ISOLATION OF BACTERIAL PATHOGENS FROM DEAD-IN-SHELL CHICKEN EMBRYOS FROM LOCAL HATCHERIES

By

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Declaration

I declare that the work presented in this dissertation was done by myself and has not previously been presented to this or any other University.

Name. **Date.**

Dr. Swithine Hameenda Kabilika **.** ***22nd May, 1996.***

Dedication

I dedicate this work to my wife ***Harriet Muchimba Kabilika*** *for her understanding, support and love, to my daughter* ***Buumba Kabilika*** *for the joy she brought in the family and to my late parents Mr and Mrs* ***Kabilika*** *for their desire and dedication to see me attain higher education.*

APPROVAL

This dissertation of Dr. H. S. Kabilika is approved as fulfilling part of the requirements for the award of the degree of Master of Veterinary Medicine by the University of Zambia

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Abstract

Commercial hatcheries in Zambia have been experiencing losses due to dead-in-shell embryos for quite sometime. Numerous *Salmonella* serotypes have been frequently recorded in poultry population in Zambia, but no information is available on bacterial association with dead-in-shell mortality in hatcheries. Hence investigation into three commercial hatcheries of Zambia was made to find out the role of bacterial pathogens in embryonic mortality and hatchability. These hatcheries had an average dead -in-shell losses between 15.2% and 31.1%

One thousand dead-in-shell chicken embryos from each hatchery formed samples. Ten pooled yolk sac contents or whole egg contents formed one sample. The homogenised samples were cultured on suitable media. Isolation and identification of bacteria was done according to standard bacteriological methods.

Three hundred and eighty three (383) cultures were isolated. More than half (208) were isolated from hatchery A, the other two hatcheries B and C had 118 and 57 cultures isolated respectively. Majority of the isolates were enteric group of bacteria. Out of all the isolates *Escherichia coli* was predominant with 18.28% (70) followed by *Staphylococcus* species 14.10% (54) and *Pseudomonas* species 11.75% (45). *Klebsiella* species recorded 9.40% (36). *Salmonella* species, *Enterobacter* and *Citrobacter* species recorded 8.87% (34) each. *Acinetobacter* recorded 6.79% (26), *Proteus* species had 6.26% (24), *Enterococcus* species recorded 2.87% (11) and *Streptococcus* sp. had 1.04% (4). *Alcaligenes faecalis* and *Aeromonas hydrophila* both recorded 0.78% (3). *Neisseria dentrificans* recorded 0.52% (2) and *Edwardsiella* species., *Micrococcus*

and *Providencia* recorded 0.26% (1) each. Out of the fifty four (54) *Staphylococcus* species, forty three (43)were *Staphylococcus aureus*.

Serotyping of *Escherichia coli* revealed that six (6) cultures were serotype 08:k47, five (5) were serotype 09:k28, two (2) belonged to serotype 032/33:k- and one (1) each were serotype 088:k-, 016:k- and 0101:k-. Out of seventy cultures of *Escherichia coli* isolated, fifty four (54) were rough untypable. All these cultures were found to be less or more pathogenic to thirteen (13) day old chicken embryos and white mice.

It was also interesting to note that all of the thirty four (34) *Salmonella* cultures isolated were *Salmonella enteritidis*. However on phage typing twenty (20) belonged to phage type 4 (PT4), four (4) were phage type 1 (PT1), two (2) were phage type 1b (PT1b) and another two (2) were phage type 7(PT7). One was phage type 7a (PT7a) and five were not typed. The isolation of *Salmonella enteritidis*, especially most invasive phage type 4, in breeding flocks for the first time in Zambia may open a new page in the epidemiology of *Salmonella* and a need for effective control measures in this country.

A few cultures of *Escherichia coli, Salmonella enteritidis* and *Staphylococcus aureus* were subjected to antimicrobial sensitivity. The results of the antimicrobial sensitivity test showed high levels of antimicrobial resistance. Antimicrobial agents used in the test were Penicillin G, Tetracycline, Gentamycin, Sulphamethoxazole, Furazolidone and Streptomycin. The results showed that *Escherichia coli* serotypes were resistant to most Antibiotics (52.7%). Most cultures were resistant to Penicillin G followed by Streptomycin and Furazolidone. Antibiotics in Zambia have been used in feeds (especially nitrofurans) as a prophylactic measure to diseases like *Salmonellosis* and to boost production. The enforcement of legislature regulating

the use of antibiotics hasn't been effectively done. This may partly explain the high levels of antimicrobial resistance observed in this limited study.

The presence of these highly pathogenic bacteria in dead-in-shell associates them with production losses and are an indication of hatchery hygiene. The significance of these isolates in causing embryonic mortality and lower hatchability is discussed. There was significant correlation between dead-in-shell and hatchability. However direct relationship between bacterial prevalence or contamination and hatchability could not easily be shown. The probable reasons are discussed in this present dissertation.

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CHARTER ONE

1.0.0. Introduction.

Bacterial pathogens which find their way into eggs by different means are one of the main factors which cause losses in poultry industries at different levels of poultry production. Losses may be at hatchery level through lowered hatchability due to spoilage of incubating eggs and death of embryos which these bacteria may cause. Orajaka and Mohan (1985) reported 14% dead-in-shell chicken embryos from which they isolated different species of bacteria in Nigeria. Losses may continue through diseases in day old chicks caused by some of the egg contaminating bacteria. Hall et al. (1949) from one outbreak reported a 33.4% losses due to mortality from Fowl Typhoid in every 4th hatch from twenty five (25) hatches from Typhoid reactors in their second year of laying. The same authors reported losses in two lots of chicks hatched from Typhoid reactors as having reached 92.8% within sixteen (16) days in one lot and 93.5% within eleven (11) days in the other lot. Egg transmission was implicated.

Contamination and penetration of eggs by bacteria take place in many ways (Zander 1984):

1. Some bacteria like non-motile *Salmonella* species and *Mycoplasma* species either get included in the eggs prior to addition of shell or through the yolk from infected ovaries.
2. Motile bacteria like *Escherichia coli* and motile *Salmonella* species can penetrate the egg through natural pores of the egg shell.
3. Other bacteria find their way into eggs at the time of laying due to pressure

differential caused by temperature difference between newly laid egg and the environment it comes into contact. This pressure difference causes bacteria on egg shell and pores to be drawn inside the egg. In this way even non-motile bacteria, fungi etc. are able to penetrate. Clark and Bueschkens (1985) managed to introduce *Campylobacter jejuni* in the eggs using temperature or pressure differential.

Once these bacteria have found their way inside the egg they may cause egg spoilage and lowered hatchability (Graham and Michael 1936) or embryonic mortality (Goren 1978, Seuna 1979) which has an ultimate effect on hatchability. Limited research (Bruce and Johnson 1978, Orajaka and Mohan 1985, Lalithakunjamma and Sudharma 1991, Nazer and Safari 1994) has been done to identify the bacterial pathogens responsible for egg spoilage and embryonic mortality in hatcheries. Bacteria that have mainly been isolated are: *Salmonella* species (Barbour and Nabbut 1982), *Escherichia coli* (Falade 1977, Orajaka and Mohan 1985, 1986), and *Staphylococcus* species, *Streptococcus* species, *Micrococcus* species (Bruce and Johnson 1978). In recent years, concerted efforts were made in several countries to identify more bacterial pathogens responsible for embryonic mortality in hatcheries.

In Zambia since the early eighties poultry industry has been on the increase with small scale farmers coming up. Due to economic hardships faced by many families today, another poultry keeping system, ''Backyard system'' has been adopted by many house holds more especially urban and periurban house holds. This has further increased the demand for day old chicks. Four hatcheries have been trying to meet the ever increasing demand for the expanding poultry industry. However, from time to time, because of embryonic mortality which ranged between 15.2 and 31.1% according to hatchery records in Zambia, the hatcheries are unable to fulfil the supply of day old chicks to poultry farmers. As such many commercial farmers have resorted to importing day old chicks from neighbouring countries.

Salmonellosis, a major bacterial disease known for high embryonic mortality, has existed in breeding farms and hatcheries in Zambia for quite sometime (Anon 1976-1992, Sharma et al. 1991). Control measures instituted against Salmonellosis, however gave a considerable success from time to time but the problem has continued with higher magnitude in the past two years.

The isolation of bacterial pathogens from poultry in Zambia has been reported from fowls either from clinical cases or necropsy samples (Gasper and Hrabeta 1978, Falade et al. 1989, Sharma et al. 1991). No information is available on the isolation of bacterial pathogens from embryonated eggs and dead-in-shell chicken embryos in Zambia. The only reference on isolation of bacteria from eggs is that of Chima (1991). However, this work was confined to isolation of bacterial and fungal pathogens from table purpose eggs, collected from a few farms and shelves of retail shops.

Thus, considering the losses in Zambian hatcheries through lowered hatchability, a timely probe into various facets of this problem is needed. A systematic study of the role of bacterial pathogens connected to hatchery losses, as envisaged in the proposed study, may help as a useful index in developing hygienic hatcheries in the country. The results might also assist poultry farmers to control early chick mortality caused by bacterial pathogens carried through the eggs.

CHAPTER TWO

2.0.0 Literature Review.

2.0.1 *General*

Embryonic mortality accounting for a considerable percentage in lowered hatchability in hatcheries have been associated with bacterial infection specific and non specific (Harry 1957, Orajaka and Mohan 1985). An assessment of the incidence and type of bacteria contaminants occurring in eggs produced and hatched in commercial hatcheries is essential for understanding the role that micro-organisms play in influencing hatchability (Bruce and Johnson 1978). Pathogenic bacteria known to induce embryonic mortality are *Salmonella* species (Barbour and Nabbut 1982, Barbour et al. 1984), *Escherichia coli* (Falade 1977, Orajaka and Mohan 1985, 1986), *Mycoplasma* and *Staphylococcus aureus* (Harry 1957, Orajaka and Mohan 1985). Bruce and Johnson (1978) in an assessment on the contamination of eggs which had failed to hatch from commercial hatcheries, reported an average of 12.7% contamination. The contaminating flora were *Streptococcus* species and *Staphylococcus* species. The flora composed predominantly of *Micrococcus* species and *Enterobacteriaceae* with others at lower levels. This picture was also observed by Seviour et al. (1972) in incubated eggs of water fowl. Orajaka and Mohan (1985) isolated *Escherichia coli* (23 isolates), *Staphylococcus aureus* (25 isolates), *Micrococcus* species.(15 isolates), *Klebsiella* species (13 isolates), *Pseudomonas* species (9 isolates) *and Proteus* species (7 isolates) from 632 dead-in-shell chicken embryos in Nigeria. They suggested that the isolates may have contributed to the embryonic mortality and reduced hatchability in the farms investigated.

Barbour and Nabbut (1982) while examining the prevalence of *Salmonella* species on two breading farms recovered 2.87% *Salmonella* species from dead-in-shell embryos.

In Zambia the only work is on table eggs done by Chima (1991). He showed the presence of *Staphylococcus, Micrococcus, Pseudomonas* bacteria and *Mucor, Penicillium, Rhizopus* and *Tricophyton* fungi on the egg shells. He reported less microbial contamination on eggs at the farm compared to the eggs examined from the shelves of the shops. Much of the published work on microbiology of eggs is concerned with table eggs (Board 1966, 1969). However, some of the observations on table eggs may be applicable to hatching eggs while others may not (Bruce and Johnson 1978).

2.0.2. *Contamination of Eggs by Bacteria*

The concept that an egg is free from micro-organisms at the time of laying has remained a vexed question (Board 1966). However, the difficulties encountered in ascertaining this state, has led to the generalisation that circa 90% of newly laid eggs are free from bacteria (Brooks and Taylor, 1955). The exact value may be higher (Board 1966). However, Miller and Crawford (1953) found 170 eggs negative for bacterial contamination out of 200 eggs examined.

The eggs may become contaminated by different micro-organisms due to unfavourable conditions they may be subjected to. The sources of these micro-organisms are contaminated environment and laying hens.

1. **Contaminated environment**

When the egg is laid it may come into contact with contaminated surroundings, usually litter of breeder houses, nests etc. which may be contaminated in turn with faecal material

(Jordan 1956), dust, contaminated feed etc. from sick birds or apparently healthy birds which are carriers. Environmental contamination can also be through other sources like wild mice and wild birds. Barbour and Nabbut (1982) and Davies and Wray (1995) while investigating the problem of *Salmonella* in breeder farms in which *Salmonella* had been a persistent problem in Saudi Arabia and Britain respectively found wild mice and their faecal material positive for *Salmonella*. Different workers have isolated different types of bacteria from both the environment and eggs and later related the bacterial isolates to diseases in farms and hatcheries. Harry (1964) investigating on the survival of coliforms in broiler houses dust obtained $2x10^5$ to $8x10^6$ counts of coliforms per gram of dust. Harry and Hemsley (1965) isolated pathogenic *Escherichia coli* serotypes from feed in tube feeders, air, dust and litter. Some of these serotypes were also isolated at the hatchery waste, egg contents, yolk sac of embryos and dead chicks and killed chicks' caeca. They correlated the isolated *Escherichia coli* serotype with Colisepticaemia in chickens. Bhatia et al. (1979) found 17.1% contamination of new straw litter with different *Salmonella* serotypes before the chicks arrived. The incidence of these serotypes increased in the litter at 5 days and 6 weeks. They used these results as an indicator for flock infection. Barbour and Nabbut (1982) isolated different species of *Salmonella* from litter (4%) and shells of hatching eggs (1.4%) from one farm. Dead breeders and dead-in-shell embryos were also positive in the following proportions, breeders 7.41%, in shell embryos 2.87% and egg contents of hatching eggs 1.47%. From these eggs *Staphylococcus,* Coliform *and Bacillus* species were also found to have contaminated and penetrated eggs in different proportions depending on the source of the eggs (clean nest, dirty floor). Soloven et al. (1946) reported that 2% of clean eggs and 16% of dirty eggs contained *Salmonella* on the shell and in pores.

Board (1966) in his review paper reviews that the bacterial contamination of the egg shell averages between 9.5-3,100 x 10^3/shell and that the contaminants on the egg shell are dominated by gram positive bacteria (Haines 1938, Zagaevsky and Lutikova 1944, Board et al.1964). The level of contamination depends on the cleanliness of the nest boxes (Smeltzer et al. 1979, Barbour et al. 1984) and handling of the eggs.

The survival of these bacteria in the environment varies. Tucker (1967) observed that *Salmonella pullorum* could survive from 3 weeks in old litter to 11 weeks in new dry litter. Botts et al. (1952) reported a rapid disappearance of *Salmonella pullorum* in a built up litter than new corncob dry litter. Moisture content played a major role on the survival period. A similar observation was also made by Harry (1964) on *Escherichia coli* in samples of dust kept at their original moisture content of 8.3-11.4%. *Escherichia coli* survived over 32 weeks the lethal effect of humidity (Relative humidity). Harry (1964) could also still isolate *Escherichia coli* at 29 weeks from dust collected from broiler houses subjected to different levels of humidity. Morse and Duncan (1974) found that *Salmonella* could survive for 28 months in natural infected faeces and 5 years in hatchery fluff stored at room temperature. The longer the bacteria survive in the environment the more exposed the eggs and indeed chickens are.

2. Laying Hens

Poultry could be the major source of bacterial flora affecting or contaminating eggs. Normal bacterial flora of the alimentary tract, skin and feathers of the hen contaminate eggshells in large numbers (Haines 1938). These bacteria can also penetrate eggs and infect embryos (Harry 1957). In the case of *Salmonella*, poultry itself is regarded as the largest reservoir of *Salmonella* organisms existing in nature (Snoeyenbos and William 1991). Carrier state in chickens which have gone through an infection systemic or enteric and even apparently normal chickens is the most dangerous state as these birds can continue shedding respective micro-organism through faeces and hence infect not only eggs but other birds as well. Carrier state is more common in Salmonella Infection than clinical disease (Borland 1975). This could be true with other micro-organism as well.

Eggs may get contaminated during laying as they pass through the reproductive system which is infected, or during egg formation, bacteria may get included in the egg contents. For this to happen the bird must have gone through systemic infection in which the ovary or the entire reproductive system gets infected. *Salmonella* species and *Mycoplasma* species are one of the known bacteria which often infect ovaries and reproductive system and are transmitted through eggs. (Gast and Beard 1990a, Barnhart et al. 1993).

About 0.5%- 6% eggs from normal hens contain *Escherichia coli* bacteria and experimentally infected hens produce about 26% eggs positive of *Escherichia coli* (Gross 1991).

2.0.3. *Bacterial penetration of eggs*

There seems to be no information on the exact mechanisms through which bacteria penetrate eggs. Much of the published work is based on the results of experiments in which eggs were immersed in a suspension of bacteria or smeared with contaminated faeces, litter etc. and egg spoilage, embryonic mortality and isolation of bacteria from the eggs and embryos are used as indicators of bacterial penetration (Board 1966, Barbour and Nabbut 1982, Stokes et al. 1956, Smeltzer et al. 1979). However Zander (1984) describes the following ways through which bacteria can penetrate eggs:

1. **Transovarian penetration**

Some bacteria like *Salmonella species* and *Mycoplasma* are able to infect the ovaries and oviducts and while the egg is being formed bacteria get included in the egg prior to addition of the shell. Barnhart et al. (1993) found about 15.8% of ovaries positive for *Salmonella species* from 19 flocks. Gast and Beard (1990b) isolated *Salmonella enteritidis* from the ovaries and oviducts (17% each) of experimentally infected birds and eggs from these birds were also positive for *Salmonella enteritidis*. Mcllroy et al. (1989) in Northern Ireland confirmed vertical transmission of *Salmonella enteritidis* in five broiler outbreaks aged between two (2) days and one (1) week as the ovaries and oviducts from parent flocks were positive and yolk sac and internal organs from hatchery chicks were also positive. Jiroj sasipreeyajan et al. (1987) isolated *Mycoplasma gallisepticum* from the oviduct of experimentally infected chicks and control groups. Eggs from these chickens were also positive of *Mycoplasma gallisepticum* indicative of vertical transmission.

2. **Penetration due to motility**

Motile bacteria like *Escherichia coli*, motile *Salmonella species* etc. can penetrate the egg through natural pores of the egg shell under different conditions. Mario Padron (1990b) demonstrated that *Salmonella typhimurium* was able to penetrate egg shells when he exposed the eggs to different conditions (spraying, moisture, contact). He further noted that water facilitated the penetration of the organisms, although not a condition.

The speed at which these bacteria penetrate eggs seems to be variable. In one research William et al. (1968) noted that penetration of eggs by *Salmonella typhimurium* occurs as early as six minutes after contamination.

3. Penetration due to temperature differential.

Temperature difference between the egg and the environment it comes into contact, can cause bacteria and other micro-organisms to be drawn inside the egg. This is most likely to happen at the time of laying when the newly laid egg has a completely different temperature with the outside. This temperature difference creates a pressure difference between the inside and the outside of the egg. So as pressure gets equalised the contaminating micro-organisms if present on the surface of the egg are able to penetrate. Clark and Bueschkens (1985) managed to introduce *Campylobacter jejuni* in the eggs using temperature or pressure differential. Stokes et al. (1956) had eggs infected with *Salmonella species* by immersing warm eggs in cold bacterial suspension.

2.0.4. *Factors influencing Bacterial penetration.*

Bacterial penetration of the eggs would be influenced by certain factors. The egg shell is coated with Mucin or Cuticular plug (Simkiss 1961) .This coat plays an important role in protecting the egg from microbial penetration. A high incidence of penetration (Gordon and Tucker 1954) and increased incidence of egg spoilage, when eggs are rubbed with sand paper or steel wool before placing them into a bacterial suspension is a clear indication of the cuticular's importance as a mechanical barrier (Board 1966).

Egg membranes contain bactericidal components that protect the egg content and developing embryos from microbial infection. The membranes allow only a limited microbial growth and Board (1969) attributed this to antimicrobial substances in the albumen. Korotkova (1957) detected lysozyme in shell membranes which play an important role in the defence of developing embryo. Ovotransferrin the principle bacteriostatic present in the albumen has its effect more on the Gram-positive than on the Gram-negative organisms. This may account for a low number of Gram-positive bacteria isolated from dead embryos and contents of spoilt eggs (Board 1966, Seviour and Board 1972). Shell membranes have also been considered as bacterial filters and mechanical barrier (Haines and Moran 1940, Miller and Crawford 1953). This was considered so through observations that there was a lag phase of up to 20 days between shell penetration and bacterial multiplication (Miller and Crawford 1953, Stokes et al. 1956). Further more, liquid sucked through the membrane from eggs in which egg contents had been replaced with bacterial suspension did not contain bacteria (Walden et al. 1956, Garibaldi and Stokes 1958). If these components of the egg that play a major role in the defence mechanisms of the egg are compromised, bacterial penetration occurs. Miller and Crawford (1953) reported penetration by bacteria in cracked thin eggs compared to none of thicker shelled eggs. They suggested shell membrane breaking as having facilitated bacterial penetration. These findings were also supported by the same authors when they further observed that, egg shell membranes when washed free of white in vitro still delayed multiplication of egg spoiling bacteria.

Temperature has also an effect in influencing bacterial penetration. Most important is the temperature difference between the newly laid egg and the environment it comes into contact. This temperature difference has been used by many workers (Stokes et al. 1956, Clark and Bueschkens 1985) to introduce bacteria into eggs. They immersed warm eggs in cold bacterial suspension. Lower temperature seems to disfavour some bacterial penetration. Stokes et al. (1956) could not isolate *Salmonella oranienburg* from eggs inoculated and incubated at 1^{0}C for 6 months, but gave positive results when incubated at 29^{0}C. The conclusion was that *Salmonella oranienburg* could not only penetrate but failed to survive at that low temperature. Humphrey and Whitehead (1993) showed that *Salmonella enteritidis* could not penetrate and grow rapidly in the yolk in majority of the eggs held at 20^{0}C for three weeks where as the growth occured rapidly in eggs held at fluctuating temperatures which reached occassionally 30^{0}C. However *Pseudomonas* species seems to do well at lower temperature, room temperature and below (Seviour and Board 1972).

Time of exposure to contaminating bacteria also increases the chances of bacterial penetration (Board 1966).

Deformed eggs are more vulnerable to bacterial penetration and eggs with thin shells offer less resistance to bacterial penetration (Orel 1959).

Dirty environment (contaminated poultry houses, nests etc.) also predisposes eggs to contamination and eventual penetration. Smeltzer et al. (1979) investigating bacterial penetration of eggs laid on floor and in nests had 15.3% penetration and 10.5% penetration respectively. Barbour and Nabbut (1982) also had a higher percentage of bacterial penetration in dirty floor eggs than clean nest eggs from two farms investigated.

Moisture/humidity though not a prime factor in influencing bacterial penetration, somehow facilitates the penetration of bacteria into eggs (Miller and Crawford 1953, Mario Padron 1990b).

2.0.5. *Effect of bacterial pathogens on eggs, embryos and chickens*

Egg contaminating bacteria have been associated with egg spoilage, embryonic mortality, lowered hatchability (Harry 1957, Goren 1978, Seuna 1979, Orajaka and Mohan 1985). The causative bacteria isolated from dead-in-shell embryos have been predominantly Gram-negative, majority being enteric bacteria (table 1). Barbour et al. (1985) in a research in which hatchery waste eggs were highly contaminated (69.1%), enteric bacteria were predominant 26.6%. Seviour and Board (1972) observed that the behaviour of contaminating bacteria differ once they have found their way in the egg.

A mixture of contaminating bacteria collected from egg shells which were predominantly Gram-positive when inoculated in eggs, the composition changed in favour of Gram-negative bacteria (Board 1964). Further more the composition of bacteria differ in eggs kept at different temperatures. At +4^{0}C *Pseudomonas* species dominated and at 22^0 C and 37^{0}C Coliforms were dominant. This observation was made also by Lahellec and Coli (1979) who noted that *Pseudomonas* increased by twofold with refrigeration of processed carcasses for 12 days while counts of *Acinetobacter* decreased by fivefold. This may explain why *Pseudomonas* species have been found in high proportions in table eggs stored at lower temperatures and coliforms are predominant in incubated eggs.

Elucidation of the role of micro-organisms in causing embryonic mortality and lower hatchability is quite difficult as embryonic mortality can be due to other factors other than bacterial presence (Harry 1957, Bruce and Johnson 1978). However the presence or isolation of known pathogenic bacteria must be viewed with caution.

TABLE 1: COMPARISON OF SOME OF THE BACTERIAL FLORA ISOLATED FROM CHICKEN, TURKEY AND WATERFOWL EMBRYOS.

	Percentage of isolates from			
Bacteria	Waterfowl[1]	Chicken[2]	Chicken[3]	Turkey[4]
Gram-positive				
Staphylococcus species.	8.5	27.18	9.2	7.7
Micrococcus species.	15	16.34	34.6	
Bacillus species.	3		1.2	3.9
Streptococcus species.			15.3	8.5
Others			2.3	
Gram-negatives				
Coliforms	64	25		
Proteus species.		7.61	2.5	
Klebsiella species.		14.13		
Enterobacter species			31.5	71.4
Pseudomonas species.	7	9.78	1.5	
Acinetobacter species.	2			
Others			2.8	

1. Seviour et al 1(972), **2**.Orajaka and Mohan (1985), **3**. Bruce and Johnson (1978), **4**. Bruce and Drysdale (1983).

Pseudomonas species

Bruce and Johnson (1978) showed significant correlation between *Pseudomonas* species and lowered hatchability. *Pseudomonas* species can invade fertile eggs causing death of embryos and hatched chicks and reduce shelf life of contaminated meat (Barnes 1991). *Pseudomonas* species have also been shown to have penetrated eggs and caused embryonic mortality after dipping eggs in contaminated antibiotic solution (Wages 1988). In conditions of high humidity *Pseudomonas* is capable of digesting egg shell cuticle (Board et al. 1979). In addition, *Pseudomonas aeruginosa* derived from dead-in-shell showed 88% mortality rate on chicken embryos infected experimentally (Shawabkeh and Tarazi 1993). *Pseudomonas* also affects egg albumen and according to Seviour and Board (1972) they grow well in eggs at lower temperature. This could be the reason among others that *Pseudomonas* is one of the major causative agent of egg spoilage as well as low hatchability.

Escherichia coli.

Escherichia coli infection is one of the common contaminants found in incubated eggs. Faecal contamination of eggs is the most common source of infection although ovarian infection can be a source of infection as well (Gross 1991). When *Escherichia coli* infects the embryos, it has the predilection of the yolk sac, and causes embryonic mortality and early chick mortality (Harry 1957, Pathak et al.1960, Gross 1991). In one research 245 *Escherichia coli* isolates were made from dead-in-shell embryos, out of which, 43 belonged to the pathogenic serotypes (Harry and Hemsley 1965). These serotypes were as well associated with embryonic mortality and colisepticaemia in hatched chicks. Mortality occurs late in incubation and many embryos die before hatching (Gross 1991). This observation was also made by Orajaka and Mohan (1985,86) when they isolated a higher proportion of *Escherichia coli* and *Staphylococcus aureus* from samples at the end of incubation than those taken at the eighteenth day of incubation.

After infection normal yolk sac contents change from viscid yellow green to watery, yellow brown or caseous material (Harry 1957, Gross 1991). In day old chicks *Escherichia coli* causes yolk sac retention and omphalitis (Harry 1957, Pathak et al. 1960), and the common causes of chick mortality up to 10 days has been reported to be omphalitis and yolk retention (Watts and Rac 1958, Rudy 1991). In a condition called 'Mushy chick disease'(omphalitis), *Escherichia coli* was the predominant isolate in large proportions (82%). However other bacteria were also isolated and they included *Streptococcus* 53%, *Micrococcus* 25%, *Bacillus* 18%, *Proteus* 16%, *Salmonella* 18% and *Aerobacter* 6% (Harry 1957).

Salmonella species.

Salmonella species are also known for embryonic mortality and early chick mortality (Barbour and Nabbut 1982). Losses in Salmonella infection begin at hatcheries and continue up to laying hens. Lowered hatchability, dead embryos and piped eggs are often noticed in hatcheries. In one research where embryonated eggs were infected with *Salmonella typhimurium* at 6 and 14th days of incubation, Glavits et al.(1984) noted that primarily the chorioallantoic membrane was damaged resulting in 10-40% embryonic mortality. Salmonellosis as a disease is most common in young birds. The most affected ages are one day old shortly after hatching to 3 wks. Mortality is variable from nothing to 100%. Morbidity is usually higher than mortality (Snoeyenbos 1991a). Hall et al. (1949) reported outbreaks of fowl typhoid in every fourth hatch from 25 hatches from typhoid reactors in their second year of laying. Losses up to 6 months amounted to 33.4 %. Mario Padron (1990a) described one outbreak of *Salmonella typhimurium* in Mexico involving 1 to 2 weeks broiler chickens. Mortality was between 1.7% to 10%.

In older birds, Salmonellosis is more prevalent as carrier than clinical disease (Borland 1975). Variable degree of infertility, lowered egg production and hatchability may be observed (Snoeyenbos 1991b, Pomeroy and Nagaraja 1991). Salle et al. (1975) had 10% bacteriological positive samples out of 104 samples tested each year from 1972 to 1975 in Brazil. Both layers and broiler flocks were infected. Williams and Tucker (1980) in an experiment on the virulence of *Salmonella species*, reported that *Salmonella typhimurium* killed 79% one day old and only 3% two days old chicks. They also noted that birds quickly gain resistance as they grew older. These birds which pass through disease remain carriers of *Salmonella* organisms and continue shedding the organisms in faeces. If these carriers are used for breeding they will recycle the infection, infect slaughter houses and environment (Nagaraja et al. 1991). Ianieri (1984) found 4% bacterial isolates out of which 0.4% were *Salmonella typhimurium* from 224 traditional poultry farmers. Outbreaks could also occur even in older birds. Luthgen (1979) reported heavy losses in laying hens in Germany due to *Salmonella pullorum* infection. Mortality was high involving 800 laying hens of a farm of 1600. Egg production went down to 50%.

One species of *Salmonella* which has become increasingly important especially as a public health problem is *Salmonella enteritidis,* more important the invasive phage type 4 (PT4) strains. *Salmonella enteritidis* has been isolated from eggs, yolk sac of baby chicks, ovaries, carcasses from abattoirs and sick birds (El-Amin et al. 1984, Laszlo et al. 1985, O'brien 1988, Paul and Batchelor 1988, Rampling et al. 1988, Shivaprasad et al. 1990, Barrow and Lovell 1991). The trasmission of this *Salmonella occurs* both vertically and laterally. In UK the first case of *Salmonella enteritidis* in chickens was reported in 1987 in East Anglia in which 5% of the flock failed to grow and were culled (O'brien 1988). Up to then *Salmonella enteritidis* especially PT4 has been implicated more as a public health problem than a veterinary problem, and human cases increased from 1101 to 6858 between 1982 and 1987 in England and Wales (Anon 1988a). However the isolation of *Salmonella enteritidis* (PT4) from poultry and poultry products linked poultry as the source of food poisoning which had increased during 1988 (O'brien 1990). Rampling et al. (1988) found 58% incidence of

Salmonella enteritidis PT4 from 81 broiler chickens that had been condemned at processing factories because of macroscopic pericarditis. They also isolated the same organism from 8 out of 20 fresh chilled chickens and Paul and Batchelor (1988) also isolated *Salmonella enteritidis* PT4 from 5 out of 10 eggs and faeces of 6 members of a family who kept their own chickens. In USA since the late seventies human cases of *Salmonella enteritidis* infection has been on the increase and has been associated with egg consumption (St. Louis et al. 1988). In a survey on unpasteurised liquid egg in USA, Eric et al. (1993) showed that out of 561 (53%) samples found positive for *Salmonella,* 162 (15%) samples were *Salmonella enteritidis* positive.

In veterinary medicine *Salmonella enteritidis* has been implicated in several outbreaks involving baby chickens. In Sudan El-Amin et al. (1984) reported an outbreak of Salmonellosis in which *Salmonella enteritidis* was isolated involving 11,325, three (3) weeks old chickens. Mortality rose from 0.9 to 1.6%. In a flock of ducks, Shahata et al. (1983) observed lowered egg production which had fertility rate of 66%, hatchability rate of 52.3% and mortality rate of 7.8% of newly hatched ducklings. They isolated *Salmonella enteritidis* from 10 cloacal swab samples out of 3000 samples taken from ducks. Other *Salmonella* species isolated by them included *Salmonella typhimurium and Salmonella thompson*. Pathogenecity test on all the isolates had mortality rate of between 70 and 100%.

Salmonella enteritidis especially PT4 strains are highly invasive. Hinton et al. (1990) in a study on the invasiveness of *Salmonella enteritidis* PT4 found that all the five test strains had colonised the caecum and invaded the liver of young chicks. The authors could not correlate the involvement of 38 megadalton (MD) plasmid involved in the virulence of *Salmonella enteritidis* in some instances. In other phage types of *Salmonella enteritidis,* Shivaprasad et al.

(1990) reported infection of liver, spleen, peritoneum, ovule, oviduct in laying hens infected experimentally. *Salmonella enteritidis* PT8 (a human isolate) used in the experiment was isolated from eggs. In another experiment Barrow and Lovell (1991) reported heavy invasion of the ovary of layers infected with *Salmonella enteritidis* PT4. Some eggs were positive. The virulence of *Salmonella enteritidis* PT4 varies. In UK it has been associated with high morbidity and mortality (Anon, 1988b, O'brien 1988), however the pathogenecity of *Salmonella enteritidis* PT4 has not been demonstrated significantly in experimentally infected birds (Hinton et al. 1990, Humphrey et al. 1989,1991a, 1991b). Gast et al. (1995) found out that although *Salmonella enteritidis* PT4 was more pathogenic than other phage types, it's pathogenecity could not be compared to that of *Salmonella pullorum*.

Mycoplama

Effect of *Mycoplasma* species on poultry industry is related directly or indirectly. Down grading of meat, reduced feed and egg production efficiency, and increased medication costs make Mycoplasmosis one of the costliest disease problem of poultry (Yoder 1991).

Mycoplasma transmission occurs also quite often through eggs in chickens and turkeys. Isolation of *Mycoplasma* species has also been from ovaries, oviduct and semen of roosters (Yoder and Hofstad 1964) and infection of hens with infected semen resulted into 100% vaginal infection (Edson 1980). Egg transmission has been successfully produced experimentally (Glisson et al. 1984, Jiroj sasipreeyajan et al. 1987). In one research, of 838 batches of turkey eggs with embryonic mortality of 33.8%, out of 21 isolates 5 were *Mycoplasma* species (Metwally et al. 1984). Infection of eggs with *Mycoplasma* will lead to embryonic mortality. Inoculation of *Mycoplasma gallisepticum* in 7 days embryos produced death of embryos in 5-7 days (Yoder 1991). *Mycoplasma* also causes dwarfing, generalised oedema, liver necrosis and enlarged spleen. These lesions were produced also by McClenaghan et al. (1981) in an experiment infecting chicken and turkey embryos. The embryos died between 18 to 24 days in turkey eggs and 19 to 21 days in chicken eggs.

Staphylococcus species.

Staphylococcus species especially *Staphylococcus aureus* have been associated with embryonic mortality. *Staphylococcus* infect mostly bones, joints, tendon sheaths and skin (Hoffman 1939) and yolk sac (Harry 1957, Serviour et al. 1972, Bruce and Johnson 1978, Orajaka and Mohan 1985) causing embryonic mortality. Glavits et al. (1984) reported a 10 to 40% embryonic mortality due to *Staphylococcus aureus* in 6 and 14 days old embryos.

Transmission of *Staphylococcus* species is mainly through contaminated environment and infection usually occurs when there is a break in the first defence mechanism, like skin, mucous membrane and cilliary activity of the respiratory tract etc. In newly hatched chicks, infection in hatcheries occurs when the hatcheries are highly contaminated with *Staphylococcus* species. Penetration is usually through open navel leading to omphalitis (Metwally et al. 1984). Morbidity and mortality is low except in massive contamination of hatcheries (Skeeles 1991).

Klebsiella species.

Several researchers have isolated *Klebsiella* species. from embryos/eggs in different proportions. Orajaka and Mohan (1985) had 14.1% *Klebsiella* species out of 92 total bacterial species isolated. Others (Alaboudi et al. 1992) although do not give percentage isolation, have shown association of *Klebsiella* species with embryonic mortality. In one research on pathogenesis of dead-in-shell bacterial isolates, Shawabkeh and Tarazi (1993) reported mortality of 80% in day old chicks due to *Klebsiella pneumoniae* and 76% were due to *Klebsiella ozaenea*. In four trials Sarakbi (1989) found that *Klebsiella pneumoniae* and *Klebsiella ozaenae* were transmitted from infected individuals to uninfected chicks during brooding period. Embryonic mortality with Setters and Hatchers were 17.6% and 10% due to *Klebsiella pneumoniae* and 19.4% and 10% due to *Klebsiella ozaenae* respectively.

Enterobacter species.

Enterobacter species which are members of the natural intestinal flora are usually found in the natural environment (water, sewage, soil, vegetables). Different species have been isolated and implicated in embryonic mortality. Bruce and Johnson (1978) isolated 31.5% *Enterobacter* species from dead in shell chicken embryos and Bruce and Drysdale (1983) isolated 71.4% from candling rejected and dead-in-shell turkey eggs.

Proteus species.

Proteus species. are quite common isolates of eggs and embryonated eggs and yolk sac infection of day old chicks. Orajaka and Mohan (1985) reported 7.61% contamination while Bruce and Johnson (1978) had 2.5% contamination with *Proteus* species in dead-in-shell chicken embryos

Acinetobacter species.

Acinetobacter species occur widely in nature and are recovered frequently from water, soil, milk, food and animal specimen (John Barnes 1991). From dead embryos and chickens the species have been isolated by different workers. Seviour et al. (1972) isolated 2% of *Acinetobacter* species from incubated eggs of water fowl. Okewole et al. (1991) incriminated

Acinetobacter anitratus in a fatal enteric disease of chicks. Fifty four (54) out of 2 weeks old chicks that died were positive of the bacterium. Kaya et al. (1989) isolated *Acinetobacter lwoffi* from two (2) hens from a flock with haemorragic enteritis in which mortality was 15%. Another species of *Acinetobacter*, *Acinetobacter calcoaceticus* was found to have caused septicaemic infection in a chicken flock with 15% mortality (Erganis et al. 1988). In an assessment on the influence of methods of preparation and storage conditions of poultry carcasses on qualitative distribution of bacteria derived from cutaneous bacterial flora of live birds, Luhellec and Colin (1979) isolated *Acinetobacter* (type A and B), however at refrigeration *Acinetobacter* reduced by fivefold. It has also been isolated from the air of the poultry rearing and processing plant (Dutkiewicz 1980).

Other bacteria from eggs and embryos:

The other bacteria which have been isolated from chicken embryos and baby chicks and associated with mortality include: *Streptococcus* species (Karim and Ali 1976, Bruce and Johnson 1978, Bruce and Drysdale 1983, Borzemska et al. 1988), *Citrobacter* species (Borzemska et al. 1988, Rudy 1991), *Aeromonas* species (Karim and Ali 1976, Umoto et al. 1990) and *Micrococcus* species (Bruce and Johnson 1978, Orajaka and Mohan 1985). Some of these bacteria have been isolated from other conditions both in man and animals. Their isolation from dead embryos, sick birds has linked them to poultry problems in hatcheries and breeder farms.

Devriese et al. (1991) reported an infection with *Enterococcus herae* involving 1 to 4% broiler chicks in Belgium and Randall and Pearson (1991) reported mortality of about 1% in chicks with Enterococcal related endocarditis in UK. In both cases the affected birds were between the age of 3 to 28 days. *Enterococcus faecalis* was reported in 3 batches of 3-10 days old chicks with 4.5, 12.3 and 16.6% mortality respectively (Ilieva et al. 1987).

2.0.6. *Dissemination of egg contaminating bacteria*

Dissemination of egg contaminating bacteria take place through different ways. Incubating eggs from carrier birds or sick birds contaminate embryos in hatcheries causing embryonic mortality or may exhibit their effect after the chicks have been born.

Contaminated egg shells, infected chicks and dead hatches are a source of incubator contamination and through aerosols and workers micro-organisms can be distributed through out hatchery environment and other poultry farms. Chart et al.(1992) managed to infect commercially reared chickens with *Salmonella enteritidis* PT 4 by aerosol challege. Samples of various types (dust, litter, fluff, faeces etc.) taken from hatcheries and poultry houses have been found positive of some of these bacteria. Bhatia and McNabb (1990) had fluff and/or meconium at hatchery contaminated with the same serotype of *Salmonella* species which contaminated litter and carcasses at the farm and processing plants. They suggested the isolation of *salmonella* species as an indicator of flock infection. To demonstrate the transmission of bacteria from parent flocks to hatchery products, Barbour et al. (1984) used the isolation of *Salmonella* species and *Escherichia coli* as indicator of this fact. They correlated the presence of *Salmonella* species in litter of breeding flocks and products of the hatchery. *Escherichia coli* was also correlated in septicaemia of the same flock. On the other hand Harry (1964) failed to demonstrate the involvement of the same serotypes of *Escherichia coli* in repeated outbreaks although he had isolated different isolates including pathogenic ones from dust of the poultry house. However, later Harry and Hemsley (1965) isolated pathogenic

Escherichia coli from dust, feed, air, water and litter and hatchery products (embryos, chicks, egg contents) which correlated with colisepticaemia in chickens. These findings were suggestive of the involvement of an external source of some serotypes in the septicaemia outbreaks. The variable periods of survival of these bacteria in the poultry houses makes it easier for continuous distribution through fomites and infecting subsequent population of chickens.

Carrier birds which have been through infection are one of the major ways through which contaminating bacteria can be disseminated, through shedding of the organisms. The shedding of organisms can be persistent for a long time. Gast and Beard (1990a) reported shedding of the organism for over five (5) months in some birds infected experimentally with *Salmonella enteritidis*. From these carrier birds some other birds were infected by contact as well and their eggs were positive for *Salmonella enteritidis*. McMartin et al. (1987) demonstrated 100% infection of other birds after experimentally infecting only one bird in seven experiments.

Dissemination through fomites (Foot wear, feed bags, shipping crates or brooding equipments) is one other important way of bacterial distributions. Rigby and Pettit (1980) found that carriage of *Salmonella typhimurium* was higher among birds placed in clean crates than uncrated controls, mainly as a result of increase in caecal carriers (from 23.5 to 61.5%). The other group of birds placed in crates contaminated with *Salmonella alachua* became carriers. Plesser et al. (1975) isolated *Klebsiella pneumoniae* from 1972 to 1975 from hands of hatchery workers, work rooms, eggshells and hatchery fluff. The same isolate was isolated in sick and dead turkeys and liver of chickens. The spread of *Klebsiella pneumoniae* obviously could have been through chicks, aerosol and personnel.

Wild animals and birds are another source and way of bacterial dissemination. Wild mice caught in poultry farms have been found positive of some organism (Barbour and Nabbut 1982, Davies and Wray 1995). In one research investigating the possibility of cockroaches as vectors of *Salmonella species*, Kopanic et al. (1994) found five (5) of forty five (45) feed mill and eight (8) of forty five (45) hatchery, cockroaches positive for *Salmonella typhimurium*. Eggs which were exposed for 24 hr to infected cockroaches became also infected. These results clearly implicate cockroaches in acquiring micro-organism and contaminating food and other objects in poultry production and processing facilities.

Dissemination of bacteria through feed can lead to distribution of micro-organisms over a wide area and in certain instances may affect the whole poultry industry. Patnaik and Panda (1986) examining 66 feed mixtures and 16 separate feeds showed that 9.09% were contaminated with *Shigella* species, 12.12% with *Proteus* species, 10.60% with *Pseudomonas* species, 19.69% with *Streptococcus* species and 7.57% with *Staphylococcus aureus* Groundnut oil cake contained *Escherichia coli, Klebsiella, Enterobacter* species, *Pseudomonas* species, *Shigella* species, *Streptococcus* species and *Staphylococcus aureus*. Ten fish meals contained *Escherichia coli, Klebsiella* species, *Enterobacter* species, *Pseudomonas* species, *Shigella* species, *Proteus* species *Streptococcus* and *Staphylococcus aureus*. Cox et al. (1983) reported an average *Enterobacteriaceae* contamination in mash, pelleted and meal samples of log 4.1, 0.8, and 1.8 per gram respectively. Members of the *enterobacteriacea* were present in 100, 60, and 92% where as *Salmonella* was present in 58, 0, and 92% of the mash, pelleted and meal samples respectively. The common isolates were *Enterobacter agglomerans*, *Enterobacter cloacae* and *Klebsiella pneumoniae.*

Ansuimi et al. (1992) found that after feeding 20, one (1) month old SPF birds with feed contaminated with *Salmonella enteritidis* for a week, 17 out of 20 became positive of *Salmonella enteritidis* as early as 14 days.

CHAPTER THREE

3.0.0. MATERIALS AND METHODS

3.0.1. *Source of samples*

Dead-in-shell chicken embryos were collected from three big hatcheries in Zambia which supply day old chicks. These hatcheries are situated in Lusaka about 20 km from city centre. One identified as hatchery A is situated in the east and two identified as hatcheries B and C are situated in the south. Hatcheries A, B and C hatch one day old chicks of 30,000, 20,000 and 8,000 per week respectively.

The rearing of chickens (breeding flock) is done on open ground floor houses. Laying nests are distributed centrally on the floor throughout the chicken house.

3.0.2. *Type and size of sample.*

Collection of samples was done randomly once a week at the end of a hatch, which is 22 days of incubation. A week's sampling made a lot. Nine (9), Eleven (11) and ten(10) such pooled samples/lots were analysed for hatcheries A, B and C respectively.

Dead-in-shell embryos(fully grown and early dead embryos, Germs) were collected as samples. Cracked and piped eggs were not collected to avoid extraneous contamination. Ten pooled yolk sac contents from fully grown dead embryos or whole egg contents from germs made one sample. An average of ten pooled samples were cultured per week. One thousand (1000) dead-in-shell chicken embryos from each hatchery were sampled, which made one hundred (100) pooled samples.

Along with each sample the following information was compiled from the hatcheries.

1. Number of eggs incubated

2.Fertility

3. Hatchability

4. Age of breeding flock from which the incubated eggs were collected.

5. Any other physical factors like electricity failure, machine breakdown during incubation of eggs.

6. Method of collection of eggs from breeding farms, whether hand picked and how many times.

7. Method of cleaning the eggs before setting for incubation..

3.0.3. ***Preparation of inoculum***

The collected eggs were cleaned of the shell contamination (faeces, stains, soil) by wiping with swabs dipped in warm water. The eggshells were then disinfected with 70% alcohol by wiping with swabs. The eggs were then transferred into the safety cabinet class II for drying. The eggs were opened for sampling with a sterile scissors by making a circular cutting on the side of the air space. Using sterile forceps the embryo was removed by the neck suspended over a sterile beaker. The yolk sac was cut open aseptically and contents were let to flow in the sterile beaker. From eggs which had germs (early dead embryos) whole egg contents were poured into the sterile beaker. The contents of ten sampled eggs were then homogenised and used as inoculum. All these operations were done in the safety cabinet. Spoilt/rotten eggs were not sampled.

3.0.4. ***Culturing and isolation of bacteria***

A wire loop full of inoculum was streaked on blood agar (Oxoid) and on MacConkey agar (Oxoid). MacConkey agar plates were incubated aerobically at 37^0C for 24 hr and blood agar plates were incubated aerobically and anaerobically at 37^0C for 24 hr and 48hrs respectively. One millilitre (1ml) of the inoculum was also cultured in selenite broth for *Salmonella* isolation at 37^0C for 24 hr then subcultured on MacConkey. The impure cultures from all the plates were subcultured and purified into single colonies. Distinct pure single colonies from each agar plate were cultured on nutrient agar slants and kept at $^+4$°C until at the end of sampling and isolation from all the lots/samples from each hatchery. The cultures were then revived on fresh nutrient agar slants and characterisation and identification was then done.

3.0.5. ***Identification of isolates***

Routine bacteriological methods of identifying bacteria viz. appearance of colonies, staining characteristics, biochemical tests were performed as detailed by Cowan and Steel (1977) and Carter (1984).

In addition to biochemical tests, all the *Staphylococcus* species were tested for DNase and coagulase. Coagulase test was done on glass slide with rabbit plasma.

3.0.6. ***Serotyping of Escherichia Coli and Salmonella Species***

Further more, serotyping of *Escherichia coli* and *Salmonella* species was done in South Africa at Onderstepoort Veterinary Institute. Serotyping assisted in further identification of *Escherichia coli*. Seventy (70) isolates of *Escherichia coli* were serotyped. Both antisera

'O' and 'K' were used to type *Escherichia coli* cultures. *Salmonella isolates* were both phage typed and serotyped. Thirty four (34) *Salmonella* isolates were tested.

3.0.7. *Antimicrobial sensitivity test.*

All isolates of *Salmonella* species, *Escherichia coli* and *Staphylococcus aureus* were subjected to antimicrobial sensitivity test. The antimicrobial agents used in the test were selected on the basis of the frequency of usage by poultry farmers and availability on the local market. Six antimicrobial agents were therefore used and these were; Penicillin 10units, Tetracycline 30μg, Gentamycin 10μg, Sulphamethoxazole 25μg, Furazolidone 50μg, and Streptomycin 10μg. All of them were obtained from Oxoid.

The method used was a single disk diffusion susceptibility test known as Kirby Bauer method based on a standardised disc (Bauer et al. 1966). Each isolate was first cultured in one millilitre (1ml) trypticase soy broth (Oxoid) for 5 hours. Then the isolates were subcultured on sensitivity test agar (Oxoid), on which the antimicrobial discs were placed and incubated at 37°C overnight. After overnight incubation diameters of clear zone of no bacterial growth around the disk including the diameter of the sensitivity disk were measured and recorded for each isolate and antimicrobial agent. The results were analysed as described by Kirby Bauer. The isolates were categorised as resistant, intermediate and sensitive.

3.0.8. *Pathogenecity of Escherichia Coli*

To determine the significance of *Escherichia coli* isolated from the three hatcheries, fourteen (14) isolates were tested for their pathogenecity. The isolates included all typed serotypes and five (5) untypable rough strains. The serotypes and the total number of each serotypes tested were; serotypes 09:k28 four (4) isolates, 016:k- one(1) isolate, 032/33:k- one (1) isolate, 0101:k- one(1) isolate, 088:k- one (1) isolate and 08:k 47 two (2) isolates.

The pathogenecity tests employed were as follows;

Congo red medium. (Berkhoff and Vinal 1985),

Congo red medium was trypticase soy agar supplemented with 0.03% Congo red dye (Kanto) and 0.15% bile salts (BDH). Each isolate was cultured on a separate plate of the Congo red agar and incubated for 24 hr. After 24 hr incubation they were left at room temperature for two days for better results. Invasive *Escherichia coli* were identified by their ability to take up the Congo red dye.

Mouse inoculation

To determine the pathogenecity of *Escherichia coli,* mouse lethal dose (MLD_{50}) was first determined on a 24 hr culture of a known poultry invasive *Escherichia coli,* 016:k- cultured in trypticase soy broth at 37^0C. Serial dilutions of culture were made in phosphate buffered saline (PBS). Mice of 20g body weight were inoculated intraperitoneally with 1.0 ml diluted cultures per mouse. Five mice were assigned to each dilution starting with undiluted culture, to 10^{-6}. Undiluted culture of serotype 016:k- gave 50% mortality, hence, the rest of the fourteen (14) isolates including the test isolate were used as undiluted culture. All the 14 isolates were cultured for 24 hr as in the test isolate. Five mice were asigned to each isolate and inoculated with 1.0ml of culture intraperitoneally. Five other mice were uninoculated and served as controls. Pathogenecity determination was based on the mortality of the mice. Bacteriological cultures from dead mice were made to determine the specificity of the test.

Embryo inoculation

Embryo inoculation to determine the pathogenecity of the *Escherichia coli* isolates was done by first determining the ELD_{50} (Embryo lethal dose) of one of the isolates known pathogen to poultry, 016:k-. To determine the ELD_{50} the test isolate was first cultured in trypticase soy broth for 24 hr at 37^0C as described for mouse inoculation. Thirteen (13) day old embryos were used. After 24 hr incubation serial dilution of the culture was done using PBS. Embryos were inoculated with 0.2ml diluted cultures into the chorio-allantoic sac. Five uninoculated embryos remained as controls. After determining the ELD_{50} which fell on undiluted culture, the rest of the isolates were subcultured in trypticase soy broth incubated for 24 hr as mentioned above and five embryos at a dose of 0.2ml/embryo were inoculated for each culture. After inoculation the embryos were observed for three days, at the end of which the eggs where opened to determine mortality. Mortality of the embryos was taken as an indicator for pathogenecity. Dead embryos were cultured bacteriologically to determine the specificity of the test.

3.0.9. ***Statistical Analysis***

To try and correlate the relationship between bacterial contamination of incubated eggs and hatchability, two parameters were used to carry out the statistical analysis.

1. The relationship between dead-in-shell (embryonic mortality) and hatchability.

2. The relationship between bacterial contamination (contaminated dead-in-shell) and hatchability.

The data was analysed using correlation relation or regression method. A simple linear regression was used to analyse the data on a computer package C.stat for windows. Eight (8) representative lots of samples whose dead-in-shell and bacterial contamination were calculated for each hatchery with corresponding hatchability percentage were obtained and analysed. It was sought to determine whether bacterial contamination in incubated eggs that led to dead-in-shell (embryonic mortality) have an effect on the levels of hatchability. The P values were used to reach a decision at 95% confidence interval ($\propto$ 0.05).

CHAPTER FOUR

4.0.0. Results.

4.0.1. *Inference on performance of the hatcheries.*

From the data collected from the hatcheries it was noted that the hatcheries under investigation had average fertility of between 80.7% and 96%, average hatchability between 69% and 84.8% and average dead-in-shell (eggs that failed to hatch) of between 15.2% and 31.1%. The age of the breeding birds averaged between 40 and 43 weeks (Table2)

TABLE 2: AVERAGE FERTILITY, HACTHABILITY, DEAD-IN-SHELL AND AGE OF THE BREEDING BIRDS

Hatchery	Average age of breeding birds	Average fertility (percent)	Average hatchability (percent)	Average dead-in-shell (percent)
A	43	80.7	72.6	27.4
B	42	95	84.8	15.2
C	40	88.5	69	31.1

There was no significant difference in age of the breeding flocks between the hatcheries, however hatchery B had better fertility levels (95%) and it's hatchability was also much higher (84.8%) than the the other two hatcheries. Although hatchery C has fairly high fertility it's hatchability percentage is but the least (69%) and it's dead-in-shell is the highiest (31.1%) (Table2).

4.0.2. ***Samples collected.***

Three hundred (300) pooled samples were cultured for bacterial isolation. These samples contained 3000 dead-in-shell chicken embryos. Out of the three hundred samples analysed, 77(39+17+21) yielded pure cultures, 73(39+25+9) yielded more than one bacteria and 150(22+58+70) were sterile respectively (Table 3A,B,C).

TABLE 3A: TOTAL NUMBER OF POSITIVE AND NEGATIVE SAMPLES FROM HATCHERY A.

Lot	**Pure Cultures**		**Impure Cultures**		**Sterile Cultures**		**Total Samples**
	Number Postive	Percent positive	Number Positive	Percent positive	Number Negative	Percent Negative	
1	9	45	1	5	10	50	20
2	8	53	6	40	1	6.67	15
3	5	50	5	50	0	0	10
4	2	20	8	80	0	0	10
5	1	10	7	70	2	20	10
6	5	50	5	50	0	0	10
7	3	60	1	20	1	20	5
8	4	40	3	30	3	30	10
9	2	20	3	30	5	50	10
Total	39		39		22		100

TABLE 3B: TOTAL NUMBER OF POSITIVE AND NEGATIVE SAMPLES FROM HATCHERY B.

Lot	Pure Cultures		Impure Cultures		Sterile Cultures		Total Samples
	Number Positive	Percent positive	Number Positive	Percent Positive	Number Negative	Percent Negative	
1	0	0	1	10	9	90	10
2	0	0	4	40	6	60	10
3	2	40	2	40	1	20	5
4	1	20	2	40	2	40	5
5	2	20	2	20	6	60	10
6	4	40	2	20	4	40	10
7	2	20	2	20	6	60	10
8	1	10	3	30	6	60	10
9	1	10	2	20	7	70	10
10	2	20	2	20	6	60	10
11	2	20	3	30	5	50	10
Total	17		25		58		100

TABLE 3C: TOTAL NUMBER OF POSITIVE AND NEGATIVE SAMPLES FROM HATCHERY C.

Lot	Pure Cultures		Impure Cultures		Sterile Cultures		Total
	Number Positive	Percent Positive	Number Positive	Percent Positive	Number Negative	Percent Negative	
1	1	10	1	10	8	80	10
2	2	20	1	10	7	70	10
3	3	60	1	20	1	20	5
4	1	20	0	0	4	80	5
5	3	30	1	10	6	60	10
6	2	20	0	0	8	80	10
7	2	20	0	0	8	80	10
8	1	5	1	5	18	90	20
9	4	40	3	30	3	30	10
10	2	20	1	10	7	70	10
Total	21		9		70		100

In general the samples which yieded no bacteria at all were found to be half 150 (50%). In case of hatchery C these samples recorded 70% of the total samples analysed (Table3C). It was also enteresting to note that more than half the number of pure cultures recorded came from hatchery A and C, 39(39%) and 21(21%) respectively (Table3A and C). Apparently these two hatcheries had lower hatchability and higher percentage of dead-in-shell as compared to hatchery B (Table 2).

4.0.3 *Bacterial isolates.*

The prevalence of bacteria per hatchery were as follows; hatchery A 208 (54.3%), hatchery B 118 (30.8%) and hatchery C 57 (14.9%) (Table 5).

Three hundred and eighty three (383) cultures were isolated through out the present investigation. Seventeen (17) different bacterial species were identified in all, totalled from all the three hatcheries. Majority of the isolates belonged to the enteric group of bacteria (Table 4). The prevalence of *Escherichia coli* was the highest 70(18.28%) followed by *Staphylococcus* species 54(14.09%) and *Pseudomonas* species 43(11.22%). Another species of Enterobacteriacea, *Klebsiella* species recorded 36(9.40%) and *Enterobacter* species, *Salmonella* speceis and *Citrobacter* species recorded 34(8.62%) each. *Acinetobacter* species recorded 26(6.79%) and *Proteus* species recorded 24(6.26%). The rest, 8 isolates formed less than 5%.

Distribution of the isolates per hatchery also showed high incidence of *Enterobacteriaceae* (Table5). In the case of hatchery A, sixteen (16) different bacterial species were identified and out of these *Enterobacteriaceae* recorded about 56%, *Escherichia coli, Enterobacter* species and *Klebsiella* species were limited to one half of that. *Salmonella* species was isolated from one sample(0.48%) out of the 208 total bacterial isolates. However even though *enterobacteriaceae* were the most frequent isolates, *Pseudomonas* species was the predominant single isolate (20.19%)

Hatchery B revealed that, *Staphylococcus* species and two *Enterobacteriaceae* species, *Escherichia coli* and *Salmonella* species contributed over 70% of the total bacteria isolated.

TABLE 4: PREVALENCE OF BACTERIAL ISOLATES FROM DEAD-IN-SHELL CHICKEN EMBRYOS.

Isolate	Number Isolated	Incidence percent	
Escherichia coli	70	18.28	Enterobacteriacea
Staphylococcus species.	54	14.10	
Pseudomonas. species.	45	11.75	
Klebsiella. species.	36	9.40	Enterobacteriacea
Enterobacter species.	34	8.87	Enterobacteriacea
Salmonella species	34	8.87	Enterobacteriacea
Citrobacter species.	34	8.87	Enterobacteriacea
Acinetobacter species.	26	6.79	
Proteus species.	24	6.26	Enterobacteriacea
Enterococcus species.	11	2.87	Enterobacteriacea
Streptococcus species.	4	1.04	
Alcaligenes faecalis	3	0.78	
Aeromonas hydrophila	3	0.78	
Neisseria dentrificans	2	0.52	
Edwardsiella species.	1	0.26	Enterobacteriacea
Micrococcus species.	1	0.26	
Providencia species.	1	0.26	Enterobacteriacea

Staphylococcus species was slighly higher (26.27%) than *Escherichia coli* and *Salmonella* species which were isolated with the same percentage (22.8%).

Hatchery C revealed a total predominance of *enterobacteriaceae*. Out of over 70% of the total *enterobacteriaceae* isolates, *Escherichia coli* was found the highiest 15(26.31%) followed by *Citrobacter* species and *Salmonella* species (Table 5).

The other two batcerial species which had significantly high prevalence were *Proteus* species (10.28%) from hatchery A and *Acinetobacter* species (17.54%) from hatchery C (Table 5).

Out of 54(14.09%) *Staphylococcus* species isolated from the three hatcheries, 43 (79.62%) were *Staphylococcus aureus,* 10(18.5%) were *Staphylococcus epidermidis* and 1(1.85%) was *Staphylococcus hyicus* (Table 6). Only one (1) isolate was derived from hatchery C. More than fifty percent (50%) of the total *Staphylococcus* species were from hatchery B (31) of which 23 were *Staphylococcus aureus* (Table 6A).

On coagulase and DNase tests, 37 cultures tested positive for coagulase, 28 tested positive for DNase and 21 tested positive to both coagulase and DNase. The tests helped in differentiating the *Staphylococcus* species (Table 6B).

TABLE 5: PREVALENCE OF BACTERIA PER HATCHERY.

Hatchery/	A		B		C		Total
Isolate	Number isolated	Percent	Number isolated	Percent	Number isolated	Percent	
Escherichia. coli	28	13.46	27	22.88	15	26.31	70
Staph. sp.	22	10.58	31	26.27	1	1.75	54
Pseud. sp.	42	20.19	3	2.54	-	-	45
Klebsiella. sp	21	10.10	12	10.17	3	5.26	36
Enterobacter sp.	25	12.02	5	4.24	4	7.02	34
Salmonella sp	1	0.48	27	22.88	6	10.53	34
Citrobacter sp	19	9.13	3	2.54	12	21.05	34
Acinetobacter sp	10	4.81	6	5.08	10	17.54	26
Proteus	22	10.28	-	-	2	3.51	24
Enterococcus sp	4	1.92	3	2.54	4	7.02	11
Streptococcus sp	4	1.92	-	-	-	-	4
Acaligenes sp	3	1.44	-	-	-	-	3
Aeromonas	3	1.44	-	-	-	-	3
Neisseria sp.	2	0.96	-	-	-	-	2
Edwardsiella sp	1	0.48	-	-	-	-	1
Micrococcus sp		-	1	0.85	-	-	1
Providencia sp	1	0.48	-	-	-	-	1
Total	208	54.3	118	30.8	57	14.9	383

TABLE 6A: DIFFERENTIATION OF *STAPHYLOCOCCUS* SPECIES ISOLATED FROM DEAD-IN-SHELL EMBRYOS

Hatchery	Staph. aureus	Staph. epidermidis	Others	Total
A	19	3		22
B	23	7	1	31
C	1			1
Total	43	10	1	54
Percent	79.62	18.5	1.85	100

TABLE 6B: RESULTS OF COAGULASE AND DN-ASE TESTS ON THE *STAPHYLOCOCCUS AUREUS.*

Isolate	Test Performed			
	Coagulase		DNase	
	Number Positive	Percent Positive	Number Positive	Percent positive
Staphylococcus species	37	86	28	65
Total tested	43		43	

4.0.4. *Serotypes* of *Escherichia Coli* and *Salmonella Species*

Details of the Serotyping of *Escherichia coli* are shown in table 7A. Out of the seventy(70) isolates, one (1) belonged to serotype 0101:k-, one (1) belonged to serotype 016:k-, one (1) was serotype 088:k-, two (2) were serotype 032/33:k-, five (5) belonged to serotype 09:k28 and six (6) were serotype 08:k47. Fifty four (54), about 77% of the total isolates were rough type and untypable.

Serotyping of *Salmonella* isolates revealed that all isolates of *Salmonella species* were Enteritidis. Out of the twenty nine (29) *Salmonella enteritidis* which were phage typed, twenty (20) isolates (about 69%) belonged to phage type 4 (PT 4), four (4) were phage type 1 (PT 1), two (2) were phage type 1b (PT 1b), two (2) belonged to phage type 7 (PT 7) and one was phage type 7a (PT 7a). The remaining five (5) species were not phage typed (Table 7B).

TABLE 7A: SEROTYPES OF *ESCHERICHIA COLI* FROM DEAD-IN-SHELL CHICKEN EMBRYOS.

Serotype	Number isolated
0101:k-	1
016:k-	1
088:k-	1
032/33:k-	2
09:k28	5
08:k47	6
Rough-untypable	54
Total	70

TABLE 7B: PHAGE TYPES OF *SALMONELLA ENTERITIDIS* FROM DEAD-IN-SHELL CHICKEN EMBRYOS.

Phage type	Number isolate
4	20
1	4
1b	2
7	2
7a	1
Not phage typed	5
Total	34

4.0.5 *Sensitivity test*

The diameters of the sensitive zones were considered to classify the bacterial isolates into resistant, intermediate sensitive or sensitive using the following key. Penicillin G. Tetracycline and Streptomycin standard cut points were obtained from the publication of Bauer et al. (1966). Gentamycin, Sulphamethoxazole and Furazolidone were analysed using our own set standards shown in table 8A

TABLE 8 A: DIAMETER RANGE FOR CONSIDERING SENSITIVE ZONE.

Antibiotic agent	Amount	Diameter range in Millimetres (mm)		
		Resistant	interm. sensitive	sensitive
Penicillin G.	10units	> 20	21-28	< 29
Tetracycline	30µg	> 14	15-18	< 19
Gentamycin	10µg	> 10	11-15	< 15
Sulphamethoxazole	25µg	> 10	11-15	< 15
Furazolidone	50µg	> 10	11-15	< 15
Streptomycin	10µg	> 11	12-15	< 15

The results of susceptibility tests for the three cultures viz *Escherichia coli, Salmonella enteritidis* and *Staphylococcus aureus* are presented in Tables 8B, C and D. *Escherichia coli* was found to be more resistant, 52.7% on an average to all antibiotics used. Most cultures were highly resistant to Penicillin G, to which 100% of the *Escherichia coli* and *Salmonella enteritidis* and 13.9% of *Staphylococcus aureus* isolates were resistant. *Escherichia coli* and

Salmonella enteritidis isolates also showed high resistance to Streptomycin. Eighty six (86%) percent of *Escherichia coli cultures* and all (100%) isolates of *Salmonella enteritidis* were found resistant to Streptomycin. *Staphylococcus aureus* were also highly resistant to Tetracyclin (53.5%) and Sulphamethoxazole (34.9%) (Tables 8B, C and D).

TABLE 8B: ANTIMICROBIAL SENSITIVITY OF *ESCHERICHIA COLI* FROM DEAD-IN-SHELL CHICKEN EMBRYOS

Antimicrobial agent	Amount	Results					
		Resistant		Intermediate		Sensitive	
		Percent	*	Percent	*	Percent	*
Penicillin	10u	100	70/70	0	0/70	0	0/70
Tetracycline	30μg	50	35/70	29	20/70	21	15/70
Gentamycin	10μg	17	12/70	71	50/70	11	8/70
Sulphamethoxazole	25μg	30	21/70	17	12/70	53	37/70
Furazolidone	50μg	34	24/70	60	42/70	5.7	4/70
Streptomycin	10μg	86	60/70	10	7/70	4.3	3/70
Average		52.7		31.2		15.7	

TABLE 8C: ANTIMICROBIAL SENSITIVITY OF *SALMONELLA ENTERITIDIS* FROM DEAD-IN-SHELL CHICKEN EMBRYOS

Antimicrobial Agent	Amount	Results					
		Resistant		Intermediate		Sensitive	
		Percent	*	Percent	*	Percent	*
Penicillin G	10u	100	34/34	0	0/34	0	0/34
Tetracycline	30μg	0	0/34	17.7	6/34	82.4	28/34
Gentamycin	10μg	0	0/34	0	0/34	100	34/34
Sulphamethoxazol	25μg	2.9	1/34	0	0/34	97.1	33/34
Furazolidone	50μg	0	0/34	52.9	18/34	47.1	16/34
Streptomycin	10μg	100	34/34	0	0/34	0	0/34
Average		33.8		11.8		37.8	

TABLE 8D: ANTIMICROBIAL SENSITIVITY OF *STAPHYLOCOCCUS AUREUS* FROM DEAD-IN-SHELL CHICKEN EMBRYOS

Antimicrobial Agent	Amount	Results					
		Resistant		Intermediate		Sensitive	
		Percent	*	Percent	*	Percent	*
Penicillin G.	10u	13.9	6/43	55.8	24/43	30.2	13/43
Tetracycline	10μg	53.5	23/43	0	0/43	46.5	20/43
Gentamycin	30μg	0	0/43	0	0/43	100	43/43
Sulphamethoxazol	10μg	34.9	15/43	30.2	13/43	34.9	15/43
Furazolidone	25μg	2.3	1/43	46.5	20/43	51.2	22/43
Streptomycin	50μg	13.9	6/43	4.7	2/43	81.3	35/43
Average		19.8		30		57.4	

*Numerator-total number resistant, intermediate and sensitive; denominator-total number tested.

In general all the three species of bacteria tested showed high levels of antimicrobial resistance. However all three bacterial species were highly sensitive to Gentamycin, Sulphamethoxazole and Tetracyclin (Table 8B, C and D).

4.0.6. *Pathogenecity of Escherichia coli*

Pathogenecity of selected isolates of *Escherichia coli* serotypes were examined by Congo red, Mouse inoculation and Embryo inoculation (Table 9).

With congo red dye test all 14 serotypes proved invasive. Only three serotypes 016:k-, and two rough untypable killed less than 50% of the embryos and only one isolate of 09:k28 and 0101:k- killed less than 50% inoculated mice (Table 9). The remaining isolates caused more than 50% mortality to embryos and mice. One isolate of 08:K47 killed 60% of the embryos and 100% mice while the other isolate killed 100% embryos and mice. Serotype 016:K- killed 40% embryos and 60% mice.

Bacteriological examination of liver of dead mice and yolk sac contents of dead embryos revealed pure cultures of *Escherichia coli.* All the controls remained negative for bacterial isolation.

TABLE 9: PATHOGENECITY OF *ESCHERICHIA COLI* SEROTYPES FROM DEAD-IN-SHELL CHICKEN EMBRYO.

Isolate Number	Serotype	Congo red	Mice		Embryos	
			Percent	*	Percent	*
8	016:k-	+	60	3/5	40	2/5
42	088:k-	+	100	5/5	100	5/5
27	09:k28	+	20	1/5	100	5/5
154	09:k28	+	100	5/5	100	5/5
136	09:k28	+	100	5/5	80	4/5
304	0101:k-	+	40	2/5	80	4/5
323	08:k47	+	100	5/5	100	5/5
345	08:k47	+	100	5/5	60	3/5
235	032/33k-	+	100	5/5	100	5 /5
53	Rough	+	100	5/5	100	5/5
49	Rough	+	100	5/5	100	5/5
91	Rough	+	100	5/5	40	2/5
385	Rough	+	100	5/5	40	2/5
386	Rough	+	80	4/5	100	5/5

* Numerator- Number died; denominator-number inoculated

4.0.7. *Statistical Analysis*

1. Relationship between hatchability and dead-in-shell embryos (eggs that failed to hatch).

The relationship between dead-in-shell and hatchability for each hatchery are presented in figures 1A, B, C. Dead-in-shell embryos had an effect on the levels of hatchability in all three hatcheries

Statistical calculation showed significant relationship between hatchability and eggs that failed to hatch or dead-in-shell . See statistical calculations for each hatchery below.

1. Data

hatchery A

	Y	X
Lot	Hatchability Percent	Percent of eggs that failed to hatch
1	74	26
2	74.4	25.6
3	75.6	24.4
4	74.2	25.8
5	71.1	28.9
6	73	27
7	70	30
8	66.4	33.6

Hatchery B

Lot	Y Hatchability Percent	X Percent of egg that failed to hatch
1	86.3	13.7
2	85.1	14.9
3	84.7	15.3
4	85.5	14.5
5	86.2	13.8
6	88.4	11.6
7	87	13
8	79.5	20.5

Hatchery C

Lot	Y Hatchability Percent	X Percent of eggs that failed to hatch
1	67	33
2	68.7	31.3
3	69.4	30.6
4	66.5	33.5
5	66.6	33.4
6	74.6	25.4
7	75.3	24.7
8	72.2	27.8

2. Assumptions

It was asumed that the simple linear regression model and it's underlying assumption are applicable to the data.

Dead-in-shell embryos might have died due to bacterial contamination.

3. Critical level $\propto$ 0.05

4. Hypothesis

H_0: $\beta=0$ (Eggs that fail to hatch has no effect on hatchability)

H_A: $\beta \neq 0$ (Eggs that fail to hatch has an effect on hatchability)

5. Test Statistic :Variance ratio

6. Distribution of test statistic is by P (Ztable>Zcalculated) value with 95% confidence Interval

7. Decision rule. Reject Ho if the calculated P value is less than ∝ 0.05 critical value

8. Statistical calculations

hatchery A

	sum of squares	variance	mean
hatchability(%)	63.82	9.12	72.34
dead-in-shell embryos(%)	62.40	8.91	27.56
covariance	-62.83	-9.04	
regression		0.06	

degrees of freedom (df)=6, P value=< 0.0001, r= -0.9956

	value	standard era(se)	95% confidence interval
slope	-1.01	0.04	-1.10 to -1.91
Intercept	100.09	1.07	97.48 to 102.70

Hatchery B

	sum of squares	variance	mean
hatchability(%)	48.38	6.91	85.34
dead-in-shell embryos(%)	48.80	6.97	14.64
covariance	-48.57	-6.94	
regression		5.66E-03	

degrees of freedom (df)=6, P value=< 0.0001, r= -0.9996

	value	standard era(se)	95% confidence interval
slope	-1.00	0.01	-1.02 to -0.97
Intercept	99.91	0.16	99.52 to 100.30

Hatchery C

	sum of squares	variance	mean
hatchability(%)	82.88	11.84	70.13
dead-in-shell embryos(%)	90.00	12.86	30.00
covariance	-86.00	-12.29	
regression		0.12	

degrees of freedom (df)=6, P value =< 0.0001,

	value	standard era(se)	95% confidence interval
slope	-0.96	0.04	-1.04 to -0.87
Intercept	98.79	1.08	96.14to 101.45

9. Statistical Decision

Reject null hypothesis for all the three hatcheries.

10. Conclusion Eggs that failed to hatch/dead-in-shell embryos have an effect on hatchability levels P< 0.0001

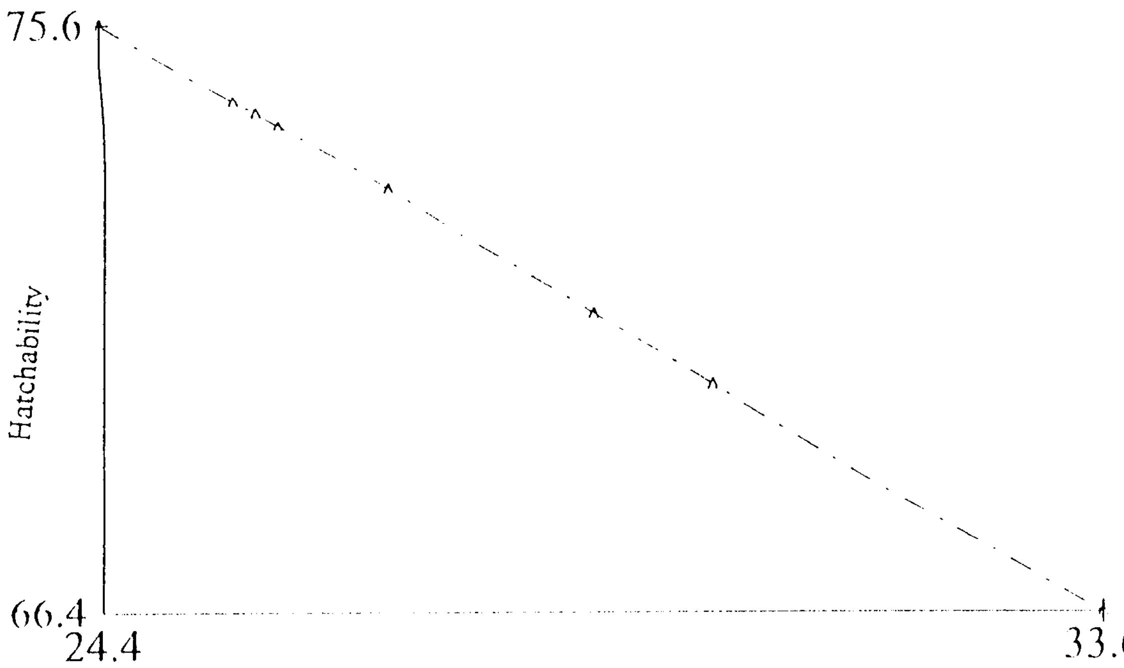

Figure 1A. Hatchery A. Relationship between hatchability and dead-in-shell embryos(eggs that failed to hatch). Each point represents the level of hatchability in percentage for each level of dead-in-shell embryos.

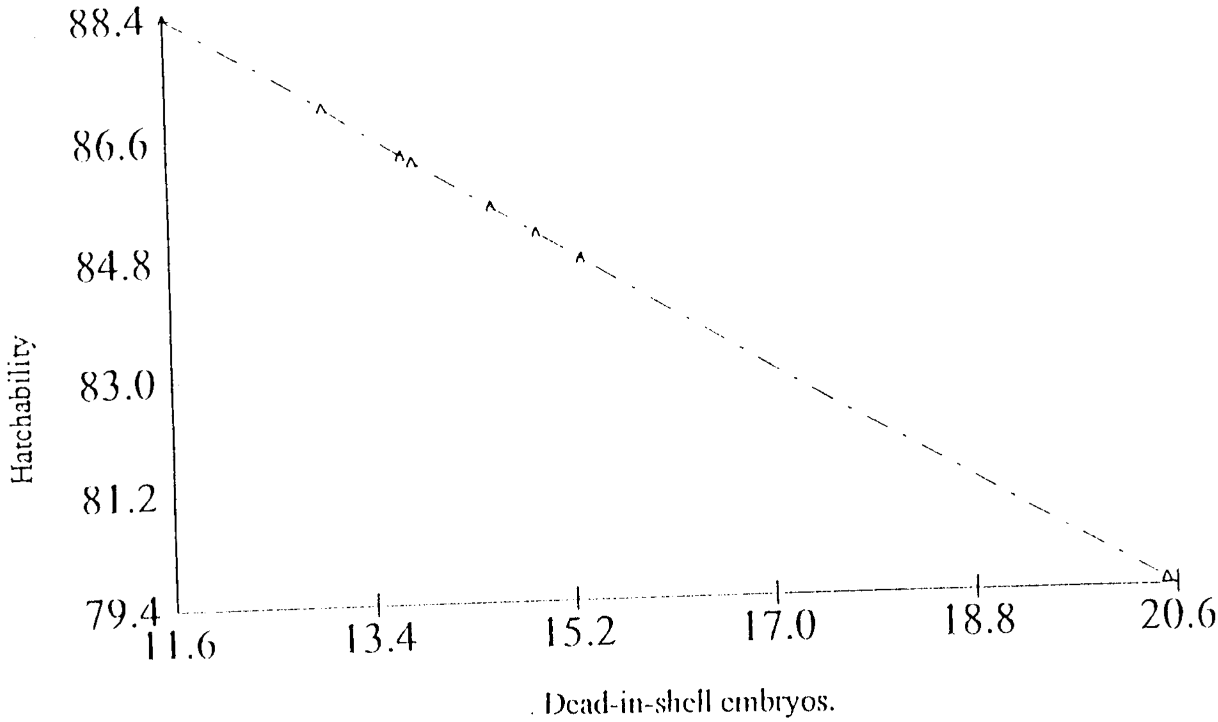

Figure 1B. Hatchery B. Relationship between hatchability and dead-in-shell embryos(eggs that failed to hatch). Each point represents the level of hatchability in percentage for each level of dead-in-shell embryos.

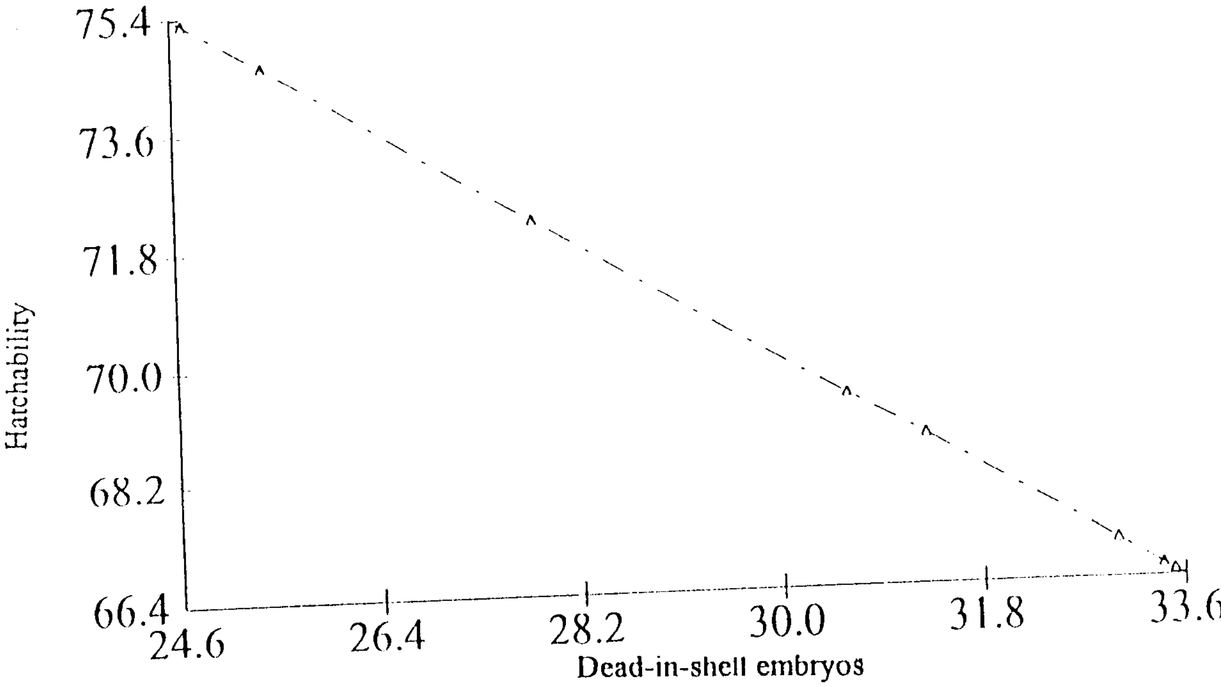

Figure 1C. Hatchery C. Relationship between hatchability and dead-in-shell embryos(eggs that failed to hatch). Each point represents the level of hatchability in percentage for each level of dead-in-shell embryos.

2. Direct relationship between hatchability and bacterial contamination/contaminated dead-in-shel.

There was no direct relationship between hatchability and bacterial contamination in all the hatcheries (Figures 2A, B, C). The statistical calculations showed no relationship between bacterial contamination and hatchability (See stastical calculations).

1. Data

hatchery A

	Y	X
Lot	Hatchability Percent	Percent, bacterial contamination
1	74	70
2	74.4	93.3
3	75.6	100
4	74.2	100
5	71.1	80
6	73	100
7	70	80
8	66.4	70

Hatchery B

	Y	X
Lot	Hatchability Percent	Percent, bacterial contamination
1	86.3	10
2	85.1	40
3	84.7	80
4	85.5	60
5	86.2	40
6	88.4	60
7	87	40
8	79.5	40

Hatchery C

Lot	Y Hatchability Percent	X Percent, bacterial contamination
1	67	20
2	68.7	30
3	69.4	80
4	66.5	20
5	66.6	40
6	74.6	20
7	75.3	20
8	72.2	10

2. Assumptions

It was asumed that the simple linear regression model and it's underlying assumption are applicable to the data.

3. Critical level $\propto$ 0.05

4. Hypothesis

H_0: $\beta=0$ (bacterial contamination has no effect on hatchability)

H_A: $\beta \neq 0$ (bacterial contamination has an effect on hatchability)

5. Test Statistic :Variance ratio

6. Distribution of test statistic is by P (Ztable>Zcalculated) value with 95% confidence Interval

7. Decision rule. Reject Null hypothesis (Ho) if the calculated P value is less than $\propto$ 0.05 critical value

8. Statistical calculations

hatchery A

	sum of squares	variance	mean
hatchability(%)	63.8	9.1	72.3
bacterial contamination(%)	1221.8	174.5	86.7
covariance	185.9	26.6	
regression		5.9	

degrees of freedom (df)=6, P value=>0.0715, r=0.6659

	value	standard era(se)	95% confidence interval
slope	0.2	6.96E-02	-1.82E-02 to 0.3
Intercept	59.15	6.1	44.2 to 74.1

Hatchery B

	sum of squares	variance	mean
hatchability(%)	48.4	6.9	85.3
bacterial contamination(%)	2987.5	426.8	46.3
covariance	10.1	1.4	
regression		8.1	

degrees of freedom (df)=6, P value= 0.9501, r= .0266

	value	standard era(se)	95% confidence interval
slope	3.39E - 03	5.19E - 02	-0.1 to 0.1
Intercept	85.2	2.6	78.8 to 89.7

Hatchery C

	sum of squares	variance	mean
hatchability (%)	88.9	12.7	70.0
bacterial contamination(%)	3400.0	485.7	30.0
covariance	-142.0	-20.3	
regression		13.8	

degrees of freedom (df)=6, P value =>0.5717, r= 0.2582.

	value	standard era(se)	95% confidence interval
slope	-4.18E -02	6.38E - 02	-0.2 to 0.1
Intercept	71.3E	2.3	65.6 to 77.0

9. Statistical Decision

Failed to reject null hypothesis for all the three hatcheries.

10. Conclusion

Bacterial contamination could have had no effect on hatchability P>(0.0715, 0.9501 and 0.5717) for hatcheries A, B and C respectively.

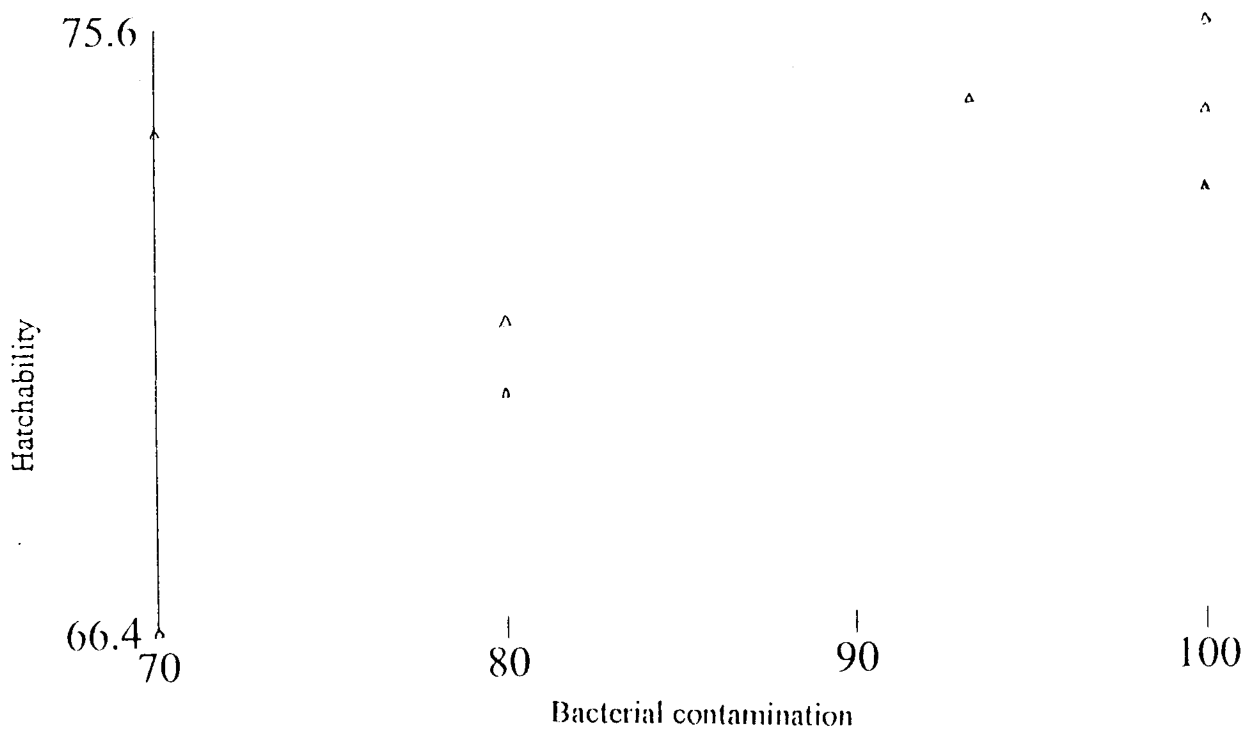

Figure 2A. Hatchery A. Relationship between hatchability and bacterial contamination. Each point represents the level of hatchability in percentage for each level of bacterial contamination from a lot sample of 10 pooled dead-in shell.

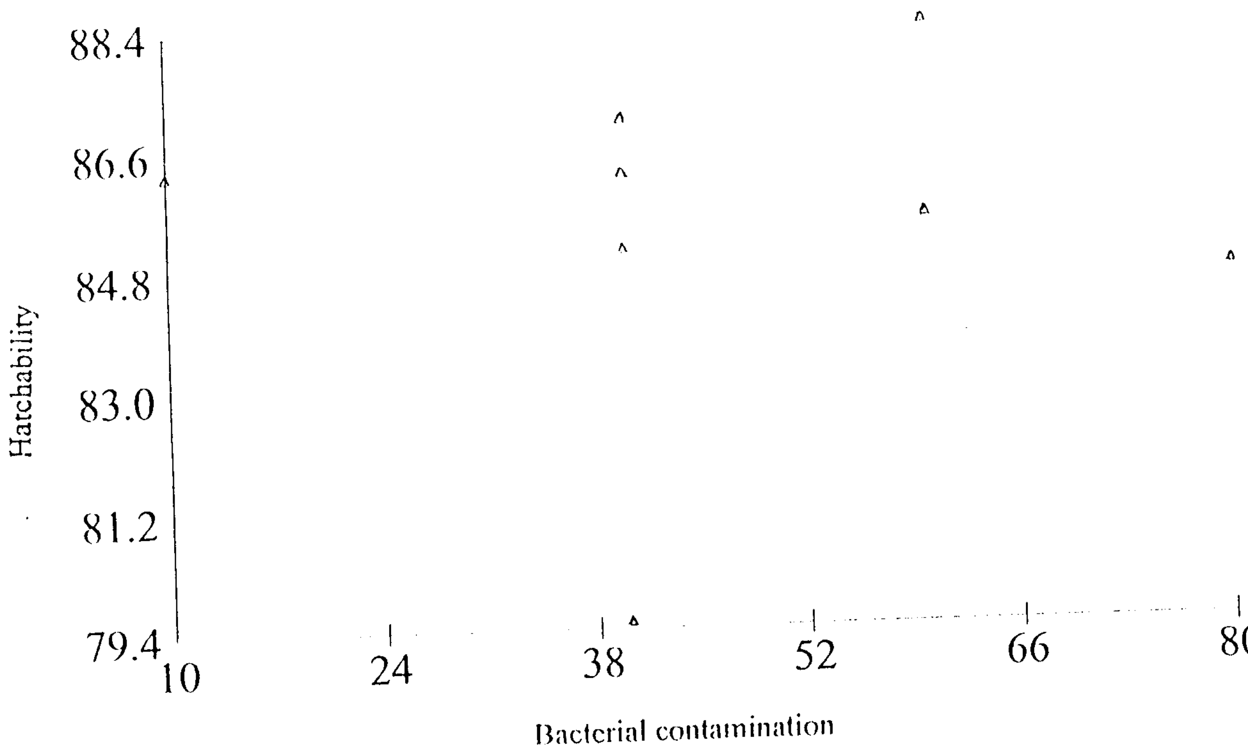

Figure 2B. Hatchery B. Relationship between hatchability and bacterial contamination. Each point represents the level of hatchability in percentage for each level of bacterial contamination from a lot sample of 10 pooled dead-in shell.

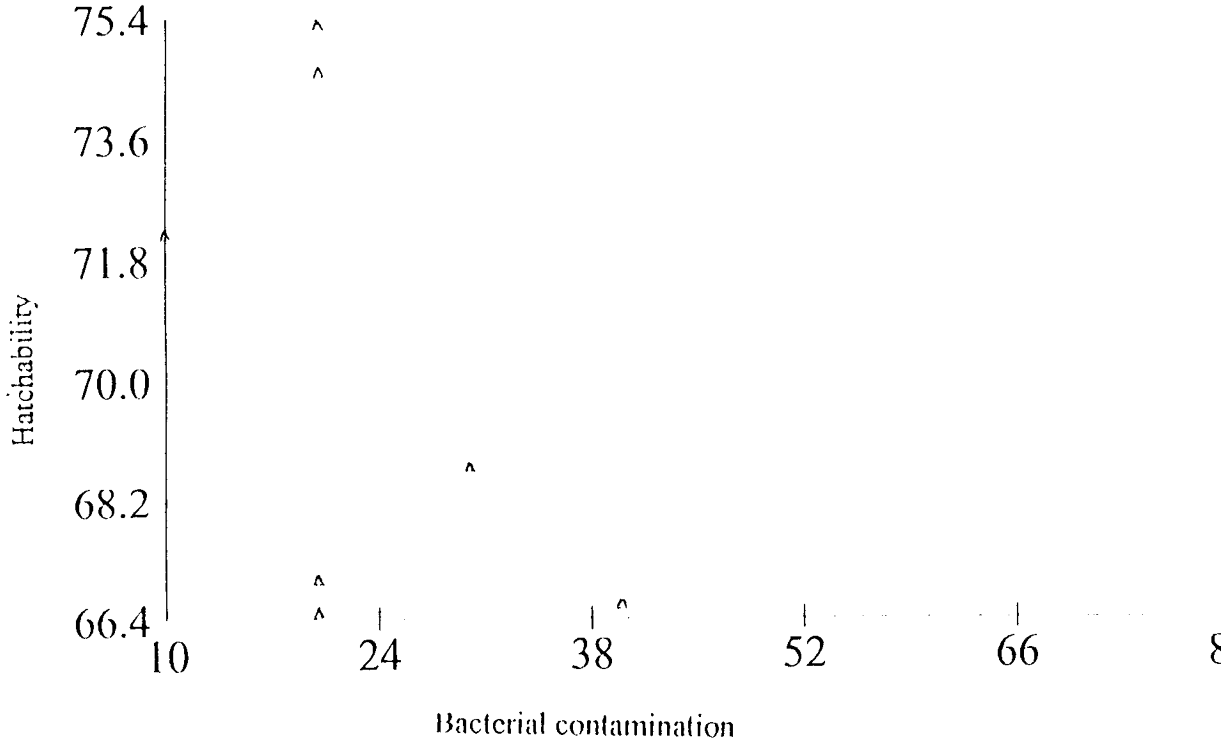

Figure 2C. Hatchery C. Relationship between hatchability and bacterial contamination. Each point represents the level of hatchability in percentage for each level of bacterial contamination from a lot sample of 10pooled dead-in shell.

CHAPTER FIVE

5.0.0 DISCUSSION.

5.0.1. *Inference on the performance of the hatcheries.*

Data collected from the three hatcheries revealed a significant difference in average hatchability and dead-in-shell between them although the breeding systems were the same and there was no significant difference in the age of the breeding flocks (Table 2). It is known that hatchability reduces with the increase in age of the flock probably due to increase in infertility (Kirk et al. 1976, Bruce and Johnson 1978). However, in this investigation the differences in production performance between hatcheries could not be attributed to the age of the breeding flocks as it was insignificant. It could however be due to differences in management of the breeding farms and hatcheries. Hatchery B was better managed than the other two hatcheries, A and C, which had occasional electricity failure and break down of other important machine (chiller machine) for regulating humidity during the research period. This could have led to high embryonic mortality and hence lowered hatchability.

5.0.2 *Inference on bacterial prevalence*

The results from the three hundred pooled samples from the three hatcheries showed a predominance of *Escherichia coli* 70(18.28%) and *Staphylococcus species* 54(14.10%) (Table 4 and 6). The findings of this work are in agreement with the findings of Orajaka and Mohan (1985), Lalithakunjamma and Sudharma (1991) and Nazer and Safari (1994) who also isolated predominantly *Escherichia coli* and *Staphylococcus aureus* in Nigeria, India and Iran. *Escherichia coli* was also found to be predominant by Seviour et al. (1972) in dead-in-shell embryos from turkey eggs (Table 1). Although the findings of this work agrees with the findings of other workers (Bruce and Johnson 1978, Bruce and Drysdale 1983, Orajaka and Mohan 1985 Lalithakunjamma and Sudharma 1991 Nazer and Safari 1994), that enteric bacteria are the common isolates from such samples, there was significant differences in the frequency of individual bacterial isolates encountered by various researchers. This difference could be attributed to the disease situation in a particular country and hatchery hygiene. Similar situation was observed between the hatcheries investigated which clearly show the differences in the frequency of individual bacterial isolates (Table 5). Even among these three hatcheries the distribution of the isolates per hatchery still showed a predominance of enteric bacteria (Table 5).

5.0.3. ***Inference on the role of bacterial isolates from dead-in-shell embryos***

The high incidence of bacteria isolated from the hatcheries (A 54.3%, B 30.8% and C 14.9%) (Table 5) corresponds well with high incidence of embryonic mortality and probably lowered hatchability experienced in the hatcheries investigated. *Escherichia coli, Salmonella enteritidis* and *Staphylococcus aureus* which have been isolated in high proportions in this research from the three hatcheries(Table 5) are known bacterial pathogens which cause embryonic mortality and early chick mortality (Harry 1957, Barbour and Nabbut 1982, Orajaka and Mohan 1985).

<u>Escherichia coli</u>

In all the three hatcheries *Escherichia coli* was isolated in high proportions (Table 5). It is evident from the results that hatchery A and C which revealed quite high incidence of *Escherichia coli* (13.46%,26.31%) had lower hatchability and high embryonic mortality (Table

2). This finding is in conformity with earlier workers (Harry 1957 Orajaka and Mohan 1985,86, Nazer and Safari 1994). On pathogenecity test of the selected isolates of *Escherichia coli* from the present investigation, *Escherichia coli* proved to be highly pathogenic to thirteen (13) days old embryos and white mice (Table 9). When *Escherichia coli* invades the embryo it has the predilection of yolk sac which it infects and causes omphalitis leading to embryonic mortality (Harry 1957, Pathak et al. 1960). Almost in all the researches on causes of ompalitis and yolk sac retention, *Escherichia coli* has been amongst the predominant isolates. The pathogenecity of *Escherichia coli* is not simply in it's ability to multiply but to also invade tissues (Nabbut and Khatib 1977). This ability to invade tissues was tested using Congo red dye. According to Berkhoff and Vinal (1985), the invasive pathogenic *Escherichia coli* have the ability to bind to Congo red dye incorporated in culture media. On the test of selected isolates of *Escherichia coli* for this ability, all the isolates where positive to the test (Table 9).

In the present work, serotyping of *Escherichia coli* revealed that, out of the seventy(70) isolates, one (1) belonged to serotype 0101:k-, one (1) belonged to serotype 016:k-, one (1) was serotype 088:k-, two (2) were serotype 032/33:k-, five (5) belonged to serotype 09:k28 and six (6) were serotype 08:k47. Fifty four (54), (about 77%) of the total isolates were rough type, non typable. The serotypes isolated in this work differed with those of Orajaka and Mohan (1986) and Falade (1977) in similar works in Nigeria, except for serotype 08:k47 which was also isolated by Orajaka and Mohan (1986) and has been associated with hatchery losses.

Some of the *Escherichia coli* serotypes isolated in this work (08:k47, 016:k-) are known poultry pathogens (Table 7A). Others, 0101:k- are pathogenic in cattle and pigs and

09:k28 are pathogenic in cattle sheep and horses. However the pathogenecity test of these isolates on 13 days old embryos proved that they are also pathogenic to chicken embryos as well (Table 9). Serotype 088:K- also isolated in this work (Table 7A) often carries CFN2 toxin (not tested) which could be detrimental to developing embryos. Serotypes 032/33:K- were becoming rough on serotyping and cross-reacted and are usually considered non pathogenic however, on pathogenecity test in the present work the two isolates proved highly pathogenic and killed 100% of the chicken embryos and mice (Table 9). *Escherichia coli* serotypes 0101:k-known pathogen to pigs and cattle, 09:k28 pathogen of cattle, sheep and horses and 08:k47 poultry patogens isolated in this work are also known pathogens to piglets (Timoney et al. 1988). These serotypes are usually refereed to as enterotoxigenic *Escherichia coli* because they produce enterotoxins. According to Smith and Gyles (1970) enterotoxigenic strains produce Lta (heat-label) toxin. Such toxins are always present in association with colonising antigens such as F4 or F5 in subjects with diarrhoea. Although the test for the production of toxins by these serotypes of *Escherichia coli* was not done in this present investigation, it could be inferred that these isolates are probably toxigenic basing on the results of pathogenecity test (Table 9). Pathogenecity test revealed that all the serotypes including some rough untypable isolates are pathogenic to both mice and 13 day old chicken embryos (Table 9), and Harry (1957) reported that yolk sac infection is also caused by toxigenic bacteria capable of stimulating inflammatory exudate from yolk sac lining and some of the *Escherichia coli* strains are some of such bacteria. In this study 16 (23%) out of 70 *Escherichia coli* isolates belonged to 'O' serogroup and 54 (77%) out of 70 *Escherichia coli* isolates were rough non typable (Table 7A). On pathogenecity test of selected serotypes and non typable strains both types killed on average 81% of the 13 day old chicken embryos (Table 9). The findings of this work seem to confirm the conclusion of Rosenberger et al. (1985) that 02 serogroups and non typable *Escherichia coli* of avian origin are among common virulent avian *Escherichia coli*. This conclusion of Rosenberger and others was later supported by Nolan et al. (1992).

Our findings of isolation of '0' serogroup *Escherichia coli* gets further support from the work of Harry and Hemsley (1965) who also isolated *Escherichia coli* belonging to pathogenic '0' serogroups from embryos which had died during incubation and from chicks killed shortly after hatch.

Salmonella enteritidis

Although *Salmonella species* isolated in this work were amongst the most prevalent, Orajaka and Mohan (1985), Bruce and Johnson (1978) could not isolate any *Salmonella species* in similar work. However Nazer and Safari (1994) reported *Salmonella typhimurium* in high proportions (10 out of 32) from dead-in-shell embryos. It is interesting to note that during the present investigation only *Salmonella enteritidis* was isolated and majority of the *Salmonella enteritidis* isolates were PT4(Table 7B).

The prevalence of this Serotype, PT4 has been on the increase since the seventies. *Salmonella enteritidis* PT 4 has primarily been associated with food poisoning in human beings through the consumption of contaminated eggs (O'brien 1988, St. Louis et al. 1988, Humphrey et al.1989). *Salmonella Enteritidis* PT4 is increasingly being recognised as a frequent pathogen of poultry (O'brien 1988). It's frequent isolation from breeding birds' ovaries (Shivaprasad et al.1990) and egg (Humphrey et al. 1989) makes it a potential pathogen to developing embryos and baby chicks. Shawabkeh and Tarazi (1993) testing the pathogenecity of bacterial pathogens isolated from dead-in-shell embryos reported 32% mortality rate in one day old broiler chicks due to *Salmonella enteritidis.*

In Zambia *Salmonella enteritidis* has been isolated from poultry samples other than dead-in-shell embryos (Gasper and Hrabeta 1978, Sharma et al. 1991) and has not been so significant in poultry diseases as *Salmonella pullorum/gallinarum* which have been known in the poultry industry for quite sometime(Anon 1976-1992, Sharma et al. 1991). In the present study all the *Salmonella* isolates were *Salmonella enteritidis* and over 50% were isolated from hatchery B. Hatchery A only recorded 1 isolate and Hatchery C recorded 6 isolats (Tables 5 and 7B). On phage typing majority belonged to PT4 (20) (Table 7B). Pathogenecity of these isolates was not done. However earlier workers (O'brien 1988, Shawabkeh and Tarazi 1993) have shown that this pathogen is highly pathogenic to embryos and chicks as well. In a flock of ducks which had hatchability as low as 52.3%, *Salmonella enteritidis* was positive in 10 out of 3000 cloacal samples and mortality rate of newly hatched ducklings was 7.8% (Shahata et al. 1983). Hinton et al. (1990) showed that *Salmonella enteritidis* especially PT4 in it's pathogenecity was highly invasive. Although *Salmonella enteritidis* PT4 is more pathogenic than other phage types, it's pathogenecity can not be compared to that of *Salmonella pullorum* (Gast et al. 1995). The Isolation of this serotype, especially PT4 from dead-in-shell chicken embryos in this work for the first time in Zambia need epidemiological studies and formulation of control measures.

Staphylococcus species

Among the *Staphylococcus* species isolated, more than 50% were *Staphylococcus aureus* (Table 6). Out of these over 50% were isolated from hatchery B and about 44% were isolated from hatchery A (Table 6). These two hatcheries had better hatchability (84.8%, 72.6%) and relatively lower dead-in-shell (15.2%, 27.4%) than hatchery C which had only one (1) unidentified *Staphylococcal* isolate and whose dead-in-shell was the highiest (31.1%) and hatchability the lowest (69%) (Table 2). *Staphylococcus aureus* is one such bacteria that cause embryonic mortality. Glavits et al. (1984) produced 10-14% embryonic mortality in 6-14 days embryos. Some *Staphylococcus aureus* possess enzymes like lecithinase, lipase and haemolysin capable of degrading the yolk of embryos. Majority of the *Staphylococcus* cultures isolated by Orajaka and Mohan (1985) from dead-in-shell embryos possessed these enzymes. In this work pathogenecity of *Staphylococcus aureus* was not done. However the coagulase test which was used to differentiate the isolates has been often used as an indicator for pathogenecity and about 86% isolates were positive for coagulase (Table 6). It can therefore be assumed that *Staphylococcus aureus* might have been associated with the death of the embryos in hatchery A and B.

The other *Staphylococcus* species, *Staphylococcus epidermidis* has been isolated in the proportion of 2.64% predominantly from hatchery B (Table 6). The pathogenecity of this serotype for embryos is not known. Hence there is need of investigating it's role in embryonic mortality.

Pseudomonas species

Pseudomonas species have been predominantly isolated from hatchery A (Table 5) which had quite high dead-in-shell embryos (27%). On overall *Pseudomonas* species recorded the third highest (11.75%) from these hatcheries(Table 4). The isolation of *Pseudomonas* species in relation to embryonic mortality is in agreement with Lalithakunjamma and Sudharma (1991). Other researchers who even isolated *Pseudomonas* species in small percentages from dead-in-shell embryos have also shown it's correlation with embryonic mortality. The work of Bruce and Johnson (1978) has also proved the correlation of *Pseudomonas* species and hatchery losses. *Pseudomonas* species are capable of invading fertile eggs and cause embryonic mortality (Barnes 1991). *Pseudomonas* has been associated with egg spoilage (rotten eggs) as they can easily grow well at lower temperatures mostly used for storage (Orel 1959, Board 1965).

In this present work pathogenecity test of *Pseudomonas* isolates was not performed. However *Pseudomonas aeruginosa* has been shown to be highly pathogenic with 88% mortality to chicks by Shawabkeh and Tarazi (1993).

Klebsiella species

Klebsiella species have recently been implicated as hatchery killers causing heavy losses in hatcheries through embryonic mortality (Sarakbi 1989). Shawabkeh and Tarazi (1993) reported 80% and 76% mortality of chicks experimentally inoculated with *Klebsiella pneumoniae* and *Klebsiella ozaenae* respectively. Other workers have also isolated *Klebsiella* species and associated them with embryonic mortality and lowered hatchability (Orajaka and

Mohan 1985, Alaboudi et al. 1992, Nazer and Safari 1994). In the present study *Klebsiella* species has been isolated in quite high percentages from hatchery A and B (Table 5). However hatchery B in which *Klebsiella* species were the third highest isolates had highest hatchability and lowest embryonic mortality (Table 2). Hence the role of *Klebsiella* species in the present investigation is not very clear.

Micrococcus species

Micrococcus species has been isolated predominantly by some workers (Bruce and Johnson 1978, Orajaka and Mohan 1985) from dead-in-shell embryos. However in the present work *Micrococcus* was isolated from one sample only.

The death of embryos and chicks can result from yolk sac infection by a number of bacteria (Harry 1957). Yolk sac retention and/or omphalitis which have been associated with bacterial infection has been shown to be the common cause of mortality in chicks up to ten days old (Watts and Rac 1958). Other bacterial isolates in the present work were *Enterobacter* species, *Citrobacter* species, *Acinetobacter* species, *Proteus* species, *Enterococcus* species, *Streptococcus* species *Alcaligenes faecalis, Aeromonas hydrophila, Neisseria dentrificans, Edwardsiella* species, and *Providencia* and some of which have been isolated in quite high percentage (Table 4). Most of these bacteria isolated in this work have also been isolated from yolk of embryos by Seviour et al. (1972), Orajaka and Mohan (1985), Lalithakunjamma and Sudharma (1991), Shawabkeh and Tarazi (1993) and Nazer and safari (1994) and yolk sac of baby chickens (Harry 1957 Pathak et al. 1960). Their presence in yolk sac of both embryos and baby chicks, have associated them with embryonic mortality hence reduced hatchability and yolk sac infection in baby chicks (Harry 1957, Seviour et al. 1972 Bruce and Johnson 1978, Orajaka and Mohan 1985, Nazer and Safari 1994).

Some of the isolates like *Providencia* species, *Neisseria dentrificans* and *Edwardsiella* species recorded during this investigation have not been isolated by other workers. Hence, there is no information available on these isolates regarding their role, in embryonic mortality, and therefore further work could be done on this aspect.

The high incidence of bacteria from these hatcheries clearly shows the poor levels of hygiene existing in these hatcheries and breeder farms. In breeding flocks of these hatcheries, the eggs meant for hatching are laid in nest boxes on the floor making them exposed to contamination through soiling and faecal material. Eggs laid on the floor have been found to be highly contaminated with egg penetrating bacteria (Smeltzer et al. 1979, Barbour and Nabbut1982) and if they are used for incubation they do not only have low hatchability but are a source of bacterial contamination for newly hatched chicks (Quarles et aL.1968). Harry (1957) recognised low standard of hygiene on the supply farms and hatchery as factors contributing to fatal yolk infections. The source of yolk infection in day old chicks in the Zambian farms may be on a large scale from these hatcheries.

The source of the bacteria isolated in this work was not determined, as it was not the purpose of this study. However, it is known that bacteria of the alimentary canal of chickens and those that inhabit the skin of chickens are able to contaminate the shell and contents of eggs (Haines 1938).

Looking at the relationship between hatchability and dead-in-shell (eggs that failed to hatch) for each hatchery (Figure 1A,1B,1C), there was simple linear relationship between hatchability and the eggs that failed to hatch ($P<0.0001$). As the number of eggs that failed to hatch increased so did hatchability reduce. These findings agree with the findings of Bruce and Johnson (1978). However a direct relationship between hatchability and bacterial contamination is rather difficult to determine (Figures 2A,B,C) although it is evident that some bacteria are able to cause embryonic mortality and hence reduce hatchability. These observations were also made by Bruce and Johnson(1978) and Orajaka and Mohan(1985).

The statistical inference however showed no relationship between bacterial contamination and hatchability for all the hatcheries (hatchery A $P>0.0715$, hatchery B $P>0.9501$ and hatchery C $P>0.5369$). During the present investigation 150 (50%) samples revealed high levels of bacterial contamination. The main isolates were *Escherichia coli, Pseudomonas* species, *Staphylococcus* species, *Klebsiella* species and *Salmonella* species. However, the frequency of these bacteria differed amongst the three hatcheries. The contamination of incubated eggs with these bacteria could have had pathogenic effect on embryos as expressed in the number of dead-in-shell. These bacteria have also been isolated by other workers and associated with embryonic mortality (Bruce and Johnson 1978, Orajaka and Mohan 1985, Shawabkeh and Tarazi 1993).

High level of bacterial contamination from the hatcheries and breeder farms have been used as an indicator for poor hygiene and managemental problems (Barbour et al. 1984). Hatcheries A and B had quite high levels of bacterial contamination, 54.3% and 30.6% respectively (Table 5). However the two hatcheries showed different levels of hatchability and fertility (Table 2). In contrast hatchery C had relatively low bacterial contamination (14.6%) but had lowest hatchability although fertility was relatively high. These differences could have possible explanation that other factors like genetic-malformation, nutritional and physical mask the effect of bacteria. This explanation gets support from the fact that 50% of the samples were sterile, where other factors played significant role in embryonic mortality other than bacterial effect. About 77 (51.33%) of these sterile samples came from hatchery A and C which had highiest dead-in-shell (Table 2, 3A and 3C). The analysis of data from the hatcheries further showed that hatcheries A and C had often electricity failure and breakdown of chiller machine. Hatchery B also had breakdown of chiller machines but was relatively better managed. It could thus be inferred that bacterial contamination coupled with bad managemental practices in hatcheries result in lowered hatchability. Hence, Bacterial contamination alone could not be correlated significantly to lowered hatchability in the present investigation.

5.0.4 ***Inference on Antimicrobial Susceptibility.***

From the results of Antimicrobial sensitivity test on *Escherichia coli, Salmonella enteritidis* and *Staphylococcus aureus*, it was noted that on average *Escherichia coli* was the highest resistant to all Antimicrobial agents (52.7%) followed by *Salmonella enteritidis* (33.8%) and *Staphylococcus aureus* (19.8%).(Tables 8B, C and D). Nazer and Safari (1994) also observed high resistance (50%) of *Escherichia coli* isolated from dead-in-shell chicken embryos to Tetracycline. The most susceptible bacteria on an average was *Staphylococcus aureus* (57.4%) followed by *Salmonella enteritidis* (37.8%) and *Escherichia coli* (15.7%) (Tables 8B, C, D). The efficacy of individual Antimicrobial agents varied. The most effective was Gentamycin followed by Sulphamethoxazole and then Tetracycline (Tables 8B, C, D). Most bacterial isolates were resistant to Penicillin G and Streptomycin which recorded between 13.9% and 100% resistance (Tables 8B,C,D). Furazolidone recorded an efficacy between 5.7% and 52.3%.

Although there is no record on Antimicrobial susceptibility on *Escherichia coli* and *Staphylococcus aureus* in Zambia, the results obtained here are more or less similar to those of Pandey and Sharma(1994) and Falade et al. (1989) who worked on different species of *Salmonella,* and reported a general increase in Antimicrobial resistance. This rather sad situation may be attributed to lack of enforcement of regulatory measures on the use of antibiotics in the country. Antibiotics in Zambia have frequently been used in feeds (especially Nitrofurans) as a prophylactic measure to boost production (Pandey and Sharma 1994). This may also explain the high levels of drug resistance obtained and probably the high level of egg contaminating bacteria isolated from hatcheries.

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6.0.0. *CONCLUSION*

1. The results of the present investigation revealed high level of bacterial contamination in hatcheries in Zambia. The presence of highly pathogenic bacteria from dead- in-shell chicken embryos in Zambian hatcheries clearly indicates their association to production losses in hatcheries as well as the poultry farms receiving the day old chicks from these hatcheries.

2. The isolation of *Salmonella enteritidis* TP4, a potent *Salmonella* serotype, for the first time from dead-in-shell embryos in Zambia during the present investigation is a significant finding. This serotype being of public health importance could have epidemiological implication not only in Zambia but in the Southern African region as a whole. Therefore there is a need to carry out epidemiological studies to formulate appropriate control measures.

3. The role of bacterial species like *Salmonella, Escherichia coli* and *Staphylococcus aureus* in causing embryonic mortality is well known. However in this present work in addition to common bacteria isolated from dead-in-shell embryos there has been high incidence of other bacteria as well. Their role in causing embryonic mortality is not clear. There is, therefore, need for investigations to establish the role of these other egg contaminating bacteria.

4. A few selected isolates of *Salmonella enteritidis, Escherichia coli* and *Staphylococus aureus* were subjected to antibiotic sensitivity test. They showed high level of antibiotic resistance. This could be attributed to indiscriminate usage of Antimicrobial agents and non enforcement of legislature on the usage of antibiotics in the country. Hence, poultry farmers,

especially breeder farms should be made aware of antibiotic resistance against certain bacteria so that appropriate measures could be taken to combat the situation.

5. The results reflect that, the hatcheries under present investigation were in poor hygienic condition. Hence there is an urgent need by the concerned to make the hatcheries and poultry production in a clean and health status. The following measures are suggested.

a) Breeding flocks should be sourced from disease free suppliers.

b) Regular monitoring of breeding flocks for diseases like *Salmonellosis* should be done. In other words the disease situation of the breeding flock should be regularly checked for the appropriate measures to be taken.

c) High and strict levels of cleanliness in both breeding farms and hatcheries should be maintained.

6. From the results it is clear that other factors other than bacterial contamination also play a role in embryonic mortality. These factors are mainly due to poor management. The situation could be improved if such management problems like electricity failure, breakdown of important machines are avoided.

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