EFFECTS OF SOME COMMERCIAL DISINFECTANTS ON AEROBIC BACTERIA ASSOCIATED WITH DEAD-IN-SHELL CHICKEN EMBRYOS IN KADUNA STATE, NIGERIA

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DECLARATION

I declare that the work in the thesis entitled "Effects of some commercial disinfectants on aerobic bacteria associated with dead-in-shell chicken embryos in Kaduna State, Nigeria" has been performed by me in the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University under the supervision of Dr. H. M. Kazeem, Professor J. U. Umoh and Dr. F. B. Mosimabale. The information derived from the literature have been dully acknowledged in the text and the list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

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CERTIFICATION

This thesis entitled "EFFECTS OF SOME COMMERCIAL DISINFECTANTS ON AEROBIC BACTERIA ASSOCIATED WITH DEAD-IN-SHELL CHICKEN EMBRYOS IN KADUNA STATE, NIGERIA" by Mamman Paul Habila (MSc/Vet.Med./36253/02-03) meets the regulations governing the award of the degree of Master of Science of Ahmadu Belo University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

The demand for poultry products in Nigeria is on the increase. Losses in the chicken 'factory' (hatchery) are associated with embryonic mortality which in turn may result from non-specific bacterial infections of incubated eggs. This study was therefore set out to investigate the likely bacteria that may be responsible for these losses in commercial hatcheries in Kaduna State, Nigeria with the aim of suggesting possible control measures. Six hundred (600) dead-in-shell chicken embryos from these hatcheries were sampled and cultured individually following recommended laboratory procedures for culture, isolation and identification of bacteria. From these samples, 62 (54.9 %) isolates of *Escherichia coli* and 21 (18.6 %) isolates of *Proteus spp* were recovered. Other isolates were *Pseudomonas spp* 6 (5.3 %), S*taphylococcus aureus* 11 (*9.7 %*), *Staphylococcus spp* 8 (7.1 %) and *Micrococcus spp* 5 (4.4 %). The various hatcheries investigated recorded 8-30 % losses of eggs at each completed incubation process and this was mainly as a result of death of the embryos in the shells. A high incidence of pathogenic strains of bacteria from these dead-in-shell chicken embryos was observed. This is suggestive of the fact that these isolates may have contributed to the embryonic mortality and reduced hatchability recorded in the farms investigated. On these isolates, the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and kill-time of two commonly used and one relatively new disinfectants were determined. The two commonly used disinfectants were Purit® and Z-germicide®; the new one was carcil®. The results revealed that purit® was most effective against *E. coli* since it required the lowest concentration of 0.00026% for 1 minute to kill the bacteria. Z-germicide® was

found to be effective at 0.00063% for 5 minutes and carcil® required 0.0031% for 10 seconds. Purit® was also found to be the most effective of the three disinfectants on *Pseudomonas spp* but Z-germicide® showed the best results on *Proteus spp*.

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ABBREVIATIONS AND DEFINITIONS

AC-ELISA : Antigen-Capture Enzyme-Linked Immunosorbent Assay

AOAC : Association of Official Analytical Chemists

BACTERIA : A group of diverse and ubiquitous prokaryotic single-celled organisms

BACTERICIDAL : Refers to any agent (chemical or physical) which is able to kill (at least) some types of (vegetative) bacteria

CARCIL® : Trade name for the disinfectant with the chemical name of Alkyl-benzyl dimethyl ammonium chloride

DEAD-IN-SHELL EMBRYO : This refers to death of the developing chick (embryo) while still in the shell i.e. before hatching

DISINFECTANT : Chemical agent used for disinfection. Disinfectants for general use should be active against a range of common pathogenic microorganisms and should be microbicidal and not microbistatic

KILL-TIME : This is the time interval between contact and cidal effect of a chemical agent and microorganisms

MBC : Minimum Bactericidal Concentration; which means the least concentration of the active ingredient of a chemical agent that is capable of killing the microorganisms in contact

MIC : Minimum Inhibitory Concentration; which means the least concentration of the active ingredient of a chemical agent capable of stopping the growth of the microorganisms in contact

PATHOGENIC BACTERIA : these are bacteria that are capable of invading the tissues of living organisms and causing disease

PCR : Polymerase Chain Reaction

PURIT® : Trade name for the disinfectant with the chemical composition of Chlorexidine gluconate bp 0.3 % w/v and cetrimide bp 3.0 % w/v.

WBP : Whole Blood Plate

Z-GERMICIDE®: Trade name of the disinfectant with the active principles of 7 % Tar acid phenol

CHAPTER ONE

1.0 INTRODUCTION

When an embryo is said to be dead in the shell it means there is a loss of the life of a chick that was still developing within the egg shell of a fertile incubated egg. This is a common occurrence in Nigerian hatcheries which results in great and untold losses. For instance, the hatcheries that were sampled in Kaduna State for this work reported a loss of between 8 and 30 % of incubated eggs after each hatching process and this loss resulted from inability of eggs to hatch because the embryos died before the hatching time (information from questionnaires served to the hatchery managers and attendants, Table 4.1).

This work was a cross-sectional study carried out to establish the prevalence of aerobic bacteria that cause dead-in-shell chicken embryos in Kaduna State, Nigeria. Disease-causing microorganisms can enter the egg by two major routes, either transovarially or via the egg shell, (Ghazikhanian, 1980). In transovarian transmission, infectious agents can pass down from either the ovum in the hen or sperm in the cock which can cause embryonic death or infection in the hatched chicken. Those disease-causing agents that are already lodged in the eggs before they are laid are called “true egg transmitted diseases” such as those caused by *Mycoplasma spp, Salmonella pullorum, Salmonella gallinarum, Avian encephalitis virus.* The shell-transmitted diseases are those caused by

Salmonella spp, Arizona spp, Escherichia coli, Pseudomonas spp, Proteus spp, Coliforms, Aspergillus sp), (Ghazikhanian, 1980).

A chicken egg shell may have between 7,000 and 17,000 tiny pores out of which 1% are open, and through which infectious agents can enter if the eggs are not handled properly (Fussell, 1987). They can pass through these pores either actively with the help of their tiny "leg" (flagella) or passively, since as a warmly laid egg cools down, it creates a suction pressure. Sizes of these pores are 3-12 times as large as the length of large bacteria (Ghazikhanian, 1980). Sources of the above mentioned infections include: feedstuffs, wild birds, rodents, reptiles, man, pets, flies, soil and air. Other environmental sources are windows, roots, floors, drinkers, feeders, cages, faeces, egg trays and feathers (Bale *et al.,* 2002).

An egg can pick up the contaminations while passing through the cloaca, or when laid in a dirty nest, or laid on the ground or rubbed with a dirty towel or contaminated steel wool used to clean excessively dirty eggs on the farm and or by other means of mishandling the egg (Ghazikhanian, 1980). These can all result in a contaminated egg which might or might not be obvious as one looks at the egg.

1.1 RESEARCH PROBLEM

The poultry industry is faced with the challenges of meeting increasing demands for white meat in place of red meat. Red meat is known to contain more cholesterol thus

predisposing people to heart problems. There is an increasing need for raising the quality and quantity of the chicks being produced and thus high performance for increased general yield of poultry and poultry products. To meet this challenge the "chick factory" (hatchery) has to give maximum output. This output has been affected by the death in the shell of embryos when incubated. This study, therefore, was undertaken to determine the likely causes of these losses with particular reference to bacterial agents.

At the various hatcheries, preliminary studies revealed that a number of disinfectants were being used, so there is the possibility of development of resistance to the disinfectants already in use, a need to just increase the concentration or perhaps the contact time.

1.2 AIMS AND OBJECTIVES

The objectives of this study are:

1. To isolate and identify bacteria associated with dead-in-shell chicken embryos.
2. To determine the susceptibility of the isolates to commonly used disinfectants with the aim of finding the most effective disinfectant(s) and thus recommending the best disinfectant.

1.3 JUSTIFICATION

1. Poultry and poultry products contribute about 30% of the protein consumption of Nigerians therefore the importance of protecting and improving such a sector can not be overemphasized.
2. Dead-in-shell chicken embryos constitute one of the major factors accounting for low hatchability of incubated eggs. Hatchery losses associated with embryonic mortality may affect the poultry industry significantly if not controlled.
3. There is paucity of information on the etiology of dead-in-shell chicken embryos and disinfectant effects on microbes particularly in Northern Nigeria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 GENERAL REVIEW ON DEAD-IN-SHELL EMBRYOS

Death in the shell results when a chick starts to breathe but dies before it can escape from the egg. The chick does not have to pip the shell to start breathing; it will begin to breathe using air in the air sac. This is the most common problem among incubation complications. Death in the shell can be caused by several factors such as wrong temperature, wrong humidity, wrong turning and of course infectious diseases (Davis, 1999).

A careful examination of egg samples is useful to provide quality assurance or to diagnose hatching problems. Even during problem-free periods, chicken, pheasants, partridge and quail eggs should be candled after 5-7 days of incubation; while turkeys, ducks and goose eggs should be candled after 8-10 days of incubation (Ernst *et al*., 2005). A normal living embryo shows clearly defined blood vessels with no hemorrhagic areas evident. Some body movements occur when stimulated by the candling light. Generally "health" appearance is obvious (Ernst *et al*., 2005). Periodic candling of the eggs helps to determine when, in the incubation period, death occurs. The blood vessels that are visible from about the 3rd to the 4th day should slowly increase in density. One should then be

able to see the small embryo slowly increasing in size. The egg darkens as the embryo/chick takes up more and more of the available space. A large increase in the air sac size or a change in shell colour to dull gray suggests a dead embryo. A stop in the increasing opacity of the developing egg or cloudiness suggests the death of embryos (Heesen and Wilson, 1999).

If the eggs show no sign of developing, it is probably infertile. On the other hand, it might be due to excessive chilling in the first few days of incubation. It is hard to recognize any development until the third or the fourth day of incubation.

2.1.1 Early Death

During candling, the appearance of a thin blood ring is recognizable in an otherwise clear egg. This is caused by the incubation temperature being too high or the egg being chilled. A stored egg that has been left for too long can also cause early death (Davis, 1999).

2.1.2 Late death

Late death occurs when the egg dies after eleven days (40 %) of incubation. The two most likely causes of this are likely wrong temperature and incorrect turning. Another reason could be infective bacteria which can cause death at any time of incubation. Heavy inbreeding has also been found to cause death in the shell (Davis, 1999). Also, some characteristics in poultry such as tufts in Araucana chickens and the short leg gene

in Japanese bantams are connected to lethal genes. When both parents carry these characteristics (short leg is bred for short leg, for example), approximately 25 % of the fertile eggs will result in late deaths in the shell (Davis, 1999).

A very important cause of dead-in-shell embryos is the nutrition of the parent birds (Gallagher, 1997). If the parental diet is lacking in any of the essential elements (i.e. proteins, fats, vitamins, minerals, water), the developing egg and embryo will suffer and death may result at any point of incubation depending on the type of deficiency (Gallagher,1997).

The solution to the problem is sorted out by the process of elimination. If the temperature is kept in check, then you know it is not the cause. Very early hatching could mean too high temperature while late hatching is probably too low temperature. If the chick has difficulty pushing out of the shell after it has chipped all the way around the egg and the chick is sticky feeling, the most common problem is simply that the hatcher is not humid enough. The difficulty of the chick to rotate in the shell may be attributable to drying out. This is what causes it to be sticky. During hatching, if the chick seems too big for the shell, then the humidity was too high during the whole incubation period (Davis, 1999).

The impact of incubation temperature on egg weight loss, embryonic mortality, incubation period, hatchability and chick weight in 394 ostrich (*Struthio camelius*) eggs was studied from three farms in Texas. Results showed that incubation of fertile eggs at

36.5 ^{0}c from the 3 farms increased hatchability in comparison with other treatments (Hassan *et al., 2*004).

Table 2.0 below shows the different incubation periods for different species of birds where the pigeon recorded the lowest with 17 days, this was followed by the chicken with 21 days. Turkey and duck had 28 days each, while the goose had 28 – 34 days and peafowl 28 – 30 days. There were no much variations in the temperature and humidity requirements within species.

Table 2.0: Incubation periods and incubation operation characteristics

Item	Chicken	Turkey	Duck	Goose	Pigeon	Peafowl
Incubation period (days)	21	28	28	28 – 34	17	28 – 30
Temperature (^{0}F dry-bulb)	100	99	100	99	100	99
Humidity (^{o}F, wet-bulb)	85 – 87	84 – 46	85 – 86	86 – 88	83 – 87	84 – 86
No egg turning after	18th day	25th day	31st day	25th day	22nd day	25th day

(Haynes and Smith, 2003)

Many different bacteria can kill the chick in the shell. The egg shell is a very porous membrane and is full of small holes that allow moisture as well as bacteria to pass through. It is through this porous membrane that the embryo exchanges gases and acquires needed moisture (Heesen and Wilson, 1999). Most deaths caused by bacteria and viruses will occur in the first half of incubation. Bacterial problems resulting in embryonic death are uncommon in dry nests with clean eggs.

2.2 REVIEWS ON SOME BACTERIA ASSOCIATED WITH DEAD-IN-SHELL CHICKEN EMBRYOS

2.2.1 *MYCOPLASMA SP*

Mycoplasmas are small procaryotic organisms lacking a cell wall. Numerous species of avian mycoplasmas have been described and characterized (Jordan, 1983). Four avian mycoplasmas are commonly recognized as poultry pathogens: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma meleagridis* and *Mycoplasma iowae*. *M. gallisepticum* is a cause of respiratory disease and egg production drops in chickens, turkeys and other avian species. It is the most economically important of the avian mycoplasmas (Ley and Yoder, 1997).

Severe air sacculitis, coughing, rales and poor growth may be observed in broilers or turkeys infected with *M. gallisepticum*, with heavy contaminations from air sacculitis at processing. Swollen sinuses are commonly seen in turkeys. *M. synoviae* causes lesions

of synovitis in chicks, turkeys and other species and is also involved in respiratory disease (Kleven *et al.*, 1997). *Mycoplasma meleagridis*, found only in turkeys, causes respiratory disease in young turkeys and is involved in stunting, poor feathering and leg problems (Yamamoto and Ghazikhanian, 1997). *Mycoplasma iowae* is a cause of late embryo mortality and reduced hatchability in turkeys (Bradbury, 1983). *M. gallisepticum*, *M. synoviae*, *M. iowae* and *M. maleagridis* are egg-transmitted and may be spread laterally by direct or indirect contact. The mode of egg transmission of *M. maleagridis* is venereal; infection of the phallus of the male results in contaminated semen, which contaminates the oviduct of the female (David *et al.*, 1998).

For culture of embryonating eggs for *M. gallisepticum*, *M. synoviae* or *M. meleagridis*, samples of yolk that contain a portion of the yolk membrane should be included. For *M. iowae*, culture of the esophagus of pipped embryos or 1-day-old poults should be obtained (David *et al., 1998).*

Tiny, smooth, 0.1 – 1 mm in diameter with dense, elevated centred colonies are suggestive of *Mycoplasma species*. Although biochemical reactions may be useful at times, identification of isolates is by serological methods, using hyperimmune serum prepared in rabbits (Senterifit, 1983).

A commonly used method for *Mycoplasma spp* identification is growth inhibition (Clyde, 1983). Where the surface of agar plates is evenly coated with a moderate innoculum (usually a 1:10 to 1:1000 dilution of a broth culture) and filter paper discs impregnated

with specific hyperimmune serum are placed on the surface. After incubation at 37 ^{0}C for several days, a zone of inhibition of colony formation surrounding the disc indicates a positive test.

In many laboratories, direct immuno florescence is the preferred method of identification of Mycoplasmas of avian origin (Gardella *et al.,* 1983).

Polymerase chain reaction (PCR) can be used to identify the species of avian mycoplasma isolates. The PCR product is cut with one or more restriction enzymes and the pattern could be used to identify the species (Fan *et al.,* 1995).

2.2.2 *SALMONELLA SP*

Avian salmonella infections are important as the cause of clinical disease in poultry and sources of food-borne transmission of diseases to humans. Host-adapted salmonellae are responsible for pullorum disease (*Salmonella pullorum*) and fowl typhoid (*Salmonella gallinarum*), severe systemic diseases that have become relatively rare in countries with testing and eradication programs (Pomeroy and Nagaraja, 1991; Snoeyenbos, 1991). Infections with non-host-adapted (paratyphoid) salmonellae are common in all types of birds (Nagaraja *et al*., 1991). Paratyphoid salmonellae can also cause human illness, while food-borne salmonellosis can lead to severe economic losses to poultry producers as a result of regulatory actions, market restrictions or reduced consumption of poultry products. Most salmonellae are transmitted horizontally, but some are transmitted

vertically and often produce highly persistent flock infections. Pullorum disease primarily affects chickens, turkeys and other fowls during the first few weeks of life. Fowl typhoid is also observed in mature poultry.

Histopathologic lesions associated with avian salmonellosis include filbrino-purulent perihepatitis and pericarditis; purulent synovitis, focal fibrinoid necrosis, lymphocytic infiltrations and small granulomae in various visceral organs; and necrosis of the pericardium, the pleuroperitoneum, and the serosa of the intestinal tract and mesentery (Nagaraja *et al.*, 1991; Pomeroy and Nagaraja, 1991).

It is critical that strict bio-security measures be followed anytime individuals visit a farm or a hatchery. Samples taken from the internal organs of birds with systemic salmonellosis are typically free of competing bacteria. In such cases, isolation is usually relatively simple and may only require direct plating on non-selective media. Liver, spleen, heart, heart blood, ovary or yolk sac, synoviae, eye and brain (if torticolis is observed) are excellent sources for isolation. Sterile cotton swabs are preferred to wire inoculating loops, except for small organs in young birds, because swabs transfer more tissue. Cloacal swabs have been shown to be an unreliable means of detecting salmonella from birds (Fanelli *et al.*, 1971). Generally, salmonellae are excreted intermittently, and often in low numbers.

Egg culture

The surface of intact eggs can be sampled by immersion in 30 – 50 ml of selective enrichment broth in plastic bag, with soaking or manual rubbing for at least 30 seconds before removal of the egg. After disinfection of egg shells (generally in 70 % ethanol or 2 % tincture of iodine), egg contents can be cultured at a standard 1:10 ratio in selective or non-selective media. The content of 10 – 30 eggs are often pooled for culturing because of the usual low incidence and level of salmonella contamination.

Environmental monitoring

Environmental monitoring or surveillance has become a useful method for predicting potential infection or colonization of flocks with the paratyphoid salmonellae. Although environmental monitoring may show good predictive evidence of salmonella in flock, it is an indirect indicator and flocks should not be diagnosed as infected based solely on environmental sampling. These samples could be taken from the floors as drag swabs, also from floor litters, nest boxes, dust, cage housing, hatcheries (open air), feed and feed ingredients, etc. (Douglas *et al.*, 1998)

Rapid salmonellae detection techniques

A number of rapid tests have been approved for detecting salmonellae in various samples. Typically these have been approved based on comparison to Association of Official Analytical Chemists (AOAC) and International Culture Procedures (AOAC, 1996). The rapid tests have several advantages over conventional culture, such as obtaining results in

less than 48 hours, being less labour intensive, and having the possibility of automation. For many sample types, especially clinical, processing plants and food samples, this rapid test appears to be very effective. However, some sensitivity and specificity problems are apparent with feed and environmental samples.

A variety of rapid detection systems are commercially available including antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) systems, DNA probes, polymerase chain reaction systems, immunodiffusion, immunofluorescence, magnetic bead systems, bioluminescence and other novel applications. The membrane filtration technique (Mallinson *et al.*, 1995) appears to combine rapid detection of salmonellae with quantitative results. As technology advances, the sensitivity and specificity of these systems will surely increase and make these systems more useful.

Basic Identification Screening Media

The combined use of triple Sugar Iron (TSI) and Lysin iron (LI) agar slants is generally sufficient for presumptive identification of most *salmonellae*-suspect colonies. On TSI, most *Salmonellae* produce an alkaline (red) slant and acid (yellow) butt with gas bubbles in the agar and a blackening due to H_2S production that often obscures the acid reaction in the butt of the tube. *Salmonella gallinarum* does not form gas in TSI, whereas *S. pullorum* may show weak gas production apart from being motile. Both of these S*almonellae* may or may not show H_2S production. Some paratyphoid strains (10 – 15 %) may be H_2S-negative in this medium. On LI agar, *Salmonellae* will show lysine

decarboxylation, with a deeper purple (alkaline) slant and alkaline or neutral butt with slight blackening due to H_2S production.

Further biochemical tests may be necessary to confirm an isolate as *Salmonella*. Key tests for differentiating *Salmonellae* from other bacteria include those for urea (*Proteus* and most *Providencia* and *Morganella* are urease-positive), betagalatocidase (salmonellae with the exception of *S. arizonae*, are negative whereas *C. freundii* and other coliforms are positive and include (*Escherichia coli* and *E. tarda* produce indole, *Salmonellae* do not) (Nagaraja, *et al*., 1991).

Serological testing of poultry flocks for Salmonellae

Several serological tests have been developed for detecting antibodies to salmonellae. The commonly used tests for pullorum-typhoid include the whole blood plate (WBP) (not approved for use in turkeys), rapid serum plate, standard macroscopic tube agglutination, microagglutination and microantiglobulin tests (Nagaraja *et al*., 1991; Snoeyenbos, 1991 and USDA, 1996). Agglutination tests have also been used for detecting antibodies to various paratyphoid salmonellae, especially *S. typhimurium*, *S. arizonae* and S. *enteritidis*. These tests have met with varying degrees of success due to sensitivity and specificity problems and have not found widespread commercial application. Because *S. enteritidis* and *S. pullorum* share somatic antigens, pullorum-typhoid antigen preparations have sometimes been applied for detecting antibodies to *S. enteritidis* (Snoeyembos, 1991).

Enzyme-linked immunosorbent assays (ELISAs) have been developed and used mostly in detecting antibodies to *S. enteritidis* particularly in Europe but concerns about their specificity still persist (Snoeyembos, 1991).

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S. gallinarum and *S. enteritidis* possess the same O antigen 1,9,12 but *S. enteritidis* possesses flagella antigens, whereas *S. gallinarum* does not (Shivaprasad, 1997). It is known that the same O antigen structure has cross immune response, so various types of salmonellae have been tested to immune chickens and protect against the shedding of these organisms (Anderson, 1995; Collins *et al*., 1996). Generally, *S. gallinarum, S. pullorum* and *S. enteritidis* share a common immunodominant surface antigen (O9). The competitive exclusion against *salmonella gallinarum* of *S. enteritidis*-infected chickens was reported to have *S. enteritidis* preinfected poultry being protected against colonization with *S. gallinarum* (Young-Ju, *et al*., 2001).

2.2.3 ESCHERICHIA COLI

E. coli is the causative agent of colibacillosis in poultry which is a common systemic infection. The disease is economically important to poultry production world wide. For *E. coli* infections to become clinically apparent, adverse environmental factors or other infectious agents are usually required.

The clinical signs of colibacillosis are non-specific and vary with the age of bird, duration of infection, organs involved, and concurrent disease conditions. In young (4-to-8-wk-

old) broilers and poults dying of acute septicemia, death is preceded by a brief period of anorexia, inactivity and somnolence. At necropsy, lesions are sparse except for swollen, dark-coloured liver and spleen and increased fluid in all body cavities. Birds surviving the septicemia phase of the disease are unthrifty and develop subacute fibrinnopurulent airsacculitis, pericarditis, perihepatitis, and lymphocyte depletion in the bursa and thymus. Airsacculitis, a classic lesion of colibacillosis, occurs following respiratory exposure to large numbers of *E. coli* but also occurs as a sequel to bacteremia. Other less common lesions are arthritis, osteomyelitis, salpingitis and pneumonia (Arp, 1982; Gross, 1991).

Sample Collection

Only internal organs or blood, not faeces or intestines are useful samples. Because normal intestinal *E. coli* flora readily invade other tissues after death, specimens from fresh carcasses are preferable. When acute colisepticemia is suspected, heart blood and liver should be sampled aseptically, (Lee and Arp, 1998).

Identification on laboratory media

On MacConkey's agar, most *E. coli* and some Enterobacter isolates produce 1-2 mm diameter pink colonies (lactose fermenting) whereas *Klebsiella spp* and *Enterobacter aerogenes* form large mucoid non-lactose fermenting colonies (Quinn *et al.,* 1994*).* On tergitol 7, *E. coli* produces 1-2 mm diameter yellow colonies with yellow zones. Blood

samples should be diluted 1:10 in brain-heart infusion broth then inoculated directly onto MacConkey's or tergitol agar plates. If primary cultures reveal large number of a predominant colony type suggestive of *E. coli*, pick several of these colonies from MacConkey's agar (or tergitol 7) and use them to inoculate triple sugar iron agar, sulfide-indole–motility (SIM) medium and blood agar plates. A Gram stain and the oxidase reaction can be performed on colonies from blood agar plates. *Escherichia coli*, Enterobacter and Klebsiella are oxidase-negative, gram-negative rods. On TSI (or K.I.) agars, these organisms produce acid and gas but not H_2S. On SIM medium *E. coli* is positive for indole reaction for positive or negative for motility and negative for H_2S whereas Enterobacter and Klebsiella are usually indole negative (Lee and Arp, 1998).

No molecular identification methods are in current use for the rapid detection of *E. coli* isolates that cause colibacillosis. Isolates of public health importance can be identified by polymerase chain reaction using primers specific for the toxins involved in the human enteric disease (Jones and Bej., 1994).

2.2.4 *STAPHYLOCOCCUS SP*

In recent years, staphylococcosis has become one of the most important bacterial diseases of poultry. This has resulted from the common practice of rearing chickens and turkeys to a greater weight because of product demands for further reprocessing. Chickens and turkeys also have been highly selected for their ability to achieve rapid growth. Such

selection has added to stress by creating various leg problems that predispose poultry to the development of staphylococcosis (Jensen and Skeeles, 1998).

Historically, staphylococcosis has been a significant problem because of the ubiquitous nature of the bacterium in the poultry farm environment. When "the door is left open" S*taphylococcus aureus* has been able to cause disease. The portal of entry is the open navel of newly hatched chicks from contaminated hatching eggs. The sources of infection progress to wounds associated with debeaking, detoeing, toe scratches, wood splinters, and sharp metal edges with increasing opportunities for injury as birds rapidly gain weight.

Numerous S*taphylococcus spp* are found in the poultry environment, but *S. aureus* is the most frequent disease-causing agent. *Staphylococci* are normal inhabitants of skin and mucous membranes and readily reside in all the environments where poultry are hatched, reared and even processed. *S. aureus* definitely can cause disease, but, along with other S*taphylococcus spp* especially *S. epidermitidis* is thought to suppress other possible pathogens through interference or competitive exclusion. Other *Staphylococcus spp* that have been isolated from lesions or possible disease situations include *S. hyicus* (fibrins heterophilic blepharitis) (Cheville *et al.,* 1988).

Identification of Staphylococci

On agar cultures, staphylococci produce 1-3 mm diameter, circular, opaque, smooth, raised colonies in 18-24 hr. *S. aureus* isolates are haemolytic on blood agar, and colonies are often pigmented in various yellowish hues. On mannitol salt agar, *S. aureus* colonies are surrounded by a yellowish halo. Typical colonies are examined microscopically to confirm that they are composed of Gram-positive *cocci* in bunches. The coagulase tests, where *S. aureus* is coagulase positive is sufficient in most cases to differentiate it from other staphylococci which are coagulase negative. Commercially available desiccated rabbit plasma containing either citrate or ethylene diamine-tetracetic acid is commonly used for the coagulase test. A rapid slide coagulase test can be performed by making a heavy, smooth suspension of a colony in one drop of reconstituted plasma on a glass slide and observing for clumping in 10 sec, this detects the clumping factor (cell-bound coagulase). A tube coagulase test is more accurate than the rapid slide test and detects extracellular coagulase. The tube test uses 0.5 ml of reconstituted plasma that is thoroughly mixed with either 0.1 ml of an overnight broth culture or a portion of a well isolated colony. The mixture is incubated for 4 hours at 37 ^{0}c, and any degree of clotting represents a positive test.

Various species of coagulase-negative staphylococci can be isolated from poultry. As yet, none of these species has been incriminated as a cause of major diseases in poultry. Serologic tests are not used for the diagnosis of staphylococcosis.

2.3 REVIEWS ON EFFICACY OF DISINFECTANTS ON ISOLATES FROM THE HATCHERIES

In today's commercial poultry industry, there is an increased demand for maximum yield with minimal input. Producers desire low chick mortality, high chick quality, and overall high chicken performance. From the time of oviposition, an egg shell may become dirty or contaminated with organic material or bacteria. Haines in 1988 found that bacteria that are normal flora to the hen's alimentary tract, skin and feathers are in large numbers on the shells of eggs. Microorganisms present on the outer surface of an egg have the potential to penetrate the egg-shell, decreasing hatchability and affecting chick quality resulting in inadequate passive antibody absorption, poor flock livability and chronic infection in growing birds (Sarma *et al*., 1985, Willinghan, 1995). Commercial chick hatcheries provide an ideal environment for the hatching of fertile eggs. The moisture and warm temperatures characteristic of the hatchery environment also provide an excellent

opportunity for microbial proliferation. As a result, these bacteria may infect newly hatched chicks (Mostellar and Bishop, 1993).

Moulding and Wilson, (1988) concluded that part of good sanitation practices often includes the treatment of eggs before and during incubation. Currently, most sanitation programmes require the use of some type of disinfectant. Control of microbial populations present on the shell and in the hatcheries requires a disinfectant that is effective in decreasing contamination but not toxic to the developing embryos.

Willinghan *et al. (1996)* investigated bacterial resistance to hatchery disinfectants where three commercial chicken hatcheries were sampled for environmental bacteria. The bacteria isolated were tested for resistance to commercial preparations of quaternary ammonia, phenolic and glutaraldehyde liquid disinfectants. Approximately 8 % of the isolates from two of three hatcheries were resistant to disinfectant concentrations at and above the manufacturers recommended dilution and time of exposure. Resistant bacteria included *Serratia marcescens*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus badius*, *Enterococcus faecalis*, *Enterococcus faecium*, *Pseudomonas stutzeri* and *Enterobacter agglomerans*.

In 2002, a study was carried out on the efficacy testing of commercial disinfectants against foodborne pathogenic and spoilage microbes in biofilm- constructs. Here, chilled poloxamer solutions (30 % w/v) were made up in a tryptone soy broth and inoculated with stationary-phase cultures of 14 food-borne spoilage microbes, including

Pseudomonas, *Baccillus*, *Staphylococcus*, *Micrococcus*, *Enterobacteria* and a yeast as well as pathogens test-strains, including *Listeria* and *Salmonella*. Incubated poloxamer gels and their discs were tranfered to solutions of commercial disinfectants, hydrogen peroxide with peracetic acid or silver ions, sodium hypochlorite, or alcohols with and without additives. After 5 minutes at 25 ^{0}c all formulations effected a >5-log kill of planktonic challenges within 5 min. Patterns of susceptibility varied as a function of the organism, disinfectant type and concentrations (Wirtanen *et al.*, 2001).

The *in vitro* efficacy of a quaternary ammonia disinfectant (Biosentry 904) and/or ethylenediamine tetracetic acid – tris (EDTA-Tris) was tested against 5 field commercial broiler hatchery isolates of *Pseudomonas aeruginosa* at 10^3, 10^6 and 10^9 colony forming units (CFU)/ml. At 10^9 CFU/ml concentration, most compounds fail to achieve a total kill with a contact time of 15 min. When tested at bacterial concentrations of 10^3 CFU/ml, the combination of EDTA – Tris mixed at a 1:1 ratio with Biocentry 904 killed the bacteria upon initial contact ($\leq$ 0.05 min). This disinfectant mixture exhibited antagonistic, indifferent, or synergetic effects when exposed to different bacterial isolates at a concentration of 10^6 CFU/ml (Walker *et al.*, 2002)

Luppens *et al* latter in the same year, 2002, studied the effect of the growth phase of *Staphylococcus aureus* on the resistance to the surface-active agents benzalkonium chloride and deodecylbenzyl sulfonic acid and the oxidizing agents sodium hypochloride and hydrogen peroxide. The resistance of cells in different growth phases was compared to those of solid medium cells grown according to the European phase 1 suspension tests.

Using cells from different growth phases ($\pm$3 x 10^7 CFU/ml), it was found that decline phase cells were the most resistant cells. However, the decline-phase cell suspension contained more than 90 % dead cells. A 10-fold-diluted suspension with a total concentration of cell equal to that of the other cell suspensions still revealed decline-phase cells to be generally the most resistant cell type. However, the resistance was drastically reduced, indicating that the large proportion of dead cells provided significant protection to the viable decline phase cells. Hydrogen peroxide resistance could be partly explained by the high catalase activity in the dead-cell fraction. Exponential-phase cells were less resistant than decline-phase cells, and surprisingly, stationary phase cells were the least resistant of the three. These findings showed that the solid medium cells currently used in disinfectant tests are not the most resistant cells that can be used. Earlier in the year Sander *et al.*, (2002) investigated the resistance of bacteria from commercial poultry sources to commercial disinfectants. Here, they considered that the methods available in published literature to detect the efficacy of disinfectants are labour intensive and do not consider how bacteria behave when adhered to a solid surface. They used a then recently developed technique, which utilizes the actual surfaces on which the disinfectant is to be applied, to evaluate the degree of resistance to four commercially available disinfectants of 17 bacterial isolates from poultry hatcheries. They found that bacterial isolates within the same genus and species have different sensitivities to the same disinfectant. In addition, disinfectants with similar but not identical chemical formulations have different efficacies against the same bacteria (Sander, *et al., 2002)*.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Sources of Samples

The study area is Kaduna State which is located in the North-western region of Nigeria. Four major functional commercial hatcheries were identified in this area from which eggs sampled for this work were obtained.

3.2 Study Design

A cross sectional study was carried out involving information from hatchery managers and attendants using questionnaires. Verbal interactions as well as personal observations, including the routine practices carried out in the various hatcheries with the sanitary conditions of the eggs included in the questionnaires.

3.3 Sampling method and culturing

On day 18 of incubation, eggs whose embryos had died, as detected during candling, were removed. These were sampled as well as those that failed to hatch after day 21 of

incubation. Sampling of the eggs was done through normal random selection. A total of 600 samples were collected from all the hatcheries. (Table 4.2).

The Kaduna-based hatcheries were each visited 2 times while those in Zaria were visited 3 times owing to proximity. Eggs were collected in containers and taken to the laboratory with minimum delay. The egg shells were washed and disinfected properly to avoid surface and extraneous contamination. The eggs were then allowed to dry after which the shells were broken using a sterile pair of scissors after mopping the surface with 70 % ethanol and the content of each egg was taken by the use of a sterile cotton swab and cultured for bacteria. Culturing was done on Blood, MacConkey and brilliant green agar. Incubation was done aerobically at 37 ^{0}C for 24 hours. Plates were further incubated for 48 or even 72 hours where growths were not too evident.

3.4 Biochemical characterization of isolates

Identification of growths on solid media using biochemical characterization was done following standard procedures of Cowan, (1974). Colonies on MacConkey that were lactose fermenters were placed on eosine methylene blue to find out if they were *E. coli* by exhibiting the characteristic greenish metallic sheen. Preliminary identification of these organisms includes colonial morphologies and microscopic appearance.

Biochemical tests carried out on the isolates include oxidative-fermentative medium, triple sugar iron agar, nitrate broth, indole reaction, urease test, citrate agar, motility medium, methyl red and Voges Proskauer medium.

3.5 Sugar fermentation

Preparation of sugars for fermentation tests were according to standard procedures of Cheesbrough, (1991)

Sugars used for the fermentation tests include glucose, lactose, maltose, rhamnose, sucrose, xylose, sorbitol, galactose, rafinose and mannitol. Reactions of the sugars were read and compared with the standards in Cowan, (1974).

3.6 Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and the kill-time of disinfectants on the isolates.

The disinfectants used were Z-germicide® (phenol + cresol), purit ® (Chlorhexidine gluconate B.P. 0.3 % w/v, cetrimide B.P. 3.0 % w/v) and Carcil® (Alkyl-dimethyl-benzyl ammonium chloride). The working concentrations of 1 %, 0.03 % and 10 % respectively of these disinfectants were made by diluting them in sterile distilled water. These working solutions were then subjected to a double serial dilution in 11 test tubes each

containing 3 ml of double-strength nutrient broth. With these dilutions, the recommended concentrations by the manufacturers fell in the middle i.e. on the 6^{th} test tube each. The double strength nutrient broth was to make room for the serial dilutions. Aliquots (0.1 ml) of 10^9 CFU/ml of bacterial suspension was added to each test tube and incubated at 37 ^{0}c for 24 hours after which the minimum inhibitory concentration (MIC) was read as the concentration equivalent to the test tube that showed visibly complete clearance. The contents of 3 consecutive test tubes that showed clearance were plated on solid nutrient agar and incubated at 37 ^{0}c for 24 hours, then the 1^{st} plate that showed no growth completely was taken as the minimum bactericidal concentration (MBC). The first tube had the highest concentration of the disinfectants and thus served as the negative control; while the last tube which had the lowest concentration of the disinfectants served as the positive control in each case.

To obtain the kill-time, a dilution of the disinfectant using normal saline was made to the MBC equivalence that was obtained. Aliquots (0.1 ml) of 10^9 CFU/ml of the bacterial suspension was inoculated into this concentration and then plating of the content using a loop-full at a time, was done unto fresh nutrient agar plates at time intervals of 10 sec, 30 sec, 1 min, 2 min, 5 min and 10 min. This was done to determine the contact time of the disinfectant with the bacterium needed in order for the disinfectant to act on the bacterium by killing it. The various plates were again incubated and results read after 24 and 48 hours at 37 ^{0}c. The first plates that yielded no growth completely were recorded.

CHAPTER FOUR

4.0 RESULTS

Table 4.1 gives a summary of information obtained from the hatcheries through questionnaires served the hatchery workers. On the number of eggs that were often set on a weekly basis, Hatchery D recorded an average of 30 % loss as the highest while hatchery A recorded 8 % as the lowest. The two other hatcheries (B and C) had almost same value averaging 16.5 % loss. These losses were mainly as a result of eggs failing to hatch because the embryos died in the shells.

One hundred and thirteen Gram +ve and Gram –ve bacterial isolates were obtained from this study. 35 isolates were obtained on the 18^{th} day of incubation; while 78 were at the termination of incubation. The distribution of the isolates is reflected in Table 4.2. Table 4.3 shows the frequency of bacterial isolation in the four hatcheries and at the two sampling periods during incubation. Thirty two (28.3 %) isolates were obtained from dead-in-shell embryos collected from the hatcheries on the 18^{th} day of incubation. The remaining 81 (71.7 %) isolates came from samples collected on the day of hatching. Of the 113 isolates, 89 were Gram-negative bacteria comprising of 62 *Escherichia coli*, 21 *Proteus sp* and 6 *Pseudomonas spp*. The remaining 24 isolates were Gram-positive bacteria made up of 11 S*taphylococcus aureus*, 8 other *Staphylococcus spp* and 5

Micrococcus spp. On the 18th day of incubation, there was a total of 32 isolates out of which 18 were *E. coli* making the highest

Table 4.1: Average number of eggs often set and number that failed to hatch

Hatchery	Av. No. of eggs Often set	Av. No. of eggs Often hatched	Av. No. of failed To hatch eggs	Av. % loss Recorded
A	5,000	4,000	4,00	8
B	12,000	10,000	2,000	16.7
C	3,000	2,500	500	16.3
D	10,000	7,000	3000	30

Table 4.2: **Distribution of isolates by sampling periods and the hatcheries**

Source of Samples	18th day of incubation		21st day of incubation		Total	
	Dead-in-shell Embryos (%)	Total No. Of isolates (%)	Failed to Hatch eggs (%)	Total No. Of isolates (%)	No. of Eggs	No. of Isolates
HatcheryA	70(35)	8(30.8)	130(65)	18(69.2)	200	26
HatcheryB	60(37.5)	9(29)	100(62.5)	22(71)	160	31
HatcheryC	54(40.3)	5(25)	80(59.7)	15(75)	134	20
HatcheryD	46(43.4)	13(36.1)	60(56.6)	23(63.9)	106	36
Total	230(38.3)	35(31)	370(61.7)	78(69)	600	113

Table 4.3: Bacterial Isolates and their distribution

Bacteria	Bacterial isolates on 18th day of incubation		Bacterial isolates on day of hatching		Total isolates (%)
	No isolated	% of sp total	No isolated	% of sp total	
Escherichia coli	21	33.9	41	66.1	62(54.9)
Proteus sp	5	23.8	16	76.2	21(18.6)
Pseudomonas sp	0	0	6	100	6(5.3)
Staph. aureus	5	45.5	6	54.5	11(9.7)
Staph. sp	1	12.5	7	87.5	8(7.1)
Micrococcus sp	3	60	2	40	5(4.4)
Total	35	31	78	69	113

frequency while *Pseudomonas spp* was lowest with no isolate at all. On day 21, *E. coli* was still the highest with 44 isolates while *Micrococcus spp was* the lowest with 2 isolates. The total number of isolates on the day of hatching was 81.

The distribution of the various bacterial isolates according to the hatcheries showed that hatchery D had the largest number (35 isolates) followed by hatchery B with 31 isolates then hatchery A with 31 and the least was hatchery C with only 20 isolates as presented on Table 4.4. Hatchery D also recorded the highest number of *E. coli* isolates (22) followed by hatchery B (20) then hatchery A (14) and hatchery C (6). The highest number of *Proteus spp* was recorded by hatchery A (10) while hatchery D had the least (2). Hatcheries A & B had no isolates of *Pseudomonas spp*, so also hatcheries A & C had no isolates of *Micrococcus spp*. Hatchery D still recorded the highest number of *Staphylococcus aureus* (4) followed by hatchery C (3) while hatcheries A & B had 2 isolates each.

The biochemical characteristics of the Gram positive bacterial isolates are shown on Table 4.5. These characteristics formed part of the criteria that were used for the identification of the bacteria. The *Micrococcus spp* were oxidase positive, were oxidizers on oxidative-fermentative medium (OF) and they did not ferment mannitol anaerobically. These served to distinguish them from members of the genus staphylococcus which were oxidase negative, fermenters on OF medium and they fermented mannitol anaerobically. *Staphylococcus aureus* was also distinguished using the hemolysin test where only *Staphylococcus aureus* was positive.

Table 4.4: Distribution of bacterial Isolates by the various hatcheries

Species of isolates	Hatchery A	Hatchery B	Hatchery C	Hatchery D	Total
Escherichia coli	14	20	6	22	62
Proteus sp	10	5	4	2	21
Pseudomonas sp	0	0	3	3	6
Staph. aureus	2	2	3	4	11
Staph. sp	1	2	4	1	8
Micrococcus sp	0	2	0	3	5
Total	27	31	20	35	113

Table 4.5: Some biochemical reactions of Gram positive bacteria isolated from dead-in-shell chicken embryos.

Tests carried out	Percentage giving positive reactions		
	Staph aureus	*Staph spp*	*Micrococcus spp*
Catalase	100	100	100
Oxidase	0	0	100
Coagulase	100	0	0
Oxidative-Fermentatve	100 F	100 F	100 O
Nitrate	100	100	100
Urease	100		
Mannitol fermentation aerobically	100	100	100
Mannitol fermentation anaerobically	100	100	0
Hemolysin test (sheep blood)	100	0	0

F = fermenter, O = oxidizer

The biochemical characteristics as well as sugar fermentation of the Gram negative isolates are presented on Table 4.6. These also served as part of the criteria that were used for the identification of the Gram negative bacteria. All the *E. coli* isolates grew on MacConkey agar, were fermenters on oxidative-fermentative medium, nitrate positive, produced indole, were motile, methyl red positive; they tested negative on urea, citrate and Voges Proskauer media and produced no hydrogen sulphide on TSI. There was a 100 % fermentation of all the sugars used except for sucrose which was fermented by only half the number of isolates. Also, sorbitol, rhamnose and raffinose were fermented by all the isolates except a few.

All the Proteus isolates grew on MacConkey with non-lactose fermenting characteristics. They were all fermenters on OF medium, were positive for nitrate, urea and methyl red, they all produced H_2S on TSI. Only 3 isolates were positive for indole and citrate. All the isolates fermented glucose, sucrose, galactose and mannitol. The *Pseudomonas spp* also grew on MacConkey and were oxidizers on OF medium. They all showed a positive reaction for indole test, citrate utilization, motility and catalase test; but negative reaction for nitrate urease and H_2S production. Non of the sugars was fermented by any of the Pseudomonas isolates

Table 4.6: Some biochemical properties of Gram-negative bacteria isolated from dead-in-shell chicken embryos

Tests	Isolates		
	E. coli	*Proteus spp*	*Pseudomonas spp*
Growth on MacConkey agar	62/0	21/0	6/0
Reaction in Oxidative-fermentative medium	F	F	0
Nitrate production	62/0	21/0	0/6
Indole production	62/0	3/18	6/0
Urease production	0/62	21/0	0/6
Citrate utilization	0/62	3/18	6/0
Motility	62/0	21/0	6/0
Catalase production	62/0	NT	6/0
Methyl Red test	62/0	21/0	NT
Voges Proskauer test	0/62	2/19	NT
Hydrogen sulphide production	0/62	21/0	0/6
Carbohydrate acid production			
Glucose	62/0	21/0	0/6
Sucrose	23/39	21/0	0/6
Lactose	62/0	0/21	0/6
Galactose	62/0	21/0	0/6
Maltose	62/0	20/1	0/6
Sorbitol	55/7	0/21	0/6
Rhamnose	58/4	10/11	0/6
Mannitol	62/0	21/0	0/6
Raffinose	54/6	6/15	0/6
Xylose	62/0	19/2	0/6

Number of strains giving positive reaction/number of strains giving negative reaction
NT = Not tested, F = Fermenter, O = Oxidizer

Table 4.7 shows the results of Z-germicide® indicating that *E. coli* required the least concentration of $63x10^{-5}$ (i.e. 1:1600) w/v of the disinfectant to kill the organisms and also at the least contact time of 5 minutes. *Proteus* and *Pseudomonas* on the other hand required higher concentrations of $12x10^{-4}$ (1:800) w/v for 10 minutes and $12x10^{-4}$ (1:800) w/v needing more than 10 minutes respectively.

Purit® produced a similar result of $26x10^{-5}$ (1:3840) m/v for 1 minute on *E. coli*, $21x10^{-4}$ (1:480) w/v for 5 minutes on *Proteus* and $42x10^{-4}$ (1:240) w/v for 5 minutes on *Pseudomonas* (Table 4.8). Carcil® yielded $31x10^{-4}$ (1:320) w/v for 10 seconds on *E. coli*, $31x10^{-4}$ (1:3200) w/v for 30 seconds on Proteus and $62x10^{-4}$ (1:160) w/v for 10 seconds on Pseudomonas (Table 4.9).

The three disinfectants used (Carcil®, Purit® and Z-germicide®) were found to be effective against the bacterial isolates but at different concentrations and contact time. Purit® was found to be most effective against *E. coli* since it required the lowest concentration of 0.00026% and 1 minute contact time to kill the bacteria. This was followed by Z-germicide® requiring 0.00063% for 5 minutes then Carcil® requiring 0.0031% and 10 seconds. On *Proteus*, Z-germicide® was the most effective requiring 0.0012% for 10 minutes followed by Purit® at 0.0021% for 5 minutes and then Carcil® at 0.0031% for 0.1 minute. In the same vein, Z-germicide® acting on *Pseudomonas* was found most effective since it acted at 0.0012% but for > 10 minutes and followed by Purit® at 0.0042% for 5 minutes then carcil® at 0.0062% for 10 seconds.

Table 4.7: MIC, MBC and kill-time of Z-germicide® on hatchery bacterial isolates

Isolate	MIC (w/v)	MBC (w/v)	Kill-time (Seconds)
Escherichia coli	1.6×10^{-4}	6.3×10^{-4}	300
Proteus sp	3.1×10^{-4}	1.2×10^{-3}	600
Pseudomonas sp	3.1×10^{-4}	1.2×10^{-3}	> 600

Table 4.8: MIC, MBC and kill-time of purit® on hatchery bacterial isolates

Isolate	MIC (w/v)	MBC (w/v)	Kill-time (Seconds)
Escherichia coli	$6.5x10^{-5}$	$2.6x10^{-4}$	60
Proteus sp	$5.2x10^{-4}$	$2.1x10^{-3}$	300
Pseudomonas sp	$1.0x10^{-3}$	$4.2x10^{-3}$	300

Table 4.9: MIC, MBC and kill-time of carcil® on hatchery bacterial isolates

Isolate	MIC (w/v)	MBC (w/v)	Kill-time (Seconds)
Escherichia coli	$7.8x10^{-4}$	$3.1x10^{-3}$	10
Proteus sp	$7.8x10^{-4}$	$3.1x10^{-3}$	30
Pseudomonas sp	$1.6x10^{-4}$	$6.2x10^{-3}$	10

The fermentation of xylose by the Gram negative organisms is shown on plate I, where the *E. coli* strains and *Proteus spp* except 1, fermented the sugar but the *Pseudomonas spp* didn't ferment it at all. Plate II shows the fermentation of galactose by all the *E. coli* strains. Plate III shows the determination of MIC of carcil® on *E. coli* where tube 7 was the first tube that showed cloudiness indicating the presence of bacterial growth and therefore was the MIC.

Plate IV shows the kill-time determination of Z-germicide® on *E. coli* where there was growth up to 30 sec of contact time. Plate V also shows that there was growth up to 2 min of contact time between the microbes and disinfectant. Plate VI shows that growth of *E. coli* was inhibited only after 10 min of contact with Z-germicide®.

Plate I: Fermentation of Xylose by the G-ve organisms
Tubes 1 – 4 *Pseudomonas sp*
Tubes 5 – 8 *Proteus sp*
Tubes 9 – 12 *E. coli* strains

Plate II: Fermentation of Galactose by the G-ve organisms

Galactose was fermented by all the *Proteus spp* *and all* *the* strains of *E. coli*

Tubes 1 – 4 *Proteus sp*

Tubes 5 – 8 *E. coli* strains

Plate III: MIC determination of carcil® on *E. coli*
MIC at tube 7
Tube 12 is negative control

Plate IV: Z-Germicide® acting on *E. coli* at 10 Sec & 30 Sec to determine kill-time. There was no effect even at 30 sec. of contact

Plate V: Z-Germicide® acting on *E. coli* at 1 Min. & 2 Min. to determine kill-time. There was no effect even at 2 min of contact

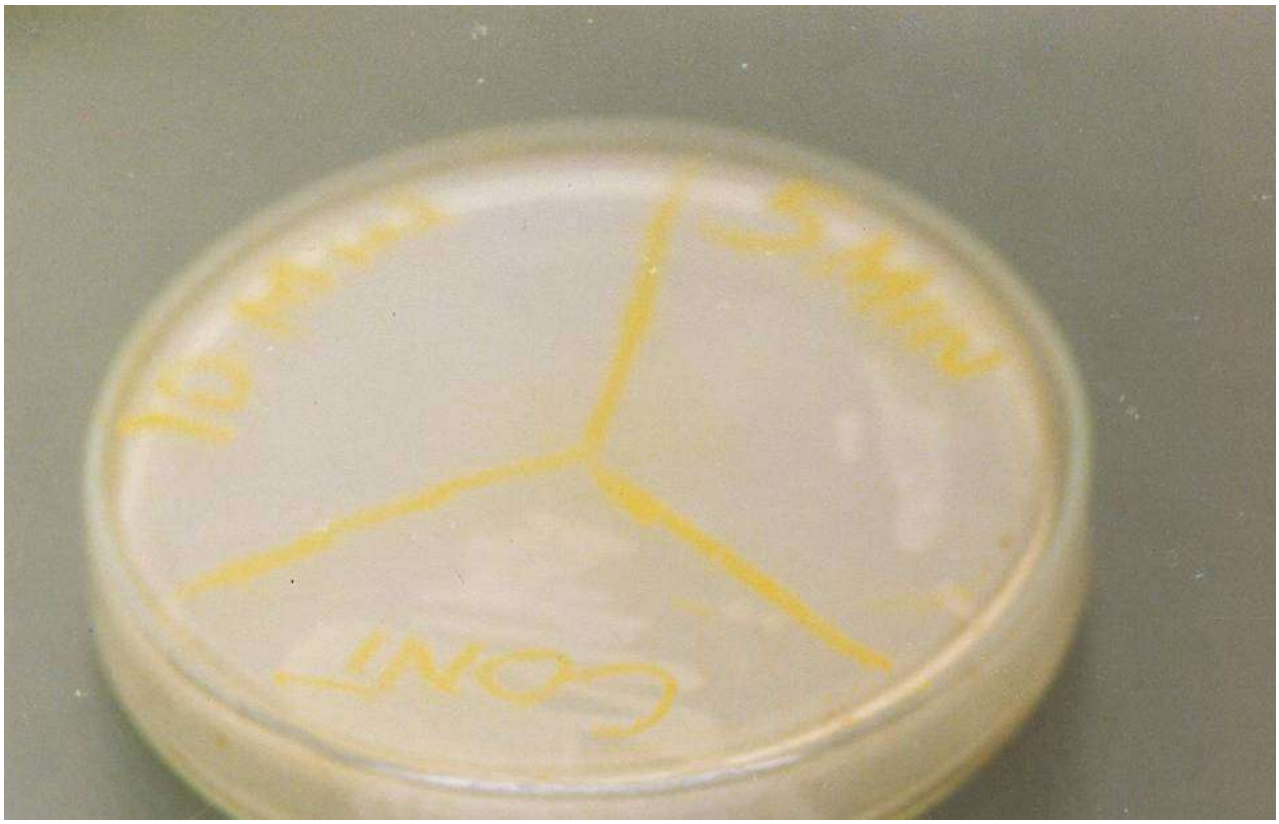

Plate VI: Z-Germicide® Acting on *E. coli* at 5 Min. & 10 Min. to determine kill-time. Effect only showed at 10 min contact time. Therefore was 60 Sec.

CHAPTER FIVE

5.0 DISCUSSION

Information gathered from the various hatchery workers through questionnaires that were administered to them and also information through personal observations of the most important conditions of hatching that the set eggs were subjected to, showed conformity with approved standards as practiced in other places (Haynes and Smith, 2003).

The results of this study have shown that the higher the frequency of isolation of bacteria the higher also the losses recorded by the hatcheries. Hatchery D which recorded the highest losses (30 %) had the highest frequency of isolation of bacteria (on day 18 and day 21 of incubation). Hatchery A had the lowest percentage loss (8 %) and this was supported by the low isolation rate of microbes. This has further confirmed that the bacteria isolated from the egg samples might have contributed to death in the shell of embryos.

The most predominant species of bacteria that was isolated in this study was *E. coli* which constituted 54.9 % of the total isolates. This was followed by *Proteus sp* with 78.6 %. These results conflict with those of Bruse and Johnson (1978) and Orajaka and Mohan (1985) where Bruce and Johnson found *Micrococcus* (34.6%) as the most predominant followed by *Enterobacteriae (*31.5 *%).* Orajaka and Mohan (1985) found *Staphylococcus*

aureus (27.18 %) as the most predominant followed closely by *E. coli* (25 %). These differences could be due to differences in environmental factors, personnels handling these eggs and other possible sources of contamination.

The results of tests shown by *S. aureus* in respect of the production of acid in mannitol aerobically and anaerobically; also the production of delta and alpha hemolysins agreed with results of Devriese and Ocding (1976) for the strains of *S. aureus* isolated from poultry. It is significant that none of the isolates produced beta hemolysin, and this is in agreement with the results obtained by other workers (Bajljosov, *et al.,* 1974 and Takeuchi and Suto, 1973).

The most likely mode of transmission of the organisms implicated in this study was horizontal and therefore the source of contamination was the environment. This deduction was reached due mainly to the mode of transmission of the organisms that were isolated. *E. coli* is known to cause yolk-sac infection in embryos and early chick embryonic mortality (Akinyemi *et al*., 1983). *Staphylococcus aureus* which inhabits the skin surface of the birds and their litters, has been reported to be capable of gaining access to the yolk sac through shell penetration and to cause embryonic death (Harry, 1957).

Several workers have commented on the probable role played by *Pseudomonas spp, Proteus spp* and *Micrococcus spp* in causing embryonic death and reduced hatchability (Akinyemi *et al*., 1983). However, their precise role in causing embryonic death has not

been very well understood; but it is known that *Pseudomonas* causes pathology to animal tissues by the release of enzymes and hemolysins especially where the immunity has been compromised (Jawetz, *et al.*, 1989).

The effects of disinfectants were determined only on G-ve bacteria because works by Mamman *et al*., (2005) showed that G-ve bacteria were generally more resistant to effects of disinfectants than G+ve bacteria probably due to their having a more complex cell wall.

The results of the effects of the disinfectants on the isolates were at variance with those of Mamman *et al.*, (2005) where carcil® was seen to kill *E. coli* isolates from clinical cases handled at the Ahmadu Bello University Veterinary Teaching Hospital at 0.039 % for 1 minute but on *Proteus*, concentration had to be raised to 0.625 % for 20 seconds. This was due probably to the fact that the isolates for the two different experiments were from different species of animals.

The most important bacteria recovered in this study were *E. coli* and *S. aureus*. Purit® (chorhexidine gluconate and cetrimidide) had proved to be the most effective of the three disinfectants used. It is, therefore, safe to recommend this to the hatcheries for use. It has been reported that chlorhexidine gluconate has been recommended as a water pan additive in incubators and borders because it has been found to be effective against *Aspergillus* fungus, Newscatle disease virus, it is not corrosive to equipments, readily available and with medium cost (Johnson, 1996). However its attending disadvantages are poor efficacy against other viruses and Gram -ve bacteria especially *Pseudomonas*, it

must be discarded and remixed daily, not effective in the presence of organic debris and not effective against bacterial spores (Johnson, 1996).

In efficiency, Purit® was closely followed by Z-germicide® which has phenol and cresol as its active ingredients. The most common disinfectant reported to be used in the various hatcheries investigated is Morigad® which has only phenol as its active ingredient. It is thus reasonable to say that the hatcheries could continue to use this disinfectant but at a little higher concentration than recommended by the manufacturers. The major disadvantages of the phenols are that they are toxic to tissues so users have to wear protective gloves, it may not work well in the presence of organic debris, not effective against bacterial spores, must be used with adequate ventilation, must be rinsed off surfaces before allowing birds contact with them (Johnson, 1996). Since we are dealing with eggs under incubation (a process which takes a long time to complete), the hatcheries therefore require a disinfectant that has a long and safe residual effect. The incorporation of cresol in Z-germicide® which is known to have long residual effect and also reported to be effective even in the presence of organic matter makes Z-germicide® a superior phenolic disinfectant than the morigad® used. The major disadvantage of cresol is the release of noxious gases.

In view of the disadvantages associated with the aforementioned disinfectants, an alternative was sought which is carcil®, a quaternary ammonium compound. The 'Quats' may be the most commonly used disinfectants for equipments like incubators and hatching trays because they are relatively non-irritating, non-corrosive, of low toxicity

and reasonably effective in the presence of organic matter. Since the incubator and its components should be cleared free of organic matter before applying a disinfectant, the quarts are a good choice (Haynes and Smith, 2003).

Results obtained and presented here showed that the disinfectants were effective at higher concentrations than the recommended levels by the manufacturers. This may be due partly to some level of resistance that may have been developed by the microbes against these disinfectants as a result of continuous use or inappropriate applications.

CHAPTER SIX

6.0 CONCLUSIONS

- Embryos could get infected with microorganisms at any point of the incubation process since microbes were isolated on both days 18 and 21 of incubation.

- Infection of embryos with pathogenic strains of bacteria could be responsible for death of embryos in the egg shell since in this study a good number of pathogenic strains of bacteria (*E. coli and Staph. aureus*) were recovered.

- Bacterial agents contaminating eggs leading to death of embryos may have developed some level of resistance to commonly used disinfectants since the effects of disinfectants to these microbes was seen only at concentrations above recommended values by the manufacturers.

6.1 RECOMMENDATIONS

- Appropriate sanitary measures are demanded of hatchery personnel while handling eggs through out the incubation process

- Hatchery managers are hereby advised to use purit®, Z-germicide® and carcil® routinely and rotationally in order to avoid the development of resistance to the disinfectants by microbes.

- These disinfectants recommended for use should be reconstituted to concentrations slightly higher than the manufacturers' recommended values. For purit®, use at a minimum concentration of 4.2 ppm ($4.2x10^{-3}$ m/v) for 10 min, Z-germicide®, at 1.2 ppm ($1.2x10^{-3}$ m/v) for 10 min and carcil® 6.2 ppm ($6.2x10^{-3}$ m/v) for 1 min.

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