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The effect of florfenicol on egg hatchability in fowls

By

Dr. Saeed M. Al-Shahrani

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Submitted in partial fulfilment of the requirements for the degree of Masters in Veterinary Science (MSc.) Pharm

Section of Pharmacology and Toxicology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria.

Promoter: **Prof V. Naidoo**

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Declaration

I declare that the experimental work described in this thesis is my original work (except where the input of others is acknowledged), conducted in the Section of Pharmacology and Toxicology, Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria, and has not been submitted in any other form to any University or academic institution. I Saeed M. Al-Shahrani declare the above statement to be true.

Sign: --
Saeed M. Al-Shahrani

Sign: --
Prof V. Naidoo (Promoter)

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Abstract

The effect of florfenicol on egg hatchability in fowls

By

Saeed M. Al-Shahrani

Supervisor: Prof V Naidoo
Department: Paraclinical Sciences
Degree: MSc (Veterinary Science) Pharm

While florfenicol is not registered for use in poultry, the product is used on a limited basis in broiler breeders for the treatment of *E. coli* by means of compounded solutions. While apparently efficacious, one unpublished adverse reaction report from the field suggest that florfenicol may interfere with embryogenesis of the developing egg. With the side effects of the product largely unknown in breeder birds, the aim of this study was to evaluate the effect of florfenicol on egg fertility in a fowl model. In this study 20 week old commercial layer breeder hens (n=30) and cockerels (n=4) in 4 groups were exposed to florfenicol in a phased manner, with the same groupings used for all phases. Prior to each phase, a wash-out period of three weeks was allowed.

In phase 1, only the hens were treated with florfenicol at 0, 10, 20 and 30 mg/kg, respectively, while in phase II, only the cockerels were treated at doses of 0, 30, 60, 90 mg/kg. In phase III only hens again were treated at doses of 0, 30, 60, 90 mg/kg. In all phases, treatments were administered once daily for 5 days directly into the crop. Eggs were collected from all groups on days 0, 2, 4, and 5 of dosing and on days 1, 3, 4, 6, and 8 days post-treatment for incubation. Fertility was evaluated by candling, egg break-outs and number of chicks hatching. In phase III, five hens from each group were slaughtered at 0, 1, 2, 3 and 4 days after drug withdrawal to ascertain the lungs concentrations achieved for the florfenicol, while eggs (n=5) were collected on days 0, 1, 2, 3, and 4 for the same reason. Florfenicol was quantified in the tissues using a validated HPLC method.

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Doses of 0, 10, 20 or 30 mg/kg of florfenicol had no major effects on the embryo and hatchability. No fertility effects were noted when the cockerels were treated. However, the hens treated at 60 and 90 mg/kg showed signs of embryonic toxicity with a complete absence of hatchability (0%) being evident soon after treatment, which only returned to normal 5 days of treatment cessation. Florfenicol had no other overt toxic effects on the treated birds. The concentration of florfenicol in eggs at the dose of 90 mg/kg was 4.27 µgmg after five days of treatement. Based on the presence or absence of toxicity, the threshold egg concentration for toxicity appears to be 0.6 ug/mg. The safe period for the consumption of eggs after treatment was estimated to be 6 days. In conclusion, florfenicol is toxic to the embryo when the hens are treated with doses of 60 or 90 mg/kg for five consecutive days.

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ABBREVIATIONS USED

ADI	Average Daily Intake
AE	Avian Encephalomyelitis Vaccine
AUCC	Animal Use and Care Committee
^0C	Degree Celsius
CO_2	Carbon Dioxide
EDS	Egg Drop Syndrome
EMA	European Medicines Agency
FAO	Food and Agricultural Organisation
GIT	Gastro-intestinal tract
HPG	*Haemophilus paragallinarum*
HPLC	High Performance Liquid Chromatography
IB	Infectious Bronchitis
IBD	Infectious Bursal Disease
IBV	Infectious Bronchitis Virus
ILT	Infectious Laryngotracheitis
IM	Intramuscular
LLQ	Lower Limit of Quantification
Ln	Natural Logarithmic
MCC	Medicines Control Council of South Africa
MG	*Mycoplasma gallisepticum* vaccine
MIC	Minimum Inhibitory Concentration
MRL	Maximum Residue Limit
ND	Newcastle Disease
R^2	Coefficient of Correlation
SA	South Africa
SAPA	Southern African Poultry Association
SC	Subcutaneous
SD	Standard Deviation
TMDI	Theoretical Maximum Daily Intake
UP	University of Pretoria
WHO	World Health Organisation

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CHAPTER 1: INTRODUCTION

The poultry industry in South Africa is a well-established industry that has been estimated to contribute 24% of the total gross value of local agriculture market (Anon, 2010b). At present this market is estimated to contribute R13.5 billion (US$2.1billion) to the economy. Driven by demand poultry production has become big business in South Africa and appears to be growing from strength to strength on an annual basis. At present the country's requirement for poultry meat is estimated to be increasing at approximately 7% per annum, far outstripping the performance of the other protein sources on the market. While numerous factors probably played a role in overall demand, the main reason for the steady growth of South Africa's economy is believed to be the improvement in living standards with an increased health awareness resulting thereof and convenience. South Africans now eat more than double the amount of poultry as they did in the 1970's, with the result that even imports have increased substantially over the past few years. During the first quarter of 2012, about 104,019 tons of poultry meat was imported into South Africa. This is 8,379 tons (or 8.8%) more than previous quarter and 27,520 tons (or 36%) more than the same quarter in the previous year (Anonymous, 2012a).

As with any production industry the supply of rich protein is directly dependent on a well-managed breeding programme and associated farm quality i.e. the success of rearing programmes depends primarily on the ability of the farming system to supply large quantities of chicks from breeding stocks that perform competitively in an increasing range of environments (Anonymous, 2011a). There are certain factors that affect production in the poultry industry. These factors include amongst others, non-infectious conditions such as aged hens and lack of certain nutrients in poultry diets; Infectious diseases caused by bacteria, viruses, or parasites; and management defects like house temperature, feed quality and light requirements. Other problems affecting production could be caused by predators (e.g. snakes), and excessive egg breakage during lay (Ahmadi and Rahimi, 2011).

Drugs represent another factor used in treating various diseases as they could also significantly affect fertility in the breeder birds. Sulphamethazine, used for treating coccidiosis in cockerels, was shown to cause premature development of combs and

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wattles together with enlarged testes with hypertrophy of the seminiferous tubules (Asplin and Boyland, 1947). Aspirin fed to White Leghorn layer breeders at different concentrations demonstrated significantly lower fertility as seen by the decline in hatchability of fertile eggs (McDaniel et al., 1993). Similarly, chicks originating in hens fed low concentration of aspirin weighed more than chicks from hens that received higher concentration of aspirin (McDaniel et al., 1993). More recently the use of florfenicol in for the management of *Escherichia coli* pneumonia in broiler breeders has been tentatively associated with massive decrease in egg hatchability (V Naidoo, personal communication, 2011). While the reason for the toxicity is unknown, we speculate that the effect may be due to early embryonal death.

1.1. Hypothesis

1. Florfenicol decreases egg hatchability by inducing early embryonic death.
2. Florfenicol has no effect on cockerel fertility.

1.2. Objectives

The objectives of the study are to:

a. determine the effect of three doses (10, 20 or 30 mg/kg) of florfenicol on embryonic hatchability when administered to hens in comparison to a control group.

b. determine the effect of three doses (10, 20 or 30 mg/kg) of florfenicol on embryonic hatchability when administered to cockerels in comparison to a control group.

c. determine the effect of three high doses of florfenicol on embryonic hatchability when administered to hens at a dose of 30, 60 and 90 mg/kg.

d. to establish the potential toxic concentrations of florfenicol to the developing embryo

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e. to determine lung concentration of florfenicol reached in comparison to the MIC_{50} and MIC_{90} for *E. coli*.

f. to establish the safety levels of florfenicol in eggs from treated flocks for human consumption.

1.3. Benefits of the study

- Results generated from this study will add to the ever increasing knowledge on the effect of florfenicol on the fertility and hatchability of eggs in broiler breeders. Proper knowledge on these effects will help in the selection of dosage regimens that could be safer and more efficacious in the control of susceptible infectious agents in broiler chickens, in the absence of an effect on egg hatchability.

- The study will contribute to a Masters in Science degree in Veterinary Pharmacology.

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CHAPTER 2: LITERATURE REVIEW

2.1. Importance of poultry

Poultry is by far the largest livestock production group with an estimated 50 billion birds reared annually as a source of food, for both their meat and their eggs (Anonymous, 2011a). On a continent basis Europe, North and Central America, and Oceania produce considerably more per person than, Africa, Asia and South America with production *per capita* being 6.5 times greater for meat and 5.4 times greater for egg production in these first world countries (Anonymous, 1999a). In total, poultry products (egg and meat) constitute 30% of all animal protein consumed globally, which translates to a 10% increase in just ten years (Anonymous, 1999a). More importantly it has been estimated that by 2015, 40% of all animal protein consumed will arise from poultry production (Anonymous, 1999a). When only meat consumption is evaluated, Mack et al. (2005) estimated that 25% of the world's meat supply is derived from poultry, i.e. chicken, turkey, duck, geese, domesticated quail, etc.

In South Africa, the poultry industry is an important component of agricultural sector with more poultry products (egg and meat) being consumed per annum than all the other animal protein sources combined (Anonymous, 2011a). The South African Department of Agriculture, forestry and fisheries estimated the annual production of white meat and eggs in 2010 in South Africa to be 1.4 million and 450,000 tonnes, respectively (Anonymous, 2011b). The average number of broilers placed per week in the country has increased from 9.83 million in 1998 to 14.64 million in 2007. Of these, the average number of broilers slaughtered per week for the same period has increased by 8.65 million to 14.60 million broilers, representing a total increase of 6.2 %. Apart from being the primary source of protein, the poultry industry also provides jobs to the hundreds of thousands of South Africans. Approximately, 10% of all agricultural sector workers in the country are employed in the poultry industry. Furthermore, the poultry industry supports other manufacturing industries such as the poultry feed producing industry (Anonymous 2008).

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2.2. Role of breeder birds in poultry production

As a result of the high demand for broiler meat, the broiler industry has to maximise productivity by firstly producing sufficient chicks to enter into the cycle, but also to optimize growth of the chicks in the shortest possible period (Havenstein et al., 1994). For this reason, management of the broiler breeder flocks is an extremely important component in the system. For the latter, the industry has relied on the genetic selection based on important economic traits such as growth rate, body size, edible meat yield, and feed conversion (Gous, 1986; Havenstein et al., 1994). Not surprisingly the breeding of the birds is strictly controlled, with the commercial broiler chicks arising from local parent flocks under very high hygiene conditions, which in turn arise from grand-parent flocks. Both the parent and grand-parent flocks are usually provided by breeder companies from pure line they run or develop (Anonymous. 2008).

2.3. Challenges in the broiler breeder flock industry

With the global need for massive poultry productivity, the poultry industry has had to become an extremely intensive system, with large numbers of animals being housed under very defined and confined conditions. As a result of the complexities of the rearing of broiler breeders, the production of poultry products does come at a fair price in terms of both production costs and factors that could interfere with the production system, which are.

2.3.1. Nutrition

Proper nutrition is necessary to support both homeostasis and egg production. As with all animals the birds nutritional requirements is dependent on both the correct concentration, but also the correct ratios of macronutrients to micronutrients (Lopez and Leeson, 1994). While deficiencies in both these types of nutrients will interfere with metabolism, deficiencies in the micronutrients which are active in enzyme and hormone systems are usually the cause of breeding and laying problems e.g.

- Vitamin deficiencies: Vitamin D_3 deficiency causes rickets, a very painful skeletal disorder in poultry (Vieth, 1999). Vitamin C deficiencies appear to have a specific effect on metabolism by reducing metabolic rate with resultant increase in susceptibility to diseases due to reduced free radical scavenging action of

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vitamin C (Burgos et al., 2006). Free radical-induced diseases may also be associated with a deficiency of vitamin E or selenium as a result of free radical build-up and subsequent damage to cell membranes (Mezes et al., 1997). Myopathy (heart, skeletal, gizzard or intestinal) is commonly seen in selenium/vitamin E-deficient birds as a result of focal hyaline degeneration and mineralisation of myofibrils. Damage to capillary endothelium followed by fibrin thrombi, haemorrhage and ischaemic necrosis of the brain (encephalomalacia) as also described lesions resulting from oxidative damage (Swayne, 1996). The clinical signs of riboflavin deficiency include peripheral neuropathy and paralysis (Swayne, 1996).

- Mineral imbalances: A deficiency in iodine can result in thyroid dysfunction (McNabb et al., 1985). With phosphorus, zinc, chromium, manganese and copper being co-factors for enzyme function, deficiencies can result in various pathological changes such as swelling of the limbs, anaemia, rubbery beak and displacement of hock tendon leading to permanent lameness (Lund and Farr, 1967). Because birds excrete semisolid urine, high calcium in young birds may block the renal tubules whereas high sodium may cause kidney disease most likely due to an increase in blood viscosity and subsequent interference with blood-flow through the capillaries of the glomerulus (Julian and Brown, 1997).

- The acid-base balance (usually low pH) of the feed can also affect poultry health, egg-shell quality, kidney function and the incidence of leg problems in broilers (Crespo and Shivaprasad, 2003). While the decreased pH results from the strong chloride anion, other anions like sulphates may also interfere with the anion/cation ratio present in the food. Conversely high cations can affect urine pH and lead to concretions in the renal tubules and ureters and subsequent hyperuricaemia.

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2.3.2. Environmental factors

2.3.2.1. Cold stress

Maintaining proper temperature to promote efficient growth is a key to any profitable poultry production as well for the rearing of the chicks which become the parent flocks. South Africa (Fig. 2.1), which geographically resides between the 22nd and 34th degrees south of the equator, is part of the Southern Hemisphere's subtropical zone (Anonymous, 2007a). Nonetheless the country experiences a wider diversity of climatological (temperature, precipitation, humidity and sunshine) changes than most other countries in sub-Saharan Africa in addition to having lower average temperatures than other countries at the same latitude.

The South African climate is typically described as temperate and pleasant with warm sunny days predominating for the majority of the year (Anonymous, 2011c). The temperate climate refers to zones in a range of latitudes between 40° and 60/70° and is characterised as being not as hot as the subtropical climate and milder than the polar climate. The climate has distinct winter and summer seasons, typical of regions found between the Tropics of Cancer and Capricorn and the Arctic and Antarctic circles. The summer period runs from November to February, when most of the country is characterised by warm to hot weather, with the coldest days occurring in July-August. The country is usually broken down into temperate zones as the Benguela current, a cold current that moves from the lower South Atlantic Ocean, causes moderate temperatures on the West Coast throughout the year. On the central plateau, which includes Free State and the Gauteng provinces the altitude usually keeps the average temperatures below 30 °C in summer (Fig. 2.2). On the eastern seaboard, the warm Mozambique current ensures that the coastal cities have a subtropical climate for most of the year. The tip of the country, being the Cape provinces, tends to have cooler temperatures due to cooler oceanic currents that come from the South Pole.

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Fig. 2.1. Political map of South Africa showing its nine provinces namely; Western Cape, Northern Cape, Eastern Cape, North West, Free State, Kwazulu Natal, Gauteng, Mpumalanga and Limpopo. Source: Downloaded from http://www.mapsofworld.com/south-africa/southafrica-political-map.html

As a result of the changes in the weather and the different temperate zones of the country, the heating and cooling of poultry house (Fig. 2.4 and 2.5) is extremely important from a performance, meat production and economic standpoint. In order to prevent the losses that can result from poor housing condition, it is imperative that animals are provided with the correct temperatures (Payne, 1966a). For the first 14 days of life, the most important requirement is the supply of heat as the birds are not able to completely maintain their body temperature. Until then, it is crucial that floor temperature be maintained between 32.2-35 ^{0}C with little variation, which is where the economics resides due to the expense of purchasing, running and maintaining such systems. Generally, supplementary heat can be discontinued at the end of the fourth week, but in winter it may be necessary to provide heat on very cold nights. Table 2.1 shows the temperature requirements of chickens at different stages of growth.

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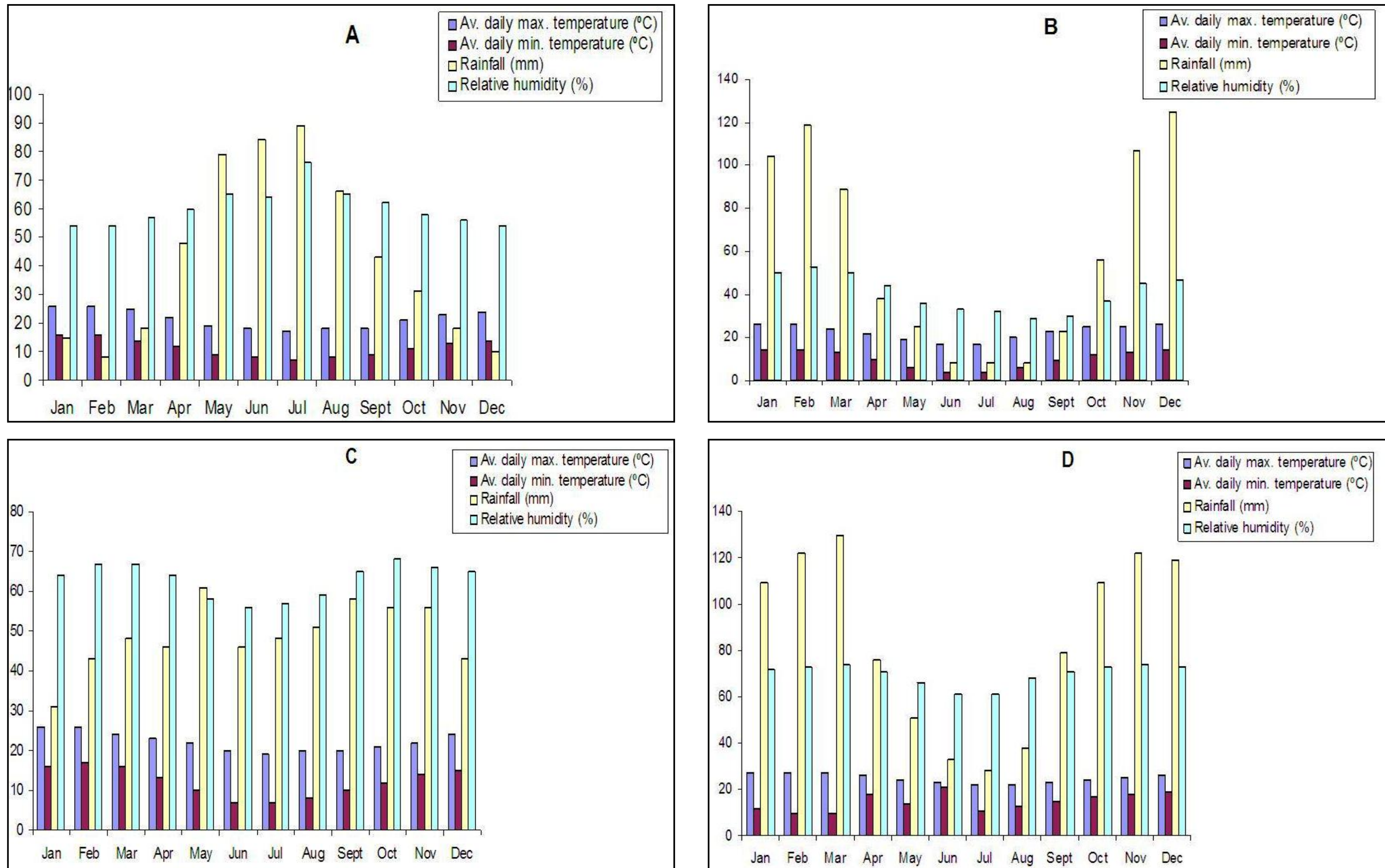

Fig. 2.2. Average min/max temperatures, rainfall and relative humidity for the four major cities of South African (A = Cape Town; B = Johannesburg; C = Port Elizabeth; D = Durban) over 14 years (1995-2009). Source: Anonymous, 2011c.

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Table 2.1. Temperature requirements of young chicks up to 28 days after hatching

Age (days)	**Temperature requirement (^{o}C)**
1	33
2	33
3	33
4	32
7	30
14	26
21	22
28	20

Source: Payne, 1966a.

The easiest way to heat a poultry house would be to use:

- Forced-air furnace. This type of heat source uses an open flame to heat air being pulled through the unit (Czarick and Fairchild, 2008). Although they are very successful in providing heat for older birds, these heaters are problematic during brooding, as the hot air produced rises quickly to the ceiling of the house away from the birds. The only manner to overcome this would be to saturate the room with hot air to force air down to floor level. The extra heat required therefore comes at an additional cost in fuel.

- Radiant heaters have been an alternate method used for the heating of houses at the floor level of the houses (Czarick and Fairchild, 2008). The most common types of radiant heat sources are pancake and radiant brooders. One of the advantages of radiant heat is that roughly 50% of the heat energy is directed to the floor, making it possible to maintain a floor temperature well above air temperature at a lower cost than heating the entire house. Another

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advantage of this system is that the system allows the floor to be warmest directly under the brooder, while it decreases as the distance from the radiant centre increases i.e. it gives the birds the advantage to self-regulate their heat requirements as they move closer or further away from the heat source.

2.3.2.2. Heat stress

High temperature is also stressful for poultry and frequently causes death from hyperthermia, especially in the older/adult animals (Payne, 1966b). Hyperthermia may occur in various parts of the breeding cycle from the incubator, the hatchery, during transfer to the farm or in the pen. The effect of ambient temperature on body temperature is not constant and varies with body heat production which is directly related to body mass and feed intake (metabolism) viz. the upper critical ambient temperature varies from just below to just above body temperature in young birds to 8-10 ^{0}C below body temperature in heavy broilers (Moulsley, 1984). The lethal high body temperature is 46.7 ^{0}C for chicks and 47.2 ^{0}C for adult birds.

Methods that are used to reduce body temperature in birds include:

- Wind-chill: For this method rapidly moving air is used to cool the birds (Daghir, 2001). To get the desired cooling effect, wind speed needs to be between 122 to 183 metres/min, depending on factors such as bird age, house temperature, and bird density. In typical wind-ventilated houses, exhaust fans are located in one end of the building which allows air to be drawn through the building via large openings at the opposite end (Fig 2.3).

- Evaporative cooling: Is a modification of the wind-chill method as evaporative cooling pads are located at the air inlets, mentioned in the above system (Daghir, 2001). The energy released during evaporation reduces the air temperature, and the resulting airflow creates a cooling effect, which can reduce the house temperature by 10 °C or more, depending on humidity. Maximum evaporation is achieved when water pumps are set to provide enough pad moisture to ensure optimum water evaporation. If too much water is added to the pads, it is likely to lead to higher relative humidity and temperatures in the shed.

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2.3.2.3. Hypoxia

Oxygen is required for metabolism and reduced oxygen levels affect the growth rate and health of the developing embryo, growing birds and adults (Christensen and Bagley, 1988). Hypoxia has its greatest effect on rapidly growing broilers in which even a slight reduction in available oxygen will slow growth. This occurs through reduced tissue respiration which causes fatigue and enhances the formation of free radicals. Free radicals are involved in reduced performance of chickens and makes them prone to many disease conditions.. Hypoxia is usually easily managed by ensuring adequate ventilation within the house (See section 2.3.2.5).

2.3.2.4. Light

Hens need about 14 hours of day length to maintain egg production, with intensity sufficient to allow a person to read newsprint newspaper. Decreasing day length during autumn and shorter day lengths in winter are therefore factors that lead to a severe decline in egg production during these periods unless supplemental light is provided. When production ceases, the birds may also undergo a feather molt, and thereafter can be expected to only resume egg production in the spring (Ahmadi and Rahimi F. 2011).

Fig. 2.3. Fans located at one end of a poultry house, to allow for cooling by with wind chill and/or evaporative cooling (For this specific house, the air was cooled by a water drive heat exchange system)(Source: Czarick and Fairchild, 2008).

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Fig. 2.4. Inside of a modern poultry broiler breeder house (Source: Czarick and Fairchild, 2008).

Fig. 2.5. A modern poultry house as seen from outside (Source: Czarick and Fairchild, 2008).

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2.3.2.5. Ventilation

Proper ventilation is essential in poultry operations to bring in fresh air (and oxygen) and to exhaust carbon dioxide due to the high stocking densities in modern breeder houses (Fig. 2.3, 2.4 and 2.5) (Payne, 1966b). Important environmental challenges include weather extremes and rapidly changing weather conditions. Colder months can result in difficulties in providing adequate ventilation while still heating the house, while in hot weather it can be difficult to provide sufficient ventilation to disperse the excess heat. Numerous methods of ventilation are available:

- Natural Ventilation: The simplest way to provide fresh air is to use the natural ventilation systems which take advantage of basic principles of warm, humid air wanting to rise and wind producing pressures on building surfaces while using windows, panels and low hanging doors to allow the fresh air in (Oluyemi and Roberts, 1979). Determinations for how far to open windows and doors can be made on the basis of air temperature, prevailing wind direction, age of birds, amount of moisture and the level of gaseous build-up within the house. The initial placing of the house is important as the house needs to take advantage of prevailing winds e.g. warm-weather openings need to be exposed to them. The house also needs to be situated away from any windbreaks, buildings or other obstacles that might impede natural air flow. If done correctly, natural ventilation is the cheapest and simplest method of ventilating a house. A degree of modernisation can also be implemented with control systems that automatically adjust the size of openings using fully adjustable curtains, panels, baffles, etc. The system does however come at a disadvantage of poor biosecurity as the infectious agent can enter with the wind.

- Automated ventilation systems. The most popular ventilation systems include:

  1. Cross ventilation: For this system the fans are installed on one side of the house and the air inlets on the opposite side (Oluyemi and Roberts, 1979). This system is commonly used when high stock density is in place and relies on a large number of fans. The system may be modified to suit

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prevailing circumstances; so that during the winter the jets will blow over the warm batteries thereby providing warm air, while during summer cold air currents will be blown at different air speeds viz. low speed air stream to recycle bird body heat in winter and high speed in summer to create a cooling effect.

2. Tunnel ventilation: In this system, fans are installed in one end wall and air inlets are installed in the opposite end wall (Oluyemi and Roberts, 1979). This system also features low air speed in winter and high air speed in summer. It is a perfect solution for areas with very cold or very warm climate. The system permits control of the minimum air quantity, so it is an ideal solution when necessary to heat and to recycle indoor air. The difference is that this system uses a polypropylene perforated duct which runs the length of the barn, to uniformly distribute the required volume of air.

3. Positive pressure ventilation: The main feature of this system is the low air speed at bird level (Jones and Dawkins, 2010). Unlike the other two systems, the fans are installed in chimneys and air outlets are in the pit, with the whole house being included in the ventilation pattern, allowing for temperature uniformity. Air circulates first in the bird's area, then in the manure pit underneath with a dehydrating effect. Positive ventilation is one of the best systems to employ, particularly when farms are close to residential areas as no fan noise is perceptible outdoors, the dust and feathers stay inside the barn and the dry manure stays odourless.

2.3.2.6. Food and Water Quality

Drinking water for poultry breeders is an important dietary requirement. Under normal conditions, poultry will consume by weight approximately twice the amount of water than food. Drinking water temperatures should be between 10°C to 15°C for the most comfortable consumption by mature birds, but some studies have indicated that water temperatures of about 25°C reduce mortality in chicks and poults (Pearson and Herron, 1980). Temperatures over 30°C will reduce consumption and birds will refuse

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to drink if water temperatures are over 44°C. Physiologically, water is the most important fluid as it helps remove waste, lubricates joints, is a major component of blood, and a necessary medium for many chemical reactions that help form muscle and eggs. In addition to being an important physiological solvent; water is important in the gastrointestinal tract (GIT) as it softens food after eating; aids in digestion and absorption; and cools the body as it evaporates through the bird's lungs and air sacs (Pearson and Herron, 1980).

Poor water quality, can retard growth, curtail egg production, or produce lower egg quality (Pearson and Herron, 1980). Body weight is positively influenced by water hardness and dissolved oxygen, and negatively influenced by total bacteria and a pH less than 6.0. While several elements can cause poor water quality, the interaction between elements is more significant in water quality problems than the simple fact of their presence (Anonymous, 2012b). Routine water analysis is required to determine if water treatment procedures are necessary. Some basic treatment techniques are:

- **Filtration:** May be required for some surface water sources to remove organic matter and/or turbidity (Anonymous, 2009).

- **Chlorination:** May be required to remove bacteria; prevent slime and algae build-up in water lines, precipitate out nitrites, iron, manganese and sulphur. Continual use of chlorine may cause corrosion of steel fixtures, residue build-up, and reduce effectiveness of any medications applied through the water system (Anonymous, 2009).

2.3.3. Disease factors

Despite the numerous factors mentioned above, the most important and limiting factor in the breeder poultry production system is the occurrence of diseases either metabolic, infectious or from toxins produced by fungi (Biggs, 1982). While these diseases are important in the adult bird and their reproductive health, they can also target the growing chick before it reaches breeding maturity.

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2.3.3.1. Metabolic diseases

Metabolic disorders may be classed as illness associated with a failure in one of the body hormone or enzyme systems, storage disease related to lack of metabolism of secretory products, or the failure or reduced activity of some metabolic function (Julian, 2005). Some important metabolic disease syndromes that generally affect poultry are discussed below.

2.3.3.1.1. Fatty liver and kidney syndrome of chickens

Fatty liver and kidney syndrome (FLKS) is a condition that occurs in growing chickens during the first few weeks of life with high mortality. The syndrome is characterized by a low blood glucose level which has led to suggestions that death is due to an absence of glucose for essential functions (Whitehead et al., 1978). The disease results from reduced activity of the biotin dependent enzyme pyruvate carboxylase which controls hepatic gluconeogenesis. At present the exact reason for the reduced enzyme activity is not known, but it has been speculated that it may be associated with high fat level in the food or changes to the food (Butler, 1976). In addition the gluconeogenic enzyme fructose-l,6-diphosphate-l-phosphohydrolase is decreased. Clinical signs include aphagia, lethargy and weakness with uncoordinated behaviour and head movement (sometimes classed as nervous signs). The chicks may lie on their breast with their neck and legs extended (Butler, 1976). Lethality can vary from 5% to 35%. At necropsy the liver and kidneys are markedly enlarged, pale and fatty. Generally, adequate dietary biotin will prevent the condition in breeder and broiler birds fed commercial rations.

2.3.3.1.2. Metabolic disorders due to management defects

The effect of cold on metabolism affects all ages of poultry (Biggs, 1982). The increase in metabolic rate induced by temperatures below the ‘comfort zone’ (the temperature below which metabolism is increased to maintain body temperature in homoeothermic species) is a significant cause of increased mortality. The lethal low body temperature is 15 ^{0}C on day of hatch and 22.2 ^{0}C in mature chickens (Robertshaw, 2004). Young birds cannot control their body temperature for the first few days of life and, as a result, become hypothermic when exposed to a cold environment. Young birds that are hypothermic may not move to feed or water and

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usually die from dehydration and/or starvation. Older birds that are cold appear dull, uncomfortable and may shiver.

At first the cold birds conserve heat by non-shivering thermogenesis (huddling). As the ambient temperatures continue to fall, non-shivering thermogenesis is no longer adequate to offset heat loss and maintain body temperature and shivering thermogenesis (involuntary contractions of skeletal muscles) results to increase heat production (Robertshaw, 2004). Further declines in the ambient temperatures, will eventually exceed the ability of non-shivering and shivering thermo-genesis to maintain body temperature. Commercial poultry rarely die from hypothermia unless buildings are damaged by wind or snow-load, the birds are not properly protected during transportation to processing in cold climates or if young birds get wet. Normally these processes have the ability to reduce production by breeder birds.

2.3.3.2. Toxins

Numerous substances have been associated with toxicity:

- Heavy metal: Many heavy metals and other toxins such as cyanide exert their effect by disabling enzyme systems. Hence inhibiting essential physiological processes like digestion, excretion, memory, etc (Biggs, 1982).

- Fungal toxins: Mycotoxins are toxic secondary metabolites produced by fungi growing on crops in the field, during handling and/or during storage (Julian, 2005). They enter the animal production system via feed (concentrate, silage or forage) or via bedding. Mycotoxins negatively affect animal performance, animal health and product quality. Mycotoxin control is therefore crucial for production economics, animal welfare, food quality and food safety. The toxic effect of most mycotoxins is mediated through the disruption of enzyme systems while other may cause storage disease (Julian, 2005).

  Mycotoxins exert their effects through four primary mechanisms viz. intake reduction or feed refusal; alteration in nutrient content of feed in terms of nutrient absorption and metabolism; effects on the endocrine and exocrine systems; and/or the suppression of the immune system (Julian, 2005). These effects often lead to rather unspecific symptoms, which makes it difficult to

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properly diagnose mycotoxicosis. Further complications in mycotoxicosis diagnoses are the possible presence of secondary clinical signs resulting from opportunistic disease related to immune suppression. In order to effectively identify mycotoxicosis, experience with mycotoxin-affected animals is important and in usually dependent on feed and tissue analyses for the presence of the toxin (Julian, 2005).

- Veterinary Drugs: Even when used and dosed correctly veterinary drugs may be responsible for toxic effects in the birds (Julian, 2005). As an example, sulphaquinoxaline, an anti-coccidial drug, interferes with vitamin K synthesis by inhibiting the epoxide reductase enzyme that is responsible for activating vitamin K (Julian, 2005).

- Nutrient overdose: While essential for growth, nutrients may become toxic in overdose. Gizzerozine, a biogenic amine from fishmeal used at low concentrations to supplement the protein portion of the ration is one such example. When included at high concentrations, it either directly or by stimulating histamine production induces ulceration and damage to the proventriculus with resultant haemorrhage into the digestive tract (Julian, 2005).

- Toxic Plants: Some feedstuffs can contain toxic elements e.g. rapeseed and some low erucic acid rapeseed varieties can cause heart and liver problems in broiler chickens, layers and turkeys (Julian and Brown, 1997).

2.3.3.3. Infectious agents

Infectious diseases are known to interfere with growth and production (Biggs, 1982). This may result from direct tissue destruction, the resultant host inflammatory response or induced nutrient deficiencies. For the inflammatory process the monokines, acute phase proteins and other mediators released by macrophages, lymphocytes and other leucocytes increase body temperature and decrease feed consumption i.e. the body changes emphasis from growth to defence. It is also possible that the infectious agent competes with the body for available nutrients e.g.

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Mycoplasma meliagridis and other mycoplasma cause chondrodystrophy in turkey poults (turkey syndrome 65) as a result of biotin deficiency.

2.3.3.3.1. Bacterial diseases

Diseases of the respiratory tract are a significant component of the overall disease incidence in poultry (Biggs, 1982). In many cases, respiratory disease observed in a flock may be a component of a multisystemic disease or it may be the predominant disease with lesser involvement of other organ systems. In some cases, such as infectious coryza (see section 2.3.3.3.1.2) or infectious laryngotracheitis (see section 2.3.3.3.2.2.), the disease may be limited to the respiratory system at least initially. Various pathogens may initiate respiratory disease in poultry, including a variety of viruses, bacteria, and fungi. Environmental factors may augment these pathogens to produce the clinically observed signs and lesions. The most important diseases in South Africa are:

2.3.3.3.1.1. Fowl cholera

Pasteurella multocida is a common cause of fowl cholera, which afflicts many avian species, with chickens (*Gallus gallus*), turkeys (*Meleagris gallopavo*), ducks (*Anas platyrhincos*), and quail (*Callipepla squamata*) being of most economical significance (Glisson, 1998). The clinical signs of the disease include cyanosis of the comb and wattle (Fig. 2.6) anorexia, and separation from the rest of the flock on the roost, nest or floor. Birds which survive the acute phase may develop rales and a thick catarrhal nasal discharge. Diarrhoea may or may not occur. *P. multocida* is capable of multiplying in the bloodstream with a result that the organism may quickly colonize many organs, contributing to the typical purulent exudative lesions of fowl cholera seen in the joints, wattles, ovaries, brain, liver, spleen, and lungs.

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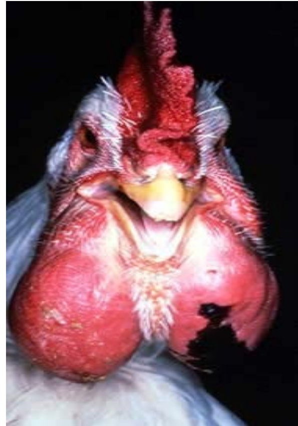

Fig. 2.6. Swollen and cyanotic comb and wattle of a chicken with fowl cholera (Source: Anonymous, 2010a).

Vaccination to prevent fowl cholera is an important aspect of controlling the disease, particularly in broiler breeders and turkeys. Fowl cholera can be effectively treated with several different antibiotics (Glisson, 1998). Tetracyclines and sulphonamides are typically effective for treating fowl cholera. Penicillin susceptibility is also common. Fluoroquinolones are the most effective class of antimicrobial compound for the treatment of fowl cholera.

2.3.3.3.1.2. Infectious coryza

Infectious coryza is an upper respiratory disease of chickens caused by infection with *Avibacterium paragallinarum* (Glisson, 1998). The disease is characterized by swollen infraorbital sinuses, nasal discharge, and depression (Fig. 2.7). The disease is most commonly seen in adult chickens and can cause a very significant reduction in the rate of egg production (Glisson, 1998).

Fig. 2.7. Swollen infraorbital sinuses, nasal discharge in a depressed chicken caused by infectious coryza. (Source: Anonymous, 2010a).

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Commercially available infectious coryza vaccines are inactivated whole-cell bacterins emulsified in an oil adjuvant or adsorbed on to aluminum hydroxide. Broilers are seldom vaccinated against infectious coryza (Anonymous, 2010a).

2.3.3.3.1.3. Colibacillosis

Respiratory colibacillosis is a respiratory disease caused by secondary infection with pathogenic *Escherichia coli* (Glisson, 1998). *E. coli,* is a ubiquitous organism in poultry production. Any insult to the respiratory tract of chickens and turkeys creates a climate for potential colonization of the respiratory tract by *E. coli*. The initial insult may be cause by a virus (infectious bronchitis virus), or bacterium (*P. multocida*) or it may be an environmental insult such as elevated ammonia levels in the poultry house (Glisson et al., 2004). At present colibacillosis is difficult to treat because of wide spread resistance (For more information refer to section 2.4 below).

2.3.3.3.2. Viral diseases

Several avian viruses have a predilection for the respiratory tract of chickens: this organ system is the primary target of infectious bronchitis virus (IBV), Newcastle disease virus (NDV), infectious laryngotracheitis virus (ILTV), avian influenza virus (AIV), and pneumovirus (Villegas, 1998).. Other viruses, such as adenovirus and reovirus, are generally considered to be secondary invaders of the upper respiratory tract of chicken. Most bacterial infections occur secondarily to viral diseases and as such it will be worthwhile to consider the two diseases together. However, for the sake of convenience, they are considered as two different entities in the discussion (Villegas, 1998).

2.3.3.3.2.1. Infectious bronchitis virus (IBV)

The Coronavirus IBV constitutes one of the most important viruses in poultry medicine with numerous serotypes being described (Gelb *et al.,* 1991). The Massachusetts (Mass) strain of IBV is considered to be the prototype strain for the group and is the representative of the Mass serotype. Infectious bronchitis (IB) is an economically important disease of poultry and has worldwide distribution, with a morbidity and mortality up to 80 and 20%, respectively (Gelb *et al.,* 1991). Although the trachea is the primary predilection site, the virus also multiplies in kidneys, intestines and

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oviducts. The disease is characterized by tracheal rales, coughing and sneezing (Fig. 2.8). Birds of all ages are susceptible to IB virus but the disease is most severe in baby chicks causing mortality. In adult flocks, the disease results in decreased egg production and malformed shells (Hofstad, 1984).

Fig. 2.8. Young chicken showing open mouth breathing, a characteristic respiratory sign of Infectious Bronchitis. (Source: Anonymous, 2010a).

Viruses or strains that differ from the Mass strain have been described in different countries of the world (Karaka et al., 1990; Bonnefoy et al., 1993; Capua *et al.*, 1994). In Australia and Hungary, genotypically In Japan, IBV strains showing additional tropism for tissues other than the respiratory tract such as the intestines, lungs and oviducts are known to exist. Several variant serotypes isolated from commercial layers and broilers were described. These strains include nephrotrophic strain (Mass 41 and H52 strains) isolated in Hungary, *IBV strain PL 84084 as a variant strain (*Bonnefoy et al., 1993) isolated by French workers, strain 624/I isolated in Italy (Capua *et al.*, 1994) and a enterotropic strains identified in the United States of America (Karaka et al., 1990).

2.3.3.3.2.2. Infectious laryngotracheitis virus

Infectious laryngotracheitis used to be of concern mainly to the layer and breeder industry with clinical signs of decreased egg production; however, during the last few years, serious outbreaks of the disease have been seen in broilers (Linares et al., 1994). Avian infectious laryngotracheitis (ILT) is a respiratory disease of chickens caused by an alpha herpesvirus. It can also affect pheasants, partridges and peafowl.

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In the virulent form, the history, clinical signs and very severe tracheal lesions characterized by difficulty in breathing and coughing (Fig. 2.9) are highly characteristic of the disease, while the mild form is indistinguishable from other mild respiratory diseases (Hadipour et al., 2011). Laboratory diagnosis depends on the demonstration of the presence of the virus or viral antigens or products (Scholz et al., 1994).

Fig. 2.9. Adult bird showing signs of difficulty breathing and coughing due to an Infectious laryngotracheitis infection. (Source: Anonymous, 2010a).

To control the condition, vaccines of chicken embryo or cell culture origin have been used with limited success (Davison et al., 2006). The recommended application route of these vaccines is the intraocular procedure, a method clearly inappropriate for the breeder industry. Several applications via the drinking water have given mixed results in broilers, where post vaccination reactions have been observed as well as a decrease in the production parameters in the vaccinated flocks. There is no effective treatment but vaccination of unaffected birds may limit an outbreak. Vaccination is effective in preventing the appearance of the disease. Work with genetically engineered vaccines and the initial studies results did look promising (Davison et al., 2006).

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2.3.3.3.2.3. Avian Influenza

Sporadic outbreaks of avian influenza were reported in several countries (Russell, 2010). In general, the highly pathogenic form of the disease is easily diagnosed as a result of high mortality and typical lesions of oedema and swelling of head, eyelids, comb, wattles, and hocks (Fig. 2.10). However, the disease may be caused by the low pathogenic strains that are not always easily recognized. Alexander and Spackman (1981) reported that a low pathogenic avian influenza infection in laying flock resulted in only transient mild respiratory signs and 2% incidence of white-shelled eggs. The control of avian influenza is based on either eliminating the virus by eradicating the disease, or by establishing a vaccination programme using inactivated vaccines to decrease the rate of virus multiplication in the field, with the final goal of eliminating the virus (Papparella et al., 1995).

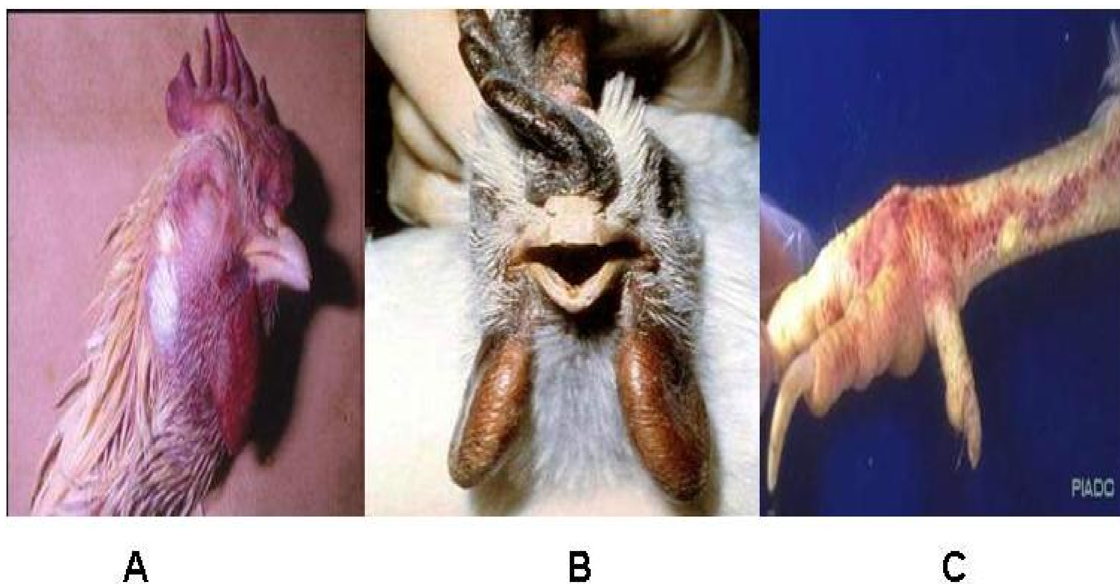

Fig. 2.10. Oedema and swelling of head and eyelids (A), comb and wattles (B), and hocks (C) of a bird with avian influenza. (Source: Anonymous, 2010a).

2.3.3.3.2.4. Newcastle disease (ND)

ND has been termed pseudo-fowl pest, pseudovogel-pest, atypische Geflugelpest, pseudo-poultry plague, avian pest, avian distemper, Ranikhet disease, Tetelo disease, Korean fowl plague, and avian pneumoencephalitis. Historically, the disease first occur in Java, Indonesia (Kraneveld, 1926) and Newcastle-upon-Tyne, England (Doyle, 1927). ND is particularly complicated in that different isolates and strains of the

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virus may induce enormous variation in the severity of disease, even in a given host such as the chicken. To simplify matters, division into forms or pathotypes of disease based on clinical signs in chickens has been made as summarized by Beard and Hanson into the following: 1. Doyle's form, which is an acute, lethal infection of all ages of chickens. Haemorrhagic lesions of the digestive tract are frequently present, and this form of disease has been termed viscerotropic velogenic Newcastle disease (VVND). 2. Beach's form, which is an acute, often lethal infection of chickens of all ages. Characteristically, respiratory and neurological signs are seen, hence the term neurotropic velogenic (NVND). 3. Beaudette's form that appears to be a less pathogenic form of NVND in which deaths usually are seen only in young birds. Viruses causing this type of infection are of the mesogenic pathotype and have been used as secondary live vaccines. 4. Hitchner's form, represented by mild or inapparent respiratory infections caused by viruses of the lentogenic pathotype (Alexander, 2003). The global economic impact of ND is enormous. It is certainly unsurpassed by any other poultry virus and probably represents a bigger drain on the world's economy than any other animal virus. In many developing countries vND is endemic and, therefore, represents an important limiting factor in the development of commercial poultry production and the establishment of trade links. Many countries rely on village chickens to supply a significant portion of dietary protein in the form of eggs and meat, especially for women and children. The constant losses from vND severely affect the quantity and quality of the food of people on marginal diets (Sen et al., 1998).

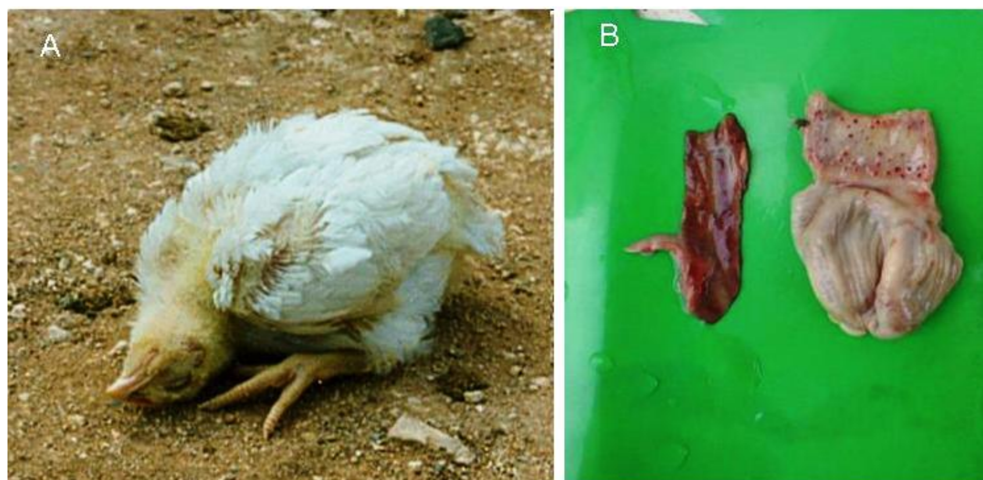

Fig. 2.11. Torticollis (A) and petechial haemorrhage on the proventriculus of a chicken with Newcastle disease. (Source: Anonymous, 2010a).

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2.3.3.3.2.5. Fowl pox

Fowlpox is a common viral disease of commercial poultry (chickens and turkeys) as well as of pet and wild birds. Of the approximately 9000 birds species, about 232 in 23 orders have been reported to have acquired a natural poxvirus infection (Bolte et al., 1999). Fowlpox is an economically important disease of commercial poultry because it can cause a drop in egg production and mortality. Avian pox viruses are members of the genus Avipoxvirus of the family Poxviridae. Morbidity rate of pox in chickens and turkeys varies from a few birds being infected to involvement of the entire flock if a virulent virus is present and no control measures are taken. Birds affected with the cutaneous form of the disease are more likely to recover than those with the diphtheritic form involving oral mucosa and the respiratory tract. Effects of pox in chickens usually involve emaciation and poor weight gain; egg production is temporarily retarded if layers are infected. The course of the mild cutaneous form of disease is about 3-4 weeks, but if complications are present, duration may be considerably longer. With virulent strains of fowlpox virus, both primary and secondary cutaneous lesions may persist for more than 4 weeks. In such cases, cutaneous lesions around the eyes or diphtheritc lesions in the mouth and upper respiratory tract interfere with normal functions resulting in significant mortality (Tripathy and Reed, 2003).

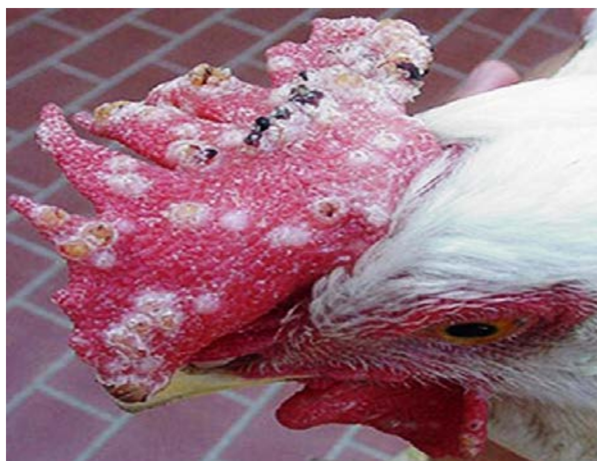

Fig. 2.12. Flaky crust caused by fowl pox virus on the comb of a chicken. (Source: Anonymous, 2010a).

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2.3.3.3.3. Protozoan Parasitic disease

Parasites which infect/infest poultry poise a serious threat in both large commercial operations or in small back-yard flocks. Parasites that affect poultry include the protozoa *Eimeria, Leucocytozoon, Haemoproteus, Toxoplasma, Sarcocystis* and *Cryptosporidium*. Flagellates such as *Histomonas, Trypanosoma, Trichomonas, Chilomastix* and *Hexamita*, and amoeba of the genera *Entamoeba* and *Endolimax* are also found. Of all the protozoan parasites mentioned, coccidiosis, histomoniasis (black head) and trichomoniasis are of special mention.

2.3.3.4.1. Coccidiosis

Coccidiosis, caused by parasitic protozoa of genus *Eimeria*, is an important disease in poultry production, leading to poor growth rate and high mortality with significant economic losses up to 3 billion US dollars annually worldwide (Dalloul and Lillehoj, 2006). Generally, *Eimeria* species responsible for coccidiosis in chickens include *E. tenella*, *E. necatrix*, *E. acervulina*, *E. maxima*, *E. brunetti* and *E. mitis* but the first four are economically important and more prevalent worldwide (Ayaz et al., 2003; Shah et al., 2009).

Coccidiosis is one of the most common diseases of poultry with chickens, turkeys, ducks and geese all being affected (Zhao et al., 2006). The coccidial parasite is, however, host-specific so fowls are not affected by duck coccidia and vice versa. The disease usually affects birds over three weeks of age with clinical signs of ruffled feathers, drooping wings, pale combs and diarrhoea sometimes streaked with blood. While most losses occur in young birds adult birds can also be affected (Lund and Farr, 1967).

Clinical signs of coccidiosis range from decreased growth rate to a high percentage of visibly sick birds, severe diarrhoea, and high mortality (Vermeulen et al., 2001). Feed and water consumption are reduced. Weight loss, development of culls, decreased egg production, and increased mortality may accompany outbreaks. Mild infections of intestinal species, which would otherwise be classed as subclinical, may cause depigmentation. Survivors of severe infections recover in 10-14 days but may never recover lost performance.

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The coccidia live in the intestine or caecum of the bird and pass out through the droppings into the litter where they remain viable for long periods of time (Lund and Farr, 1967). They become infective in damp surroundings and are spread from bird to bird when infected droppings are eaten. Fig. 2.11 shows the typical lesions seen with coccidia parasites infections. Good management will help prevent serious outbreaks of coccidiosis e.g. proper disinfection of chicken houses and adequate care not to allow the houses become wet with poultry drinking water.

Fig. 2.13. Haemorrhage in the ceca characteristic of *E. tenella* infection (A) and intestinal distention, mucoid blood filled exudate, and white spots noted on the serosa with *E. necatrix* infection (B). (Source: Anonymous, 2010a).

2.3.3.4.2. Histomoniasis (blackhead)

Both young fowls and turkeys are affected by the *Histomonas meleagridis*, with turkeys being more susceptible and more likely to contract this disease when run with poultry than when housed on their own (Reid, 1967). Histomonads live largely in the caecum (blind gut) and invade other organs from there. Symptoms of blackhead include a drooped and huddled appearance, dark colouration of the head, shrunken comb and wattles and yellowish diarrhoea. At post-mortem a good identifying sign is a liver with bulls-eye lesions as shown in Fig. 2.12. Living in the caecum places the histomonads in contact with the caecal worm (*Heterakis gallinae*) and the eggs of the worm may become infected with the protozoa (Graybill and Smith, 1920). Blackhead is transmitted when birds eat droppings containing these infected worm eggs. Drugs such as dimetridazole, ronidazole and ipronidazole are available to treat blackhead in some countries. To prevent re-infection, measures include control of caecal worms and good management (McDougald, 2005).

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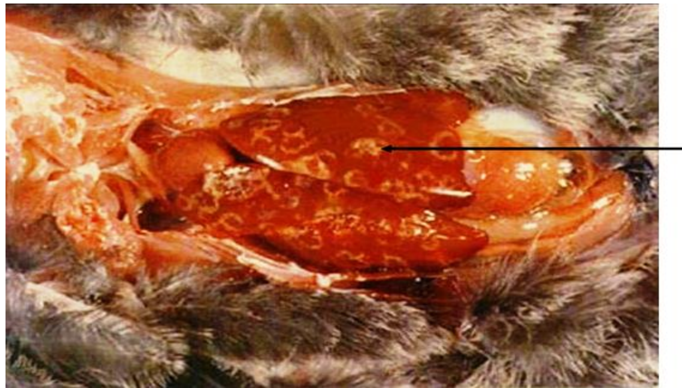

Fig. 2.14. Bulls-eye lesions found on the liver (arrow) of a turkey with blackhead disease. (Source: Anonymous, 2010a).

2.3.3.4.3. Trichomoniasis

Trichomoniasis is caused by *Trichomonas gallinae* (Reshetnyak et al., 1960). It is a disease of the upper digestive tract. It has been found in pigeons, doves and kites in the nasal tract, but may invade hens and turkeys if they drink infected water or eat infected feed. Affected pigeons will go off-feed, appear ruffled, become emaciated and die, with a green yellow fluid dripping from the beak.

2.4. *Escherichia coli* infection in South Africa

E. coli is the primary causative agent of cellulitis, septicemia, and airsacculitis in poultry and is considered one of the most significant bacterial pathogen of fowls (Cheville and Arp, 1978) due to the economic losses it causes (Barnes and Gross, 1997). Avian colibacillosis starts as a respiratory infection (airsacculitis) frequently followed by generalized infections which manifest as peri-hepatitis, pericarditis, and septicemia (Ewers et al., 2004). Clinically apparent *E. coli* infection is generally indicative of immunosuppression in poultry (McGruder and Moore 1998). *E. coli* infection damage to the immune system of the chickens includes lymphocyte depletion in both bursa and thymus (Nakamura et al., 1990).

Colibacillosis in laying hens is characterized by acute mortality without prior clinical signs of disease and without a significant effect on egg production or quality. Normally, colibacillosis is a secondary infection that appears after a situation of immunosuppression caused by another bacterial or viral infection, although Vandekerchove et al. (2004a) proposed that it may act as a primary pathogen as well.

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Furthermore, this situation is made complicated by environmental stress such as improper ventilation, temperature, and dust (Shane, 2007). The most common route of infection is via the respiratory tract frequently followed by septicemia. The majority of the outbreaks occur around the period of peak egg production, which is believed to be an important stress factor contributing to the disease, but continues as the flock ages. Cumulative mortality is normally between 5 and 10% during a single outbreak (Barnes and Gros, 1997; Vandekerchove et al., 2004b). However, the proportion of hens that suffer from the disease often reaches more than 50% (Ask, 2007). Experience has shown that *E. coli* infection can degrade live bird parameters including weight gain, feed conversion and liveability. Infection are assumed to result in a 2% decline in live weight, 2.7% deterioration in feed conversion ratio and an increase in absolute mortality from a standard 5% to 7%. Saleable product would be reduced by 9% per week (Shane and Durham, 2009). Lesions seen at post mortem are airsacculitis, pericarditis, peri-hepatitis and peritonitis (Fig. 2.13).

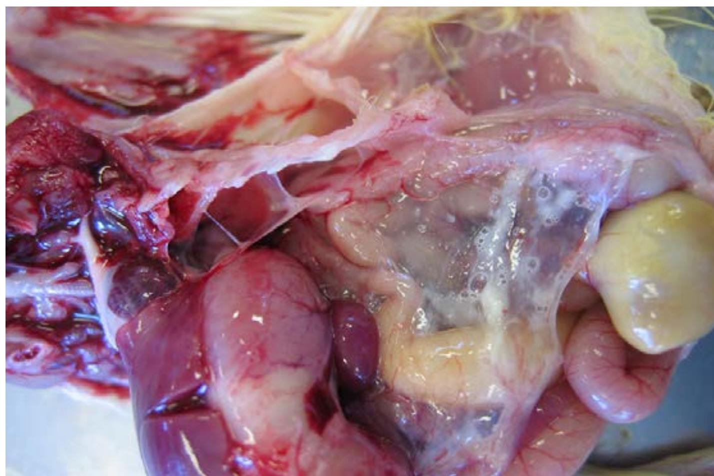

Fig. 2.15. Airsacculitis and peritonitis seen with *E. coli* infection. (Source: Anonymous, 2010a).

2.4.1. Impact of *E. coli* infection following the development of resistance

There are several antimicrobials approved for treatment of *E. coli* infections in broiler chickens such as tetracycline and streptomycin (Anonymous, 2011a). However, some are not cost effective while others are ineffective due to acquired resistance to these drugs (Bass et al., 1999). Antibacterial drug resistance has been shown to be higher in the developing world rather than the developed world. Trimethoprim resistance

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amongst pathogenic Gram-negative bacteria was found to be 64% in South India (Young et al., 1986), 49.1 % in South Africa (Wylie and Koornhof, 1989) and 63.3% in Nigeria (Lamikanra and Ndep, 1989). In contrast, the levels of such resistance were found to be only 14-19% in Finland (Heikkila et al., 1990) and 23 % in Scotland (Thomson et al., 1992). Reasons for the high levels of resistance in the developing countries may include the indiscriminate use of antibiotics without prescription and the contamination of the water supply (Dunn et al., 2004). Antimicrobials are used in animals to treat and prevent bacterial infection and to improve production efficiency in food producing animals. In veterinary practices, in most cases, there may not be any facility to identify the most sensitive antimicrobial agent. Hence empirical therapy is mostly practiced. There is an extensive use of antimicrobials in veterinary practice, which contribute much to the development of antimicrobial resistance in human beings by way of consumption of meat, milk and animal products. Moreover, the use of antibiotic growth promoters in feed is a usual practice in case of meat producing animals. Food borne pathogens are also a source of concern in human beings (Usha et al., 2010).

In a study conducted in North West province of South Africa, *E. coli* isolated from different sources (chickens, livestock and from the environment) were found to be resistant to tetracyclines, ampicillin, erythromycin and chloramphenicol (Kinge et al., 2010). Furthermore, nalidixic-acid-resistant *E. coli* strain was isolated from chickens at slaughter (Permin et al., 2006). In 2010, The German Monitoring Programme (GERMAP) reported that resistance by sulpha-trimethoprim combination against *E. coli* to reach up to 43 and 16% in adult and young broilers, respectively (Anonymous 2010c). As a result, new treatments are needed to manage this disease. To try to overcome resistance, a number of atypical antibiotics were tested using the *in vitro* Kirby Bauer technique in the Faculty of Veterinary Science, University of Pretoria (unpublished information). From this study, florfenicol was shown to be very effective i.e. the study demonstrated that little or no resistance was present for florfenicol against the tested *E. coli* bacterial strain from South Africa. With this promising *in vitro* results, the potential application of for florfenicol as a valuable therapeutic agent needs to be evaluated further.

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2.5. Florfenicol

Florfenicol is a fluorinated derivative of thiamphenicol (Budavari, 1996)(Fig 2.14) having the chemical name of 2,2-dichloro-N-[1-(flouromethyl)-2-hydroxy-2-[(methylsulfonyl) phenyl]ethyl] acetamide and belonging to the class of amphenicols. The molecular formula of the drug is $C_{12}H_{14}C_{l2}FNO_4S$ with molecular weight of 358.21 (Varma, 1994). Florfenicol has a melting point of 153 to 154 °C (Budavari, 1996) and is soluble in both water and lipids (Sams, 1994). Its chemical structure differs from that of chloramphenicol and thiamphenicol in that a fluorine atom replaces the 3' hydroxyl group. As a result, its activity is not affected by chloramphenicol acetyltransferase, which is responsible for most of the acquired bacterial resistance to chloramphenicol and thiamphenicol. While chloramphenicol, the progenitor molecule in the class of the amphenicol antibiotics, has been banned for use in food-producing animals because of its potential for human toxicity, florfenicol has not been associated with aplastic anaemia on administration (Yunis, 1988). This has been regarded as the most remarkable improvement of florfenicol over chloramphenicol and as a result florfenicol is widely used in the food producing species to treat pneumonia (Varma, 1994).

Florfenicol is a broad-spectrum, primarily bacteriostatic, antibiotic with a range of activity similar to that of chloramphenicol, including many gram-negative and gram-positive organisms (Ismail and El-Kattan, 2009) with activity against chloramphenicol-sensitive pathogens such as *P. multocida*, *P. haemolytica* and *H. somnus* (Varma 1994), and certain chloramphenicol-resistant strains of *E. coli*, *Salmonella typhimurium* and *Staphylococcus aureus* (Neu and Fu 1980, Syriopoulou *et al.*, 1981).

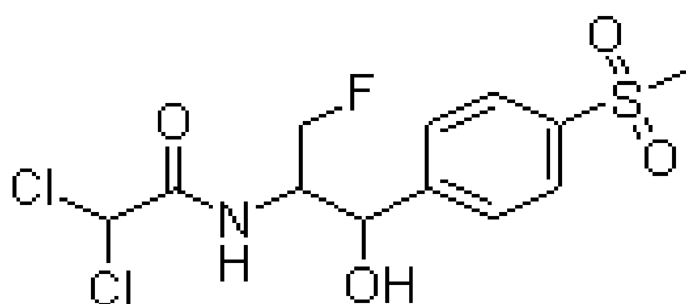

Fig. 2.16. Line diagram of the chemical structure of florfenicol

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At therapeutic doses, florfenicol is generally considered bacteriostatic. However due to preferential accumulation in the lung parenchyma, it becomes bacteriocidal therein (Ueda et al., 1995). Once within the bacterium, florfenicol functions through the suppression of protein by inhibiting the peptidyl transferase enzyme as well as ribosomal translocation (Sams, 1994; Cannon *et al*., 1990). As a result the transfer of amino acids can no longer continue with the end result being the inhibition of protein synthesis (Sams, 1994; Cannon *et al*., 1990).

2.5.1. Efficacy of florfenicol

Various published studies describe the use of florfenicol preparations as injection, food premix, implant, etc.. Its efficacy has been demonstrated against many infectious diseases of domestic animals (Nordmo *et al*., 1994; Ueda and Suenaga, 1995; Booker *et al*., 1997; Sheldon, 1997; Almajano *et al*., 1998; El-Banna, 1998; Shen *et al*., 2002). Due to its broad antibacterial spectrum, florfenicol has the potential to become a valuable antibiotic in the treatment of infectious diseases in livestock and poultry.

Following oral administration of florfenicol at 30 mg/kg in healthy ducks, plasma concentrations were maintained above the minimum inhibitory concentration (MIC) of 4 ug/ml against *E. coli* for 24 hours. In contrast, MIC of 0·25 to 1µg/ml were attained for only 12 h in *Pasteurella* infected veal calves following iv or im administration at the same dose (Varma *et al.* 1986). In a tissue kinetics study, El-Banna (1998) found high lung concentration (5.95 ± 0.31 µg/ml) above the MIC reported for natural respiratory *E. coli* infections in broilers. In addition florfenicol was also shown to have large apparent volume of distribution of 5.15 ± 0.09 L/kg in the body indicating that the drug is extensively distributed in extra-vascular tissues. Tissue concentration studies in broilers and ducks revealed similar high volume of distribution of 6.57 ± 0.30 L/kg (Afifi and El Sooud, 1997). At present no information is available to support the use of florfenicol in breeder birds. However, the high level of the drug found in the lungs of other birds may be of value in treating respiratory diseases in breeder chickens.

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2.5.2. Toxicity of florfenicol

While florfenicol has potential benefit in the management of respiratory infection in chicken breeder flocks, one additional concern with its use has been its potential to induce toxicity both systemically in the breeder birds and/or the developing embryo (Anonymous, 1999b). In a two-generation reproductive study in rats, oral administration at 12 mg/kg/day for 90 days reduced epididymal weights, decreased pup survival, and reduced milk production. In another two-generational teratogenicity study in mice, florfenicol was administered at doses of 40, 120, or 400 mg/kg by gavage on days 6-15 of gestation with toxic signs of embryo lethality at the high dose. Significant decreases in mean foetal body weight, soft tissue defects, and retarded skeletal ossification were also observed at this dose. The lower doses were not devoid of toxicity with foetal skeletal ossification being affected in a dose-related fashion, at 40 and 120 mg/kg/day (Anonymous, 1999b).

Perhaps similar toxic effects may occur in breeder cocks and florfenicol may be potentially toxic to reproduction in a breeder flock. While no published information is available, a single spontaneous pharmacovigilance report was received in South African, following the use of oral florfenicol to treat an *E. coli* pneumonia in a commercial grand-parent flock at 10 mg/kg (V Naidoo, personal communication, 2011). The spontaneous report described complete recovery in the hens with no apparent physical side effects. A major side effect was, however, reported in the chicks as hatchability declined by up to 80% for as long as one week, before returning to normal. At this stage no further information is available despite the veterinarian involved being requested to submit more information.

2.6. Conclusion

As a result of increasing drug resistance new drugs are needed for the management of *E. coli* respiratory infection in breeder birds. Based on in vitro efficacy studies florfenicol appears to be a potential candidate for further development especially since the molecule is generally regarded as safe. However, since the primary aim of farming with these birds is the production of chicks, reproductive toxicity needs to be given more rigorous attention. This study is aimed as the first step in the development of

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florfenicol as a viable drug for use in breeders. This will be evaluated by means of tissue kinetic study to ascertain if the drug can reach MIC for E. coli while at the same time ascertaining if the product has an effect on flock fertility and/or chick hatchability.

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CHAPTER 3: MATERIALS AND METHODS

3.1. Ethics

This study was approved by the Animal Use and Care Committee (AUCC) of the University of Pretoria and the Research Committee of the Faculty of Veterinary Science, University of Pretoria (V070-08).

3.2. Experimental birds

The study made use of 136 commercial cockerels and hens (Fig. 3.1). The hens were purchased from Eggspert eggs (Kempton Park, South Africa), while the cocks were purchased from HyLine SA (Midrand-South Africa) at the age 15 weeks. Five weeks prior to the administration of florfenciol the birds were randomly divided into four groups of 30 hens and four cockerels each.

Fig.3.1. The nest boxes and hanging clumps of Lucerne to enrich the environment.

The hens and cockerels were vaccinated before arrival, according to the vaccination schedule recommended for Pretoria housed birds (Table 3.1). (S.P.R. Bisschop, personal communication, 2010).

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Table 3.1. Vaccination programme for the Eggspert eggs hens used in the study.

Age	Date	Disease	Company	Vaccine	Alternate	Method Given
Day Old		Mareks	SP Intervet	Rispens	None	SC Injection
(hatchery)		ND	Merial	Avinew 2000 doses	Newhatch - SP Intervet	Coarse spray
		IB	Immunovet	Avipro IB H120	IB H120 (Anyone)	Coarse spray
		IBD	Merial	Bur 706 5000 doses	Avipro Gumboro vac - Immunovet	Coarse spray
1-3 days		Avetotal +	Baytril		90ml in 200ltr(day1 - 3) 1 mix per day	Drinking water
7 days	I	ND/IBD	Merial	IBD Broiler Plus	None	0.1mL SC Injection
10 days		IB	Immunovet	Avipro IB H120	IB H120 (Anyone)	Water
14 days		IBD	CEVA	Gumbo L - CEVA 5000 doses	Avipro Gumboro vac - Immunovet	Water
		ND	Merial	Avinew 2000 doses	Nobilis Clone 30 - SP Intervet	Water
21-23 days		Avetotal +	Baytril	80ML per mix(3 mix per day) 3 days only		Drinking water
4 weeks		ND/IB	Immunovet	Tabic VH+H120 5000 doses	Clone 30 + Ma5 - SP Intervet	Water
		IBD	CEVA	Gumbo L - CEVA 5000 doses	Avipro Gumboro vac - Immunovet	Water
5 weeks				**Bleed MG Elisa, MS Elisa.**		
6 weeks	II	ILT	SP Intervet-plough/CEVA	ILT asl	None	Eye-drop
		MS	Merial	MS vaxsafe	None	Eye-drop
		MG	Merial	MG ts 11	None	Eye-drop
9 weeks	III	AE/Pox	CEVA	CEVAC Tremor + FPL 1000	CEVAC Tremor + FPL - CEVA	Wingweb Stab (left wing)
		SG	OBP/Immunovet	Fowl Typhoid	SG9R - SP Intervet	Injection
10weeks				**(Monitor pox lesions for take - 100 birds)**		
11 weeks		ND/IB	Immunovet	Tabic VH+H120 5000 doses	Clone 30 + Ma5 - SP Intervet	Water
12 weeks	IV	ND double dose	Merial	Gallimune ND day old	Various	0.5mL im injection
		Coryza	Immunovet	Avivac Coryza	Various	0.5mL im injection
14 weeks		ND	Hipra	Hipraviar S 5000 doses	Clone 30	Fine Spray
		SE	Immunovet	AVIPRO Salmonella vac E	OBP fowl typhoid	Water
16 weeks	V	ND/IB/MG	Immunovet	Avipro 304	None	0.5 mL im injection
		Coryza/EDS	Immunovet	Avivac Coryza/EDS	None	Injection
		Pox	Merial	Diftosec	OBP, Intervet	Wingweb Stab (right wing)
		ILT	Immunovet	Avipro ILT	Intervet ILT	Eye-drop
16 weeks				**Bleed MG Elisa, MS Elisa.**		
17 weeks		**Transfer to laying**				
18 weeks		ND/IB	Anyone	ND La Sota/IB H120		
22 weeks				**Bleed MG Elisa, MS Elisa. ND HI (test)**		
(In lay vaccinate ND La Sota every 8 weeks and ND La Sota/IB H120 every 8 weeks)						
40 weeks				**Bleed MG Elisa, MS Elisa. ND HI (test)**		

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Table 3.2. Vaccination programme for the Hy-Line cockerels used in the study.

Weeks	Days	Disease	Supplier	Product	Route of Administration
1	Hatchery	MAREKS	INTERVET	RISMAVAC + CA126 (bivac)	.2ml Subcutaneous
		EXCENELL	PFIZER	EXCENELL	1.5ML/1000 d
		NEWCASTLE IB	IMMUNOVET	TAD HITCHNER ND+IB	COARSE SPRAY
	ON ARRIVAL	BAYTRIL			WATER
	1	GUMBORO	IMMUNOVET	TAD GUMBOROVAC	DRINKING WATER
	5	COCCIDIA	SCHERING-PLOUGH	PARACOX	WATER 5000 DOSES/PLACEMENT
	10	GUMBORO/ NEWCASTLE	MERIAL	IBD BROILER PLUS	**INTRAMUSCULAR 0.1ml**
2	16	GUMBORO	IMMUNOVET	TAD GUMBOROVAC	DRINKING WATER
		BRONCHITIS & NEWCASTLE	IMMUNOVET	TAD IB/ND Lasota	STIHL FOGGER
4	24	GUMBORO	IMMUNOVET	TAD GUMBOROVAC	DRINKING WATER
5	31	GUMBORO	IMMUNOVET	TAD GUMBORO	DRINKING WATER
	35	MS	MERIAL	MS Vaxsfe	EYE DROP
6		BRONCHITIS & NEWCASTLE	IMMUNOVET	TAD IB/ND Lasota	STIHL FOGGER
7		SALMONELLA	IMMUNOVET	O.P FOWL TYPHOID	DRINKING WATER
		MG	MERIAL	TS 11	EYE DROP
		IB ND MG BAC	IMMUNOVET	Avipro 304 IB/ND/MG	.5ml INTRAMUSCULAR
8		AE +FOWL POX	IMMUNOVET	Avipro Trempox	WING STAB
		EDS & CORYZA	IMMUNOVET	AVIVAC CORYZA EDS	.5ml INTRAMUSCULAR
11		BRONCHITIS & NEWCASTLE	IMMUNOVET	TAD IB/ND Lasota	STIHL FOGGER
		CAV	IMMUNOVET	TAD THYMOVAC	DRINKING WATER
		MS BAC	IMMUNOVET	Antec MS bac	.5ml INTRAMUSCULAR
		ILT	AVIPHARM	LT Ivax	EYE DROP
12					
		EDS & CORYZA	IMMUNOVET	AVIVAC CORYZA EDS	.5ml INTRAMUSCULAR
		IB ND IBD	AVIPHARM	CEVAC ND IB IBD K	.5ml INTRAMUSCULAR
15		SALMONELLA	MERIAL	Merial Gallimune SE	.5ml INTRAMUSCULAR
		BRONCHITIS & NEWCASTLE	IMMUNOVET	TAD IB/ND Lasota	STIHL FOGGER

3.2.1. Housing and management

The study was conducted within the facilities of the Poultry Reference Laboratory (house B2), Faculty of Veterinary Science, University of Pretoria at Onderstepoort. The birds were kept on cement floor with wood shavings as scratch and bedding material. Each house was supplied with 2 tier nesting box (5 boxes per row) (Fig. 3.1). The house was naturally ventilated with curtained windows that were opened daily from 7

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am till 5 pm except during times of rain (Fig.3.2). The study was completed in the summer season, where the temperatures ranged between 19 and 32 ^{0}C.

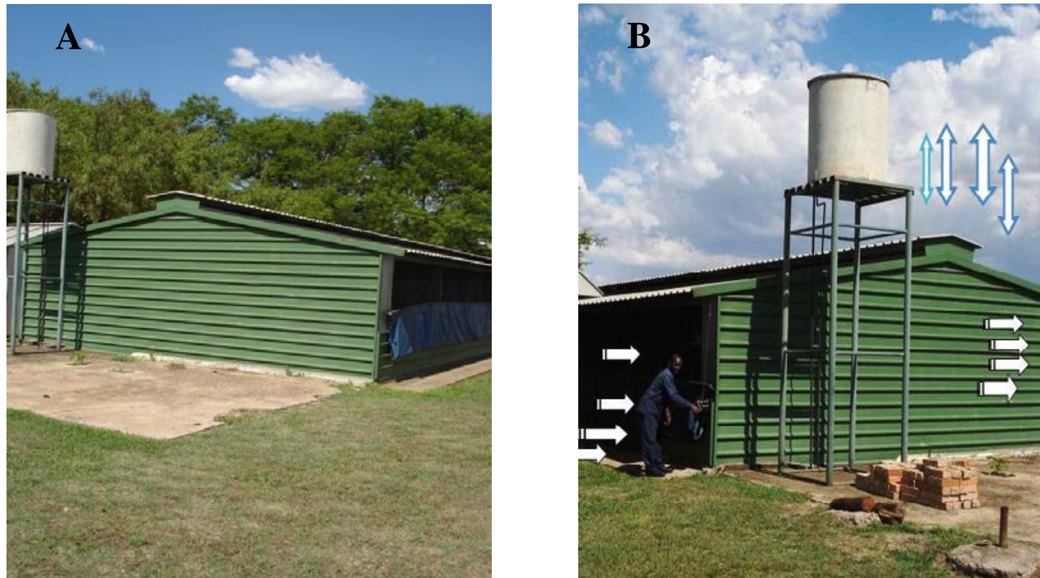

Fig 3.2. The naturally ventilated house in which the chickens were housed for the duration of the study. A: Shows the curtained sided house, while B: indicate directional flow for natural ventilation and air passage (vertical and horizontal arrows).

The allotted space per treatment group was 3.10 x 3.90 m (Fig.3.3, Fig.3.4).The birds were fed *ad lib* with a layer mash fortified with Salinomycin (coccidiostat) (Avi-Products (Pty) in two feeders per pen. Standard municipal potable water was supplied by two automatic bell drinkers (Fig.3.3). Enrichment was supplied by means of nest boxes (as mentioned above), the bedding which allowed for foraging and hanging bags of lucerne.

Fig. 3.3. Photograph of the pens inside the chicken houses. The pens were separated by mesh wire fencing (A), while the birds were housed on shavings, with water and food supplies ad lib (B).

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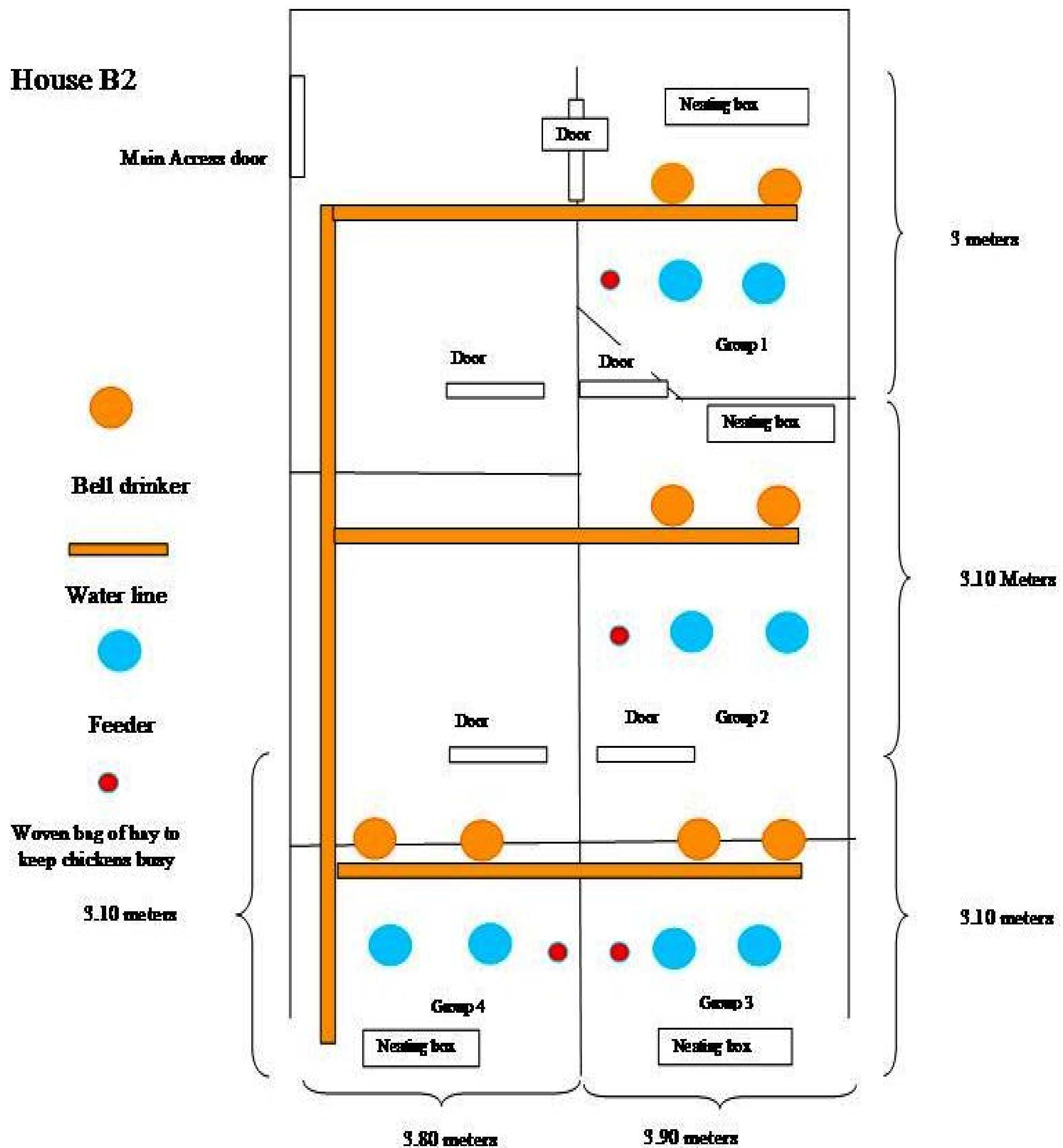

Fig. 3.4. A line diagram illustrating the layout of the pens and the equipment (size, bell drinkers, feeders and hanging clumps) therein.

Individual birds were identified by metal wing patagial tags, each with a unique number. The animals in a group were identified by colour coding using food colouring dye sprayed onto their feathers. Birds in groups 1, 2 and 3 were coloured with red, blue and yellow dyes, respectively, while those in group 4 were uncoloured (Fig.3.5).

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Fig. 3.5. Photograph showing their blue colour code (food colouring dye) sprayed on the feathers used for identification of birds in this group.

3.3. Study design

The study was divided into three phases, with the same group of birds being used in all three phases. Prior to each phase, a wash-out period of three weeks was allowed. The initiation of the next phase was based on birds being healthy, within normal weight limits, and having equivalent fertility to the control group. The study design was chosen as florfenicol was speculated to have a temporary and completely reversible effect on fertility i.e no permanent effect on fertility was expected based on the pharmacovigilance report.

Prior to the initiation of phase 1, the hens were randomly assigned into four groups of 30 birds each (n=30). Thereafter, 4 cockerels each were assigned to every hen group (n=4) making 4 cockerels to 30 hens in a group. Birds were allowed to acclimatise for 5 weeks before the start of the phase I trial.

3.3.1. Effect of florfenicol on hen fertility and egg hatchability (Phase 1)

In phase 1, only the hens were treated with florfenicol at doses of 10 mg/kg, 20 mg/kg, 30 mg/kg or pure water (non-treated control) (n=30) for five consecutive days by

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gavage. The drug at the doses mentioned gave an effective plasma concentration levels in broiler chickens in an earlier study (Affifi and El-Sooud, 1997) hence the reason for choosing those doses. Florfenicol was obtained as a 3 % solution (30 mg/ml; VTech Compounding solutions Pharmacy, Midrand). Treatments were administered once daily, though the use of a calibrated syringe attached to flexible dosing tube inserted directly into the crop. The tube was emptied of drug prior to removal from the oesophagus. The birds were not starved prior to treatment. Animals were returned to their pens immediately after dosing.

Eggs (n=30) were subsequently collected from all groups on days 0, 2, 4, and 5 of dosing and on days 1, 3, 4 ,6 and 8 post dosing (Table 3.2). The collected eggs were subsequently incubated till hatch (pipping) or point of egg-breakout (Refer to section 3.5.1).

Table 3.3. Schedule for egg collection (n=30) in the treated hens (n=30) following treatment for 5 days.

Dose (mg/kg)	Egg Collection Point		Activity*		
	Time (h)	**Relationship to treatment**	**Candling (n=30)**	**Break outs (n=30)**	**Pipping (n=30)**
0, 10, 20, 30	0	Control	✓		✓
	48	Treatment 2	✓	✓	
	96	Treatment 4	✓		✓
	120	Treatment 5	✓	✓	
	144	Withdrawal 1	✓	✓	
	192	Withdrawal 3	✓		✓
	216	Withdrawal 4	✓	✓	
	264	Withdrawal 6	✓	✓	
	288	Withdrawal 8	✓		✓

* The ticks indicate if the incubated egg fell into the candling, break-out or pipping groups.
Time: Represent the time from the day before treatment to the last point of egg collection.
Relationship to treatment: Control represents the day before treatment; Treatment 2,4,5 represented the 2nd, 4th and 5th dose, while withdrawal 1, 3, 4, 6 and 8 represents day 1, 3, 4, 6, and 8 after withdrawal of treatment
Candling: Represents the candling of the egg to determine if an embryo was present.
Break-outs: Represents those eggs that were physically open on day 18 of their incubation to ascertain the age of the embryo
Pipping: Represents those eggs taken to the point of hatch.

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3.3.2. Effect of florfenicol egg hatchability following exposure in cockerel (Phase 2)

A wash out period of 21 days was allowed between phases. In studies conducted by several authors, florfenicol was detected in tissues of animals up to 12 days after dosing (Varma et al., 1986; Afifi and El-Sooud, 1997; Shen et al., 2002). In the present study, 21 days was allowed for almost complete elimination of the drug before commencing next dosing. In phase 2, cockerels in groups 1, 2 and 3 were treated with florfenicol at the dose of 30, 60, and 90 mg/kg, respectively, while the non-treated control group received only pure water (n=30) for five consecutive days by gavage. Treatments were administered once daily, though the use of a calibrated syringe attached to flexible dosing tube that was inserted directly into the crop. The tube was emptied of drug prior to removal of the tube from the oesophagus. The birds were not starved prior to treatment. Animals were returned to their pens immediately after dosing.

Eggs (n=30) were subsequently collected from all groups on days 0, 2, 4, and 5 of dosing and on days 1, 3, 4, 6, and 8 post dosing (Table 3.3). The collected eggs were subsequently incubated till hatch or point of egg-breakout (Refer to section 3.5.1).

Table 3.4. Schedule for egg collection (n=30) following the treatment of only the cocks (n=4) following treatment for 5 days.

Dose (mg/kg)	Egg Collection Point		Activity*		
	Time (h)	Relationship to treatment	Candling (n=30)	Break outs (n=30)	Pipping (n=30)
0, 10, 20, 30	0	Control	✓		✓
	48	Treatment 2	✓	✓	
	96	Treatment 4	✓		✓
	120	Treatment 5	✓	✓	
	144	Withdrawal 1	✓	✓	
	192	Withdrawal 3	✓		✓
	216	Withdrawal 4	✓	✓	
	264	Withdrawal 6	✓	✓	
	288	Withdrawal 8	✓		✓

* The ticks indicate if the incubated egg fell into the candling, break-out or pipping groups.
Time: Represent the time from the day before treatment to the last point of egg collection.
Relationship to treatment: Control represents the day before treatment; Treatment 2,4,5 represented the 2'nd, 4'th and 5'th dose, while withdrawal 1, 3, 4, 6 and 8 represents day 1, 3, 4, 6, and 8 after withdrawal of treatment
Candling: Represents the candling of the egg to determine if an embryo was present.
Break-outs: Represents those eggs that were physically open on day 18 of their incubation to ascertain the age of the embryo
Pipping: Represents those eggs taken to the point of hatch.

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3.3.3. Influence of excessively high doses on hen fertility (Phase 3)

For this phase, the hens were treated with florfenicol at doses of 90 mg/kg (n=30), 60mg/kg (n=30) and 30mg/kg (n=30), while the control group received only pure water (n=30) for five consecutive days by gavage. Higher doses of the drug were chosen as a means of evaluating their effect of fertility in chickens. Treatments were administered once daily, through the use of a calibrated syringe attached to flexible dosing tube that was inserted directly into the crop. The tube was emptied of drug prior to removal of the tube from the oesophagus. The birds were not starved prior to treatment. Animals were returned to their pens immediately after dosing.

Eggs were subsequently collected from all groups on days 0, 2, 4 and 5 of dosing and on days 1, 3, 4, 6 and 8 after dosing (Table 3.4). The collected eggs were subsequently incubated (Refer to section 3.5.1). Unlike in the earlier two phases, the eggs collected were reduced (from 25 at the start to 5 at the end) as the hens were stagger slaughtered as mentioned below.

Hens (n=5) were stagger slaughtered by means of a CO_2 euthanasia at 0, 1, 2, 3 and 4 days post drug withdrawal from every group of treatment to determine the concentration of drug in the lung tissue. In addition the concentration of florfenciol in the eggs (n=5) were also determined on days 0, 1, 2, 3, and 4 post drug withdrawal from each group of treatment (See below for the analytical methodology).

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Table 3.5. Schedule for egg collection and lung tissue in the treated hens (n=30) following treatment for 5 days.

Dose (mg/kg)	Egg Collection Point		Activity*		
	Time (h)	Relationship to treatment	Lung concentration	Egg concentration	Incubated
0, 30, 60, 90	0	Zero Hour			✓ (30)
	48	Treatment 2			✓ (30)
	96	Treatment 4			✓ (30)
	120	Treatment 5/ Withdrawal 0	✓	✓	✓ (25)
	144	Withdrawal 1	✓	✓	✓ (20)
	168	Withdrawal 2	✓	✓	✓ (15)
	192	Withdrawal 3	✓	✓	✓ (10)
	216	Withdrawal 4	✓	✓	✓ (5)
	264	Withdrawal 6			✓ (5)
	312	Withdrawal 8			✓ (5)

* The ticks indicate if the listed activity being undertaken for the listed time point.
Time: Represent the time from the day before treatment to the last point of egg collection or slaughter.
Relationship to treatment: Control represents the day before treatment; Treatment 2,4,5 represented the 2'nd, 4'th and 5'th dose, while withdrawal 0, 1, 2, 3, 4, 6 and 8 represents day 0, 1, 2, 3, 4, 6, and 8 after withdrawal of treatment (The eggs/birds assigned to the zero withdrawal were slaughtered 1 hours after treatment 5)
Lung concentration: represents the time points animals were staggered slaughtered to ascertain concentration of drug within the lung parenchyma (n=5)
Egg concentration: represents the time points for which the concentration of drug was ascertained in the whole egg (n=5)
Incubated: Represents the time points and sample size (in parenthesis) of eggs incubated to pip.

3.4. Test products

Florfenciol was specifically compounded and donated by Vtech Compounding Solutions Pharmacy (Midrand, South Africa). The florfenicol (Batch no.VT090219) was diluted with glycerol to a final concentration 30 mg/ml. The product was supplied with a certificate of analysis (Appendix. 1).

3.5. Drug analysis

Lung tissue and egg concentrations of florfenicol were determined using a validated, specific and sensitive HPLC method that was specifically validated for this study by Mrs MG Mulders of the Department of Paraclinical Sciences (Appendix 2).

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3.5.1. Sample collection for analysis

Hens (n=5) were stagger slaughtered at 0, 1, 2, 3 and 4 days post drug withdrawal from each group of treatment in phase 3 to determine the concentration of florfenicol in the lung tissue. Both lungs from each bird were removed *in* toto at necropsy and placed in sample bottles marked with their date and group, and subsequently immediately frozen at -30 ^{0}C until analysed.

For quantification of florfenciol in the eggs (n=5), samples were collected on days 0, 1, 2, 3, and 4 post from the last treatment. For the purpose of this study, day 0 eggs were collected within 1 hour of the last treatment. Following removal from the pen, the eggs were broken and emptied (yolk and albumin) into a sampling bag and frozen at -30 ^{0}C after been properly identified.

3.5.2. Tissue extraction of florfenicol

3.5.2.1. Extraction of florfenicol from the lungs

One hundred microlitres (100 µl) of thiamphenicol (internal standard) and 9 ml ethyl acetate were added to 2 gram of lung tissue in a test tube, mixed well, and subsequently centrifuged at 2000 × G for 10 minutes. The resulting supernatant was decanted into a new tube and dried off for 30 minutes at 60^{0} C under a steady stream of nitrogen. The dried samples were subsequently reconstituted with 1.5 ml of mobile phase (as below), mixed well and re-centrifuged at 2000 × G for 10 minutes. The reconstituted samples were thereafter cleaned up using Varian BondElute C18 cartridges (200mg, 3ml) primed first with 4 ml methanol and subsequently with 4 ml MilliQ (reverse osmosis, deionised) water. After the sample was loaded, the column was washed with 2 ml MilliQ water and allowed to dry under vacuum for 5 minutes. Finally the analytes were eluted off the column with 3 ml methanol under vacuum applied for at least 5 minutes. The eluent was dried under a stream of nitrogen for 30 minutes at 60^{0} C, reconstituted in 500 µl mobile phase of which 100 µl was injected for analysis as below (Varma et al., 1986).

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3.5.2.2. Extraction of florfenicol from the eggs

One hundred microlitres (100 µl) thiamphenicol (internal standard) and 9 ml ethyl acetate were added to 2 gram of homogenised egg tissue (yolk and albumin combined in natural ratios) in a test tube, mixed well, and subsequently centrifuged at 2000 × G for 15 minutes. The resulting supernatant was decanted into a new tube and dried off for 30 minutes at 60º C under a steady stream of nitrogen, prior to being mixed with 2 ml MilliQ water and 2 ml hexane and centrifuged at 2000 × G for 15 minutes. The sample was subsequently cleaned with the same solid phase extraction method as discussed for the lung analysis (Varma et al., 1986).

3.5.2.3. Analysis of florfenicol

The HPLC method and extraction were modified from a previous method (Varma et al.1986) by the Department of Paraclinical Sciences, University of Pretoria. Thiamphenicol was used as internal standard for this analytical method. A Diode array detector on a Beckman HPLC was used for detection and quantitation of florfenicol. Detection was accomplished by detector absorption at 223 nm. A Phenomenex Security Guard Holder (KJO-4282) containing guard cartridges (AJO-4287) was fitted before the analytical column (LG reverse phase, Luna 5µaC18 (2); 100A; 150 x 4.6 mm). The mobile phase used was 30% acetonitrile in filtered water. The run time of each chromatogram was 10 min. The LLQ (lower limit of quantification) was 0.1 µg/ml (Appendix 2).

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3.6. Fertility assessment

3.6.1. Incubator protocol and specifications

Eggs were identified immediately after collection (30-35 eggs were collected daily around 8 am local time). The eggs were hereafter candled to ascertain if they had cracks in their shells prior to placement in an incubator (Buckeye egg incubator), with an automated turning platform. All eggs were incubated at a temperature of 36 ^{0}C and humidity of 25 ^{0}C for 18 days (Fig.3.6), with the turning set to turn once every hour.

Fig. 3.6. The incubator measures temperature and humidity using electronic detectors. The eggs were incubated and the hatching boxes placed at the bottom (Anonymous, 2011d).

3.6.2. Egg break-out procedure

On day 18 of the incubation period, the eggs (n=30) were evaluated at necropsy using standard egg break-out procedures. A forceps was used to break the egg shell at the top. The embryo was evaluated against a standard chart of development to ascertain if early mortality had occurred and more importantly, to determine the age of embryonic

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death (Fig.3.7). The percentage fertility was determined as the number of eggs without signs of an embryonic development to the total number of eggs broken.

Fig. 3.7. Stages showing embryonic development. Day 1 = All the cells are alike and homogenous; Day 6 = the embryo has all organs needed to sustain life after hatching, and most of the embryo's parts can be identified. The chick embryo cannot, however, be distinguished from that of mammals; Day 12 = the feathers and feather tracts are visible, and the beak hardens; Day 15 = the claws are forming and the embryo is moving into position for hatching; Day 21 = the beak has pierced the air cell, and pulmonary respiration has begun. The chick rests, changes position, and keeps cutting until its head falls free of the opened shell. The chick is exhausted and rests while the navel openings heal and its down dries. Gradually, it regains strength and walks (Anonymous, 2007)

3.6.3. Candling procedures

On day 18 of incubation, the eggs (n=30) were candled by placing a bright light against the shell (Anonymous 2007a). Eggs were considered fertile, if the embryo appeared fully developed with blood and veins being seen easily (Fig. 3.8). These fertile eggs were incubated to hatch.

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Fig. 3.8. Candling method showing the embryo and its veins (Anonymous 2007a).

3.6.4. Hatching procedure

Eggs incubated to hatch were removed from the grids on day 19 and placed into the hatching box of the same incubator under the same temperature and humidity conditions (Fig.3.9). The emerging chicks were subsequently removed from the incubator at the day 22, evaluated for their quality, such as ability to stand on their feet for more than 3 minutes, the feather cover, shape of their beak, movement and feeding behaviour prior to CO_2 euthanasia. The number of chicks hatching was recorded as % pipped.

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Fig. 3.9. Egg hatching in the incubator hatching box after 21 days of incubation. (Anonymous, 2011d).

3.7. Adverse drug reactions

The birds were observed throughout the study for any adverse drug reaction by a veterinarian from the poultry reference laboratory, Onderstepoort, South Africa.

3.8. Determination of minimum inhibitory concentration (MIC) of florfenicol

The MIC of florfenicol against *Escherichia coli* was determined by the Standard Operating Procedure (SOP) developed at the Faculty of Veterinary Science, University of Pretoria according to Schwarz et al., 2006. Briefly, the culture of the bacterial organism was prepared in 10 ml Brain Heart Infusion Broth (BHI) and incubated for 24 hours at 37°C. The concentration was adjusted until an optical density of 1 was obtained at 540 nm corresponding to a density of 10^8 colony forming units of bacterial organism. A two fold serial dilution of the drug was done from test tube 1-10 with test tube 11 and 12 serving as negative and positive control tubes, respectively. Each tube contained 2 ml BHI. Thereafter, about 20 µl of the bacterial culture was added to each tube except tube 11 while tube 12 contained only the bacterial organism. The whole preparation was further incubated for 24 hours at 37°C. The MIC was read as the lowest concentration of the drug where growth of the bacteria was inhibited. The samples used

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in the assay were obtained by Vtech Compounding Pharmacy, South Africa as part of their monitoring programme in various areas in the country. Due to confidentiality, Vtech was unwilling to provide the farm or specific area from where the samples were collected.

The MIC_{50} and MIC_{90} were determined from the results. The MIC_{50} represents the MIC value at which 50% of the isolates in a test population are inhibited; it is equivalent to the median MIC value. The MIC_{90} represents the MIC value at which 90% of the strains within a test population are inhibited; the 90th percentile. The MIC_{90} is calculated accordingly, using $n \geq 0.9$. If the resulting number is an integer, this number represents the MIC_{90}; if the resulting number is not an integer; the next integer following the respective value represents the MIC_{90}. MIC_{50} and MIC_{90} values are presented as concentrations (Schwarz et al., 2006).

3.9. Prediction of the withdrawal time

The withdrawal time was based on the method of the Medicines Control Council of South Africa (Anonymous, 2004b). According to the guideline, the 95% upper population tolerance with 95% confidence was ascertained using a non-central t-theorem. For this study the upper tolerance was calculated as: mean + 4.203 × SD (Table 3.5) on the natural logarithm (Ln) scale. The upper confidence value was converted to real values (anti-log) and multiplied by the 100 g of egg consumed that an average person of 60 kg is expected to consume according to the WHO food basket, to create the Total Theoretical Maximum Daily Intake (TMDI). (The TMDI is the amount of concentration the person could be exposed to by consuming this concentration of egg with drug therein). The TMDI obtained was compared to the average daily intake of 600 µg/person published by the European Agency for the Evaluation of Medicinal Products (EMA) (Anonymous, 1999b) to check if the concentrations reached 0% of the ADI during the monitoring period.

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Table 3.6. Factors to determine the upper population tolerance

	$p = 0.90$			$p = 0.95$			$p = 0.99$		
1-α	0.9	0.95	0.99	0.9	0.95	0.99	0.9	0.95	0.99
N									
2	10.253	20.581	103.02	13.09	26.26	131.43	18.5	37.094	185.62
3	4.258	6.155	13.995	5.311	7.656	17.37	7.34	10.553	23.896
4	3.188	4.162	7.38	3.957	5.144	9.083	5.438	7.042	12.387
5	2.724	3.407	5.362	3.4	4.203	6.578	4.666	5.741	8.939
6	2.494	3.066	4.411	3.092	3.708	5.406	4.243	5.062	7.335
7	2.333	2.755	3.859	2.894	3.399	4.728	3.972	4.642	6.412
8	2.229	2.582	3.497	2.754	3.187	4.285	3.783	4.354	5.812
9	2.133	2.454	3.240	2.65	3.031	3.972	3.641	4.143	5.389
10	2.066	2.355	3.048	2.568	2.911	3.738	3.531	3.981	5.074

p represents the population tolerance required and 1-alpha is the required confidence interval

To assess if a withdrawal time was possible, linear regression was applied to the time versus egg upper tolerance concentrations (mean + 4.203 × SD) for the highest dose on the Ln scale. The theoretical MRL was set at half the LLQ of 0.1 µg/ml, which according to the food basket (TMDI) would account for approximately 0.8 % of the average daily intake (ADI). The time point at which the best-fit line reached the value of 0.05 µg/ml was considered to be a potential withdrawal time.

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CHAPTER 4: RESULTS

4.1. Phase 1

At the dose of 10, 20 or 30 mg/kg, florfenicol had no effects on embryonic development as ascertained by candling, break-outs or pipping. The percentage of fertile eggs as determined by candling was 77-93%, 70-93% and 74-89% for the 10, 20 and 30 mg/kg groups, respectively (Table 4.1). This compared, in general, favourably with the control group which was in the range of 78-96%. However, when the differences were evaluated on a day to day basis, the eggs collected the morning before the 5th dose showed a minor reduction in the 20 and 30 mg/kg groups (82 and 74% respectively) in comparison with the 0 and 10 mg/kg groups (96 and 90%) and appears to follow a dose-response relationship from the control to the 30 mg/kg dose group (Fig. 4.1). None of the other time points demonstrated a dose-response relationship.

The fertility of the eggs as determined by egg break-out for the groups treated with 10, 20 and 30 mg/kg of florfenicol was 78-90%, 70-90% and 74-89%, respectively for the monitored period (Table 4.2). This compared, in general, favourably with the control group which was in the range of 81 to 94%. However, when the differences are evaluated on a day to day basis, the first evaluated time point after dosing all three doses showed a reduction in % viability of around 15% in comparison to the control group, with the two high doses plateauing. The eggs collected the morning before the third dose (48h) showed a minor reduction for all three dose levels (78 to 76%) in comparison to the control group (89%). By morning prior to the 5th dose (120 h), the two high dose groups had a lower fertility (73 and 74%) when compared to the 10 and 0 mg/kg group (90 and 86%). The effect on fertility appears to follow a dose-response relationship from the control to the 30 mg/kg for the 48 to the 120 h time points (Fig 4.2). None of the other time points demonstrated a dose-response relationship. At 120 hours, the 10 mg/kg group no longer appears to have an adverse effect on fertility. When the results from the candling and egg-breakouts are compared, the 120 hours of the egg-breakouts and candling appears to produce corresponding results.

The hatchability as evaluated by the number of pipped chicks (table 4.3), ranged from 70 to 93%, 66 to 89% and 61 to 78%, for the 10 mg/kg, 20 mg/kg and for 30 mg/kg

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groups respectively for the monitored period (Table 4.2 and Fig.4.2). This in general, compared favourably with the control group where the range in the same period was 74 to 88%. However, as for the other assays, when a day by day comparison is made, a difference was evident. On day 0 and day 4, the 30 mg/kg group demonstrated lower hatchability than the other groups. None of the other time points demonstrated a dose-response relationship.

No adverse effect was observed in any bird after drug administration. Most of the chicks hatched without any abnormalities and in a healthy condition. The chicks were graded as being healthy.

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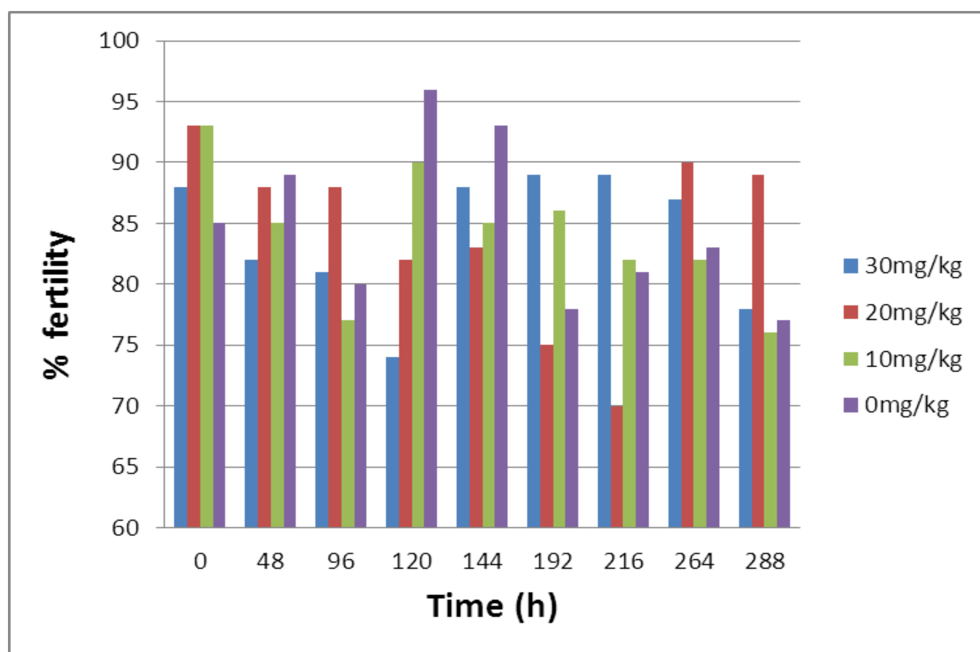
Table 4.1. The effect of different doses of florfenicol on egg fertility (%) (n=30) determined by candling.

Time (hours)	Percent fertility			
	30 mg/kg	20 mg/kg	10 mg/kg	Untreated control
0	88	93	93	85
48	82	88	85	89
96	81	88	77	80
120	74	82	90	96
144	88	83	85	93
192	89	75	86	78
216	89	70	82	81
264	87	90	82	83
288	78	89	76	77

Time: Zero hours is the day before dosing; 144h represents 24h after the last dose.



Time: Zero hours is the day before dosing; 144h represents 24h after the last dose

Fig. 4.1. The effect of different doses of florfenicol on egg fertility (%) determined by candling on day 18 post-incubation (n=30).

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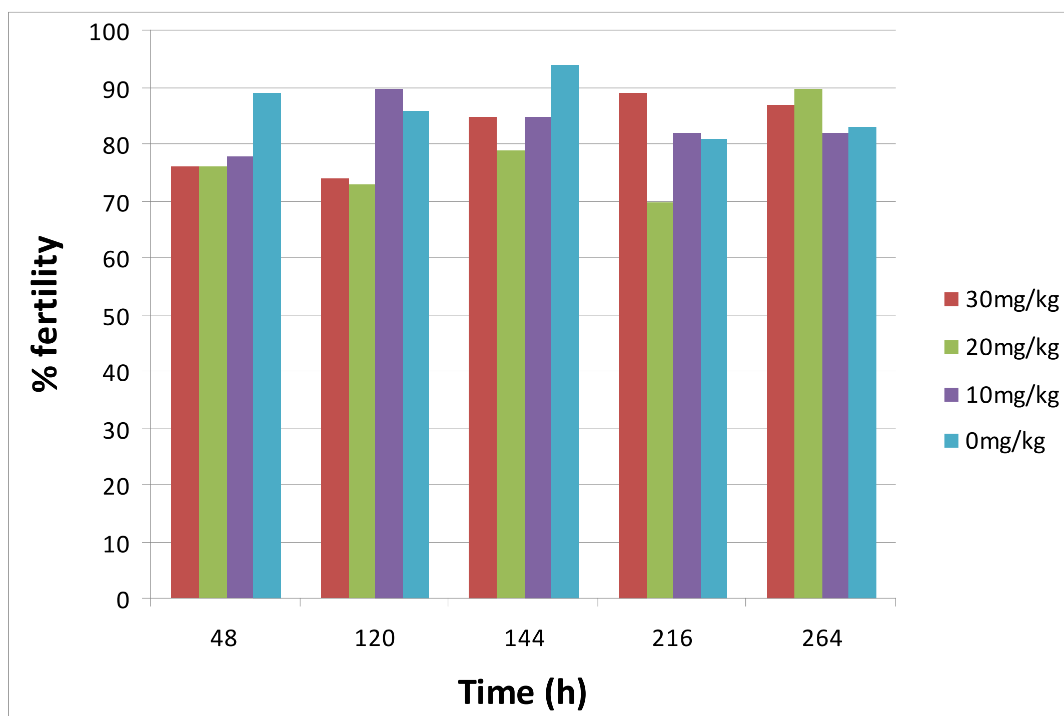
Table 4.2. The effect of different doses of florfenicol on egg fertility (%)(n=30) determined by the egg break out 18 days post-incubation.

Time (hours)	Percent fertility			
	30 mg/kg	20 mg/kg	10 mg/kg	Untreated control
48	76	76	78	89
120	74	73	90	86
144	85	79	85	94
216	89	70	82	81
264	87	90	82	83

Time: Zero hours is the day before dosing; 144h represents 24h after the last dose



Time: Zero hours is the day before dosing; 144h represents 24h after the last dose

Fig. 4.2. The effect of different doses of florfenicol on egg fertility (%) assessed by egg break out 18 days post-incubation (n=30).

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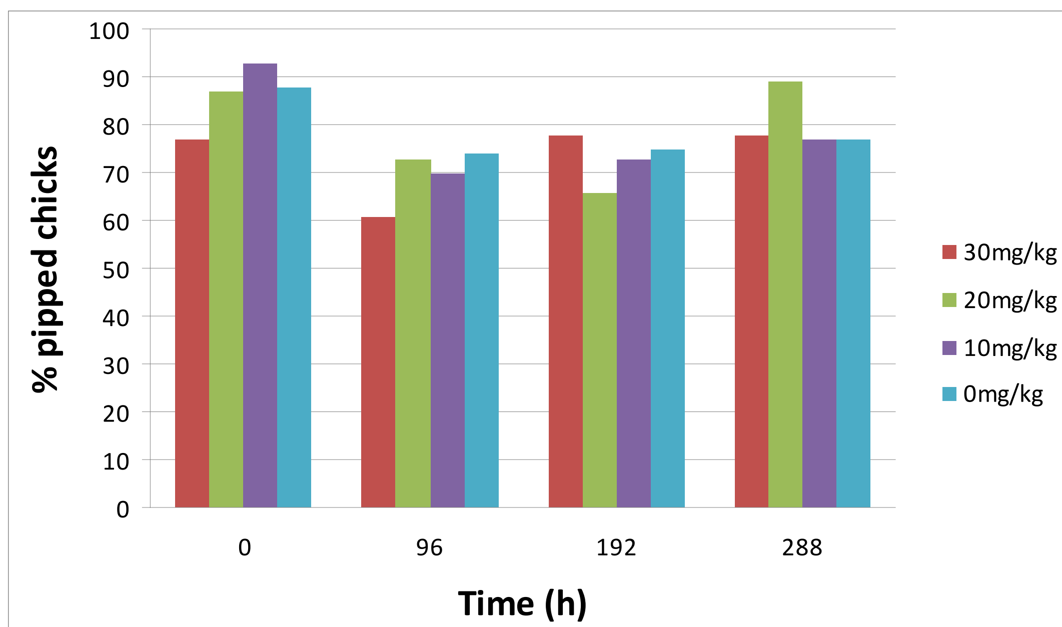
Table 4.3. The effect of different doses of florfenicol on hatchability (%) (n=30) determined by the number of chicks that pipped.

Time (hours)	Percent Hatchability			
	30 mg/kg	20 mg/kg	10 mg/kg	untreated
0	77	87	93	88
96	61	73	70	74
192	78	66	73	75
288	78	89	77	77

Time: Zero hours is the day before dosing; 144h represents 24h after the last dose



Time: Zero hours is the day before dosing; 144h represents 24h after the last dose

Fig. 4.3. The effect of different doses of florfenicol on hatchability for eggs incubated to pipping (%) (n=30).

4.2. Phase 2

The fertility of the eggs represented by egg break-out ranged from 77-92 %, 77-92% and 83-93% when treated with 30, 60 and 90 mg/kg of florfenicol, respectively for the monitored period. The non-treated control group had a percentage fertility of 70-92%. (Table 4.4, Table 4.5) (Fig.4.4, Fig.4.5). No trend was evident for the candling results.

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For egg break-out a slight dose-response relationship was evident only on the morning of the 5th treatment, which ranged from 86% for the 30mg/kg dose and 87% for the control group. The candling and egg break-out results appeared to be poorly correlated visually.

The hatchability of the eggs, represented by the number of pipped chicks for groups treated with 30, 60 and 90 mg/kg of florfenicol were between 72-92%, 62-73% and 57-76%, respectively for the monitored period, while the non-treated control group had an egg hatch of 63-81%. (Table 4.6, Fig.4.6). A dose-response relationship was evident for the eggs collected on day 0 of the study and range from 67% to 81% from the high dose to the control group. For an unknown reason the hatchability was rather poor at 192 and 288h for some of the groups in a non-dose dependent manner.

At the end of the treatment, there was no adverse effect observed for any bird as almost all chicks hatched without any abnormalities and appeared healthy.

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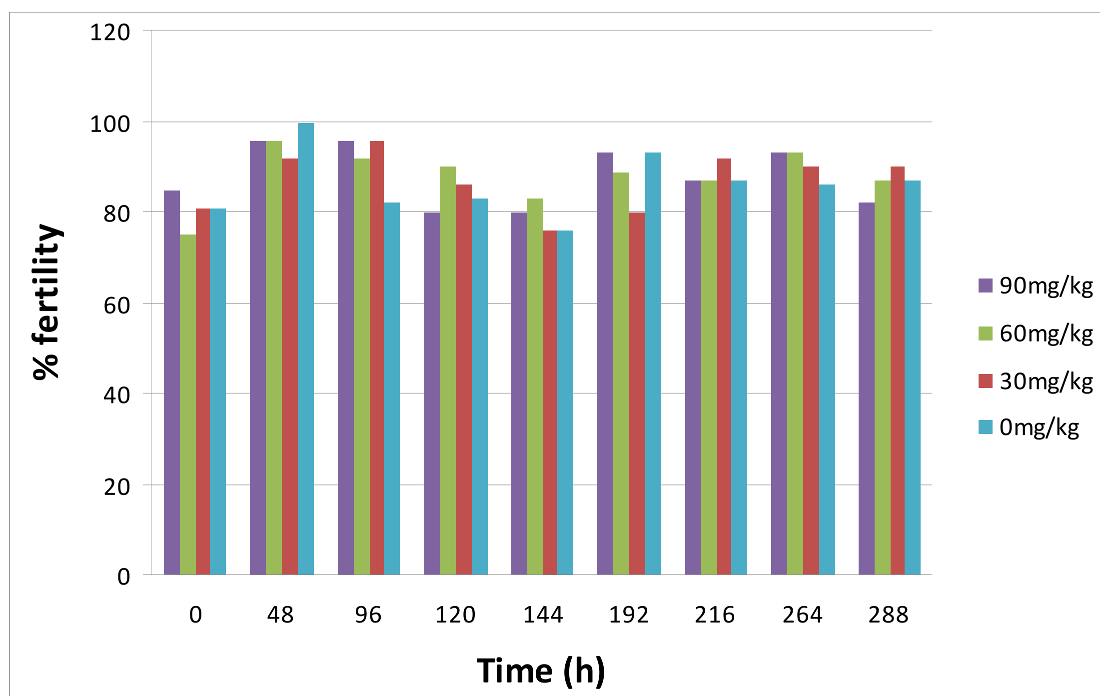
Table 4.4. The effect of different doses of florfenicol on egg fertility (%)(n=30), following treatment of only the cockerels (n=4), by candling 18 days post-incubation.

Time (hours)	Percent fertility			
	90 mg/kg	60 mg/kg	30 mg/kg	Untreated control
0	85	75	81	81
48	96	96	92	100
96	96	92	96	82
120	80	90	86	83
144	80	83	76	76
192	93	89	80	93
216	87	87	92	87
264	93	93	90	86
288	82	87	90	87

Time: Zero hours is the day before dosing; 144h represents 24h after the last dose



Time: Zero hours is the day before dosing; 144h represents 24h after the last dose

Fig. 4.4. The effect of different doses of florfenicol on egg fertility (%)(n=30), following treatment of only the cockerels (n=4). by candling 18 days post-incubation.

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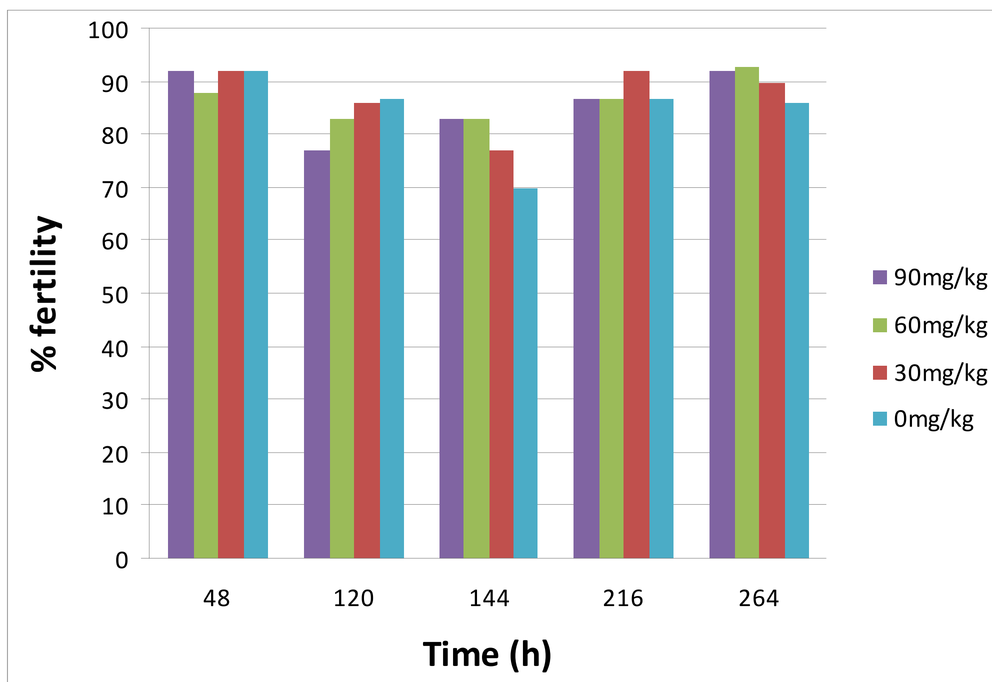
Table 4.5. The effect of different doses of florfenicol on egg fertility (%), following treatment of only the cockerels (n=4), by egg breaks (n=30) 18 days post-incubation.

Time (hours)	Percent fertility			
	90 mg/kg	60 mg/kg	30 mg/kg	Untreated control
48	92	88	92	92
120	77	83	86	87
144	83	83	77	70
216	87	87	92	87
264	92	93	90	86

Time: Zero hours is the day before dosing; 144h represents 24h after the last dose



Time: Zero hours is the day before dosing; 144h represents 24h after the last dose

Fig. 4.5. The effect of different doses of florfenicol on egg fertility (%)(n=30), following treatment of only the cockerels (n=4), assessed by egg break out 18 days post-incubation.

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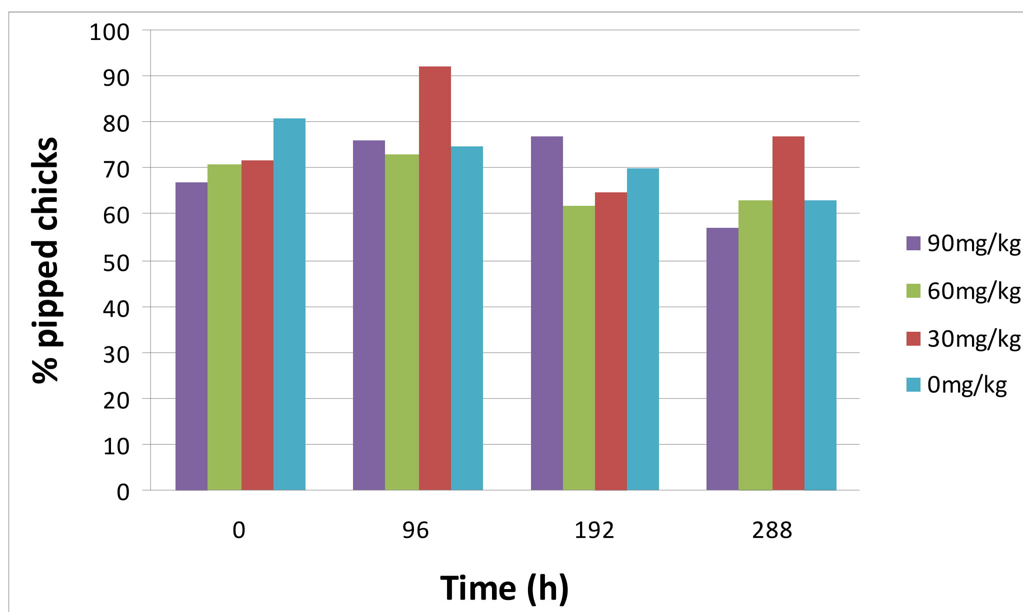
Table 4.6. The effect of different doses of florfenicol on hatchability (%)(n=30), following treatment of only the cockerels (n=4), determined by the number of chicks that pipped.

Time (hours)	Percent Hatchability			
	90 mg/kg	60 mg/kg	30 mg/kg	Untreated control
0	67	71	72	81
96	76	73	92	75
192	77	62	65	70
288	57	63	77	63

Time: Zero hours is the day before dosing; 144h represents 24h after the last dose



Time: Zero hours is the day before dosing; 144h represents 24h after the last dose

Fig. 4.6. The effect of different doses of florfenicol on hatchability (n=30), following treatment of only the cockerels (n=4) for eggs incubated to pipping (%) (n=30).

4.3. Phase 3

The fertility of the eggs represented by candling and egg break-out was 0% and 3% in the first 48 hours after the administration of florfenicol at dosages of 60 and 90 mg/kg, respectively. Complete recovery was observed by day five post-treatment. Conversely, the fertility of eggs in group treated with 30 mg/kg and the non-treated control group were 70-80 % and 64-100 %, respectively (Table 4.7; Table 4.8) (Fig. 4.7, Fig. 4.8). When a trend for a dose-response relationship was evaluated a relationship was

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present until day 5 for the egg-break-outs and day 9 for candling. From the break-out result, death occurs around day 5 of embryonic development in many eggs. When the results for the 0 and 30 mg/kg group are compared to the phase I results, no comparison was possible as the fertility for both these groups has declined by phase III.

The hatchability of the eggs as demonstrated by the number of pipped chicks was 0% 24 hours after the first florfenicol administration at doses of 60 and 90 mg/kg. The hatchability also declined in the non-treated control and 30 mg/kg groups to approximately 50% by the morning of the 5th treatment. The hatchability returned to the level that appeared to be normal 5 days post-treatment, with the 30 and 90 mg/kg group showing 100% hatchability, while the control group only achieved 80% hatchability (Table 4.9, Fig.4.9). Surprisingly, 5 days after treatment, percent hatchability in the non-treated control group and group treated with 60 mg/kg and were lower by 20 and 33%, respectively, compared with that treated with 90 mg/kg.

No adverse effect was observed in any bird after drug administration. Most of the chicks hatched without any abnormalities and under healthy conditions.

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Table 4.7. The effect of different doses of florfenicol on fertility (n=30) determined by candling at the day 18 of incubation.

Time (hours)	Percent fertility			
	90 mg/kg	60 mg/kg	30 mg/kg	untreated
0	73	80	81	93
48	0	3	67	87
96	0	0	81	91
120	0	0	81	88
144	8	12	63	71
192	0	0	89	100
216	0	0	86	100
264	0	60	100	80
288	100	60	100	100
312	75	80	80	100
336	100	67	100	80

Time, represents the day after dosing the eggs were collected

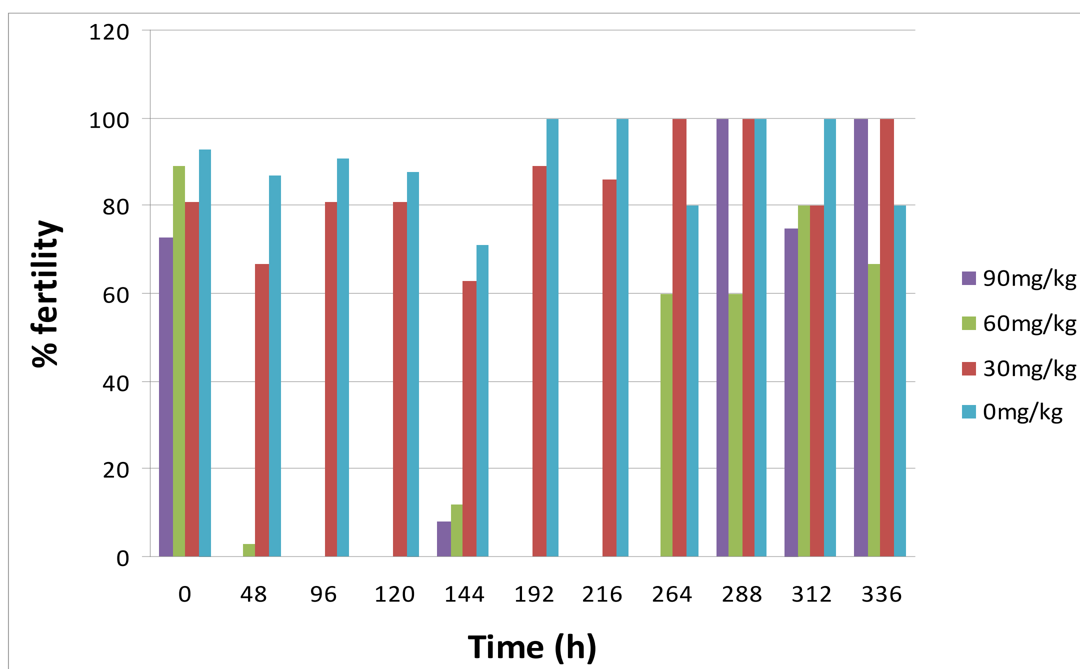

Fig. 4.7. The effect of different doses of florfenicol on fertility (n=30) determined by candling at the day 18 of incubation.

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Table 4.8. The effects of different doses of florfenicol on fertility (%) (n=30) as determined by egg break out at the day 18 of incubation.

Time (hours)	Percent fertility			
	90 mg/kg	60 mg/kg	30 mg/kg	untreated
48	0	3	69	70
120	0	0	81	71
144	8	12	64	71
216	75	60	100	80
264	75	60	100	80

Time, represents the day after dosing the eggs were collected

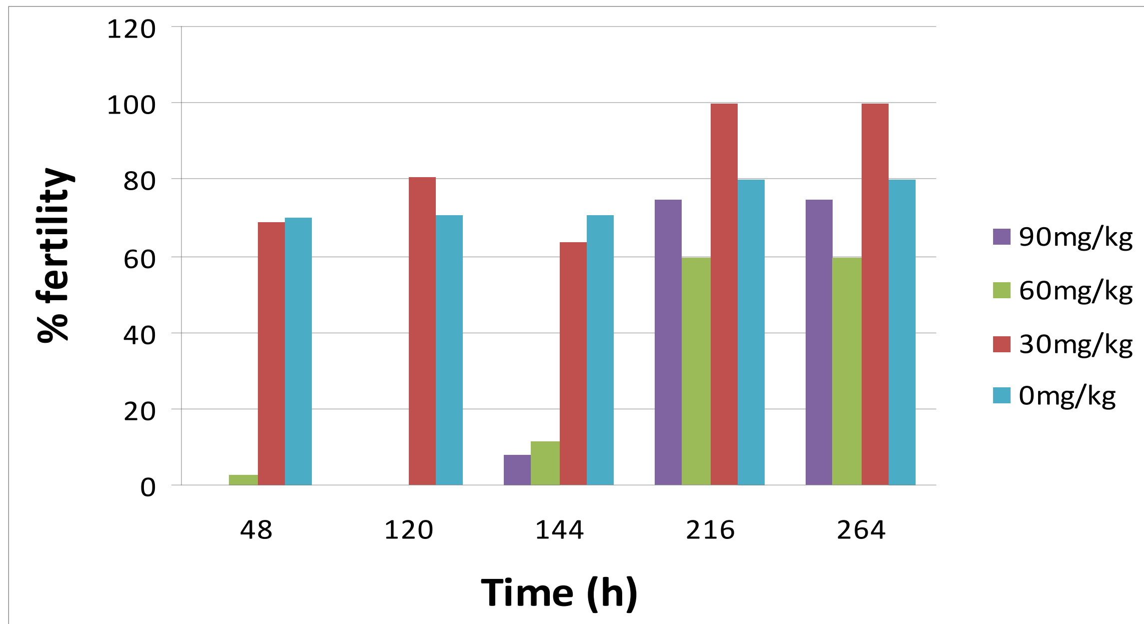

Fig.4.8. The effects of different doses of florfenicol on fertility (%) (n=30) as determined by egg break out at the day 18 of incubation.

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Table 4.9. The effect of different doses of florfenicol on hatchability (%) (n=30) determined by the number of chicks that pipped.

Time (hours)	Percent hatchability			
	90 mg/kg	60 mg/kg	30 mg/kg	untreated
0	73	90	77	70
96	0	0	52	50
192	0	0	71	66
288	100	67	100	80

Time, represents the day after dosing the eggs were collected

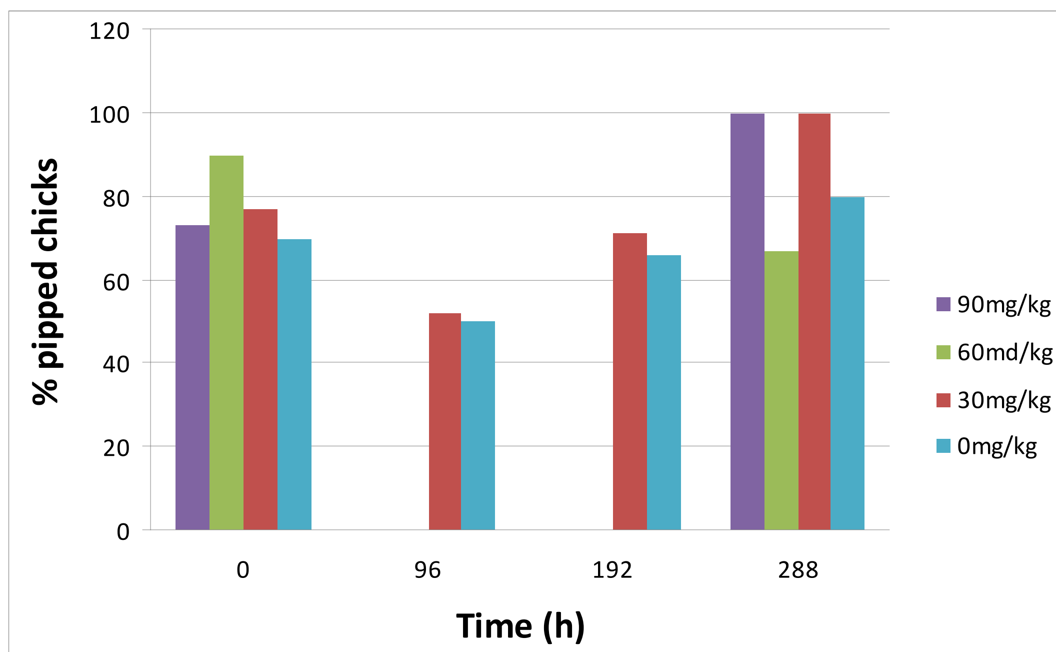

Fig. 4.9. The effect of different doses of florfenicol on hatchability (%) (n=30) determined by the number of chicks that pipped.

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4.4. Tissue concentrations of florfenicol

The laboratory analytical method was properly validated prior to analysis using both pure florfenicol in solution and spiked tissue samples (Appendix 2). The method was deemed to be specific for florfenicol as no interfering compounds were seen during the validation runs. In all cases the increase in relative response corresponded to an increase of florfenicol in the spiked samples.The method was also accurate and precise for the linear range of 0.1 to 10 µg/g, with the correlation coefficient always being above 99%. The accuracy for the lowest concentration of 0.1 µg/g was 0.11 ± 0.01µg/g and 0.10 ± 0.02 µg/g for the lung and eggs respectively, while the accuracy for the highest concentration 10 µg/g was 10.18 ± 0.28 µg/g and 10.58 ± 0.61 µg/g for the lung and eggs respectively. The extraction efficiency of the method was 114% for the 0.1µg/ml concentration in the lung tissue samples and 97% for the eggs samples, and 102% for the high concentration in the lung tissues samples and 105% for the eggs samples (Table 5.6).

The concentrations of florfenicol in lungs were determined at the doses of 30, 60 and 90 mg/kg, for five days post-treatment. Florfenicol was detectable and above LLQ (0.01 µg/g) for only 24 hours after withdrawal of treatment. The concentration of florfenicol in lungs at the dose of 90 mg/kg was 8.53 µg/g at 1h after the last dose while at 60 and 30 mg/kg, the concentration of the florfenicol achieved in the lungs at 1h after the last dose were 5.59 and 3.64 µg/g, respectively (Table 4.10, Fig.4.10). The concentrations of the drug in the lung tissue were highly variable as seen with the large standard errors achieved. The concentration achieved in the lungs of the treated birds was in linear relationship to the dose administered.

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Table 4.10. Concentration (µg/mg) of florfenicol in the lung 0, 24, 48, 72 and 96 hours after the withdrawal of treatment of florfenicol at 30, 60 or 90 mg/kg oid for 5 days.

Time after dosing (hour)	Dose		
	90 mg/kg	60 mg/kg	30 mg/kg
0	8.53 ± 2.22	5.59 ± 1.00	3.64 ± 1.08
24	0.14 ± 0.06	0.03 ± 0.01	0.21 ± 0.02
48	0.04 ± 0.01	0.07 ± 0.03	0.07 ± 0.02
72	0.02 ± 0.00	0.03 ± 0.00	0.11 ± 0.02
96	0.01 ± 0.00	0.06 ± 0.01	0.12 ± 0.03

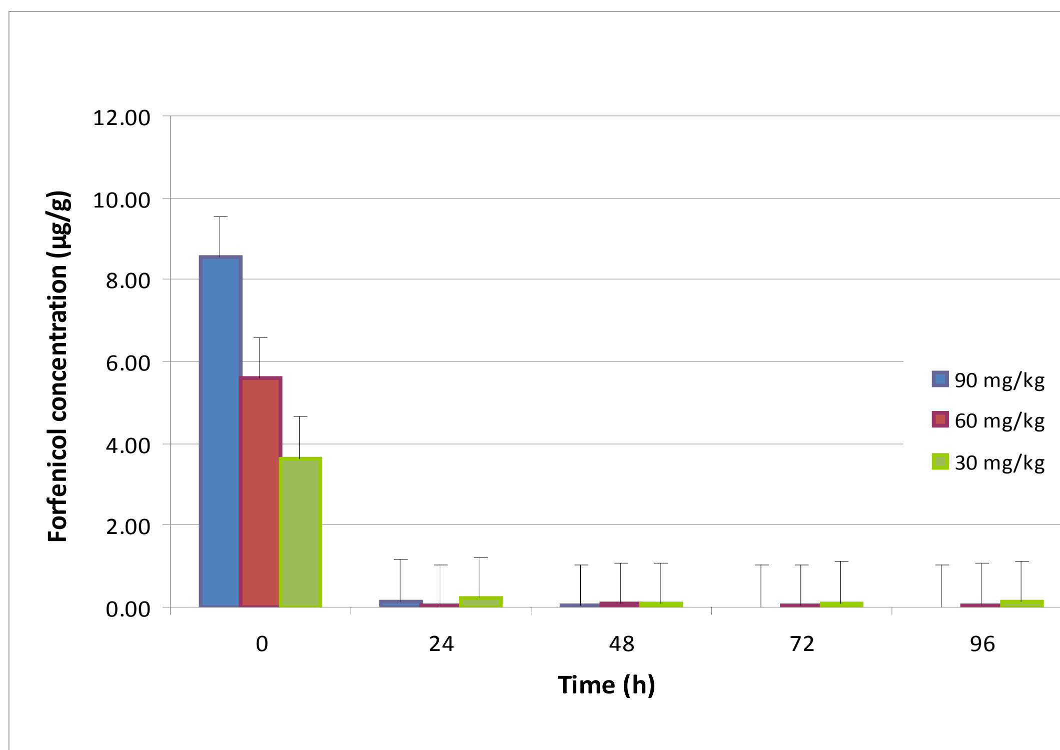

Fig. 4. 10. Concentration (µg/mg) of florfenicol in the lung 0, 24, 48, 72 and 96 hours after the withdrawal of treatment of florfenicol at 30, 60 or 90 mg/kg oid for 5 days.

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The concentrations of florfenicol in eggs were determined at the doses of 30, 60 and 90 mg/kg, for five days post-treatment. Florfenicol was detectable and above LLQ (0.01 µg/g) in the eggs for all time points. The concentration of florfenicol in eggs at the dose of 90 mg/kg was 4.27 µg/ml at 0 h post-withdrawal and gradually decreased to 0.85 by four days post-treatment. At doses of 60 and 30 mg/kg, the concentration of the florfenicol achieved in the eggs at 0 h post-dosing were 2.2 and 0.68 µg/ml/mg, respectively, and had declined to 0.59 and 0.27 µg/ml, by four days post-treatment respectively (Table 4.11, Fig.4.11). Similarly, just as what was observed in the lungs, the concentrations of florfenicol achieved in the eggs of the treated birds were dose-dependent.

Table 4.11. Concentration (µg/g) of florfenicol in the eggs 0, 24, 48, 72 and 96 hours after the withdrawal of treatment of florfenicol at 30, 60 or 90 mg/kg oid for 5 days.

Time after dosing (hour)	**Dose**		
	90 mg/kg	**60 mg/kg**	**30 mg/kg**
0	4.27 ± 0.76	2.2 ± 0.30	0.68 ± 0.05
24	3.8 ± 0.34	2.53 ± 0.24	1.08 ± 0.11
48	1.85 ± 0.28	1.8 ± 0.15	0.59 ± 0.02
72	1.12 ± 0.13	0.6 ± 0.04	0.39 ± 0.06
96	0.85 ± 0.05	0.59 ± 0.13	0.27 ± 0.01

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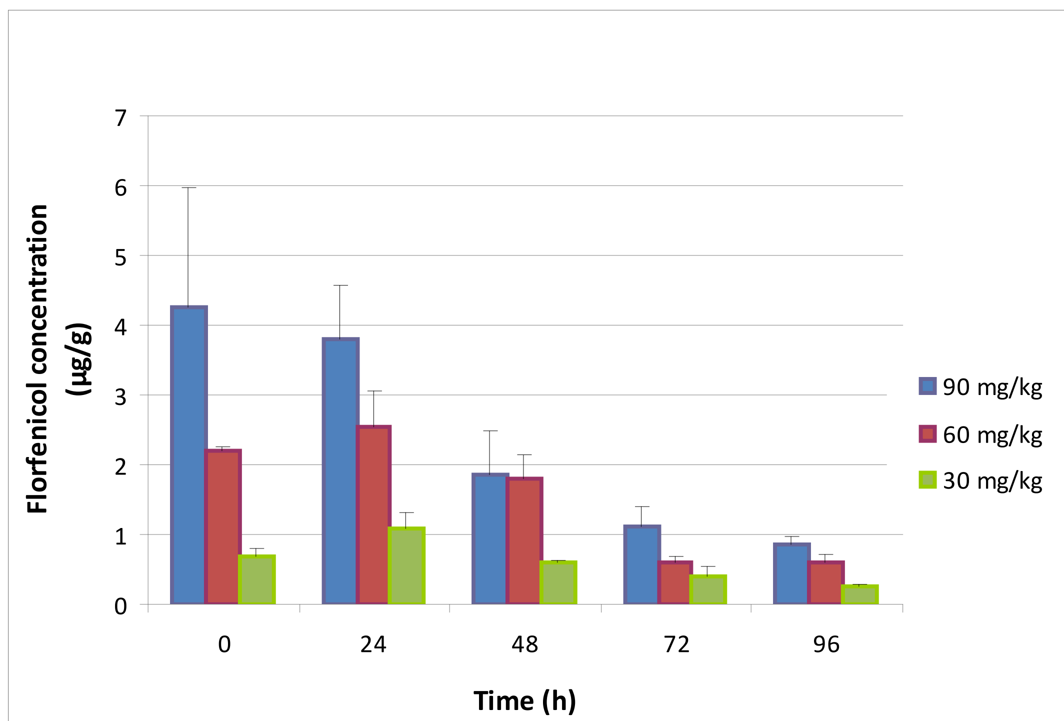

Fig. 4.11. Concentration (µg/g) of florfenicol in the eggs 0, 24, 48, 72 and 96 hours after the withdrawal of treatment of florfenicol at 30, 60 or 90 mg/kg oid for 5 days.

Table 4.12. Relationship between egg florfenicol concentration (µg/g) and fertility, after 5 consecutive days of treatment

Day	Dose (mg/kg)	Concentration (µg/ml)	Fertility (%)
0	90	4.27	0
0	60	2.2	0
3	90	1.12	0
3	60	0.6	0
0	30	0.68	52
3	30	0.39	71

Day: Represents the time period from the withdrawal of treatment

The effect of florfenicol on fertility and eggs hatchabily was significant and was detected in hens treated at 60 and 90 mg/kg. The fertility of the eggs declined to 0% after the first 24 hours of florfenicol administration, at doses of 60 and 90 mg/kg, to eventually recover by five days post-treatment. In addition, all the embryos for which a 0% hatchability was observed, embryonal death occurred at an early stage (day 2-3 of embryonic development).

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When the concentration in the egg was compared to the fertility, a trend was observable with the threshold embryonal toxic concentration being 0.6 ug/ml. This was derived from the 60 mg/kg treated group in which concentration of 0.6 ug/ml reduced fertility to 0% 3 days after treatment. The same concentration was achieved for the 30 mg/kg group at day 0 after treatment withdrawal with a 52 % fertility.

4.5. Minimum inhibitory concentration (MIC) of florfenicol

The MIC_{50} and MIC_{90} of florfenicol against *Escherichia coli* was 4 and 5 µg/ml, respectively. The MIC_{50} of the drug was only achieved in the lungs when administered at 90 mg/kg for five days.

4.6. Estimation of Withdrawal Period

For the duration of the monitoring period, the concentration in all the eggs multiplied by the predictive consumption, to get the TMDI, was always within the ADI and not below 0.5% as wanted (Fig 4.12). As such the withdrawal was extrapolated using the tolerance method. The upper tolerance best-fit equation was calculated from the Extrapolated 95% upper population tolerance at 95% confidence (Table 4.12) and defined by the following equation: $y=-0.1156x+11.942$; with a $R^2 = 0.9078$. Fitting the value of 0.05 µg/ml to the y values, an x value of 103.30 hours is achieved (Fig 4.12). It indicates that a withdrawal period of about 5 days is required from the last day of florfenicol administration at the dosage of 90 mg/kg as to the time the eggs will be fit for human consumption.

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Table 4.13. Extrapolated 95% upper population tolerance at 95% confidence and the theoretical maximum daily intake (TMDI) for a person of 60 kg.

Time	Concentration	Ln Mean	Ln SD	Upper Limit	TMDI	% ADI
0	30	-0.40	0.18	1.44	144.25	24.04
24	30	0.06	0.20	2.50	249.54	41.59
48	30	-0.53	0.09	0.85	84.80	14.13
72	30	-0.99	0.37	1.75	175.11	29.18
96	30	-1.32	0.09	0.39	39.49	6.58
0	60	0.65	0.36	8.91	890.71	148.45
24	60	0.91	0.23	6.51	651.13	108.52
48	60	0.58	0.20	4.11	411.06	68.51
72	60	-0.50	0.17	1.24	124.45	20.74
96	60	-0.55	0.22	1.45	144.83	24.14
0	90	1.39	0.41	22.59	2259.29	376.55
24	90	1.32	0.21	8.92	892.35	148.72
48	90	0.57	0.34	7.50	750.14	125.02
72	90	0.21	0.13	2.12	212.19	35.37
96	90	-0.17	0.13	1.46	146.49	24.41

Mean and SD are presented on the log scale. The upper limit and TMDI are present real values
ADI is 600 µg/person

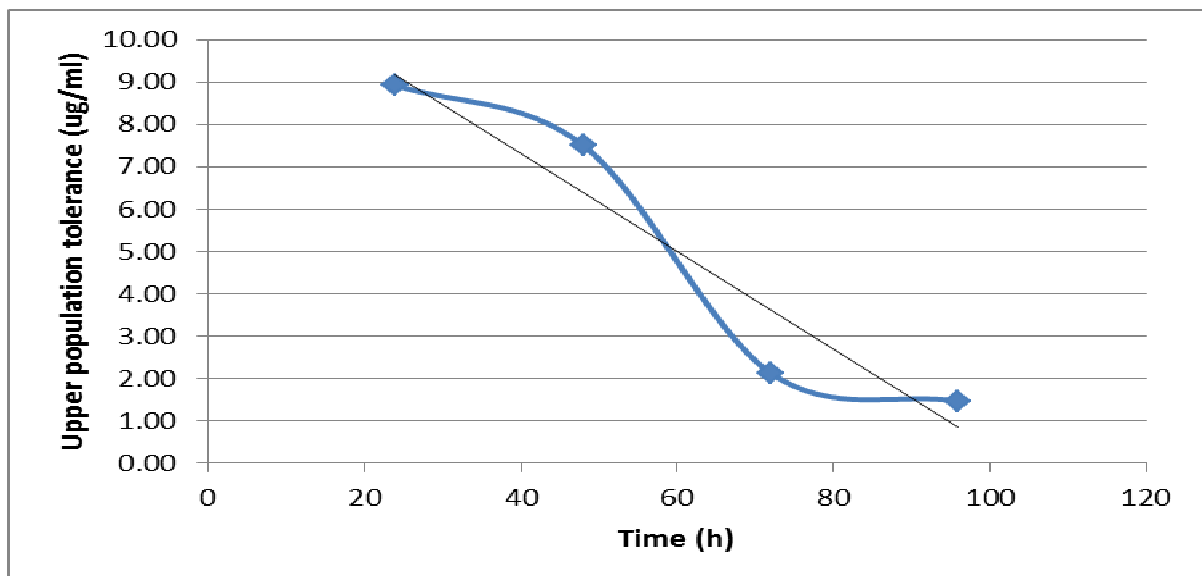

Fig 4.12. Upper population tolerance for eggs from animals treated at 90 mg/kg.

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CHAPTER 5: DISCUSSION AND CONCLUSION

5.0. Discussion

This study used different types of assessment in order to ascertain the effect of florfenicol on fertility and hatchability of chicken eggs, and to determine if concentrations of florfenicol in eggs and lungs were detectable. Florfenicol is currently the only antibiotic of the amphenicol family approved for use in food producing animals. Florfenicol functions through the inhibition of protein synthesis of susceptible bacteria by interfering with the 70S ribosomal subunits translocation and the peptidyl-transferase enzyme (Cannon *et al*., 1990; De Craene *et al*., 1997). A major benefit of florfenicol over chloramphenicol and thiamphenicol has been its ability to circumvent bacterial acetyltransferase-mediated drug resistance and also to reduce the risk of inducing drug-related aplastic anaemia in humans (Soback *et al*., 1995). It contains a fluorine atom at the 3´ carbon position instead of the hydroxyl group found in thiamphenicol. This substitution makes the antibiotic significantly more active than chloramphenicol and thiamphenicol (Lobell et al., 1994). Florfenicol is especially active against enteric bacteria such as *Enterobacter cloacae*, *Shigella dysenteriae*, *Salmonella typhi* and *Escherichia coli* (Neu and Fu, 1980; Syriopoulou et al., 1981). The potent activities of florfenicol against most of the susceptible Gram-negative and -positive bacteria frequently occurring in animal herds have been reported by several groups (Neu and Fu, 1980; Suzuki *et al*., 1989; Cannon *et al*., 1990). For *E. coli*, *K. pneumoniae*, and *S. aureus*, the MICs were reported to be >3.1 µg/ml (Neu and Fu, 1980). Because of its distinct advantages relating to safety and efficacy over thiamphenicol and chloramphenicol, florfenicol is believed to be an ideal replacement of these two drugs in animals (Jianzhong et al., 2004). These attributes of florfenicol have therefore made it a valuable antibacterial agent in the treatment of bacterial diseases particularly for the treatment of respiratory and enteric infections in food producing animals (Nordmo et al., 1994; Ueda and Suenaga, 1995; Booker et al., 1997; Sheldon, 1997).

5.1. The effect of low doses of florfenicol on the fertility of hens

The administered florfenicol did not have an effect on the hens or the number of eggs produced. The antibiotic did; however, appear to have an effect on fertility albeit minimal at 10 mg/kg. For the 20 and 30 mg/kg doses the effect was more marked with the 30

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mg/kg dose producing a 20% drop in fertility after five days of therapy. Despite the drop in fertility seen on break-out (early embryonic death), the eggs that were allowed to incubate to hatch, demonstrated no major differences in hatchability irrespective of the treatment group. This therefore tends to suggest that toxicity results in the earlier phases of embryonic development instead of later stages of development i.e. if the developing embryo was unaffected early on it would continue to develop in the expected manner.

To the best of our knowledge the mechanism of embryotoxic effect of florfenicol is yet to be ascertained. However the class of amphenicols has been previously associated with embryonic toxicity. In a controlled study with chick embryos, 14- or 20-somite embryonic stages exposed to chloramphenicol (the progenitor compound of the amphenicol group), at concentrations of 0, 200, or 300 µg/ml for 22 - 24 hours showed defects of the neural tube (failure to close) and the forebrain as well as the inhibition of haemoglobin formation (Billet et al., 1965). The study showed that the effect was due to inhibition of protein synthesis as a result of interference with the messenger RNA by chloramphenicol. In another study, chloramphenicol injected into turkey hatching eggs to eliminate *M. meleagridis* infection at doses of 2.5 and 5 mg reduced hatchability. Furthermore, the embryos died before 9 days of incubation. For the three studies described in McCapes et al (1975), embryos treated with lower dose (0.5 mg) of chloramphenicol appeared weak at candling. In a rat model of teratogenicity it was found that the chloramphenicol interfered specifically with mitochondrial protein synthesis by decreasing the production of mitochondrial cytochrome oxidase enzyme. Cytochrome C oxidase is the terminal enzyme in the electron transport chain located on the inner mitochondrial membrane. Most interestingly, the effect was time dependant as the 10 day old embryo was more susceptible than a 9 day embryo (Bass, 1999).

With florfenicol and chloramphenicol both sharing the same mechanism of protein synthesis inhibition, it is probable that florfenicol is toxic in the early stages of embryonic development via direct foetal protein synthesis inhibition.

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5.2. The effect of higher doses of florfenicol on the fertility of cocks

As a result of the minor change in fertility and hatchability seen in phase 1, only the cockerels were dosed in phase 2 to ascertain if toxicity was related to male fertility as seen for mammals. In addition since the 20 and 30 mg/kg doses appeared to have a minor effect, the doses were increased to 60 and 90 mg/kg to ensure that overt toxicity would be readily evident. In an unexpected manner none of the doses (90, 60 or 30 mg/kg) had an influence on the treated cocks, as chick embryo development and hatchability showed no abnormalities.

The reason for the difference in toxicity response between the cockerels and that reported in rats is currently unknown. Ando et al (1997) speculated that the testicular toxicity seen in rats receiving thiamphenicol, characterized by vacuolated Sertoli cells, was most likely a result of reduced cytochrome activity as seen with embryonic toxicity. With the cytochrome oxidases being important for aerobic sperm production, one can only speculate that avian Sertoli cells are less sensitive to the effects of the amphenicols or perhaps a difference in intracellular bioavailability results.

5.3. The effect of high doses of florfenicol on hen fertility

In the absence of toxicity in the cockerels at 30, 60 and 90 mg/kg, the toxic effect of the same dose in the hens was ascertained. The administered florfenicol did not have an effect on the hens or the number of eggs produced. The effects of florfenicol on fertility and eggs hatchability were significant in hens given 60 and 90 mg/kg. The egg fertility declined to 0% during the first 24 hours of florfenicol administration and eventually only recovered five days post-treatment. In addition, all the embryos for which 0% hatchability was observed, embryonic death was always recorded at an early stage in development (day 2-3 of embryonic development). When the florfenicol concentrations in eggs were evaluated in conjunction with the embryonal mortalities, an approximate concentration of 0.6 µg florfenicol per gram of raw egg content in natural proportions was found to be toxic to the chicken embryo. As mentioned above, the mechanisms behind toxicity at this stage are unknown, but may be linked to embryonic protein synthesis inhibition. The effect is also clearly due to the sensitivity of the embryo itself as maternal toxicity was not recorded nor was there any drop in egg production.

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5.4. Tissue and minimum inhibitory concentrations of florfenicol

The concentration of florfenicol in lung tissue of slaughtered birds showed the drug to accumulate in the respiratory tissue. In an unexpected manner, the drug was also rapidly eliminated from the lung tissue in all treatment groups. When compared to the MIC_{50} (4 µg/ml) only the 90 mg/kg dose was able to reach sufficiently high concentrations (0.14 ± 0.06 µg/g), making the total distribution of forfenicol into lung tissue poor for all but the high dose. The concentrations achieved in this study were also lower than previously reported. When Chang et al (2010) administered florfenicol at doses of 30 mg/kg directly into the crops of Taiwanese native chickens (*Gallus gallus domesticus*) and leghorns (*Gallus gallus)* significantly higher concentrations were measured in the brain (0.56 µg/g), lung (0.6 µg/g) and kidneys (0.9 µg/g) after 24 hours. Chang attributed the high concentrations to low plasma protein binding of the drug and extensive tissue distribution. The low concentrations achieved in the present study are difficult to explain, but may be due to an interference of the feed reducing absorption and subsequent bioavailability. While it may be possible that extraction efficiency from the tissue played a role, this seems unlikely as the method used for analysing the drug was efficient with an extraction efficiency of 102 to 114 % (0.01 µg/g and 10 µg/g samples), which is within the VICH acceptable limits (Varma et al. 1986).

The ability of the 90mg/kg dose to reach MIC nonetheless indicates the usefulness of the product in the management of respiratory tract infections caused by susceptible organisms in chickens. However the toxic effect to the developing embryo needs to be taken into consideration when opting to treat i.e. the loss in hatchability needs to be weighted against the cost of mortalities in the breeder hens.

The last question that was answered was the safety to the consumer if they were to eat the treated bird or the egg soon after the animals were treated. Unfortunately according to the ADI and MRLs proposed by the EMA, 100% of the ADI has already been distributed across various organs, with no partitioning left for the eggs. For the eggs to be acceptable for human consumption, the product would therefore need to be below the limit of detection at the time of consumption. None of the three doses tested were below the limit of quantification for any of the tested points, representing 5% of the ADI for the 30 mg/kg dose and 24% for the 90 mg/kg dose. If the MRL was to be set at the

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0.5 µg/ml, in theory it should take 6 days to reach 0.8% of the ADI. Therefore we suggest that the eggs may be safe to consume 6 days after the last treatment. The latter would obviously have to be verified by a study at the recommended dose and method of treatment as most farmers would use a continuous exposure method in the water or feed.

5.5. Conclusion

In conclusion florfenicol should not be given to breeder birds as it causes early embryonic toxicity in the absence of an effect on male fertility. Based on this result both hypotheses are accepted.

5.6. Future Trends

- It will be interesting to establish the exact mechanism of florfenicol's toxicity and the embryonic cell type most susceptable to toxicity.
- Further work should also focus on explaining the lack of cockerel fertility toxicity.
- A pharmacokinetic study to evaluate the difference in the pharmacokinetics of florfenicol in the absence and presence of food would also be of interest.

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LABORATORY REPORT | Page : 1 of 1

Bioindustrial Services
P.O Box 350
Van Riebeeck Park
1629

Tel : 0119702525
Fax : 0119702526
Cell : 0825596111
Email rajna@bioindustrial.co.za

06/03/2009

V-Tech Veterinary Solutions (P
P.O. Box 12285
Vorna Valley
Vorna Valley
1686
ATT: K Fourie

Client Tel : 0118055703
Client Fax : 0866360487

TEST ITEMS

Description	PHARMA - Injectable
Condition	Good
Date Received	04/03/2009
Date of Analysis	06/03/2009

RESULTS

Laboratory Number	Sample	Batch	Analysis	Meth No	Expected Level	UOM	Result
090398	Pharmacy	VT090219	Florfenicol 30mg/ml	HPLC	29.40 - 30.60	mg/ml	30.60

Estimated uncertainty of meaurement	2 %
Spesific test conditions	None

Responsible Analyst | Laboratory Manager

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ANALYTICAL VALIDATION REPORT

THE DETERMINATION OF FLORFENICOL IN CHICKEN LUNG TISSUES AND EGGS BY

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

(HPLC)

Analyst **: Ms MSG Mulders**

Senior Technical Assistant: Pharmaceutical Analytical Laboratory

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Introduction

The objective of this study was to determine the effect of florfenicol on egg hatchability in fowls and validate a method for determination of florfenicol in chicken eggs and lung tissue. The animal phase was conducted at the facilities of the Poultry Reference Laboratory (house B2) at Onderstepoort, Faculty of Veterinary Science to an approved protocol. Samples were transferred to the laboratory on ice within 1 hour of sample collection.

This study resulted in the need to analyse 75 eggs and 75 lung tissue and the required control samples that accompany every prepared batch. The collected samples were frozen at the analytical facility, and pooled until completion of the animal phase. This document contains the validation results obtained by the selected analytical procedure.

Materials and Methods

Reagents:

Filtered water-Milli-Q50

Ethyl acetate

Florfenicol

Thiamphenicol

Acetonitrile HPLC grade

Ethyl alcohol

Equipment:

Beckman autosampler Module 508

Beckman Programmable Solvent Module 126

Beckman Diode Array Detector Module 168

Beckman Karat 32 software package

IBM Pentium IV computer

Hewlett Packard colour laser 2600n printer

Socorex adjustable micro pipettes 50-200µl and 200-1000µl

Multitube Vortexer, Labtek Corporation, Christchurch, New Zealand supplied by Lenton, South Africa

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Zymark Turbovap LV Evaporator supplied by Microsep

Socorex dispenser, 5ml

Beckman Coulter Allegra-22X centrifuge Module 2161 Midispin R centrifuge

Consumables

Glass insert,250 µl, Separations,cat nr 053101.

Screw Caps, natural rubber, Separations cat nr 09150867.

HPLC vials 1.8 ml Separations cat nr 11090520.

Culture tubes, Merck NT Laboratory suppliers, Duran cat nr 23175145.

Suitable disposable tips.

Preparation of stock solution

Chemicals

All chemicals used were of analytical reagent grade except the acetonitrile, ethanol and ethyl acetate which was HPLC grade. Water was filtered and treated with Milli-Q50 system to obtain water suitable for HPLC. Acetonitrile (HPLC grade, PN 152516Q), Methanol (HPLC grade, PN 1.06007.2500). Ethyl acetate (HPLC grade, PN 1.00868.2500) were supplied by Merck South Africa. Ethanol (HPLC grade, PN 34870), Thiamphenicol (PN T0261) were supplied by Sigma Aldrish,South Africa.

Florfenicol was supplied by Dr Ehrenstorfer GmbH product number C13665000 Lot number 60801.

Calibration standards

Two sets of calibration standards were prepared as specified in (Table 1&2). Weigh of 1 g negative tissue sample (lung), add to 100 µl from each individual standard plus 100 µl of internal standard. The samples were subsequently extracted using the same method as described for the extraction of the test samples.

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Table 1: dilutions required for the higher range of standards.

ml of 100 µg/ml stock solution required	Final volume, ml	(standard, µg/ml)
0.25	10	2.5
0.5	10	5.0
1.0	10	10.0
C1/ 0.75	10	7.5

Table 2: Dilution required for the lower range of standards

ml of 100 µg/ml stock solution required	Final volume, ml	(standard, µg/ml)
0.1	10	0.1
0.25	10	0.25
0.5	10	0.50
1.0	10	1.0
C2/ 0.75	10	0.75

Extraction procedure for test samples:

Lungs: For the extraction of the analyte, 100 µl internal standard (Thiamphenicol) and 9 ml Ethyl acetate were added to 2 g lung tissue in a test tube, mixed well, centrifuged at 2000 *rcf* for 10 minutes. The resulting supernatant was decanted into a new tube and dried off for 30 minutes at 60º Celsius under a steady stream of nitrogen. The dried samples were subsequently reconstituted with 1.5 ml for mobile phase, mixed well and re-centrifuged at 2000 *rcf* for 10 minutes. The reconstituted samples were thereafter cleaned up using solid phase extraction.

Varian BondElut C18 cartridges (200mg, 3ml) were primed with 4 ml methanol, followed by 4 ml MilliQ50 water. After the sample was loaded, a second wash was performed using 2 ml MilliQ50 water and allowed to dry under vacuum for 5 minutes. The analytes were eluted adding 3 ml methanol, and a vacuum applied for at least 5 minutes thereafter. The eluates were dried under a stream of nitrogen for 30 minutes at 60º Celsius.

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The dried eluates were reconstituted in 500 µl mobile phase before 100 µl of each was injected onto the column.

Eggs: For the extraction of the analyte, 2 g of well mixed egg in a culture tube were added to 100µl of 10µg/ml Thiamphenicol as internal standard. 9 ml of ethyl acetate was subsequently added and vortex mixed for 5 minutes and centrifuged at 200 rpm for 15 minutes. The ethyl acetate was dried off under steam of nitrogen for 30 minutes at 60ºC. To the dried sample 2 ml MilliQ-50 water and 2 ml hexane was added and well mixed well prior to centrifugation at 200 rcf for 10 minutes. Varian BondElut C18 cartridges (200mg, 3ml) were primed with 4 ml methanol, followed by 4 ml MilliQ50 water. After the sample was loaded, a second wash was performed using 2 ml MilliQ50 water and allowed to dry under vacuum for 5 minutes. The analytes were eluted adding 3 ml methanol, and a vacuum applied for at least 5 minutes thereafter. The eluates were dried under a stream of nitrogen for 30 minutes at 60º Celsius. The dried eluates were reconstituted in 500 ul mobile phase before 100 µl of each was injected onto the column.

Chromatographic conditions:

Column: Luna 5µ C18 (2) 100A 150 x 4.6 mm separations code number 00F-4252-EO

Guard colum: Phenomenex security guard holder and guard cartridges, separation code no.KJO-4282 And cartridges AJO-4287.

Mobile phase: 30% acetonitrile in filtered water mixed thoroughly and filtered through a 45 µm Millipore filter and put in an ultra sonic bath to degas for 10 minutes.

Detection: Diode array detector, 223 nm

Inject volume: 100µl

Flow rate: 1.0 ml/min, isocratic

Run time: 10 minutes

LLQ: 0.1 µl/ml

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RESULTS AND DISCUSSION

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. The specificity of the method was determined using negative lung and egg tissue with various known concentrations of the analyte, and subjected to the same extraction procedure. In all cases, florfenicol and thiamphenicol produced clearly identifiable peaks, at a wavelength of 223 nm (Fig.1 to 14).

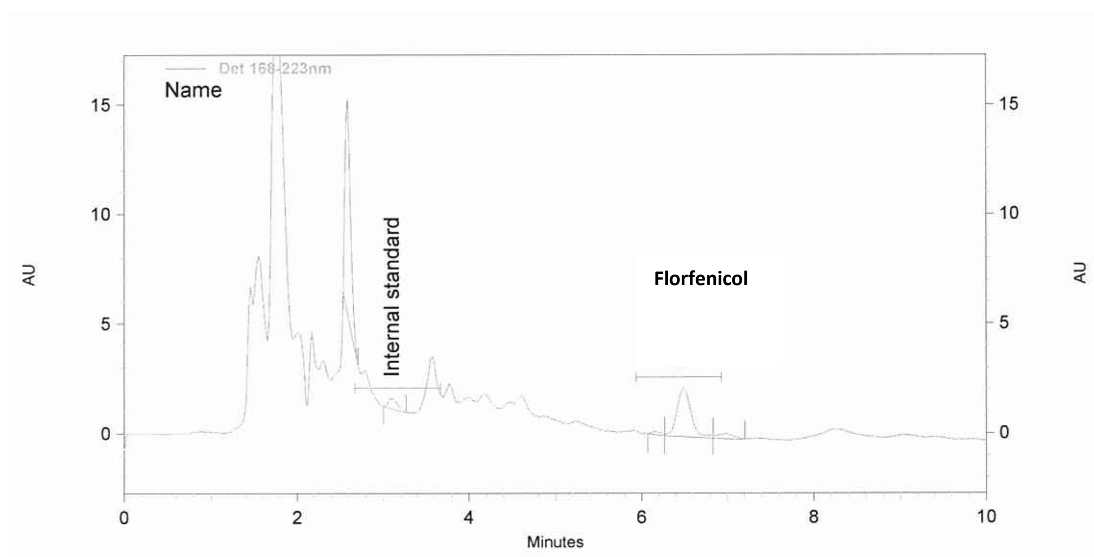

Figure 1: chromatogram for lung tissue at 0.1µg/ml.

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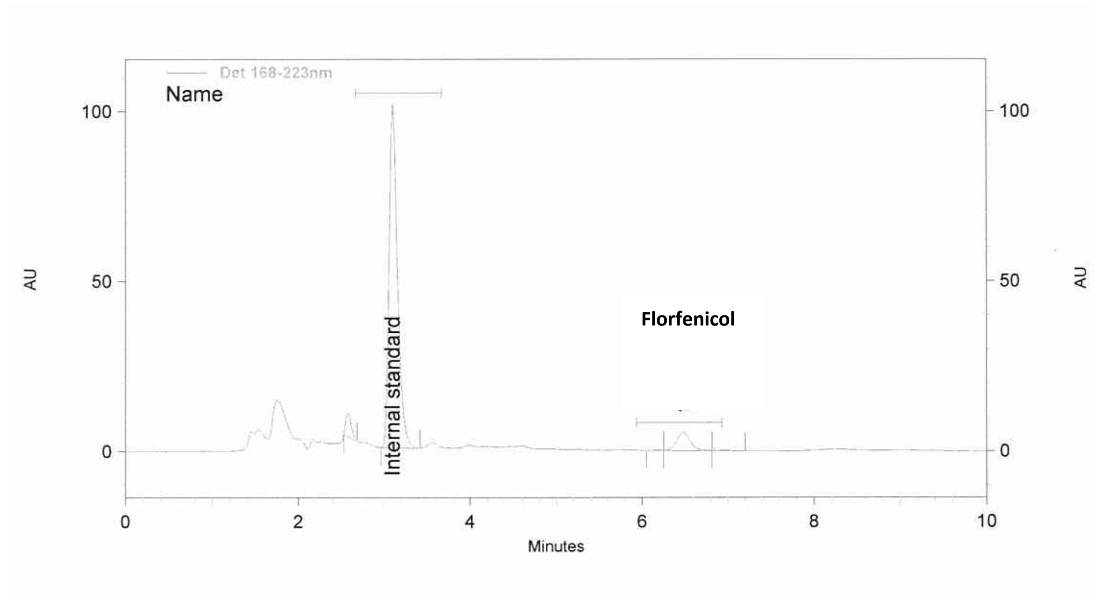

Figure 2: chromatogram for lung tissue at 0.25µg/ml

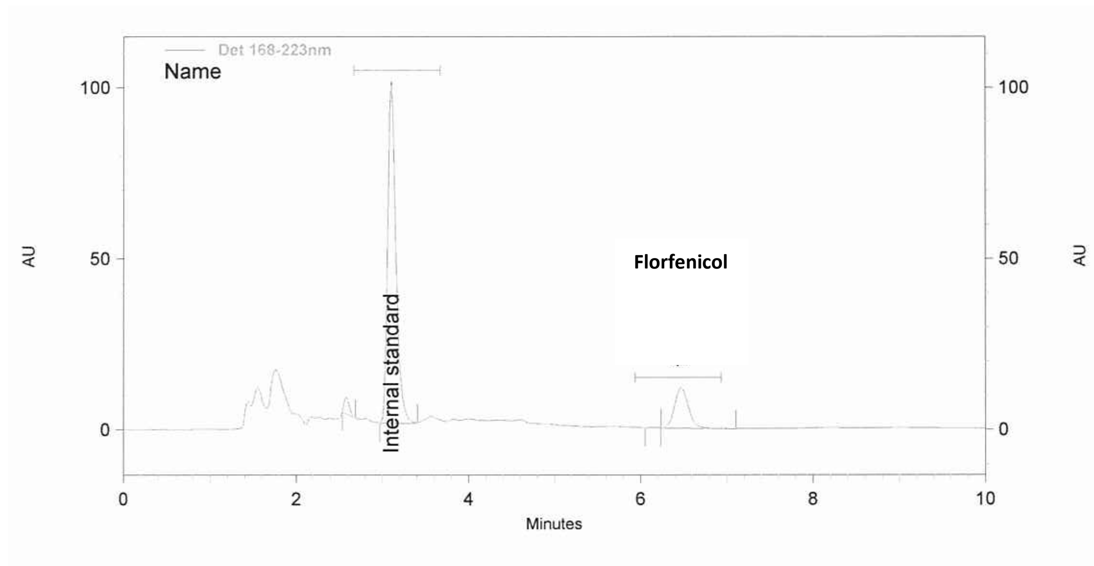

Figure 3: chromatogram for lung tissue at 0.5µg/ml

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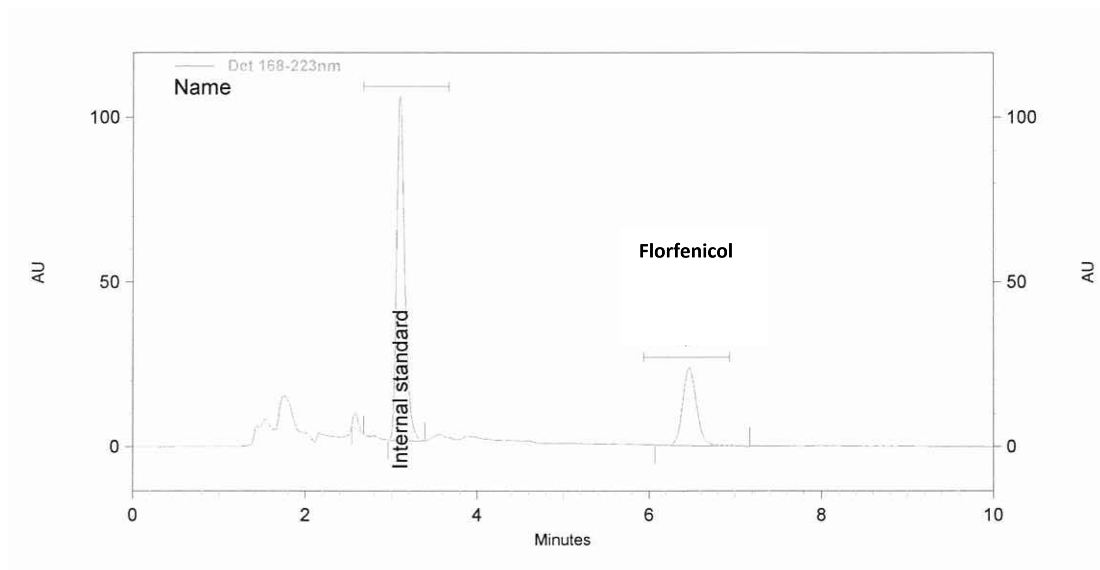

Figure 4: chromatogram for lung tissue at 1.0µg/ml

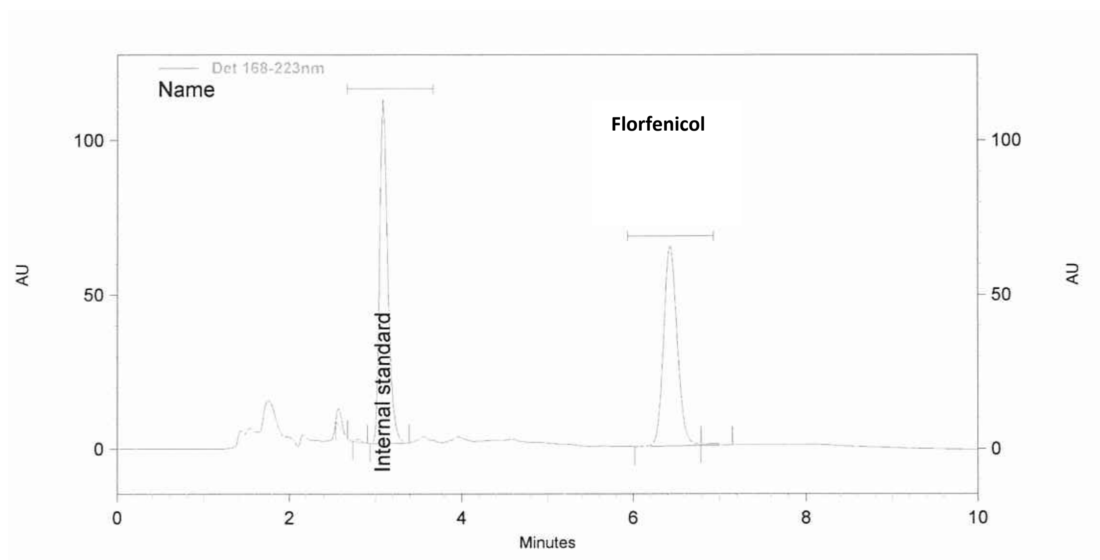

Figure 5: chromatogram for lung tissue at 2.5µg/ml

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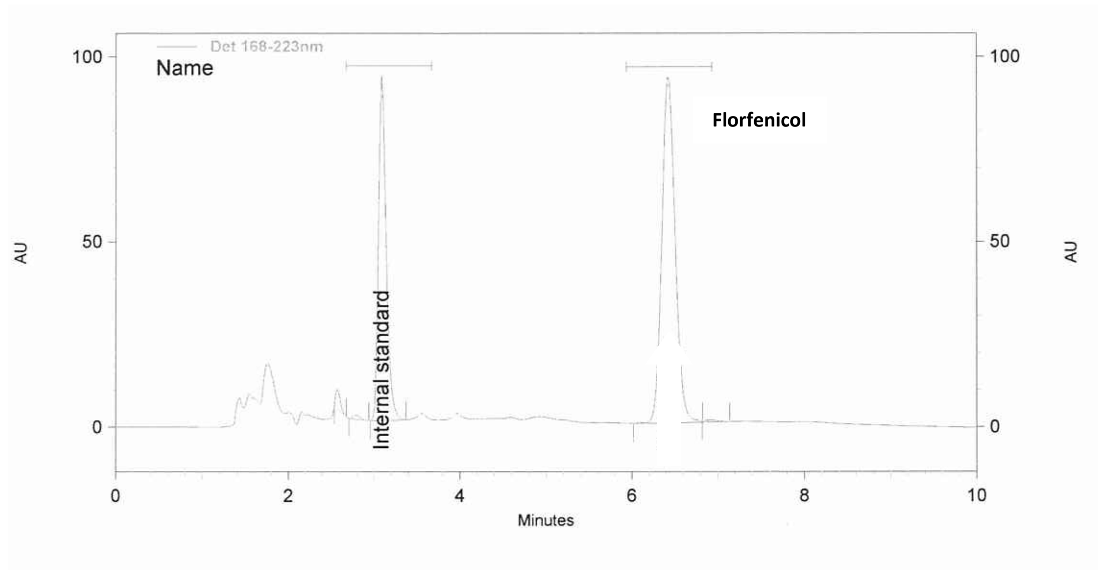

Figure 6: chromatogram for lung tissue at 5µg/ml.

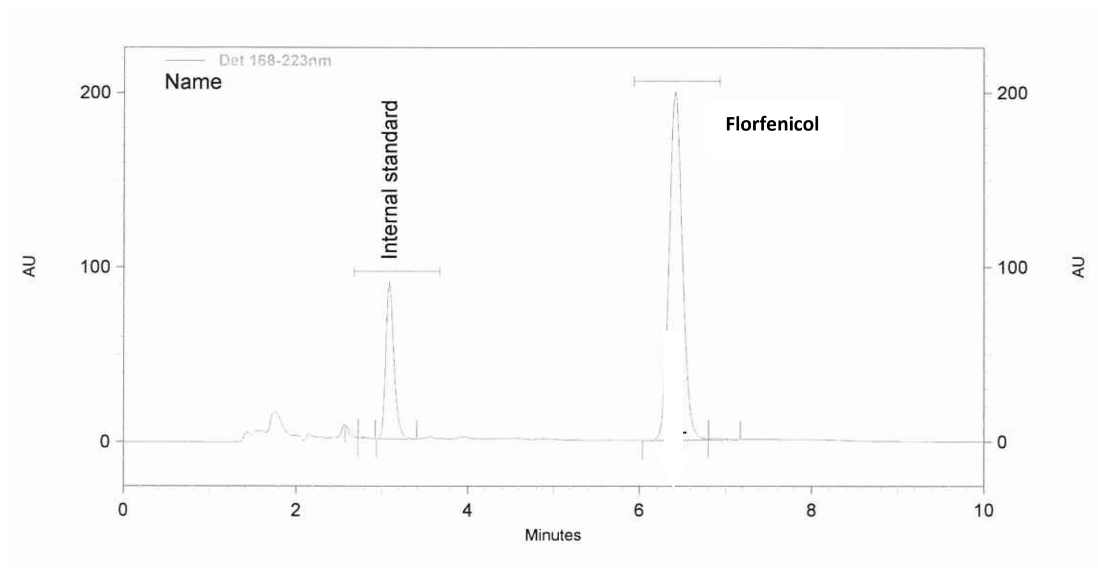

Figure 7: chromatogram for lung tissue at 10µg/ml.

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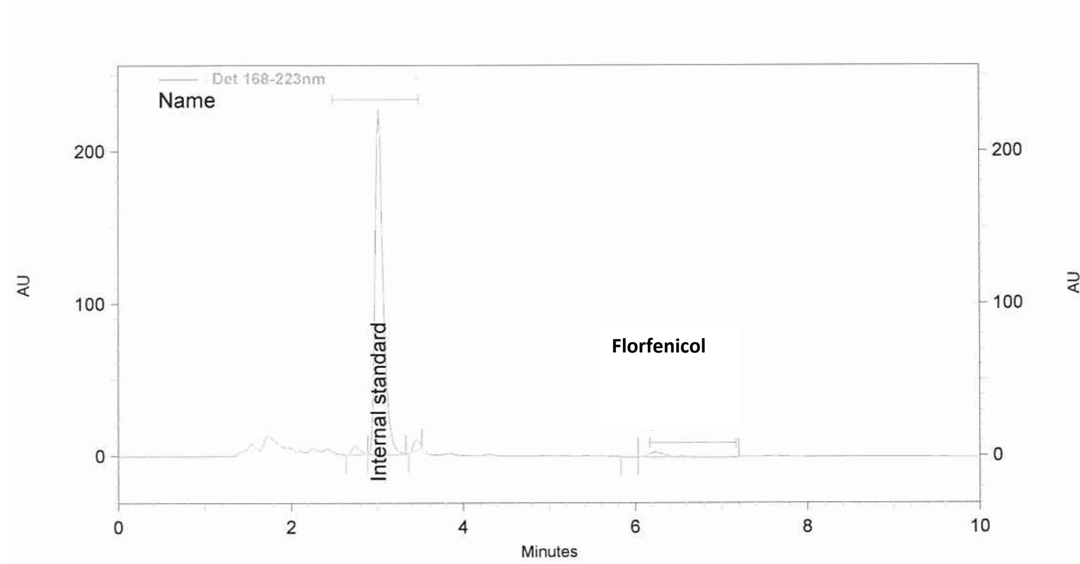

Figure 8: chromatogram for egg tissue at 0.1µg/ml

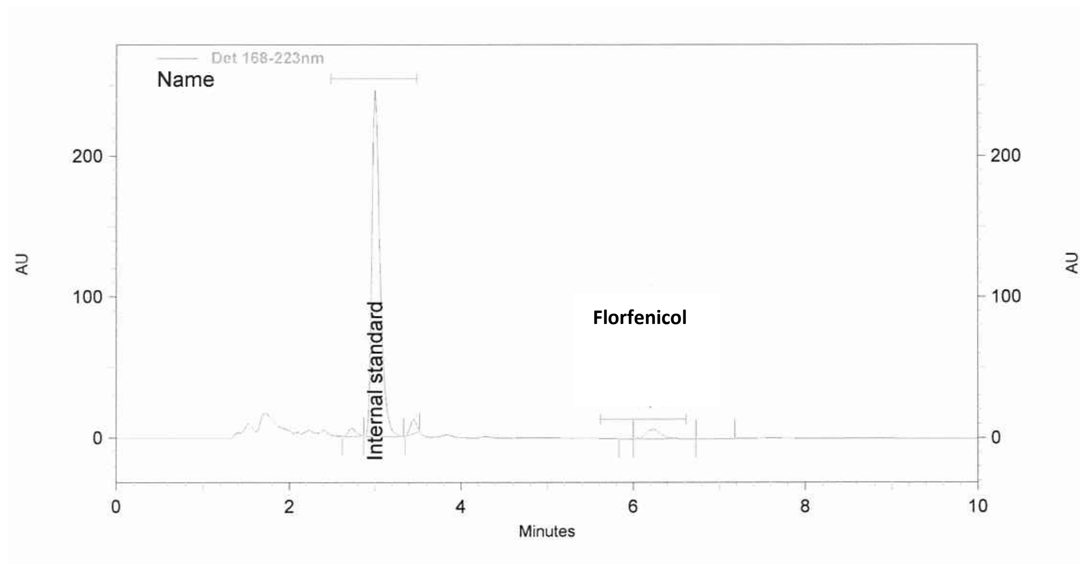

Figure 9: chromatogram for eggs tissue at 0.25µg/ml

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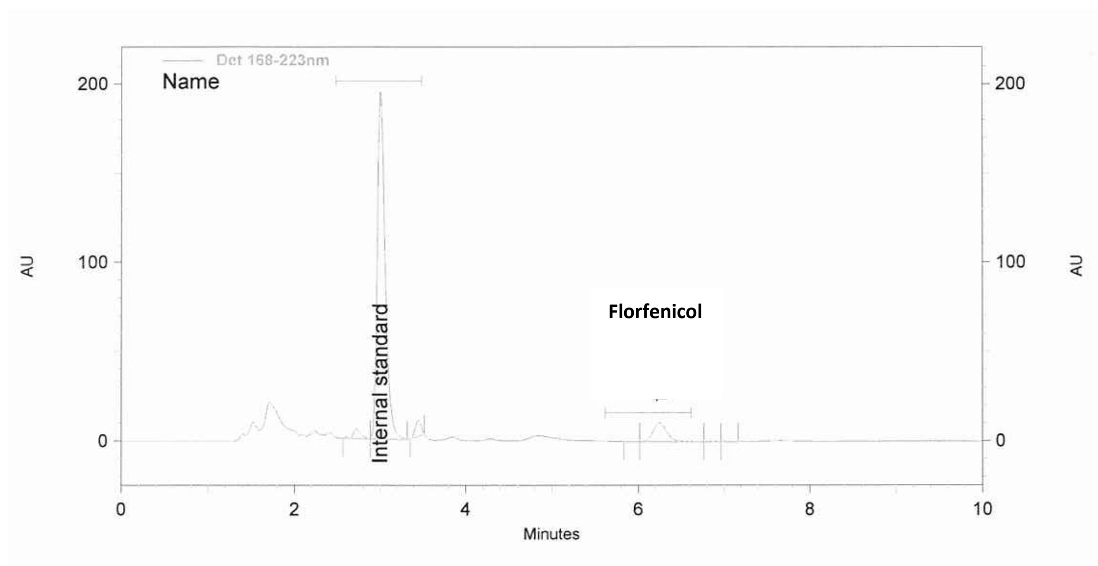

Figure 10: chromatogram for egg tissue at 0.5µg/ml.

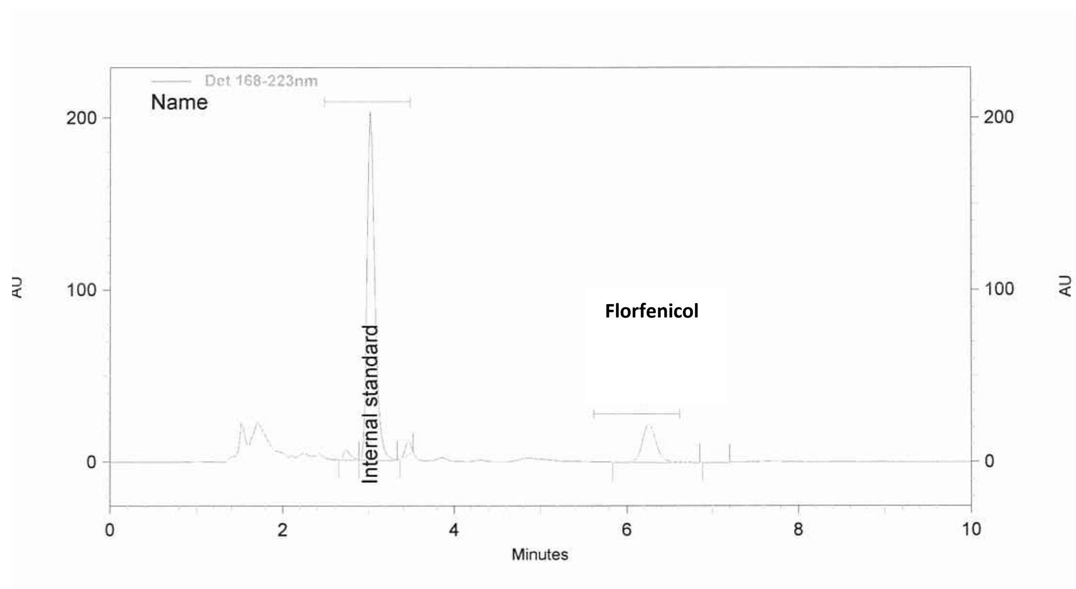

Figure 11: chromatogram for egg tissue at 1.0µg/ml.

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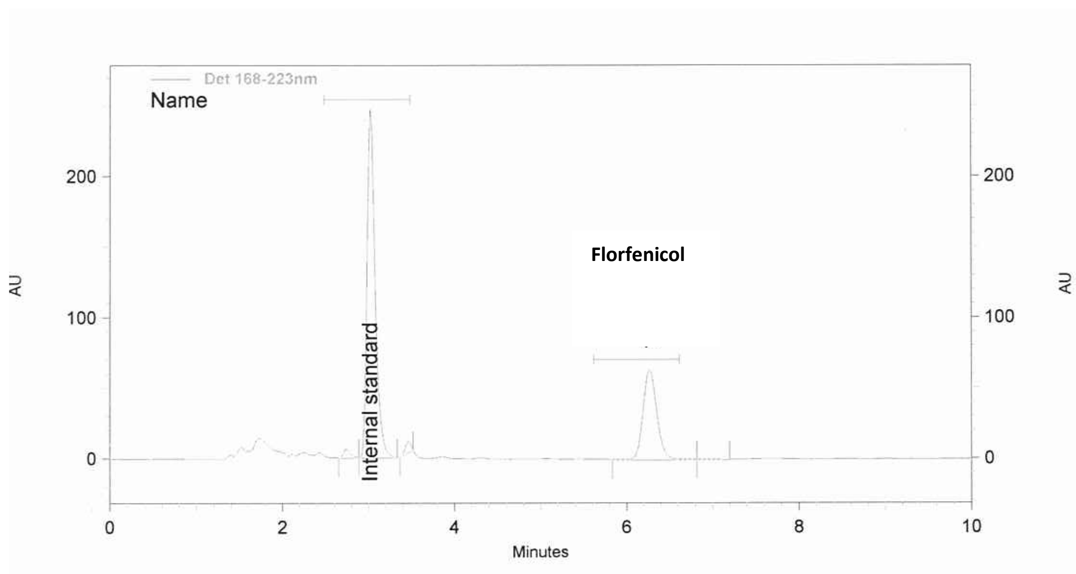

Figure 12: chromatogram for egg tissue at 2.5µg/ml.

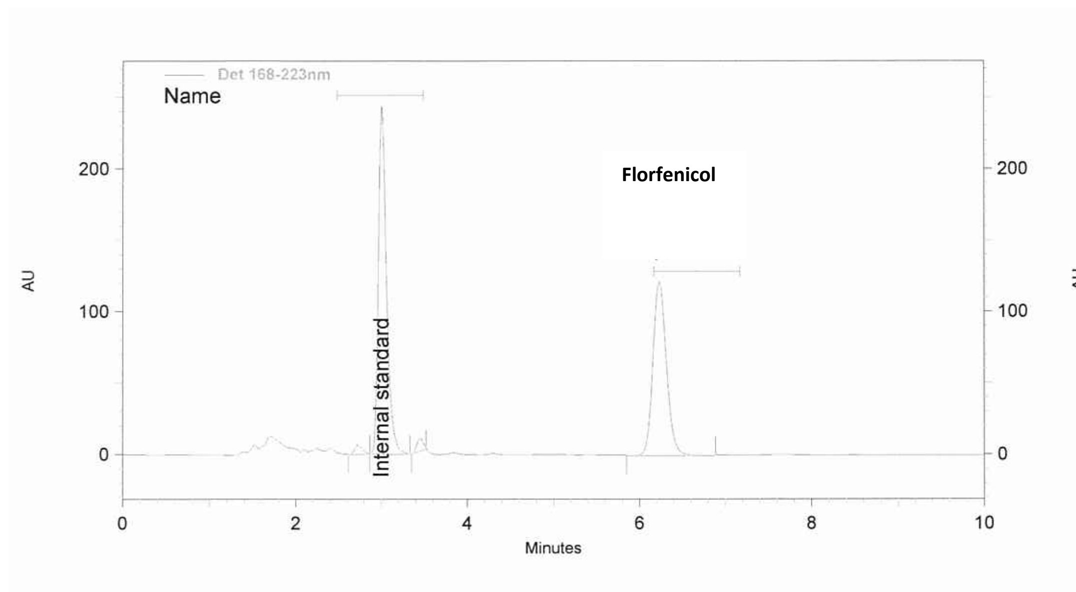

Figure 13: chromatogram for egg tissue at 5µg/ml.

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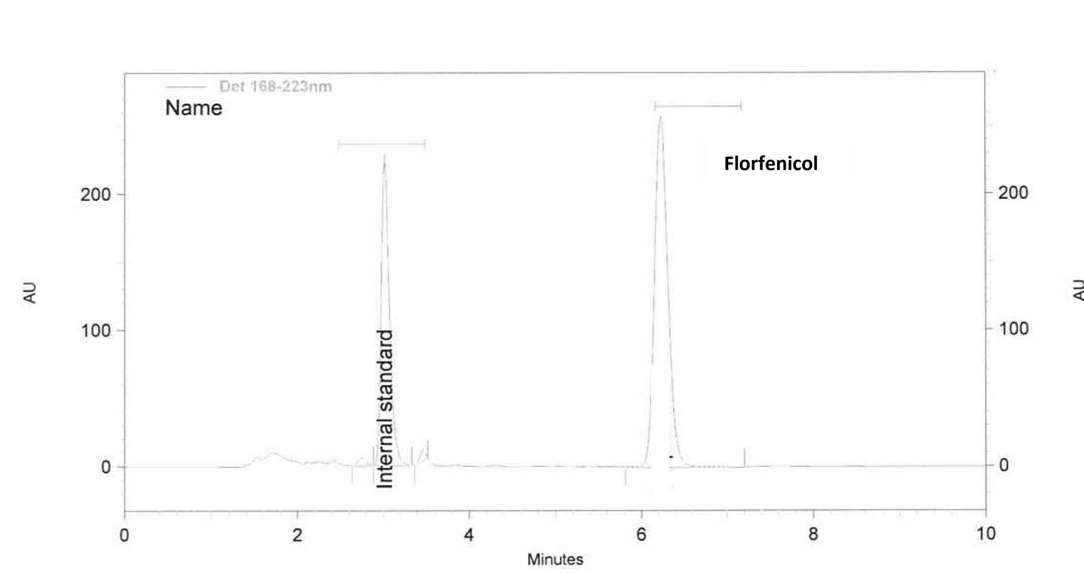

Figure 14: chromatogram for egg tissue at 10µg/ml.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as conventional true value or an accepted reference value and the found. The accuracy of the method was determined as the average of the calibration standards analyzed in the different runs, and compared to the expected concentration (Table 3 and 4). The accuracy for the lung tissue and eggs for the lowest concentration of 0.1 µg/g was 0.11 µg/g and 0.10 µg/g for the lung and eggs respectively. The accuracy for the lung and eggs tissue for the highest concentration 10µg/g was 10.18 µg/g and 10.58 µg/g for the lung and eggs respectively.

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Table 3: comparison of the measures concentration (µg/g) and expected concentration (µg/g), with a descriptive statistics for the lung tissue samples.

Date	0.1	0.25	0.5	1	2.5	5	10
20-Jul-09	0.17	0.17	0.31	0.29	0.81	1.93	4.64
21-Jul-09	0.06	0.13	0.24	0.29	0.96	1.94	4.74
27-Jul-09	0.04	0.09	0.20	0.38	0.98	1.75	3.76
28-Jul-09	0.04	0.10	0.19	0.41	1.21	1.76	3.71
Mean	0.08	0.12	0.23	0.34	0.99	1.85	4.21
GeoMean	0.06	0.12	0.23	0.34	0.98	1.84	4.18
SD	0.06	0.04	0.05	0.06	0.16	0.10	0.55
SE	0.03	0.02	0.03	0.03	0.08	0.05	0.28
%CV	82.98	29.73	22.61	18.76	16.58	5.65	13.16
95%	0.06	0.04	0.05	0.06	0.16	0.10	0.54
95%lower limit	0.01	0.09	0.18	0.28	0.83	1.74	3.67
95% upper limit	0.14	0.16	0.29	0.40	1.15	1.95	4.76

Table 4: comparison of the measures concentration (µg/g) and expected concentration (µg/g), with a descriptive statistics for the egg samples.

Date	0.1	0.25	0.5	1	2.5	5	10
18-Jun-09	0.02	0.08	0.09	0.18	0.53	0.99	1.75
3- August-09	0.02	0.04	0.09	0.19	0.43	0.82	1.87
4- August-09	0.02	0.05	0.08	0.17	0.39	0.69	2.05
5- August-09	0.02	0.04	0.07	0.18	0.42	0.63	2.44
6- August-09	0.03	0.05	0.12	0.23	0.50	0.85	2.72
Mean	0.02	0.05	0.09	0.19	0.45	0.80	2.17
GeoMean	0.02	0.05	0.09	0.19	0.45	0.79	2.14
SD	0.00	0.02	0.02	0.02	0.06	0.14	0.41
SE	0.00	0.01	0.01	0.01	0.03	0.06	0.18
%CV	20.45	29.39	22.07	11.65	13.00	17.62	18.79
95%	0.00	0.01	0.02	0.02	0.05	0.12	0.36
95% lower limit	0.02	0.04	0.07	0.17	0.40	0.67	1.81
95% upper limit	0.02	0.06	0.10	0.21	0.50	0.92	2.52

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Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts different equipment, etc. For intermediate precision, %CV and 95% confidence intervals for all runs undertaken in the study were ascertained (Table 3&4). The precision for the lowest concentration of 0.1µg/g was 9.64% and 17.50% for the lungs and eggs respectively while the highest concentration of f 10µg/g was 2.73% and 5.80% for the for the lung and eggs respectively.

Quantification limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The lower limit of quantification was determined to be less than 0.1 µg/ml.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. All standard curves used for this study showed a correlation co-efficient above 99%. Linearity was established from 0.1 to 10µg/ml (figure 15 to 23).

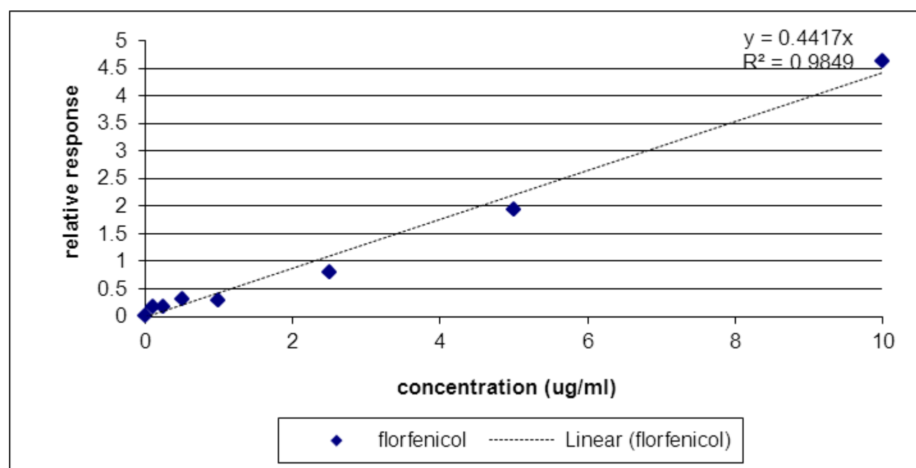

Figure 15: Standard curve obtained on the 20 July 2009 for lung tissue sample.

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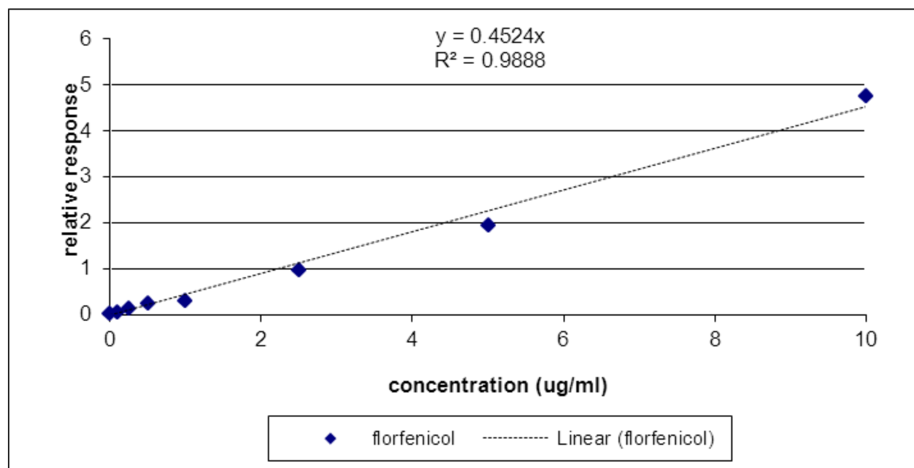

Figure 16: Standard curve obtained on the 21 July 2009 for lung tissue sample.

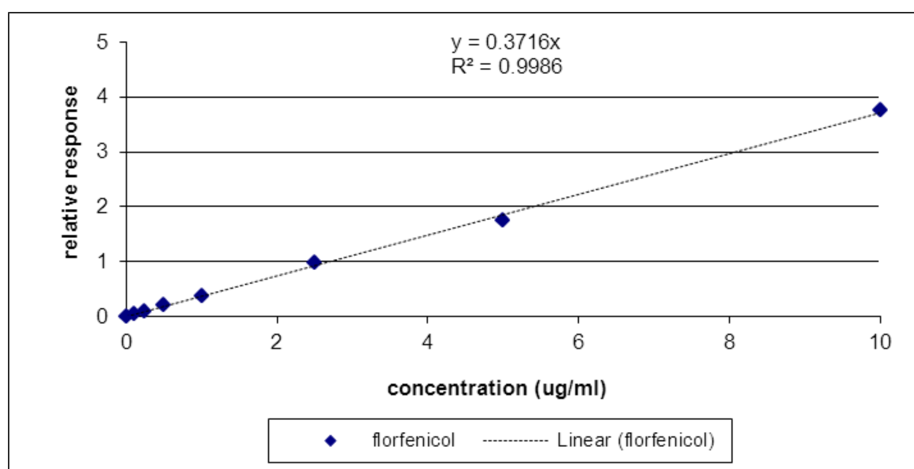

Figure 17: Standard curve obtained on the 27 July 2009 for lung tissue sample.

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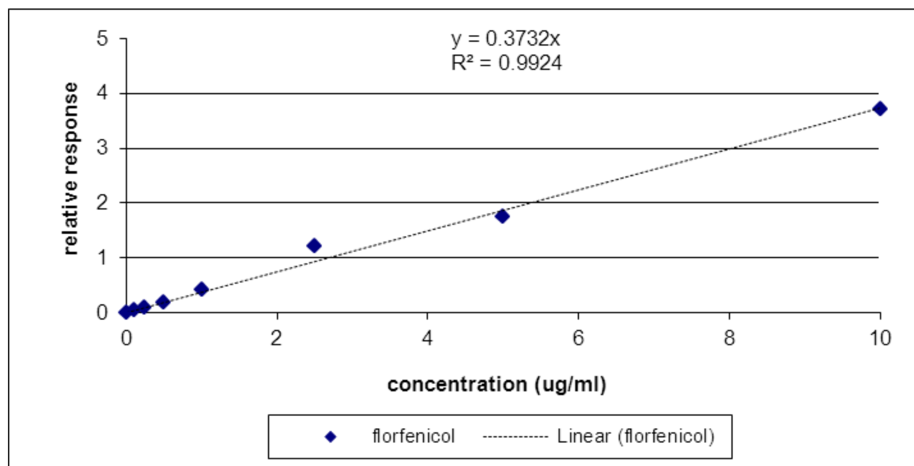

Figure 18: Standard curve obtained on the 28 July 2009 for lung tissue sample.

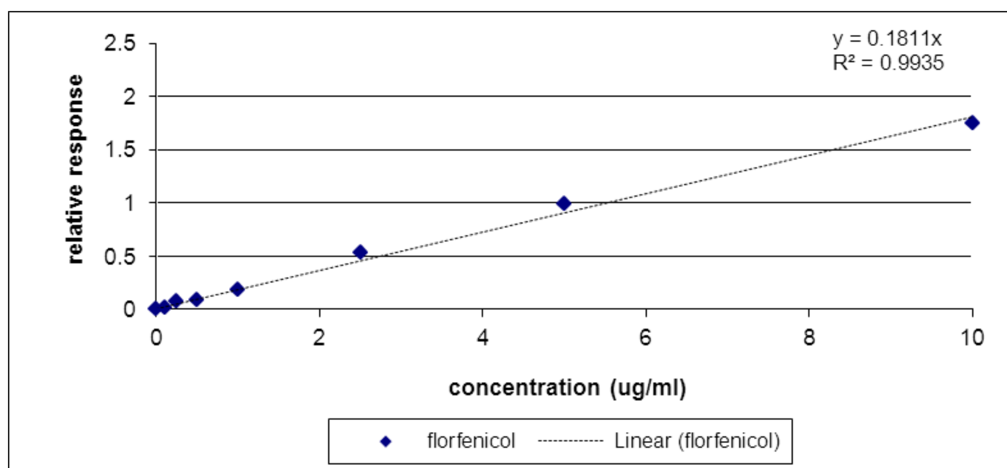

Figure 19: Standard curve obtained on the 18 June 2009 for eggs sample.

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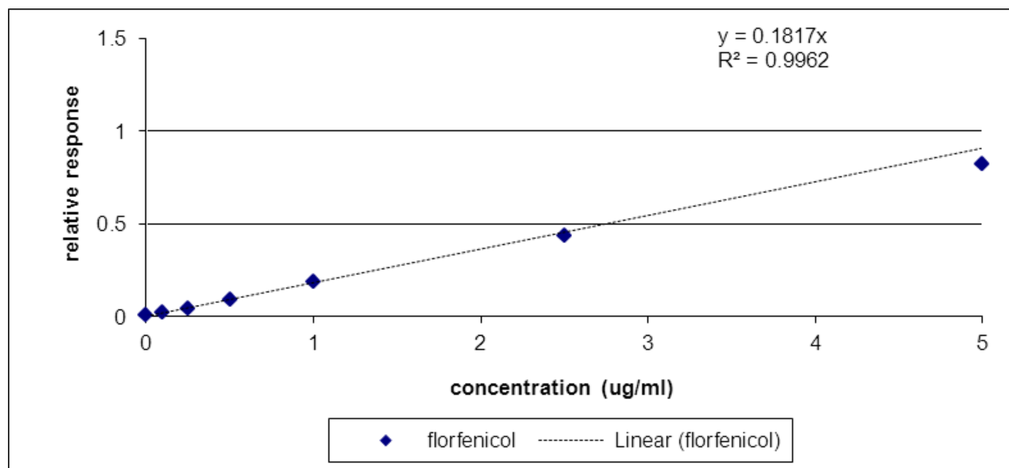

Figure 20: Standard curve obtained on the 3 August 2009 for eggs sample.

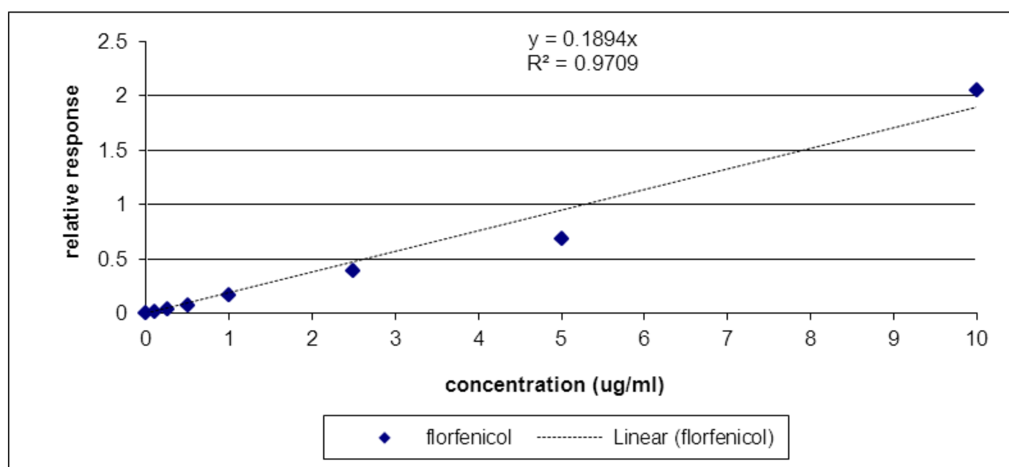

Figure 21: Standard curve obtained on the 4 August 2009 for eggs sample.

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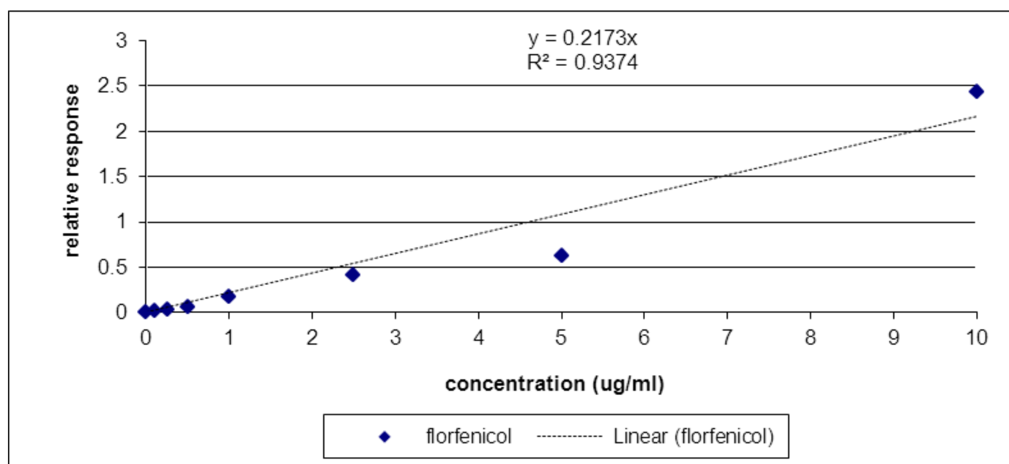

Figure 22: Standard curve obtained on the 5 August 2009 for eggs sample.

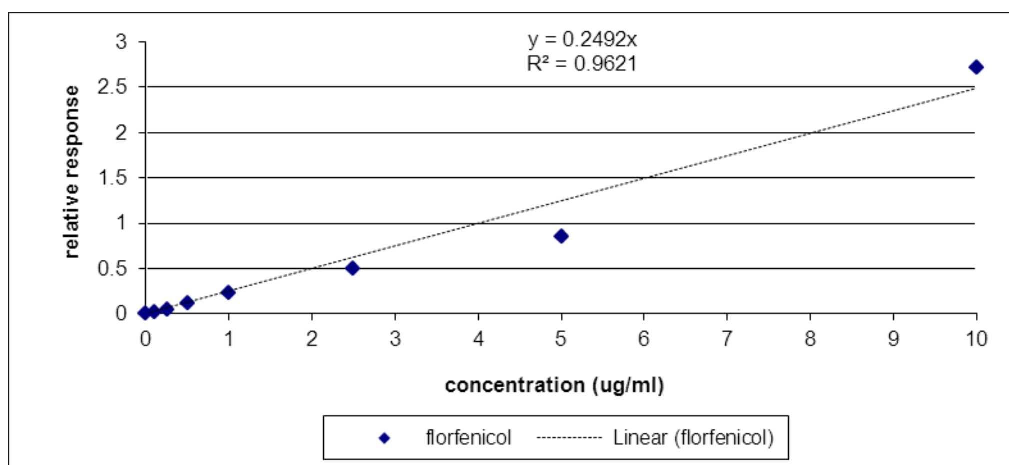

Figure 23: Standard curve obtained on the 6 August 2009 for eggs sample.

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Range

The range of an analytical procedure is the interval between the upper and lower concentration (amount) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The analytical standards ranged from 0.1µg/ml to 10µg/ml.

Recovery

The extraction efficiency of the method had a mean of 114% for the 0.1µg/ml concentration in the lung tissue samples and 97% for the eggs samples, and 102% for the high concentration in the lung tissues samples and 105% for the eggs samples (Table 5,6).

Table 5: Extraction efficiency for the various standards for the individual runs for lung tissue samples.

Date	0.1	0.25	0.5	1	2.5	5	10
21-Jul-09	126.85	119.36	105.03	63.51	85.16	85.73	104.85
27-Jul-09	107.86	94.18	107.92	101.21	105.46	94.09	101.12
28-Jul-09	107.71	106.70	103.00	110.26	129.52	94.47	99.42
Mean	114.14	106.74	105.32	91.66	106.72	91.43	101.79
GeoMean	113.80	106.25	105.30	89.16	105.17	91.34	101.77
SD	11.01	12.59	2.47	24.80	22.21	4.94	2.78
SE	5.50	6.29	1.24	12.40	11.10	2.47	1.39
%CV	9.64	11.79	2.35	27.05	20.81	5.41	2.73
95%	10.79	12.34	2.42	24.30	21.76	4.84	2.72
95%lower limit	103.35	94.41	102.90	67.36	84.95	86.58	99.07
95% upper limit	124.92	119.08	107.74	115.96	128.48	96.27	104.51

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Table 6: Extraction efficiency for the various standards for the individual runs for eggs sample.

Date	**0.1**	**0.25**	**0.5**	**1**	**2.5**	**5**	**10**
18-Jun-09	110.32	171.16	95.89	100.60	117.30	108.93	96.62
3- August-09	109.74	97.39	99.15	103.81	95.50	90.18	102.72
4- August-09	91.00	97.34	80.36	87.94	81.94	72.78	108.09
5- August-09	69.79	72.88	61.60	82.98	76.43	58.00	112.05
6- August-09	103.78	78.01	94.84	90.38	79.50	68.45	109.29
Mean	96.93	103.36	86.37	93.14	90.13	79.67	105.75
GeoMean	95.59	98.40	85.10	92.82	88.98	77.73	105.61
SD	17.05	39.50	15.61	8.77	16.84	20.06	6.13
SE	7.62	17.66	6.98	3.92	7.53	8.97	2.74
%CV	17.59	38.21	18.08	9.41	18.69	25.18	5.80
95%	14.94	34.62	13.69	7.69	14.76	17.58	5.37
95% lower limit	81.99	68.74	72.68	85.46	75.37	62.08	100.38
95% upper limit	111.87	137.98	100.06	100.83	104.90	97.25	111.13

Conclusion

The method selected is therefore considered specific, accurate and precise for the florfenicol analyte.

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