	129	142	146
62	BC	101	107	112	121	119	120
77	BC	108	112	117	121	126	129
3	BC	125	124	135	139	144	154
2	BC	124	128	129	144	145	152
60	BC	107	121	115	122	125	130
66	BC	101	106	110	116	121	123
12	BC	113	116	121	125	134	138
Arithmetic mean		114	117	122	128	134	139
Untreated group: Group A							
18	NBC	118	126	123	130	138	146
65	NBC	109	114	122	120	124	132
8	NBC	112	121	117	124	127	131
13	NBC	122	126	130	131	139	143
10	NBC	102	108	108	114	116	123
63	NBC	96	103	106	115	111	121
5	NBC	116	123	121	134	131	140
4	NBC	108	108	123	117	130	138
21	NBC	119	123	125	129	134	147
68	NBC	103	106	114	110	119	119
59	NBC	110	110	118	121	128	126
74	NBC	102	119	116	117	116	119
71	NBC	112	125	118	125	128	126
69	NBC	109	123	123	129	130	134
76		109	118	111	116	120	124
Arithmetic mean		**110**	**117**	**118**	**122**	**126**	**131**
Group B							
36	BC	123	123	126	132	140	146
34	BC	108	114	126	123	134	138
52	BC	99	107	108	112	121	128
40	BC	102	101	104	108	113	119
42	BC	103	105	107	114	120	126
54	BC	96	101	106	113	118	124
48	BC	106	104	108	115	121	127
55	BC	107	113	121	115	122	125
53	BC	99	108	119	110	122	126
32	BC	139	110	138	146	151	162
45	BC	104	113	115	124	134	140
35	BC	112	118	131	134	138	145
28	BC	122	124	128	135	134	147
46	BC	105	109	119	117	125	132
25	BC	108	110	118	125	135	137
Arithmetic mean		**109**	**110**	**118**	**121**	**128**	**135**

58	NBC	103	107	110	115	119	125
47	NBC	106	104	106	113	120	126
50	NBC	98	101	111	114	114	122
23	NBC	120	126	137	131	134	144
37	NBC	120	128	139	138	147	153
56	NBC	107	109	110	114	122	126
26	NBC	132	135	141	147	153	164
51	NBC	96	112	111	110	117	124
43	NBC	102	105	115	117	119	126
38	NBC	100	104	108	111	113	118
57	NBC	104	105	110	114	122	124
61	NBC	104	100	108	100	106	108
49	NBC	100	110	123	113	118	123
33	NBC	107	115	119	127	133	137
39	NBC	106	107	117	116	124	131
		107	**111**	**117**	**118**	**124**	**130**
Group C		no sampling					
73	NBC		121	117	123	125	130
11	NBC		136	132	139	147	155
1	NBC		121	124	120	126	135
6	NBC		137	134	133	146	145
67	NBC		125	124	130	132	137
14	NBC		120	121	126	131	145
15	NBC		124	130	132	142	145
71	NBC		110	118	125	128	133
70	NBC		104	107	108	112	113
9	NBC		117	129	135	140	147
72	NBC		107	114	121	119	125
57 or 27	NBC		100	110	123	122	124
39	NBC		107	117	116	124	131
30	NBC		108	110	118	126	131
31	NBC		104	104	111	118	123
50	NBC		101	111	114	114	122
61	NBC		100	108	100	106	108
44	NBC		114	124	121	127	135
24	NBC		110	114	113	117	125
41	NBC		109	114	122	128	134
29	NBC		113	124	124	127	137
Arithmetic Mean			**114**	**119**	**121**	**127**	**132**

Appendix 4.2.2 Average weekly weight of calves:

	Group A		**Group B**	
Week	**BC**	**NBC**	**BC**	**NBC**
1	114	110	109	107
2	116	117	110	111
3	122	118	117	117
4	128	122	123	118
5	134	126	128	124
6	139	131	135	130

BC calves treated with toltrazuril and NBC calves not treated with toltrazuril

Appendix 4.2.3. Statistical analysis of Liveweights:

The Mixed Procedure

Model Information

Dependent Variable	lw
Covariance Structure	Compound Symmetry
Subject Effect	anim
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
anim	60	2 3 4 5 7 8 10 12 13 16 17 18 19 20 21 22 23 25 26 28 32 33 34 35 36 37 38 39 40 42 43 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 68 69 71 74 75 76 77
gr	2	a b
treat	2	bc nbc
week	6	1 2 3 4 5 6

Note: gr= Group A, B, Treat=Treated with toltrazuril (BC), Not treated with toltrazuril (NBC), Week= weeks post treatment.

Dimensions

Covariance Parameters	2
Columns in X	36
Columns in Z	0
Subjects	60
Max Obs Per Subject	6
Observations Used	360
Observations Not Used	0
Total Observations	360

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	2088.37648425	
1	1	2018.21081582	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
CS	anim	8.8861
Residual		15.0825

Fit Statistics

-2 Res Log Likelihood	2018.2
AIC (smaller is better)	2022.2
AICC (smaller is better)	2022.2
BIC (smaller is better)	2026.4

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	70.17	<.0001

Solution for Fixed Effects

Effect	gr	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|

Intercept				28.4651	5.1773	56	5.50	<.0001
gr	a			-0.9468	1.7925	56	-0.53	0.5994
gr	b			0	.	.	.	.
treat		bc		2.9043	1.7898	56	1.62	0.1103
treat		nbc		0	.	.	.	.
week			1	-22.8667	1.4181	279	-16.12	<.0001
week			2	-18.9667	1.4181	279	-13.37	<.0001
week			3	-12.4667	1.4181	279	-8.79	<.0001
week			4	-11.4333	1.4181	279	-8.06	<.0001
week			5	-6.0000	1.4181	279	-4.23	<.0001
week			6	0	.	.	.	.

Effect	gr	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|
gr*treat*week	a	bc	1	-1.6157	3.2282	279	-0.50	0.6171
gr*treat*week	a	bc	2	-2.7157	3.2282	279	-0.84	0.4009
gr*treat*week	a	bc	3	-3.4157	3.2282	279	-1.06	0.2909
gr*treat*week	a	bc	4	1.3843	3.2282	279	0.43	0.6684
gr*treat*week	a	bc	5	1.7843	3.2282	279	0.55	0.5809
gr*treat*week	a	bc	6	0.8177	2.5297	279	0.32	0.7468
gr*treat*week	a	nbc	1	1.0933	2.0055	279	0.55	0.5861
gr*treat*week	a	nbc	2	4.0267	2.0055	279	2.01	0.0456
gr*treat*week	a	nbc	3	-1.1067	2.0055	279	-0.55	0.5815
gr*treat*week	a	nbc	4	1.7267	2.0055	279	0.86	0.3900
gr*treat*week	a	nbc	5	0.1600	2.0055	279	0.08	0.9365
gr*treat*week	a	nbc	6	0	.	.	.	.
gr*treat*week	b	bc	1	-2.8067	2.0055	279	-1.40	0.1628
gr*treat*week	b	bc	2	-5.1733	2.0055	279	-2.58	0.0104
gr*treat*week	b	bc	3	-4.0733	2.0055	279	-2.03	0.0432
gr*treat*week	b	bc	4	-1.7067	2.0055	279	-0.85	0.3955
gr*treat*week	b	bc	5	-0.2067	2.0055	279	-0.10	0.9180
gr*treat*week	b	bc	6	0	.	.	.	.
gr*treat*week	b	nbc	1	0	.	.	.	.
gr*treat*week	b	nbc	2	0	.	.	.	.
gr*treat*week	b	nbc	3	0	.	.	.	.
gr*treat*week	b	nbc	4	0	.	.	.	.
gr*treat*week	b	nbc	5	0	.	.	.	.
gr*treat*week	b	nbc	6	0	.	.	.	.
lw1				0.9477	0.04692	56	20.20	<.0001

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
gr	1	56	0.20	0.6583
treat	1	56	1.13	0.2932
week	5	279	315.55	<.0001
gr*treat*week	16	279	2.62	0.0008
lw1	1	56	407.92	<.0001

Least Squares Means

Effect	gr	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|
gr	a			121.25	0.6226	56	194.74	<.0001
gr	b			120.85	0.6226	56	194.10	<.0001
treat		bc		121.52	0.6199	56	196.02	<.0001

treat	nbc		120.58	0.6199	56	194.51	<.0001
week		1	109.80	0.6320	279	173.72	<.0001
week		2	113.57	0.6320	279	179.68	<.0001
week		3	118.88	0.6320	279	188.09	<.0001
week		4	122.42	0.6320	279	193.68	<.0001
week		5	127.93	0.6320	279	202.41	<.0001
week		6	133.70	0.6320	279	211.54	<.0001

Effect	gr	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|
gr*treat*week	a	bc	1	110.00	1.2762	279	86.19	<.0001
gr*treat*week	a	bc	2	112.80	1.2762	279	88.39	<.0001
gr*treat*week	a	bc	3	118.60	1.2762	279	92.93	<.0001
gr*treat*week	a	bc	4	124.43	1.2762	279	97.50	<.0001
gr*treat*week	a	bc	5	130.26	1.2762	279	102.07	<.0001
gr*treat*week	a	bc	6	135.30	1.2762	279	106.02	<.0001
gr*treat*week	a	nbc	1	109.80	1.2641	279	86.86	<.0001
gr*treat*week	a	nbc	2	116.63	1.2641	279	92.27	<.0001
gr*treat*week	a	nbc	3	118.00	1.2641	279	93.35	<.0001
gr*treat*week	a	nbc	4	121.87	1.2641	279	96.41	<.0001
gr*treat*week	a	nbc	5	125.73	1.2641	279	99.47	<.0001
gr*treat*week	a	nbc	6	131.57	1.2641	279	104.09	<.0001
gr*treat*week	b	bc	1	109.75	1.2648	279	86.77	<.0001
gr*treat*week	b	bc	2	111.28	1.2648	279	87.98	<.0001
gr*treat*week	b	bc	3	118.88	1.2648	279	93.99	<.0001
gr*treat*week	b	bc	4	122.28	1.2648	279	96.68	<.0001
gr*treat*week	b	bc	5	129.22	1.2648	279	102.16	<.0001
gr*treat*week	b	bc	6	135.42	1.2648	279	107.07	<.0001
gr*treat*week	b	nbc	1	109.65	1.2709	279	86.28	<.0001
gr*treat*week	b	nbc	2	113.55	1.2709	279	89.35	<.0001
gr*treat*week	b	nbc	3	120.05	1.2709	279	94.46	<.0001
gr*treat*week	b	nbc	4	121.09	1.2709	279	95.28	<.0001
gr*treat*week	b	nbc	5	126.52	1.2709	279	99.55	<.0001
gr*treat*week	b	nbc	6	132.52	1.2709	279	104.27	<.0001

Considering group C

The Mixed Procedure

Model Information

Data Set	WORK.FOUR
Dependent Variable	tfec
Covariance Structure	Compound Symmetry
Subject Effect	anim(group*treat)
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values

anim 76 1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23
24 25 26 28 29 30 31 32 33 34
35 36 37 38 39 40 41 42 43 44
45 46 47 48 49 50 51 52 53 54
55 56 57 58 59 60 61 62 63 64
65 66 67 68 69 70 71 72 73 74
75 76 77
group 3 A B C
treat 2 bc nbc
week 6 1 2 3 4 5 6

Dimensions

Covariance Parameters	2
Columns in X	41
Columns in Z	0
Subjects	81
Max Obs Per Subject	6
Observations Used	464
Observations Not Used	22
Total Observations	486

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	3807.62010841	
1	2	3806.29249166	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
CS	anim(group*treat)	11.8383
Residual		296.50

Fit Statistics

-2 Res Log Likelihood	3806.3
AIC (smaller is better)	3810.3
AICC (smaller is better)	3810.3
BIC (smaller is better)	3815.1

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	1.33	0.2492

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F

week	5	358	4.36	0.0007
treat	1	77	14.62	0.0003
group	2	77	4.55	0.0136
group*treat*week	20	358	2.78	<.0001

Least Squares Means

Effect	group	week	Estimate	Standard Error	DF	t Value	Pr > |t|
week		1	Non-est	.	.	.	.
week		2	Non-est	.	.	.	.
week		3	Non-est	.	.	.	.
week		4	Non-est	.	.	.	.
week		5	Non-est	.	.	.	.
week		6	Non-est	.	.	.	.
treat	bc		Non-est	.	.	.	.
treat	nbc		Non-est	.	.	.	.
group	A		12.7372	1.4325	77	8.89	<.0001
group	B		8.5477	1.4289	77	5.98	<.0001
group	C		Non-est	.	.	.	.
group*treat*week	A	bc	1	30.0660	4.5338	358	6.63	<.0001
group*treat*week	A	bc	2	2.8316	4.5338	358	0.62	0.5327
group*treat*week	A	bc	3	3.1382	4.6920	358	0.67	0.5040
group*treat*week	A	bc	4	4.3134	4.5338	358	0.95	0.3420
group*treat*week	A	bc	5	6.6543	4.5338	358	1.47	0.1431
group*treat*week	A	bc	6	8.8392	4.5338	358	1.95	0.0520
group*treat*week	A	nbc	1	13.5860	4.5338	358	3.00	0.0029
group*treat*week	A	nbc	2	13.0484	4.5338	358	2.88	0.0042
group*treat*week	A	nbc	3	28.3679	4.5338	358	6.26	<.0001
group*treat*week	A	nbc	4	18.4920	4.5338	358	4.08	<.0001
group*treat*week	A	nbc	5	15.9268	4.5338	358	3.51	0.0005
group*treat*week	A	nbc	6	7.5821	4.5338	358	1.67	0.0953
group*treat*week	B	bc	1	4.1875	4.5338	358	0.92	0.3563
group*treat*week	B	bc	2	2.6377	4.5338	358	0.58	0.5611
group*treat*week	B	bc	3	3.0023	4.5338	358	0.66	0.5083
group*treat*week	B	bc	4	2.6377	4.5338	358	0.58	0.5611
group*treat*week	B	bc	5	5.3030	4.5338	358	1.17	0.2429
group*treat*week	B	bc	6	7.6809	4.5338	358	1.69	0.0911
group*treat*week	B	nbc	1	11.4796	4.5338	358	2.53	0.0118
group*treat*week	B	nbc	2	14.2431	4.5338	358	3.14	0.0018
group*treat*week	B	nbc	3	20.9917	4.5338	358	4.63	<.0001
group*treat*week	B	nbc	4	16.8409	4.5338	358	3.71	0.0002
group*treat*week	B	nbc	5	4.8504	4.5338	358	1.07	0.2854
group*treat*week	B	nbc	6	8.7178	4.5338	358	1.92	0.0553
group*treat*week	C	nbc	2	20.1783	3.8318	358	5.27	<.0001
group*treat*week	C	nbc	3	34.2240	3.8318	358	8.93	<.0001
group*treat*week	C	nbc	4	22.9174	3.8318	358	5.98	<.0001
group*treat*week	C	nbc	5	7.5162	3.8318	358	1.96	0.0506
group*treat*week	C	nbc	6	9.4588	3.8318	358	2.47	0.0140

Appendix 4.2. 1. Live weights of calves treated with toltrazuril at weaning:
Group A: BC calves treated with toltrazuril and NBC calves not treated with toltrazuril

NO.	Treatment	7th Nov	14th Nov	21stNov	28thNov	3rd Dec	12th Dec
Group A							
75	BC	102	112.5	109	116	122.5	119.5
19	BC	130	134	141	145.5	154	157
17	BC	111	108.5	117	123.5	129	135
64	BC	103	109.5	113.5	118	121	139.5
22	BC	113	116.5	128.5	132.5	140	145
16	BC	124	107	135.5	141	150.5	156
20	BC	121	124	121.5	127.5	136	145
7	BC	120	128	128.5	129	142	145.5
62	BC	101	107	111.5	120.5	118.5	120
77	BC	108	112	117	121	126	128.5
3	BC	125	124	135	138.5	144	154
2	BC	124	128	129	144	144.5	151.5
60	BC	107	120.5	114.5	122	124.5	130
66	BC	101	106	110	115.5	120.5	123
12	BC	113	115.5	120.5	125	134	138
Arithmatic mean		**113.53**	**116.60**	**122.13**	**127.96**	**133.8**	**139.16**
Group A							
18	NBC	118	125.5	122.5	129.5	137.5	145.5
65	NBC	109	114	121.5	119.5	124	131.5
8	NBC	112	120.5	116.5	124	127	131
13	NBC	122	126	130	131	138.5	142.5
10	NBC	102	108	107.5	114	116	123
63	NBC	96	102.5	106	114.5	110.5	120.5
5	NBC	116	122.5	121	134	130.5	139.5
4	NBC	108	108	123	117	129.5	138
21	NBC	119	123	124.5	128.5	134	147
68	NBC	103	106	114	109.5	118.5	119
59	NBC	110	110	117.5	120.5	128	125.6
74	NBC	102	118.5	115.5	116.5	115.5	133
71	NBC	112	124.5	117.5	125	127.5	134
69	NBC	109	122.5	122.5	128.5	129.5	124
76		109	118	110.5	116	119.5	
Arithmetic mean		**109.8**	**116.63**	**118**	**121.86**	**125.73**	**132.43**
Group B							
36	BC	123	123	125.5	132	140	145.5
34	BC	108	114	126	122.5	134	138
52	BC	99	107	107.5	112	121	128
40	BC	102	100.5	103.5	108	113	118.5
42	BC	103	104.5	107	114	119.5	125.5
54	BC	96	100.5	105.5	113	117.5	123.5
48	BC	106	103.5	107.5	115	120.5	126.5
55	BC	107	112.5	120.5	115	122	125
53	BC	99	107.5	118.5	110	121.5	125.5
32	BC	139	110	138	146	151	162
45	BC	104	112.5	115	123.5	133.5	139.5

35	BC	112	118	131	133.5	137.5	145
28	BC	122	124	128	134.5	134	147
46	BC	105	108.5	119	117	125	131.5
25	BC	108	110	117.5	125	135	137
Arithmetic mean		**108.86**	**110.4**	**118**	**121.4**	**128.33**	**134.53**
58	NBC	103	107	110	115	119	125
47	NBC	106	104	106	113	119.5	125.5
50	NBC	98	100.5	110.5	114	113.5	121.5
23	NBC	120	125.5	137	130.5	134	143.5
37	NBC	120	127.5	139	138	147	153
56	NBC	107	108.5	109.5	113.5	121.5	125.5
26	NBC	132	134.5	140.5	146.5	153	164
51	NBC	96	111.5	110.5	109.5	116.5	123.5
43	NBC	102	104.5	114.5	117	118.5	126
38	NBC	100	103.5	107.5	111	113	117.5
57	NBC	104	105	109.5	114	122	124
61	NBC	104	100	108	99.5	106	108
49	NBC	100	110	123	113	118	123
33	NBC	107	114.5	119	126.5	132.5	137
39	NBC	106	107	116.5	115.5	124	131
		107	110.9	117.4	118.43	123.86	
Group C							
73	NBC		120.5	116.5	123	124.5	130
11	NBC		136	132	139	147	155
1	NBC		120.5	124	120	126	134.5
6	NBC		137	133.5	133	146	144.5
67	NBC		124.5	124	130	131.5	136.5
14	NBC		120	121	125.5	131	145
15	NBC		123.5	129.5	132	142	144.5
71	NBC		110	117.5	125	127.5	133
70	NBC		104	107	108	112	113
9	NBC		117	129	134.5	139.5	147
72	NBC		106.5	113.5	120.5	119	124.5
57 or 27	NBC		100	109.5	123	122	124
39	NBC		107	116.5	115.5	124	131
30	NBC		108	109.5	117.5	125.5	130.5
31	NBC		104	103.5	111	118	122.5
50	NBC		100.5	110.5	114	113.5	121.5
61	NBC		100	108	99.5	106	108
44	NBC		114	123.5	121	127	135
24	NBC		110	113.5	113	116.5	124.5
41	NBC		109	114	1211.5	128	133.5
29	NBC		113	123.5	123.5	127	137
Arithmetic Mean				**118.07**	**173.33**	**126.35**	**132.14**

Appendix 4.2.2 Average weekly weight of calves:

	Group A		Group B	
Week	Bc	nbc	bc	nbc
1	110	109.8	109.75	109.65
2	112.8	116.63	111.28	113.55
3	118.6	118	118.88	120.05
4	124.43	121.87	122.28	121.09
5	130.26	125.73	129.22	126.52
6	135.3	131.57	135.42	132.52

BC calves treated with toltrazuril and NBC calves not treated with toltrazuril

Appendix 4.2.3. Statistical analysis of Live weights:

The Mixed Procedure

Model Information

Data Set	WORK.TWO
Dependent Variable	lw
Covariance Structure	Compound Symmetry
Subject Effect	anim
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
anim	60	2 3 4 5 7 8 10 12 13 16 17 18 19 20 21 22 23 25 26 28 32 33 34 35 36 37 38 39 40 42 43 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 68 69 71 74 75 76 77
gr	2	a b
treat	2	bc nbc
week	6	1 2 3 4 5 6

Dimensions

Covariance Parameters	2
Columns in X	36
Columns in Z	0
Subjects	60
Max Obs Per Subject	6
Observations Used	360
Observations Not Used	0
Total Observations	360

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	2088.37648425	
1	1	2018.21081582	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Subject	Estimate

CS	anim	8.8861
Residual		15.0825

Fit Statistics

-2 Res Log Likelihood	2018.2
AIC (smaller is better)	2022.2
AICC (smaller is better)	2022.2
BIC (smaller is better)	2026.4

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	70.17	<.0001

Solution for Fixed Effects

Effect	gr	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept				28.4651	5.1773	56	5.50	<.0001
gr	a			-0.9468	1.7925	56	-0.53	0.5994
gr	b			0	.	.	.	
treat		bc		2.9043	1.7898	56	1.62	0.1103
treat		nbc		0	.	.	.	
week			1	-22.8667	1.4181	279	-16.12	<.0001
week			2	-18.9667	1.4181	279	-13.37	<.0001
week			3	-12.4667	1.4181	279	-8.79	<.0001
week			4	-11.4333	1.4181	279	-8.06	<.0001
week			5	-6.0000	1.4181	279	-4.23	<.0001
week			6	0	.	.	.	
gr*treat*week	a	bc	1	-1.6157	3.2282	279	-0.50	0.6171
gr*treat*week	a	bc	2	-2.7157	3.2282	279	-0.84	0.4009
gr*treat*week	a	bc	3	-3.4157	3.2282	279	-1.06	0.2909
gr*treat*week	a	bc	4	1.3843	3.2282	279	0.43	0.6684
gr*treat*week	a	bc	5	1.7843	3.2282	279	0.55	0.5809
gr*treat*week	a	bc	6	0.8177	2.5297	279	0.32	0.7468
gr*treat*week	a	nbc	1	1.0933	2.0055	279	0.55	0.5861
gr*treat*week	a	nbc	2	4.0267	2.0055	279	2.01	0.0456
gr*treat*week	a	nbc	3	-1.1067	2.0055	279	-0.55	0.5815
gr*treat*week	a	nbc	4	1.7267	2.0055	279	0.86	0.3900
gr*treat*week	a	nbc	5	0.1600	2.0055	279	0.08	0.9365
gr*treat*week	a	nbc	6	0	.	.	.	
gr*treat*week	b	bc	1	-2.8067	2.0055	279	-1.40	0.1628
gr*treat*week	b	bc	2	-5.1733	2.0055	279	-2.58	0.0104
gr*treat*week	b	bc	3	-4.0733	2.0055	279	-2.03	0.0432
gr*treat*week	b	bc	4	-1.7067	2.0055	279	-0.85	0.3955
gr*treat*week	b	bc	5	-0.2067	2.0055	279	-0.10	0.9180
gr*treat*week	b	bc	6	0	.	.	.	
gr*treat*week	b	nbc	1	0	.	.	.	
gr*treat*week	b	nbc	2	0	.	.	.	
gr*treat*week	b	nbc	3	0	.	.	.	
gr*treat*week	b	nbc	4	0	.	.	.	
gr*treat*week	b	nbc	5	0	.	.	.	
gr*treat*week	b	nbc	6	0	.	.	.	
lw1				0.9477	0.04692	56	20.20	<.0001

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
gr	1	56	0.20	0.6583
treat	1	56	1.13	0.2932
week	5	279	315.55	<.0001
gr*treat*week	16	279	2.62	0.0008
lw1	1	56	407.92	<.0001

Least Squares Means

Effect	gr	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|
gr	a			121.25	0.6226	56	194.74	<.0001
gr	b			120.85	0.6226	56	194.10	<.0001
treat		bc		121.52	0.6199	56	196.02	<.0001
treat		nbc		120.58	0.6199	56	194.51	<.0001
week			1	109.80	0.6320	279	173.72	<.0001
week			2	113.57	0.6320	279	179.68	<.0001
week			3	118.88	0.6320	279	188.09	<.0001
week			4	122.42	0.6320	279	193.68	<.0001
week			5	127.93	0.6320	279	202.41	<.0001
week			6	133.70	0.6320	279	211.54	<.0001

Least Squares Means

Effect	gr	treat	week	Estimate	Standard Error	DF	t Value	Pr > |t|
gr*treat*week	a	bc	1	110.00	1.2762	279	86.19	<.0001
gr*treat*week	a	bc	2	112.80	1.2762	279	88.39	<.0001
gr*treat*week	a	bc	3	118.60	1.2762	279	92.93	<.0001
gr*treat*week	a	bc	4	124.43	1.2762	279	97.50	<.0001
gr*treat*week	a	bc	5	130.26	1.2762	279	102.07	<.0001
gr*treat*week	a	bc	6	135.30	1.2762	279	106.02	<.0001
gr*treat*week	a	nbc	1	109.80	1.2641	279	86.86	<.0001
gr*treat*week	a	nbc	2	116.63	1.2641	279	92.27	<.0001
gr*treat*week	a	nbc	3	118.00	1.2641	279	93.35	<.0001
gr*treat*week	a	nbc	4	121.87	1.2641	279	96.41	<.0001
gr*treat*week	a	nbc	5	125.73	1.2641	279	99.47	<.0001
gr*treat*week	a	nbc	6	131.57	1.2641	279	104.09	<.0001
gr*treat*week	b	bc	1	109.75	1.2648	279	86.77	<.0001
gr*treat*week	b	bc	2	111.28	1.2648	279	87.98	<.0001
gr*treat*week	b	bc	3	118.88	1.2648	279	93.99	<.0001
gr*treat*week	b	bc	4	122.28	1.2648	279	96.68	<.0001
gr*treat*week	b	bc	5	129.22	1.2648	279	102.16	<.0001
gr*treat*week	b	bc	6	135.42	1.2648	279	107.07	<.0001
gr*treat*week	b	nbc	1	109.65	1.2709	279	86.28	<.0001
gr*treat*week	b	nbc	2	113.55	1.2709	279	89.35	<.0001
gr*treat*week	b	nbc	3	120.05	1.2709	279	94.46	<.0001
gr*treat*week	b	nbc	4	121.09	1.2709	279	95.28	<.0001
gr*treat*week	b	nbc	5	126.52	1.2709	279	99.55	<.0001
gr*treat*week	b	nbc	6	132.52	1.2709	279	104.27	<.0001

Appendix 4.3. Faecal consistency and oocyst counts:

Serial. no	Animal number.	1	Animal No.	2	Animal No.	3
1	42	0	58	150	56	0
2	36	0	46	0	61	7750
3	37	900	48	0	41	250
4	31	0	15	950	43	0
5	27	100	77	50	49	500
6	23	0	40	0	38	0
7	26	0	35	0	49	50
8	24	200	32	0	37	50
9	53	50	69	600	57	0
10	19	100	10	10	50	0
11	64	0	57	400	60	0
12	51	450	50	450	72	750
13	11	1100	39	100	59	500
14	13	50	4	950	23	
15	12	0	59	750		
16	47	50	9	100		
17	28	0	73	0		
18	22	0	20	50		
19	5	350	2	50		
20	67	1250	62	0		
21	31	0	8	200		
22	76	300	34	0		
23	30	50	72	1350		
24	25	50	44	1250		
25	29	650	45	0		
26	14	2250	18	350		
27	71	100	65	200		
28	3	0	63	1350		
29	55	0	21	0		
30	12	1200	38	0		
31	75	200	61	200		
32	60	200	42	50		
33	52	50	32	0		
34	66	50	41	200		
35	17	0	58	100		
36	7	0	46	250		
37	68	100	33	0		
38	1	250	43	0		
39	70	0	53	100		
40	6	4550	54	150		
41	74	2150	40	0		
42	35	0	36	0		
43	29	150	55	0		
44	52	50	56	150		
45	26	150	68	650		
46	51	0	10	0		
47	48	0	54	50		
48	31	0				

49	33	0		
50	34	0		
51	45	0		
52	47	0		
53	25	250		
54	30	100		
55	4	150		
56	8	0		
57	15	0		
58	18	0		
59	2	0		
60	13	0		
61	17	0		
62	16	0		
63	22	350		
64	9	50		
65	3	0		
66	20	0		
67	12	1200		
		286.56	238.51	757.69

Appendix 4.4.Statistical analysis of faecal consistency:
The CORR Procedure

3 Variables: fc foc sr_foc FC-faecal consistency, Foc=Faecal oocyst counts, sr= square root

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
fc	127	1.57480	0.67282	200.00	1.00000	3.00000
foc	126	258.01587	566.54747	32510	0	4550
sr_foc	126	10.40687	12.32553	1311	1.00000	67.46110

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	fc	foc	sr_foc
fc	1.00000	-0.06193	-0.00750
		0.4909	0.9336
	127	126	126
foc	-0.06193	1.00000	0.90842
	0.4909		<.0001
	126	126	126
sr_foc	-0.00750	0.90842	1.00000
	0.9336	<.0001	
	126	126	126

sr_foc -0.00750 0.90842 1.00000
0.9336 <.0001
126 126 126

The SAS System
The Mixed Procedure

Model Information

Dependent Variable foc
Covariance Structure Variance Components
Group Effect fc
Estimation Method REML
Residual Variance Method None
Fixed Effects SE Method Model-Based
Degrees of Freedom Method Between-Within

Class Level Information

Class Levels Values

fc 3 1 2 3
Dimensions

Covariance Parameters 3
Columns in X 4
Columns in Z 0
Subjects 127
Max Obs Per Subject 1
Observations Used 126
Observations Not Used 1
Total Observations 127
Iteration History

Iteration Evaluations -2 Res Log Like Criterion

0 1 1920.63780848
1 1 1893.24809972 0.00000000
Convergence criteria met.

Covariance Parameter Estimates

Cov Parm Group Estimate

Residual fc 1 495347
Residual fc 2 141552
Residual fc 3 69318
Fit Statistics
-2 Res Log Likelihood 1893.2
AIC (smaller is better) 1899.2
AICC (smaller is better) 1899.4

BIC (smaller is better) 1907.8

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
2	27.39	<.0001

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
fc	2	123	0.49	0.6141

Least Squares Means

Effect	fc	Estimate	Standard Error	DF	t Value	Pr > |t|
fc	1	286.57	85.9840	123	3.33	0.0011
fc	2	238.51	54.8794	123	4.35	<.0001
fc	3	175.00	76.0034	123	2.30	0.0230

Differences of Least Squares Means

Effect	fc	_fc	Estimate	Standard Error	DF	t Value	Pr > |t|
fc	1	2	48.0565	102.00	123	0.47	0.6384
fc	1	3	111.57	114.76	123	0.97	0.3329
fc	2	3	63.5106	93.7457	123	0.68	0.4994

Appendix. 5.1: Oocyst counts from Ballantrae Hill Country Research Station.

No-Chemical-1(NC-1)				Conventional -1(CO-1)			
Sep	**Dec**	**Mar**	**Calves**	**Sep**	**Dec**	**Mar**	**Calves**
100	50	100	4641	0	0	0	618
500	200	1750	4634	1500	200	50	623
0	50	600	4640	0	0	0	719
0	50	200	4637	0		50	694
0	0	150	4643	0	0	0	364
			Dams	0	0	0	467
0	0	0	307	200	200	300	693
0	0	0	414	150	50	1300	673
50	0	0	407	0	0	0	618
200	0	0	351				**Dams**
0	100	100	426	0	0	150	15
				0	0	0	12
				0	100	0	9

No-Chemical- 2(NC-2)				Conventional-2(CO-2)			
Sep	**Dec**	**Mar**	**Calves**	**Sep**	**Dec**	**Mar**	**calves**
0	0	100	6526	0	0	350	2793
150	0	3050	6519	350	50	700	2796
250	0	50	6535	0	0	350	2795
50	0	400	6527	0	50	0	381
0	0	400	6497	0	0	600	2792
100	0	300	6504	0	0	0	2794
				0	0	250	2791
			Dams				**Dams**
0	0	0	613	0	50	0	377
0	0	0	608	0	50	0	381
0	0	0	604	50	0	0	213
0	0	0	615	0	0	0	212
0	0	0	603	0	50	0	209

Appendix 5.2: Table showing the details of combined no chemicals (NC1+NC2 and combined conventional (CO1+CO2) of calves and Dams at Ballantrae:

	sep	**Dec**	**Mar**
NC1- C	120.00	70.00	560.00
NC2-C	91.66	0.00	716.66
NC- Calves	**105.83**	**35.00**	**638.33**
CO1-C	205.55	56.25	188.88
CO2-C	50.00	14.28	321.42
CO- calves	**127.77**	**35.26**	**255.15**
NC1-D	50.00	20.00	20.00
NC2-D	0.00	0.00	0.00
NC-Dams	**25.00**	**10.00**	**10.00**
CO1-D	0.00	33.33	50.00
CO2-D	10.00	30.00	0.00
CO-Dams	**5.00**	**31.66**	**25.00**

Appendix 5.3: Statistical analysis of Ballantrae farm:

10:36 Friday, February 13, 2004 102
Age=calf
The Mixed Procedure
Model Information

Data Set WORK.THREE
Dependent Variable ln_foc
Covariance Structure Compound Symmetry
Subject Effect anim
Estimation Method REML
Residual Variance Method Profile
Fixed Effects SE Method Model-Based
Degrees of Freedom Method Between-Within
Class Level Information
Class Levels Values

treat 2 CO NC
Mo 3 Dec Mar Sep
gr 4 1 2 3 4
anim 27 364 381 467 488 618 623 673
693 694 719 2791 2792 2793
2794 2795 2796 4634 4637 4640
4641 4643 6497 6504 6519 6526
6527 6535
Dimensions
Covariance Parameters 2
Columns in X 16
Columns in Z 0
Subjects 27
Max Obs Per Subject 3
Observations Used 81
Observations Not Used 0
Total Observations 81
Iteration History
Iteration Evaluations -2 Res Log Like Criterion
0 1 358.75510350

1	1	346.02149497	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
CS	anim	2.7362
Residual		3.4723

Fit Statistics

-2 Res Log Likelihood	346.0
AIC (smaller is better)	350.0
AICC (smaller is better)	350.2
BIC (smaller is better)	352.6

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	12.73	0.0004

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
treat	1	23	3.01	0.0964
mo	2	50	18.60	<.0001
treat*mo	2	50	2.70	0.0771
gr(treat)	2	23	0.24	0.7862

Least Squares Means

Effect	treat	mo	gr	Estimate	Standard Error	DF	t Value	Pr > |t|
treat	CO			2.0257	0.4972	23	4.07	0.0005
treat	NC			3.3732	0.5974	23	5.65	<.0001
mo		dec		1.5000	0.4897	50	3.06	0.0035
mo		mar		4.4813	0.4897	50	9.15	<.0001
mo		sep		2.1170	0.4897	50	4.32	<.0001
treat*mo	CO	dec		1.4084	0.6260	50	2.25	0.0289
treat*mo	CO	mar		3.1920	0.6260	50	5.10	<.0001
treat*mo	CO	sep		1.4767	0.6260	50	2.36	0.0223
treat*mo	NC	dec		1.5916	0.7532	50	2.11	0.0396
treat*mo	NC	mar		5.7706	0.7532	50	7.66	<.0001
treat*mo	NC	sep		2.7573	0.7532	50	3.66	0.0006
gr(treat)	CO		3	1.9598	0.6577	23	2.98	0.0067
gr(treat)	CO		4	2.0916	0.7458	23	2.80	0.0101
gr(treat)	NC		1	3.7822	0.8825	23	4.29	0.0003
gr(treat)	NC		2	2.9642	0.8056	23	3.68	0.0012

Differences of Least Squares Means

Effect	treat	mo	gr	_treat	_mo	_gr	Estimate	Standard Error	DF	t Value	Pr > |t|
treat	CO			NC			-1.3475	0.7773	23	-1.73	0.0964
mo		dec			mar		-2.9813	0.5161	50	-5.78	<.0001
mo		dec			sep		-0.6170	0.5161	50	-1.20	0.2375
mo		mar			sep		2.3643	0.5161	50	4.58	<.0001
treat*mo	CO	dec		CO	mar		-1.7836	0.6588	50	-2.71	0.0093
treat*mo	CO	dec		CO	sep		-0.06832	0.6588	50	-0.10	0.9178

treat*mo	CO	dec	NC	dec	-0.1833	0.9794	50	-0.19	0.8523
treat*mo	CO	dec	NC	mar	-4.3622	0.9794	50	-4.45	<.0001
treat*mo	CO	dec	NC	sep	-1.3489	0.9794	50	-1.38	0.1746
treat*mo	CO	mar	CO	sep	1.7153	0.6588	50	2.60	0.0121
treat*mo	CO	mar	NC	dec	1.6004	0.9794	50	1.63	0.1085
treat*mo	CO	mar	NC	mar	-2.5786	0.9794	50	-2.63	0.0112
treat*mo	CO	mar	NC	sep	0.4347	0.9794	50	0.44	0.6591
treat*mo	CO	sep	NC	dec	-0.1149	0.9794	50	-0.12	0.9071
treat*mo	CO	sep	NC	mar	-4.2939	0.9794	50	-4.38	<.0001
treat*mo	CO	sep	NC	sep	-1.2806	0.9794	50	-1.31	0.1970
treat*mo	NC	dec	NC	mar	-4.1790	0.7946	50	-5.26	<.0001
treat*mo	NC	dec	NC	sep	-1.1657	0.7946	50	-1.47	0.1486
treat*mo	NC	mar	NC	sep	3.0133	0.7946	50	3.79	0.0004
gr(treat)	CO	3	CO	4	-0.1318	0.9944	23	-0.13	0.8957
gr(treat)	CO	3	NC	1	-1.8224	1.1006	23	-1.66	0.1113
gr(treat)	CO	3	NC	2	-1.0044	1.0400	23	-0.97	0.3442
gr(treat)	CO	4	NC	1	-1.6906	1.1554	23	-1.46	0.1569
gr(treat)	CO	4	NC	2	-0.8726	1.0978	23	-0.79	0.4348
gr(treat)	NC	1	NC	2	0.8180	1.1948	23	0.68	0.5004

age=dam

The Mixed Procedure

Model Information

Data Set	WORK.THREE
Dependent Variable	ln_foc
Covariance Structure	Compound Symmetry
Subject Effect	anim
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
treat	2	CO NC
mo	3	dec mar sep
gr	4	1 2 3 4
anim	19	9 12 15 209 212 213 307 351 377 381 407 414 426 603 604 608 613 615 621

Dimensions

Covariance Parameters	2
Columns in X	16
Columns in Z	0
Subjects	19
Max Obs Per Subject	3
Observations Used	57
Observations Not Used	0
Total Observations	57

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	204.77202938	
1	1	203.88904901	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
CS	anim	-0.3466
Residual		3.0065

Fit Statistics

-2 Res Log Likelihood	203.9
AIC (smaller is better)	207.9
AICC (smaller is better)	208.1
BIC (smaller is better)	209.8

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	0.88	0.3474

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
treat	1	15	1.34	0.2659
mo	2	34	0.88	0.4260
treat*mo	2	34	1.61	0.2155
gr(treat)	2	15	3.15	0.0719

Least Squares Means

Effect	treat	mo	gr	Estimate	Standard Error	DF	t Value	Pr > |t|
treat	CO			1.0594	0.2956	15	3.58	0.0027
treat	NC			0.6155	0.2451	15	2.51	0.0240
mo		dec		1.2648	0.3809	34	3.32	0.0022
mo		mar		0.5527	0.3809	34	1.45	0.1559
mo		sep		0.6949	0.3809	34	1.82	0.0769
treat*mo	CO	dec		2.0540	0.5813	34	3.53	0.0012
treat*mo	CO	mar			0.5813	34	1.08	0.2862
treat*mo	CO	sep		0.4942	0.5813	34	0.85	0.4012
treat*mo	NC	dec		0.4755	0.4922	34	0.97	0.3409
treat*mo	NC	mar		0.4755	0.4922	34	0.97	0.3409
treat*mo	NC	sep		0.8955	0.4922	34	1.82	0.0777
gr(treat)	CO		3	1.0703	0.4675	15	2.29	0.0370
gr(treat)	CO		4	1.0485	0.3621	15	2.90	0.0111
gr(treat)	NC		1	1.2310	0.3621	15	3.40	0.0040
gr(treat)	NC		2	-54E-17	0.3305	15	-0.00	1.0000

Differences of Least Squares Means

Effect	treat	mo	gr	_treat	_mo	_gr	Estimate	Standard Error	DF	t Value	Pr > |t|
treat	CO			NC			0.4439	0.3841	15	1.16	0.2659
mo		dec			mar		0.7121	0.5697	34	1.25	0.2199
mo		dec			sep		0.5699	0.5697	34	1.00	0.3242
mo		mar			sep		-0.1422	0.5697	34	-0.25	0.8045
treat*mo	CO	dec		CO	mar		1.4242	0.8670	34	1.64	0.1097
treat*mo	CO	dec		CO	sep		1.5598	0.8670	34	1.80	0.0809
treat*mo	CO	dec		NC	dec		1.5785	0.7617	34	2.07	0.0459
treat*mo	CO	dec		NC	mar		1.5785	0.7617	34	2.07	0.0459

treat*mo	CO	dec	NC	sep	1.1585	0.7617	34	1.52	0.1375
treat*mo	CO	mar	CO	sep	0.1357	0.8670	34	0.16	0.8766
treat*mo	CO	mar	NC	dec	0.1544	0.7617	34	0.20	0.8406
treat*mo	CO	mar	NC	mar	0.1544	0.7617	34	0.20	0.8406
treat*mo	CO	mar	NC	sep	-0.2656	0.7617	34	-0.35	0.7295
treat*mo	CO	sep	NC	dec	0.01869	0.7617	34	0.02	0.9806
treat*mo	CO	sep	NC	mar	0.01869	0.7617	34	0.02	0.9806
treat*mo	CO	sep	NC	sep	-0.4013	0.7617	34	-0.53	0.6017
treat*mo	NC	dec	NC	mar	-278E-18	0.7394	34	-0.00	1.0000
treat*mo	NC	dec	NC	sep	-0.4200	0.7394	34	-0.57	0.5737
treat*mo	NC	mar	NC	sep	-0.4200	0.7394	34	-0.57	0.5737
gr(treat)	CO	3	CO	4	0.02178	0.5913	15	0.04	0.9711
gr(treat)	CO	3	NC	1	-0.1608	0.5913	15	-0.27	0.7894
gr(treat)	CO	3	NC	2	1.0703	0.5725	15	1.87	0.0812
gr(treat)	CO	4	NC	1	-0.1825	0.5121	15	-0.36	0.7265
gr(treat)	CO	4	NC	2	1.0485	0.4903	15	2.14	0.0493
gr(treat)	NC	1	NC	2	1.2310	0.4903	15	2.51	0.0240

Appendix 6.1. Cleaning of oocysts:

1. The oocysts in 2% H_2so_4 were washed with PBS for 4 cycles at 830g last wash with double glass distilled water.
2. Sterilise the oocysts with 50% Janola (V/V) 5ml Janola+5ml water at room temperature for one hour.
3. Wash with Double glass distilled water twice and re-suspend them in PBS or Hanks balanced s solution.

Appendix 6.2. Vortexing:

1. Take cleaned oocyst into a tube.
2. Add 0.5 ml chilled buffer and glass beads so they compose about half of the total resulting volume and place the tube on a Vortex mixer and turn to maximum speed and agitate the contents until the most of the oocysts have been mechanically fractured their sporocysts.
3. Note: toughness varied with different species.
   For robust and xx 30 - 40 strokes. One stroke - holding the test tube on with mixer for a count of one second. Check the process of the cracking by examining under a microscope. A very small sample at regular intervals after every 5 to 6 strokes until some experience is gained in this procedure. If too many strokes used a large proportion of the oocysts will be damaged.
   Remove sporocysts from glass bells with repeated addition of PBS PH 7.6 and resuspend the sporocyst in an appropriate volume. 20ml for 5 $x10^6$ sporocysts or 200ml for 400ml$x10^6$ sporocysts.

4. Wash sporozoites in medium of PBS PH 7.6 resuspend in an appropriate volume of the medium or PBS Hanks balanced solution with 5 ml of $MgCl_2$ ideal volume

Appendix 6.3.Western Blotting:

Equipment

Mini-protean II Electrophoresis cell

a) Mini Trans-blot Electrophoretic transfer cell
b) Power Supply device
c) PVDF membrane
d) 3 MM Whatman paper
e) Developing film

Reagents

a) Sterile Water

b) 0.5 M Tris HCl (pH 6.8)

60.55 g Tris base in 800-ml water adjust pH with 6N HCl then makeup 1 liter.
Store at 4 degree C
(Or Biorad Cat no: 161-0799)

c) 1.5 M Tris HCl (pH 8.8)

181.65 g Tris base in 800ml water, adjust pH with 6N HCl then make up to 1 liter
Store at 4 degree C
(Or Biorad Cat no: 161-0798)

d) 10% SDS

Dissolve 10g SDS in 100ml distilled water

e) Acrylamide/bis

Biorad agent 30% acrylamide/bis solution 37.5:1(2.5%C)
Cat No. 161-0158

f) 10% Ammonium persulfate (APS)

Make up fresh each time
0.05g APS in 0.5ml water
Biorad Cat No. 161-0700

g) TEMED

Biorad Cat No. 161-0800

h) Gel loading buffer

Deionized water	3.8ml
0.5M Tris HCl	1.0ml
Glycerol	0.8ml
10%SDS	1.6ml
2-Mercaptoethanol	0.4ml
1 %(w/v) bromophenol blue	0.40ml

Store at room temperature

i) 5X running buffer (pH 8.3)

Tris base	15.1g
Glycine	94.0g

Dissolve in 900 ml deionized water.
Add 50ml SDS and adjust volume to 1 liter
Store at 4ºC

j) Transfer buffer

Tris base	5.82g
Glycine	2.93g
Methanol	200ml

PH should be between 9 and 9.3-this is critical
Store at 4 degrees C

k) PBS-Tween

NaCl	8g

KCl	0.2g
KH_2PO_4	0.2g
Na_2HPO_4	1.16g (or Na2HPO4.12H2O 2.9g)
Tween 20	0.5ml

Dissolve in 1 liter deionized water
Store at 4 degrees C

l) 5% milk powder

Dissolve 2.5g Pams Non-fat Mil powder in 50ml deionized water
Store at 4 degrees C

m) Primary antibody

Dog anti-Neospora antibody diluted 1:1000 (10ul in 10ml)

n) Secondary antibody

Anti-bovine HRP diluted 1:10000 (2ul in 20ml)

o) Westfemto

Combine equal volumes of luminol and peroxide solutions immediately before use

Separating (resolving) Gel (12%)

(Makes 2 gels)

Distilled water	3.35ml
1.5M Tris HCl	2.5ml
SDS	100ul
Acrylamide/bis	4.0ml
(Degas 15 minutes)	
APS	50ul
TEMED	5ul

p) Staking Gel

Distilled water	6.1ml
0.5M Tris HCl	2.5ml
SDS	100ul
Acrylamide/bis	1.3ml
(Degas 15 minutes)	
APS	50ul
TEMED	10ul

Procedure

1) Assembling the glass plate sandwiches

a) Wipe the glass plates with ethanol and assemble on a clean surface. Lay the longer plate down first, and then place spacers along the short edges of the plate, Next place the shorter glass plate on the top of the spacers.

b) Loosen the four screws on the clamp assembly and stand it in the alignment slot of the pouring tray so these screws are facing away from you. Pick up the glass plate sandwich so that the longer plate is facing from you and gently slide it in to the clamp assembly gently tighten both sets of screws.

c) Ensure the rubber gasket (Grey) is on top of the red foam pads in the casting slots. Transfer the clamp assembly to the casting slots in the following way: Butt the acrylic pressure plate against the wall of the casting slot and the bottom, so the glass plate rests on the rubber gasket. Snap the acrylic plate underneath the overhang pushing with the white portion of the clamps.

2) Casting the Gels

a) Prepare the separating gel by combining all the reagents except APS and TEMED. Degas the solution for least 15 minutes.

b) Place comb into the glass plate assembly. Add APS and TEMED and pour the solution to 1cm below the teeth of the comb. Remove comb.

c) Immediately overlay the monomer solution with water.

d) Allow the gel to polymerize for 1 hr. Rinse off the overlay solution completely with distilled water.
e) Prepare the staking as in a)
f) Add APS and TEMED to the stacking gel pour the solution to the top of the shorter glass plate and inset comb.
g) Allow the gel to polymerize 45 min. Remove the comb by pulling it up slowly and gently.
h) Rinse the wells completely with distilled water.

3) Running Gels

a) Remove gel sandwich from casting stand.
b) Lay inner cooling core flat on bench. With the glass plates of the clamp assembly facing the cooling core (and the clamp screws facing out) carefully slide the clamp assembly into the locator slots at the top of the core and snap clamp assembly on to the cooling core by presenting the bottom of the clamp assembly. Place entire assembly in to the electrophoresis cell
c) Pre 500ml off running buffer and fill the inner chambers until the buffer half way between the short and long plates. Pour the remaining buffer in the outer chamber so that at least the bottom 1 cm of the gel is covered.
d) Dilute the samples 1:4 with gel leading buffer boil for 3 minutes and load into the wells.
e) Place lid on electrophoreses cell and run it to 100 V for approx. 45 min.

4) Blotting

a) Remove gel from glass plates and place in transfer buffer for 10 minutes.
b) Place two fiber pads and two sheets of blotting paper cut to size into transfer buffer for 10 minutes
c) Cut PVDF membrane and vet in methanol. Rinse in transfer buffer until membrane sinks.
d) Open blotting cassette. With black side down, first place 1 fiber pad, then 1 filter paper, then gel followed by the membrane followed by a further layer of filter paper and fiber pad. Rolling out bubbles between each layer. Secure the blotting cassette closed.
e) Place blotting cassette in the blotting unit black side facing. Place blotting unit and ice block in the electrophoresis cell.
f) Fill tank with transfer buffer and transfer it to 100 V for 1 hour.

5) Developing

a) Rinse membrane in two changes of PBS-Tween
b) Block in 5% milk powder for 1 hour at room temperature on a rocker.
c) Rinse twice in PBS Tween.
d) Dilute primary antibody in 5% milk powder, add to membrane and incubate overnight at 4 degrees C on a rocker.
e) Rinse membrane in 6 changes of PBS Tween for 5 minutes each.
f) Dilute secondary antibody in 5% milk powder, add to membrane and incubate 1 hour at room temperature on a rocker.
g) Rinse membrane in 6 changes of PBS-Tween for 5 minutes each.
h) Make up westfemto.
i) Place membrane on 1/2 a plastic sheet and covers with Westfemto.
j) Fold other half of plastic sheet over top of membrane and spread Westfemto to get an even cover of the membrane.
k) Seal membrane into plastic and place in to a photographic cassette.
l) In a dark room place x-ray film on to membrane shiny side up.
m) Expose for 1 minute and process.

Appendix 6.4.Tissue culture technique for *Eimeria*

REAGENTS REQUIRED:

1. **Foetal Bovine serum:**

Heat inactivated sterile FBS is received in 500ml bottles. Thaw bottle in 37 ◦C water bath or incubator. Aseptically aliquot into 50 ml, 10ml volumes to be added to 500ml MEM to give 10% and 2% . Ready made MEM from GIBCO needs 10% serum for cell cultures and 2% for parasite growth. Whereas, advanced media requires only 2% for cell culture. Label centrifuge tubes with date and store at -20 ◦C.

2. **L_Glutamine:**

Glutamax is received in 100ml bottles .Thaw bottle at 37 ◦C and aliquot aseptically under laminar flow into 5ml volumes .Labellled and stored at – 20 ◦ C to be added to 500 ml MEM.

3. **Pencillin and Streptomycin (Pen strep) :**

Thaw at 37C and Aliquot into 5ml volumes to be added to 500ml MEM which contains 10,000units of Pencillin and 10,000 ug of Steptomycin/ ml. Labelled and stored at -20◦C .

4. **Fungizone:**

Aseptically aliquot into 200ul to be added to 500ml MEM, labelled and stored at - 20C.

5. **Non essential Amino Acids:**

Store the bottle at 4ºC add 5ml aseptically to 500ml MEM.

6. **Sodium bicorbonate**

Store at 4ºC and add 10ml to 500ml MEM.

7.10x MEM:

Add 50ml to 400ml of sterile water and store at 4ºC.

Or else buy 1x MEM.

MEM made up as follows:

400 ml sterile water or 1X MEM

50ml-10X MEM to 400ml water to make up MEM.

10ml-sodium bicarbonate.

5ml-Non essential medium.

5ml -Glutamax.

5ml-PenStrep

200 µl-Fungizone.

Thaw all the required chemicals at room temperature or at 37ºC. Wipe all the containers with alcohol and make up the media in a laminar flow and kept at 37ºC for immediate use or stored at 4ºC for further use.

Passage of Vero cells:

Reagents Required:

1. MEM with 2% (Advanced medium) or 10% Foetal calf serum.
2. EDTA/ Trypsin (10X- aliquots of 2ml/ 18ml sterile water).
3. 18 ml sterile water in universals.
4. Sterile PBS 1x (10 ml of 10X in 100ml sterile water).

Equipment Required:

1. Sterile work station or Biohazard cabinet.
2. Incubator 37ºC, 5% CO_2.
3. Water bath.
4. Microscope.
5. Centrifuge.
6. Tissues Paper towels for wipe
7. Cell counter 8. Cell culture Flasks (75ml, 30ml, 10ml).
9. Sterile Pipettes – 10ml, 5ml, 20ml.

10. Gloves.
11. Sterile Universals (20ml).
12. Discard bucket.
13. Neubauer Counting chamber.
14. 70% ethanol.

Trypsinising cells:

Check the cell the monolayer for bacterial, fungal and foci of cell necrosis which will be seen as turbid with bacteria, white demarcated foci sometimes hyphe for fungi. Necrosis indicates cytopathic effect of virus. Discard all the flasks showing such lesions.

When monolayer is healthy then proceed to the following steps.

1. Warm media, 15 PBS, water in universals (18ml) and EDTA/ Trypsin to 37◦C in water bath or incubator (wipe all the containers with 70% ethanol before use).
2. Carefully decant old media from the flask into discarding bucket (Container with a funnel) and avoid splashing and cross contamination).
3. Wash monolayers with 1% PBS.
4. Add 2ml EDTA to 18ml sterile water (universals) and add sufficient amount to flask (75 ml/ 10ml, 30ml/7ml, 10ml/5ml). Leave for 1 minute and discard leaving enough to cover the monolayer.
5. Leave the flasks at 37C until cells detach from the surface (Check under microscope and give a gentle tap on the flask).
6. When all the cells are detached add 5ml of MEM to stop trypsinisation (lengthy trypsinisation may be toxic to cells).
7. Optional (centrifuge the cells at 1200rpm discard the supernatant and resuspend in 5ml MEM.
8. Cell counting:

A. place cover slip on cell counting Chamber, add a drop of cell suspension to counting chamber with a sterile Pasteur pipette. Allow the fluid to flow under cover slip by capillary action.

B. counts the cells in the large corner squares (WBC) each square has 16 cells.

C. take the mean number of cells per square which gives cells x 10^4/ml.

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