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ISOLATION AND CHARACTERIZATION OF BACTERIA ASSOCIATED WITH YOLK SAC INFECTION (OMPHALITIS) IN CHICKS IN BISHOFTU POULTRY FARMS, ETHIOPIA.

MSc Thesis

BY

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: Kasech Melese entitled *Isolation and characterization of bacteria associated with yolk sac infection (omphalitis) in chicks in Bishoftu poultry farms, Ethiopia* and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Science in Veterinary Microbiology.

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STATEMENT OF AUTHOR

First, I declare that this thesis is my *bona fide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
CIRAD	Centre for International Research on Agricultural Development
CSA	Central Statistical Authority
DZARC	Debre Zeit Agricultural Research Center
EEC	Endothelial epithelial cells
FAO	Food and Agricultural Organization
GI	Gastrointestinal
Ig	Immunoglobulin
IgY	Purified yolk immunoglobulin
M.A.S.L	Meter above sea level
NMSA	National Meteorological Service Agency
VM	Veterinary medicine
YSI	Yolk sac infection
YSM	Yolk sac membrane

ABSTRACT

A study was conducted from November 2014 and may 2015 at Bishoftu Poultry Farm, Ethiopia, in(1-7) days old chicken obtained from three different hatcheries to isolate and identify bacteria associated with yolk sac infection and to determine drug sensitivity pattern of the predominant isolates. A total of 341bacterial isolates were isolated and identified using biochemical tests and then tested for their susceptibility to 11 antimicrobial agents. Escherichia coli was the most common bacteria followed by Salmonella and Staphylococcus aureus. Other bacteria were also identified but in a lower proportion. Unabsorbed yolk sac was observed in 370 chicken associated with signs of septicemia in many cases. In antibacterial susceptibility testing the isolates were susceptible to Gentamicin, Chloramphenicol, Kanamycine and Amikacine indicating the potential usefulness of these antimicrobials for the treatment of yolk sac infection. The existence of multi-drug resistance bacteria isolates associated with yolk sac infection suggests that more emphasis be given towards preventing omphalitis in chicks through improvements of sanitary measures than to consider control options through the use of antimicrobials.

Key words: *Bacterial Isolation, Chicken, Drug Sensitivity, Yolk sac infection*

1. INTRODUCTION

Ethiopia has great potential for increased modern poultry production, both for local use and for export. However, expansion was constrained by an unsteady supply of hatching eggs, day-old-chicks, lack of premix or veterinary drugs, diseases, a lack of support services and absence of sufficient data on poultry for decision making (CIRAD, 2005). Infectious diseases are remaining among the major health constraints hampering its intended potential (Chanie *et al*., 2009).

Ethiopia has large poultry population estimated to be around 40.6million with native chicken of non descriptive breeds representing 96.6%, hybrids 0.55% and exotic breeds kept mainly in urban and peri-urban areas 2.84% (CSA, 2009).

The rapid expansion of poultry industry has presented many poultry diseases among which is yolk sac infection (YSI) also known as mushy chick disease or omphalitis. Omphalitis is an economically important disease since it increases first week mortality and causes poor weight gain. In addition, birds that survive to yolk sac infection show poor carcass quality Corts *et al*. (2004).Yolk sac infection can occur in all flocks resulting in decreased hatchability, increased mortality and increased culling rate due to retarded growth. It is accounting for large economic losses to the poultry industry with mortality rates reaching 5-10% (Rai *et al.,* 2005; Yassin *et al.,* 2009; Ulmer, 2011).

Yolk sac infection occurs due to the use of unhygienic equipments in the hatchery. The affected chicks manifested depression, drooping of the head and huddling near to the heat source as reported previously (Kahn *et al.*, 2008). Several bacteria such as *E coli., Salmonella* spp*., Proteus* spp*., Enterobacter* spp*., Pseudomonas* spp*., Klebsiella* spp*., Staphylococcus* spp*., Streptococcus* spp*., Clostridium* spp*., Bacillus cereus* and *Enterococcus* have been isolated from the yolk sac of the infected birds (Cortes *et al.*, 2004; Iqbal *et al.,* 2006).

There are certain factors that affect yolk absorption and in turn may lead to its retention. Once bacteria get entry to yolk, rapid bacterial growth occurs due to fat and water in the yolk which are favored nutrients for bacteria. In addition, the yolk sac is maintained at the temperature of the hatcher and then at the chick's body temperature, which are the ideal temperatures for multiplication of certain bacteria. So yolk sac retention due to any cause may lead to yolk sac infection (Anonymous, 2000; Anjum, 1997).

In Ethiopia the prevalence of YSI was reported in kombolcha poultry farms with prevalence rate of 33.1% in dead sampled White Leg horn and Rhode Island Red breeds in causing the high mortality of chicks during their first week life post-hatching and thus posing a great threat to the poultry industry (Abadi *et al.,* 2013).

Bishoftu is the area of most commercial poultry farms planted and very few works has been done in the area regarding early chicks mortality. Efforts has never been done on yolk sac infections (Omphalitis), hence the disease have got little attention. Early and accurate detection of bacteria is important to undertake appropriate control measure. Good management and sanitation as well as use of antibiotics could help reduce mortality. Therefore the objectives of this study were

- To isolate and identify the bacteria from clinical cases of omphalitis (YSI) in newly hatched chicks in three hatcheries,

- To determine the sensitivity of the isolates to most commonly used antibiotics.

2. LITERATURE REVIEW

2.1. Definition of yolk Sac infection

Omphalitis and yolk sac infection commonly cause disease in young chicks. Omphalitis is a common cause of death in chicks during the first week of life and most common with artificially hatched chicks. Contamination of unhealed navels has been suggested as a cause of yolk sac infection in newly hatched hicks (Fasenko and O'Dea 2008). Different types of bacterial agents are attributed for causation of yolk sac infection/omphalitis in chicks. It is due to the close anatomic relationship, because the avian embryo lacks a physical connection to the hen (Anjum, 1997; Anonymous, 2000).

2.2. Understanding the yolk Sac

During incubation, extra embryonic membranes encircle the yolk substance and constitute the yolk sac, which is attached to gut of the chick by yolk stalk. Just before hatching, the yolk sac is pulled from the egg cavity to abdomen of chick as an extension of intestine. Residual yolk comprises 20-25% of body weight at hatch but within the first week of life it becomes negligible in size (Ramnoff, 1960).

Growth of a chick begins in the small fertilized area at the top of the yolk. A network of blood vessels begins to develop spreading from the embryo out over the yolk. The yolk sac (YS) contains essential nutrients (fat, protein, water, minerals, and COH) that are utilized by the developing chicken embryo. The YS is comprised of the yolk sac content (YSC) and its enveloping structure the yolk sac membrane (YSM). The YSM is a layer of tissue growing over the surface of the yolk, which eventually surrounds the yolk completely. The YSM is a complex extra-embryonic structure that is responsible for the transfer of nutrients needed for energy and tissue growth from the YSC to the chick embryo (Noble and Cocchi 1990).

Nutrients pass from the YSC to the YSM by receptor-mediated endocytosis of lipoproteins (Hermann *et al*., 2000) and by transporters for peptides, glucose and minerals (Yadgary *et al*., 2011).Yolk lipids, that serve as the main energy source for the chicken embryo, are taken up at the apical surface of the YSM endothelial epithelial cells (EEC) in the area vasculosa, the lipids are then released from the basal surface of these cells into the capillaries of the YSM and transported to the growing embryo (Noble and Cocchi 1990).

2.3. Yolk Retention

2.3.1. Incidence

Incidence of yolk retention and yolk sac infection is widely reported in literature stated that it was the commonest cause of early chick mortality the most frequent cause of death in chicks (Anjum, 1997). Incidence of yolk retention and yolk sac infection was reported as 10.5, 9.9, 5.1 and 8.9% in chickens by Shrivastava (1982), Rathore *et al*. (1985), Suresh *et al*. (1988) and Bhattacharjee *et al.* (1996), respectively. Incidence was reported to be 15.20 and 20.71% in two different strains of White Leg Horn by Viswanath *et al*. (1985). Unabsorbed yolk was observed as principal lesion in quails died up to one week of age by Boado and Rojas (1990) found 7.3% incidence of omphalitis in goose. Similarly, Sainsbury (1992) reported its higher incidence towards the end of winter or in the early spring. Yolk retention is not only the cause of death in chicken but also in other species of poultry including guinea fowl, duck, turkey, quail and goose. It was reported as most frequent cause of death in indigenous guinea fowl (Rudy, 1991).

2.3.2. Effects on host

Putrefactive and offensive odor was observed as characteristic clinical sign of yolk sac infection. Abdomen of chick felt soft and distended with thickened, inflamed and moist

umbilicus. Unabsorbed yolk sac was present in the abdomen and therefore it was named yolk retention. Yolk sac contents changed from viscid yellow green to watery yellow brown due to denaturation of yolk by bacteria (Anjum, 1997; Jordan, 1990; Sainsbury, 1992). Studies shows that infected yolk sacs were, in general, larger in mass than uninfected sacs from poults of same age (Deeming, 1995). Yolk sac and subcutaneous blood vessels were dilated and engorged with blood. Infection can result in the deprivation of nutrients and maternal antibodies with resultant immunosuppression. Chicks that survive are often stunted and do poorly in general. The absorption of toxins and systemic spread of *E. coli* may ultimately result in death. Chicks surviving more than four days might have pericarditis as well as infected yolk indicating systemic spread hemorrhagic serous peritonitis was also observed. Maximum deaths occurred up to 3 days of age in some cases, there might be no mortality with retained infected yolk as only manifestation (Anjum, 1997; Jordan, 1990).

2.4. Factors Contributing towards yolk Retention

2.4.1. Yolk sac Infection

Causative Organisms

Different types of bacterial agents are attributed for causation of yolk sac infection/omphalitis. *Escherichia coli* were frequently the main one involved. Next followed by frequently found bacteria were genus Salmonella (Buhr *et al.,* 2006; Suha *et al.,* 2008). *Salmonella spp, Staphylococcus spp, protease spp, Bacillus spp, Streptococcus spp, Pseudomonas spp, Klebsiella spp, Clostridium spp, Aerobacter spp, Citrobacter spp, Achromobacter spp, Enterococci spp* are some bacteria that have been isolated from yolk sac infections in chicks in different locations all over the world (Deeming, 1995; Sainsbury, 1992; Anjum, 1997; Anonymous, 2000).

Source of infection

Farm faecal contamination of shell was reported as source of infection and poor hatcher hygiene condition was considered as another important source that the infection was the waste in the hatchery or contaminated poult boxes or poult box pads. Other source of infection includes breeder, feed, environment, feathers, human skin, floor and dirty equipment (Anonymous, 2000; Sainsbury, 1992).

Route of infection

Transmission of bacteria is through unhealed naval was revealed by Jordan (1990) and Anjum (1997). Infection through blood stream and contamination of yolk before it is internalized into the chick were reported as other routes of infection by Anonymous (2000).

Experimental infection

Sub-clinical yolk sac infection after oral administration of pure cultures of bacteria was concluded that the infection arose through translocation of bacteria across the gut wall Singh *et al.* (1997). The pathogenicity of *Escherichia coli* by intraperitoneal injection into 2-days-old chicks reported as unabsorbed yolk sac was among the main lesions. Sander *et al.* (1998) observed retained yolk in chicks which received *Enterrococcus feacalis* broth inoculation into yolk sac. Omphalitis was also observed as gross lesion in experimental infection with *Salmonella harder* and *Salmonella enteritidis* by Desmidt *et al*. (1998) and Dhillon *et al.* (2001), respectively inoculated *E. coli* broth into yolk sac of day-old chicks and observed high yolk sac weight in these chicks as compared with the chicks inoculated with sterile broth.

Disease produced by inoculating *Bacillus cereus* inside the egg shell of pipped eggs and through intrayolk, intraperitoneal, subcutaneous and oral routes. Omphalitis was reproduced only when inoculated into the yolk sac. Severe edematous swelling around

navel orifice, severe Omphalitis and incomplete withdrawal of yolk sac in chicks hatched from embryonated eggs that were dipped in 24-hours bacterial broth culture on 18th day Khan *et al*. (2002).

2.4.2. Post hatch starvation

Slow absorption of yolk due to fasting has been reported by many workers. Fasting led to a reduced uptake of yolk as compared to fully nourished birds. Fasting favored removal of moisture and lipid to a greater extent than protein while the converse was true if access to feed and water was permitted (Moran and Reinhart, 1980).

Observations showed that yolk utilization was more rapid in fed than in fasted chicks, suggesting that transport of yolk through the intestine could be increased by the greater intestinal activity found in fed chicks. Similar findings were observed that starving chicks were unable to use the yolk sac nutrients, suggesting that, yolk sac utilization seems to be correlated with activation of the digestive system (Noy *et al*., 1996).

2.4.3. Type of initial feed

Substituted commercial starter ration with ground corn in the first 12, 24 and 36 hours of life concluded that corn feeding in early life led to slow absorption of yolk sac contents. The levels of lysine and methionine also have some effect on absorption of yolk sac in chicks (Wang *et al*., 1994).

2.4.4. Brooding temperature

The incidence of unabsorbed yolk was increased by the fluctuating environmental temperature, with little difference for the cold and hot environments, compared with a control situation (Leeson *et al*., 1978).

2.4.5. Miscellaneous factors

Studies shows that the effect of prolonged exposure to hatcher environment on yolk sac size and observed enlarged yolk sac in broiler chicks (Martin,1996). In contrast, Chamblee *et al*. (1992) reported that chicks kept in hatcher for 24 hours and chicks kept in the hatcher for 12 hours and then on litter for 12 hours exhibited non-significant differences in body weight and yolk weight. Knizetova *et al*. (1989) observed rapid resorption of yolk sac contents in large duckling and goslings than in small ones.

2.5. Chicken immune response against infection

Maternal antibody transfer can be defined as the transfer of antibodies by a female to her offspring either through the placenta, colostrums, milk, or egg (Grindstaff *et al.,* 2003). Birds transmit maternal antibodies to their offspring by depositing the antibodies in the egg. Very young chicks are susceptible to many pathogens during the first few weeks of age because their immune system is not fully developed; hence, maternal antibodies are the primary means of antigen-specific protection Sharma *et al*. (1989). There are 3 classes of antibodies in chickens, namely IgY (IgG), IgA, and IgM. Chicken IgA and IgM are similar to mammalian IgA and IgM in terms of molecular weight, structure, and immunoelectrophoretic mobility. Although structural differences exist between IgY and mammalian IgG, IgY is considered the avian equivalent to mammalian IgG. In eggs, IgY is present predominantly in the egg yolk whereas IgA and IgM are present in the egg white as a result of mucosal secretion in the oviduct and Chicken eggs can be an alternative antibody source because the IgY in the chickens' blood is transported to the egg and accumulates in the egg yolk. Hens usually lay about 280 eggs per year. Egg yolk contains a considerable amount of IgY, approximately 100–150 mg per egg. Therefore, an immunized hen yields more than 40 g of IgY per year through eggs, equivalent to the production from 40 rabbits that IgY corresponding to almost 0.5 L of serum may be recovered from a chicken in 1 month. This is five to ten times more than can be obtained from the blood of a rabbit Leslie and Clem (1969),

2.6. Immunotherapeutic applications of IgY in infectious disease

Recently, there has been increasing interest in the oral administration of antibodies for localized treatment of gastrointestinal infections. Chicken egg yolk has been recognized as an economical source of polyclonal antibody for oral administration, and egg yolk immunoglobulin Y (IgY) of chickens immunized with pathogenic microbes has been extensively studied for the treatment of various gastro enteric infectious diseases. The increase in antibiotic-resistant bacteria and the desire to treat pathogens that do not respond to antibiotics, such as viral pathogens, has prompted much research into the oral administration of antibodies as an alternative to antibiotics to treat enteric infections (Girard *et al*., 2006). The possibility of the industrial-scale production of specific antibodies makes IgY technology a practical reality for the prevention of infectious diseases in the near future, with significant potential for the fortification of food products and nutraceutical applications. However, to be effective in such applications, the IgY must survive the GI environment and reach the target areas with its biological activity still intact. Unfortunately, the stability of IgY to low pH and in the presence of digestive enzymes has been demonstrated to be lower than that of immunoglobulins from other species (Shimizu *et al.,* 1992; Hatta *et al*., 1993).

2.7. Prevention

Poultry that have an infection show a variety of symptoms, such as respiratory problems, diarrhea and paralysis. It should be emphasized at the outset that prevention of infection in a poultry flock through sound management is very important. This is because although some infectious diseases can be treated, for many it is a waste of time and money and infected birds should be disposed of immediately. The intensive system of poultry keeping which birds may be continuously confined indoors until they are disposed of is

the system most suitable for profitable and commercial poultry production (Oluyemi and Roberts, 2000; Gietema, 1992).

There is no specific treatment for omphalitis most affected birds die. The use of antibiotics to treat Colibacillosis may be recommended in some cases in accordance with susceptibility testing based on the prevalent bacterial type involved, but is probably of little value. The disease is prevented by careful control of temperature, humidity, and sanitation of the incubator and nesting boxes clean and dry. Set only fresh, clean, non-porous and uncracked eggs. If it is necessary to set dirty eggs, they should be kept away from clean eggs. Gentle sanitizers are available for cleaning dirty eggs. Always wash hands, especially when turning always disinfect incubators and equipment before and after use (Merck, 2011).

2.8. Antibiotic resistance and public health concern

Many forms of resistance spread with remarkable speed.Salmonellosis is the most important zoonotic disease, causing diarrhea and systemic infections. Due to poor management in antibiotic consumption, microbial resistance has increased in the treatment of zoonotic diseases. It is among the most important food-borne pathogens in the world. Poultry and poultry products are usually incriminated in human Salmonellosis outbreaks. *Salmonella spp* the major bacterial pathogens of poultry worldwide, and most Salmonella infections in human result from the ingestion of contaminated poultry (Carli *et al.,* 2001).During the last decade, there has been an alarming increase in the appearance of antibiotic-resistant bacteria as a result of poor management in antibiotic consumption. The administration of antimicrobial agents in chickens creates selection pressure that favours the survival of antibiotic-resistant pathogens. Resistance of Salmonella to commonly used antimicrobials is increasing, both in the veterinary field and the public health sector and has emerged as a global challenge (Molla *et al.,* 2003). Recent studies from different countries reveal that Salmonella serotypes isolated from foods of animal origin have multidrug resistance profiles (Winokur *et al.,* 2000; Holt *et al*., 2007).

Usage of antibiotics in poultry is for three purposes including: therapy, prevention, and growth promotion. The classes used include: β-lactams (penicillins and cephalosporins), sulphonamides β-lactams with and without trimethoprim, tetracyclines, macrolides, lincosamides and streptogramins, and quinolones (including fluoroquinolones β-lactams), which have a variety of therapeutic and preventive applications in food animals and are the same classes as those used in human therapy. Much of the evidence relating to the potential for transfer of a resistance problem from animals to man comes from a consideration of the epidemiology of zoonoses, mainly Salmonella and Campylobacter infection. The hypothesis is that the food chain is the main means of transmission. This hypothesis is intuitively attractive, and there can be no doubt of the existence of a hazard, but neither of these consideration means that the hypothesis is correct or of universal significance (Chiu *et al*., 2002).

2.9. Diagnostic techniques

2.9.1. Culture and morphological staining of bacteria

The isolation and identification of colonies in different culture media should performs using standard bacteriological procedures as described by (Quinn *et al*., 2002; Swayne *et al*.,1998). The representative bacterial colonies in any clinical materials should be characterized morphologically using Gram's stain described by Merchant and Packer (1967).

2.9.2. Hemolytic activity

To characterize the hemolytic patterns isolated strains should be tested for hemolysis on bovine BA plate by incubating them at 37°C for 24 hours. Hemolytic patterns should categorized as: *Alpha (α) hemolysis:* a zone of greenish discoloration around the colony manifested by partial hemolysis. *(β) Hemolysis;* complete clear zone of hemolysis around the colony and *Gamma (γ) hemolysis:* no detectable hemolysis (Cheesbrough,2006).

2.9.3. Reactions of the organisms in TSI agar slants

Triple sugar iron agar (TSI agar) used to detect the lactose, sucrose and dextrose fermenter and also the bacteria which produce hydrogen sulphide. The organisms seeded over the surface of the slants and stabbed into the butt where the cases changes after an incubation of 24 hours at 37°C (Cheesbrough,2006).

2.9.4. Sugar fermentation test

The sugar fermentation test used to perform whether the bacteria utilize sugar or not, five basic sugars (e.g., dextrose, sucrose, lactose, maltose, and mannitol) separately according to the procedure described by Ryan and Ray (2004).

2.9.5. Catalase test and coagulase test

Slide catalase and tube catalase tests used to performed to differentiate the isolated bacteria whether coagulase positive or negative samples should be recorded according to the procedure described by Cheesbrough (2006).

2.9.6. Indole test, Methyl red test and Voges-Proskauer test

These tests used to differentiate the isolated bacteria from various bacteriological samples collected. The test should performed and result should be interpreted according to the standard procedure described by Cheesbrough (2006).

2.9.7. Agar slant and 25% sterile buffered glycerin

The test performed by inoculating into the nutrient agar slants to preserve the isolated bacteria as a stock culture for few months and in 25% sterile buffered glycerin for several years at 80ºC without any deviation of their original characters (Buxton and Fraser 1977).

2.9.8. Serological tests

Many potentially pathogenic bacteria are present as part of the normal flora of a host or are common in the environment. They may produce antibodies to the organisms. Antibodies as animals are frequently exposed to these bacteria, demonstrable in a serum sample are evidence of exposure to an infectious agent but they do not necessarily confirm an etiological role for that agent. Despite these limitations, serological tests are used extensively for confirming infection with particular pathogens in susceptible animals. Serological tests such as ELISA and agglutination techniques are of greatest value when used on a herd or flock basis. A rising antibody titer using paired serum samples is indicative of active infection (Quinn *et al*., 2002).

2.10. Review on the status of yolk sac infection in Ethiopia

In Ethiopia the prevalence of YSI was reported in kombolcha poultry farms with prevalence rate of 33.1% in dead sampled White Leg horn (WLH) and Rhode Island Red (RIR) breeds during the first week of life. This is the first report in Ethiopia pertaining to YSI of chicks. In the study, *E. coli* 87 (51.17 %) was the most predominant isolate followed by *staphylococcus aureus* 40 (23.53%), *Proteus mirabilis* 39 (22.94 %) and other bacteria like *streptococcus species* and *Bacillus species* in lower proportion. Mixed bacterial infections were the predominant cases recorded due to *E. coli* together with any of the three species (*Staphylococcus aureus*, *Proteus mirabilis* and rarely with *Bacillus cerus* (Abadi *et al*,. 2013).

2.11. Characteristics of the family *Enterobacteriaceae*

Bacteria belonging to the family *Enterobacteriaceae* are Gram-negative rods up to 3μm in length. which ferment glucose and a wide range of other sugars, and are oxidase-negative. They are catalase-positive, nonspore-forming facultative anaerobes which grow well on MacConkey agar because they are not inhibited by the bile salts in the medium. These enteric organisms reduce nitrates to nitrites and some species, notably *Escherichia coli,* ferment lactose. The motile enterobacteria have peritrichous flagella. The family contains more than 28 genera and over 80 species. Less than half of the genera are of veterinary importance . The term 'coliform', formerly only used to describe enterobacteria capable of fermenting lactose, is now sometimes used to describe other members of the family. Enterobacteriaceae can be arbitrarily grouped in three categories: major pathogens, opportunistic pathogens and non-pathogens. Those without pathogenic significance for animals, such as *Hafnia* and *Erwinia,* can be isolated from faeces and the environment and may contaminate clinical specimens. Opportunistic pathogens occasionally cause clinical disease in locations other than the alimentary tract. The major animal pathogens *E, coli, Salmonella* species and *Yersinia* species can cause both enteric and systemic disease (Quinn *et al*., 2002).

2.11.1.Escherichia coli

Escherichia coli is usually motile with peritrichous flagella and often fimbriate. This lactose fermenter produces pink colonies on MacConkey agar and has characteristic biochemical reactions in IMViC tests. Some strains produce colonies with a metallic sheen when grown on eosin-methylene blue agar. Haemolytic activity on blood agar is a characteristic of certain strains of *E, coli.* Somatic *(O),* flagellar (H) and sometimes capsular (K) antigens are used for serotyping *E coli.* The somatic antigens are lipopolysaccharide in nature and located at the surface of the cell wall. The specificity of these antigens is determined by carbohydrate side chains. The flagellar antigens are protein in nature and the capsular antigens are composed of polysaccharides.

Proteinaceous fimbrial (F) antigens act as adhesins facilitating attachment to mucosal surface (Quinn *et al*., 2002)

2.11.2 . Salmonellosis in poultry

Salmonella pullorum, *Salmonella gallinarum* and *Salmonella enteritidis* can infect the ovaries of hens and be transmitted through eggs. The presence of *Salmonella enteritidis* in undercooked egg dishes may result in human food poisoning. Pullorum disease or bacillary white diarrhea *(Salmonella pullorum*) infects young chicks and turkey poults up to 2 to 3 weeks of age. The mortality rate is high and affected birds huddle under a heat source and are anorexic, depressed and have whitish faecal pasting around their vents. Characteristic lesions include whitish nodes throughout the lungs and focal necrosis of liver and spleen. Fowl typhoid ***(****Salmonella gallinarum*) can produce lesions in young chicks and poults similar to those of Pullorum disease. However, in countries where fowl typhoid is endemic, a septicaemic disease of adult birds occurs, often resulting in sudden deaths. Characteristic findings include an enlarged, friable, bile-stained liver and enlarged spleen. As *Salmonella* Pullorum and *Salmonella* Gallinarum possess similar somatic antigens both have been eradicated from many countries by a serological testing and slaughter policy for Pullorum disease. Paratyphoid is a name given to infections of poultry by non-host-adapted salmonella such as *Salmonella enteritidis* and *Salmonella typhimurium*. These infections are often subclinical in laying birds (Cooper,1994).

2.12 General Characteristics of *Staphylococcus* organisms

The family *Staphylococcaceae* includes the genera *Staphylococcus* and *Micrococcus*. These organisms are Gram-positive cocci commonly occurring in grapelike clusters. Clinically, the most important genus of the *Staphylococcaceae* family is *Staphylococcus* (Rho and Schaffner, 2007).

3. MATERIALS AND METHODS

3.1. Description of study Area

The study was conducted in three different poultry farms, found in Bishoftu. Debre Zeit Agricultural Research Center, Alema and Elere poultry farm during the period between November 2014 to May 2015 in Bishoftu Ethiopia. Bishoftu is located at 47 km Southeast of Addis Ababa at an altitude of about 1900 m.a.s.l (38^{o} 58″ E 08^{o} 44″ N). It receives an annual rainfall of 1115.6 mm with two rainy seasons. The short rainy season extends from March to May, while the main rainy season extends from June to September. The average maximum and minimum temperatures are 30.5^{o}C and 8.5^{o}C, respectively (National Metrological Service Agency NMSA (2003).

3.2. Study Birds

A total of 385 layers chicks 1 to 7 day old suspected to have died from yolk sac infection were included in this study; 225 diseased and 130 dead chicks. The chickens comprise Lohmans and Kokock breeds were obtained from three successive batches from Alema, Elere and DZARC hatchery located in Bishoftu.

3.3. Study Methodology

3.3.1. Postmortem (Necropsy) Examination:

All the chicks were subjected to necropsy before sampling in order to record any gross lesion on their viscera with special reference to the yolk sac. Postmortem examination was done according to established procedures (Chauhan and Roy, 2007).

3.3.2. Sample Collection and Transportation:

All chicks with gross lesions of yolk sac were sampled with sterile cotton swab from the yolk sac. The collected swab samples were labeled, packed and transported along with portable coolant (Ice pack) to the Debre Zeit Agricultural Research Center animal health laboratory. The collected samples were stored in refrigerator at + 4 °C according to Quinn *et al*. (2002).

3.3.3 . Bacterial Isolation

The swab was aseptically cultured on blood agar and MacConkey agar for first isolation of the causative agent. All inoculated media were incubated at 37 ºC and inspected for growth after 16 to 40 hours of incubation. Based on macroscopic and microscopic appearance, the developed colonies were selected from each sample and subcultured on eosin methylene blue (EMB) agar to get pure cultures of the bacteria. Colonies from primary plates were also cultured in brilliant green agar (BGA), mannitol salt agar (MSA), salmonella-shigella (SS) agar, triple sugar iron (TSI) agar and Harlequin Salmonella ABC medium and incubated at 37°C for 16 to 40 hours.

3.3.4 . Bacterial Identification

Identification of the pure isolates was done on the basis of staining reaction, colony morphology, cultural and biochemical character of the isolates by using standard bacteriological and biochemical procedures (Swayne *et al*., 1998; Quinn *et al*., 2002).
Bacteria were identified or characterized based on morphology of colonies (size, margin, elevation and colour), Gram stain (Merchant and Packer 1967), catalase, M-R, V-P, Indole, and triple sugar iron tests for sugar fermentation (Cheesbrough, 2006).

3.3.5. Antimicrobial Susceptibility Test

Antimicrobial sensitivity was tested using 0.5 McFarland turbidity standard inoculum and freshly prepared, dried Mueller Hinton agar (Oxoid, UK) against 11 common antibiotics. The antibiotics used include Gentamicin, Amoxicillin, Penicillin, Kanamycine, Norfloxacin, Streptomycin, Chloramphenicol, Ciprofloxacin, Tetracycline, Sulphamethaxzol and Amikacine were used. disk diffusion or Kirby- Bauer method (Bauer *et al.,*1966). The antimicrobial agents were categorized into susceptible, intermediate and resistant categories according to National Committee for Clinical Laboratory Standards NCCLS (2007).

3.4. Data management and analysis

The data and laboratory results were first coded and managed into Microsoft Excel and analyzed using Statistical Package for Social Sciences software (SPSS) version 17. Descriptive analysis such as sum and frequency distribution were computed.

4. RESULTS

4.1. Cultural and morphological characteristics

In the current study, the major bacterial species isolated from yolk sac were *E. coli,* Salmonella and *Staphylococcus auras* based on cultural, Gram's stain and morphological features which were consistent with the characteristics of the respective bacterial species presented in table 1.

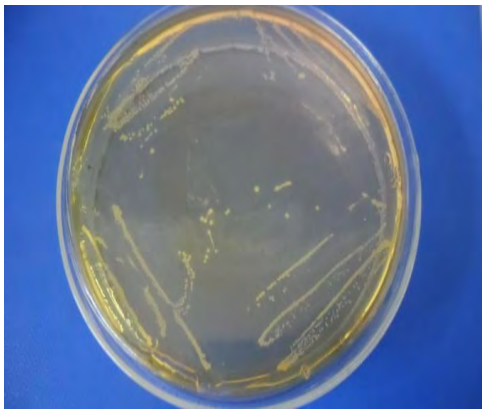

Figure 1: Growth on MSA

Figure 2: Growth on Harlequin

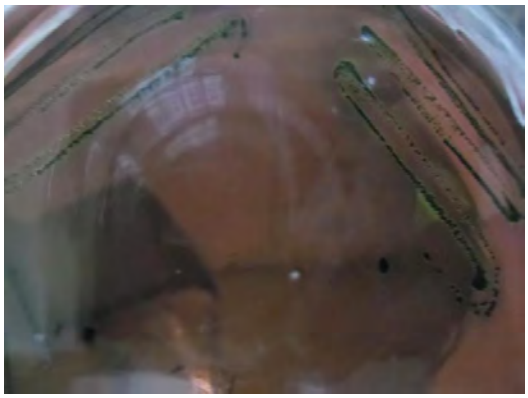

Figure 3: Growth on EMB

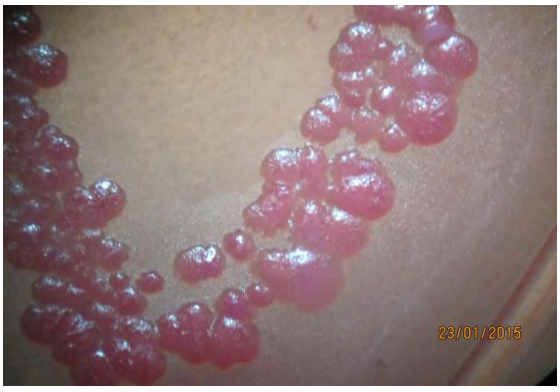

Figure 4: Growth on MacConkey

Table 1: Cultural characteristics of representative bacteria isolates from yolk sac infection in day (1-7) age chicken.

Characteristic *Growth in:*	Representative bacteria isolates			
	1	*2*	*3*	*4*
EMB	+	-	-	-
BA	+	+	+	+
BGA	+	+	-	+
MSA	-	-	+	-
XLD	+	+	-	+
SSA	+	+	-	-
Harlequin	-	+	-	-
MacConkey	+	+	-	+
Biochemical test				
MR	+	+	+	+
VP	-	-	+	+
Indole	+	-	-	-
Catalase	+	+	+	+
TSI, Yellow slant, Butt with gas	+	-	-	-
TSI, Butt yellow slant pink	-	+	-	+
H_2S	-	+	-	+
Carbohydrate fermentation tests	DX, AG SU, AG L , AG MN, AG	DX, AG MN, AG ML, AG	DX, A SU, A L, A MN, A ML, A	SU, A
Interpretation	*E. coli*	*Salmonella*	*Staphylococcus aureus*	*Proteus mirabilis*

EMBA = Eosin methylene blue agar, BA=Blood agar, BGA=Brilliant green agar, MSA=Mannitol salt agar, XLD= Xylose-lysinedeoxycholate agar, SSA= Salmonella Shigella agar, DX = Dextrose, ML = Maltose, L = Lactose, SU = Sucrose, MN = Mannitol, A = Acid, AG = Acid and gas, + = Positive, – = Negative, MR = Methyl red and VP = Voges-Proskauer, TSI = Triple sugar iron agar, H_2S = Hydrogen Sulphide production.

4.2. Biochemical characteristics

E. coli fermented dextrose, lactose, sucrose and mannitol with the production of acid and gas. *Salmonella* fermented dextrose, maltose and mannitol with acid and gas production were as *Staphylococcus auras* fermented all five basic sugars with only acid production The result of Catalase, Indole, MR and V-P of the isolates is presented in table 1.

4.3. Bacterial Isolation

The frequency of isolation of the different bacterial species obtained in this study is summarized in tables 2. More than one type of bacteria species was isolated from all chicks with the yolk sac problems revealing that mixed bacterial infection is a common scenario. Out of the total 341 different bacterial strains isolated belonging to different genera, *E. coli (N*=57; 37.5%) was the most predominant isolate followed by *Salmonella spp* (*N*=52; 34.2%) and *Staphylococcus aureus (N*=38, 25%) whereas *Proteus mirabilis* 5(3.3%) were the least frequently isolated bacteria from cases of yolk sac infections in Alema farm and *E. coli (N*=32; 34.9%) was the most predominant isolate followed by *Salmonella spp* (*N*=28; 30.4%) and *Staphylococcus aureus (N*=26, 28%) whereas *Proteus mirabilis* 6(6.5%) were the least frequently isolated bacteria from cases of yolk sac infections in DZARC poultry farm and *E. coli (N*=30, 30.9%), *Staphylococcus aureus (N*=30, 30.9%) and *Salmonella spp (N*=33, 34%) occurred in more or less similar frequency where as *Proteus mirabilis* was the least frequently isolated (*N*= 4, 4%) in Elere farm (day-1) chicks.

Table 2: The frequency of bacteria species isolated from chicken with omphalitis in Alema, DZARC and Elere hatcheries

Hatchery	Age	No Examined birds	YS Retention	Type and frequency of representative bacterial isolates								Total
				E.coli		*Salmonella*		*Staph. aureus*		*P. mirabilis*		
				N	%	N	%	N	%	N	%	
Alema	1-7 day	235	225	57	37.5	52	34.2	38	25	5	3.3	152
DZARC	1-7 day	37	37	32	34.9	28	30.4	26	28	6	6.5	92
Elere	Day -1	113	108	30	30.9	33	34	30	30.9	4	4	97
Total		385	370	119	34.9	113	33	94	27.6	15	4.4	341

N= no of isolates

* The percentage is with respect to the total number of isolates from the respective hatcheries

4.4 Necropsy (gross lesion) Findings

The major gross lesions observed in chicks died of yolk sac infection were unabsorbed yolk sac, which was observed in many cases (96%) with congestion and discoloration of the yolk (96%) greenish yellow; dark brown to bright yellow, retained caseous yolk sac (5%) and edematous yolk (especially in 3-7 days old chicks) . The signs of omphalitis were also evident(83%), as their navel was markedly thickened and dark blue in color. With the exception of the signs of omphalitis, all the remaining clinical signs were nonspecific. Yolk sac infection is observed also with peritonitis, pericarditis, peticheal and ecchymotic hemorrhages on the serosal surface of visceral organs (the intestine) were presented in table 3.

Table 3. Gross lesions observed in chicks died of YSI

	Necropsy (gross lesion) Findings							
	Unabsorbed YS	Discoloration of yolk	Omphalitis	Retained caseous YS	Peritonitis	Pericarditis	Peticheal	Ecchymotic
Examined birds	385	385	385	385	385	385	385	385
Findings	370	370	320	20	75	45	250	35
%	96	96	83	5	19	11.7	64.9	9

* The percentage is with respect to the total number of examined birds

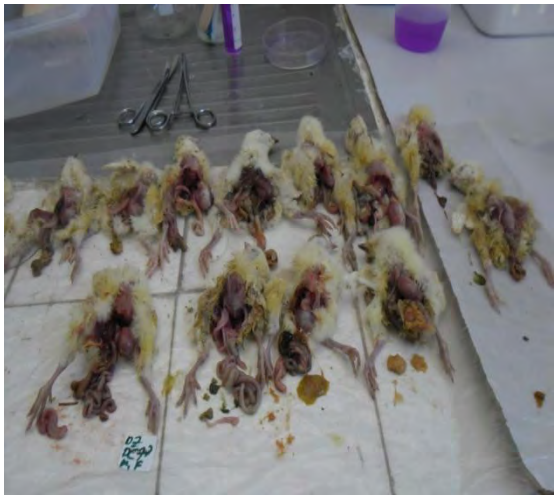

Figure 5: Peritonitis (D-1)

Figure 6 : Retained caseous YS (D-3)

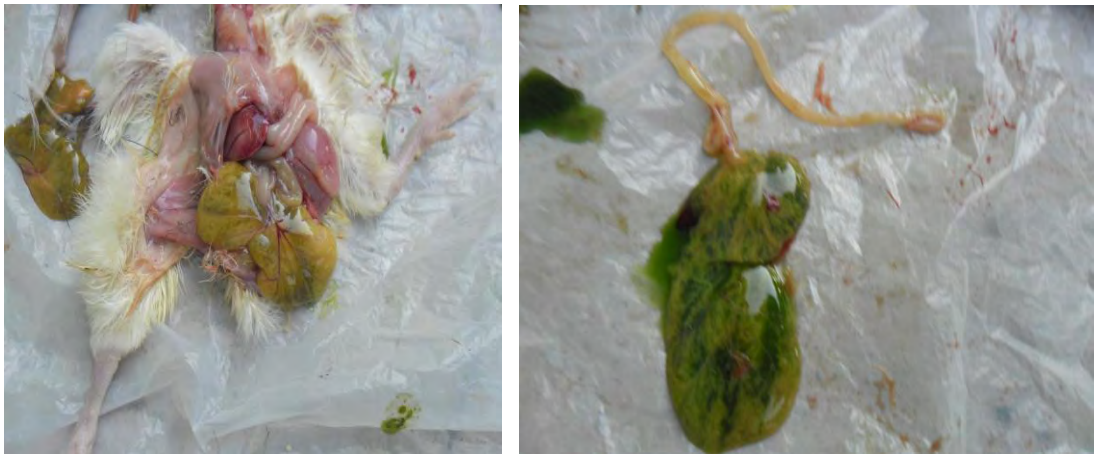

Figure 7: Unabsorbed yolk sac in day- old chick

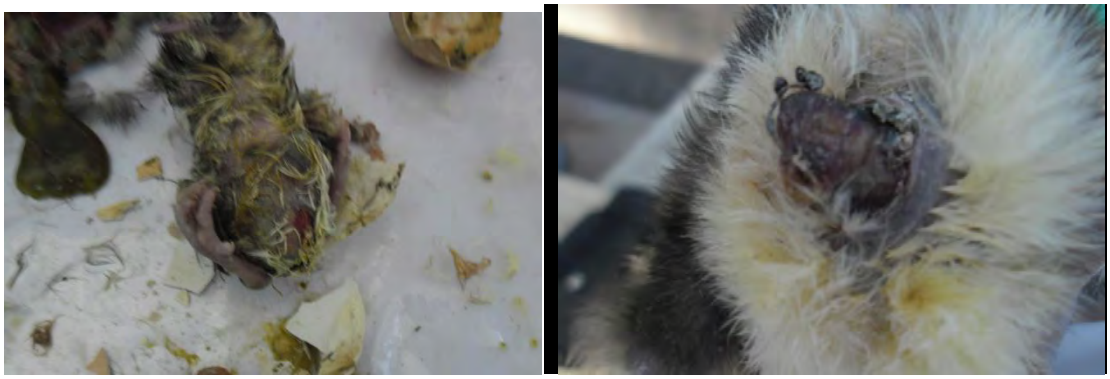

Figure 8: Open wound on navel in day old chick (omphalitis)

4.5 *In vitro* Drug Sensitivity Test Result

Antimicrobial sensitivity study of the isolates using 11 different antimicrobials showed *E. coli* and salmonella isolates were highly susceptible to Gentamicin, Chloramphenicol, Amikacine and Kanamycine and that of *Staphylococcus aureus to* Gentamicin, ciprofloxaciline, Chloramphenicol and streptomycin.

E. coli isolates were resistance to ciprofloxacin, amoxicillin, Ampicillin, tetracycline, Norfloxacin, sulphamethoxazole and that of *Salmonella* to tetracycline, ciprofloxacin, penicillin, Amoxicillin, sulphamethoxazole and Norfloxacin. *Staphylococcus aureus* to

penicillin, sulphamethoxazole and tetracycline in DZARC, Alema and Elere hatcheries are presented in table 4.

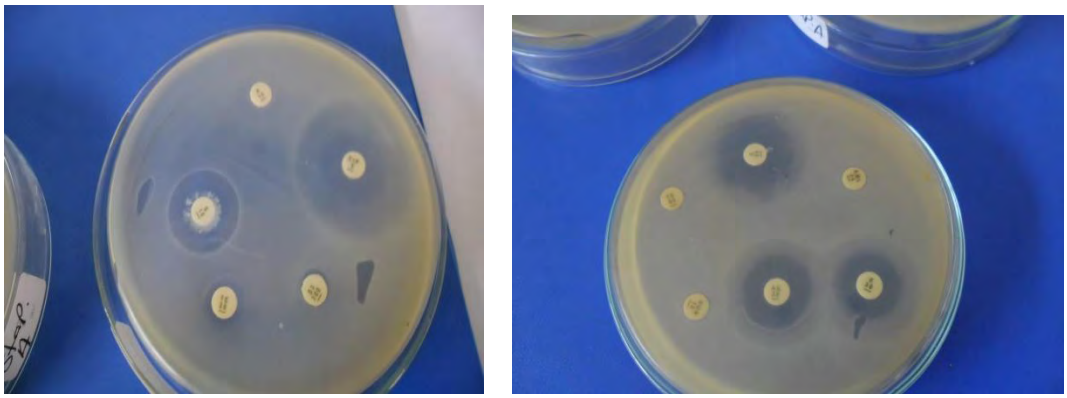

Figure 9: Single disc diffusion test

Table 4 : Antimicrobial Susceptibility pattern of the most frequently isolated bacteria involved in yolk sac infection in chicken

Antimicrobial agents	Disc content in µg	*E.coli isolates* (*n*= 119)			*Salmonella spp isolates* (*n*= 113)			*Staph. aureus isolates (n=94)*		
		R%	I%	S%	R%	I%	S%	R%	I%	S%
Gentamicin	10	0(0)	0(0)	119(100)	0(0)	20(17.7)	93(82.3)	0(0)	0(0)	94(100)
Ciprofloxacin	5	119(100)	0(0)	0(0)	113(100)	0(0)	0(0)	0(0)	0(0)	94(100)
Penicilline G	10u	119(100)	0(0)	0(0)	113(100)	0(0)	0(0)	74(78.7)	20(21.3)	0(0)
Tetracycline	30	119(100)	0(0)	0(0)	113(100)	0(0)	0(0)	94(100)	0(0)	0(0)
Sulphametazole	25	119(100)	0(0)	0(00	113(100)	0(0)	0(0)	94(100)	0(0)	0(0)
Amikacine	30	0(0)	0(00	119(100)	0(0)	0(0)	113(100)	20(21.3)	74(78.7)	0(0)
Chloramphenicol	30	0(0)	0(0)	119(100)	0(0)	0(0)	113(100)	0(0)	0(0)	94(100)
Amoxicillin	10	119(100)	0(0)	0(0)	113(100)	0(0)	0(0)	0(0)	20(21.3)	74(78.7)
Kanamycine	30	0(0)	0(0)	119(100)	0(0)	0(0)	113(100)	20(21.3)	0(0)	74(78.7)
Norfloxacin	10	119(100)	0(0)	0(0)	113(100)	0(0)	0(0)	0(0)	94(100)	0(0)
Streptomycin	10	0(0)	119(100)	0(0)	13(11.5)	100(88.5	0(0)	0(0)	94(0)	0(0)

R=resistance, I = intermediate, S = susceptible

5. DISCUSSION

Yolk sac infection is one of the health problems of poultry responsible for considerable losses (Rai *et al.,* 2005; Yassin *et al.,* 2009). It results in decreased hatchability, increased mortality and increased culling rate in affected flocks due to retarded growth following alteration in structure of immunoglobulin proteins accompanied by microbial infection finally resulting immunosuppression (Sander *et al*., 1998). It occurs mainly due to bacterial contamination of the eggshell after the egg is laid, while the cuticle is still moistened. Factors promoting contamination include lack of hygiene in the nests, presence of eggs on the floor, incubation of dirty eggs or eggs with eggshell defects, and collection of dirty and clean eggs at the same time (Rahman *et al*., 2007; Ahmed, 2009; Ulmer, 2011). The isolation of these bacteria species from yolk sac in the current study may attributed to the poor hatchery managements and un hygienic activates adopted in the farms but needs further investigation.

In this study the predominantly isolated bacteria species include *E. coli, Salmonella* and *staphylococcus aureus* which is in agreement with earlier studies (Hazariwala *et al*., 2002; Rosario *et al*.,2004; Iqbal *et al.,* 2006; Buhr *et al.,* 2006; Suha *et al.,* 2008). *Escherichia coli* has been previously reported as one of the most frequently isolated organisms involved in the development of yolk sac infection (omphalitis) (Buhr *et al.,* 2006; Suha *et al.,* 2008). *Staphylococcus aureus* has also been reported as an important cause of diseases in poultry (Hazariwala *et al*., 2002), as a common isolate from the broilers and turkeys, and as a cause of yolk sac infection in broilers (McCullagh *et al.*, 1998). Involvement of *Staphylococcus* species, *Proteus* species, *Streptococcus* and *bacillus* species has also been reported previously Rosario *et al*.(2004); Buhr *et al.,*2006; Suha *et al.,* 2008). The relatively few bacteria species isolated in the current study may be due to the use of antimicrobial agents by some of the farms for controlling early chicks mortality. It is the first report in the study area which showed that isolation and identification of the predominant bacterial species from yolk sac of chicks suffering from omphalitis.

The gross lesions observed in chicks that died of yolk sac infection which were manifested as edematous and unabsorbed/ retained yolk sac was also reported by different workers (Ahmed *et al*, 2009; Kawalilak *et al*, 2010). With the exception of the signs of omphalitis, all the remaining clinical signs were non-specific, however, they are identical with those mentioned by Sarma *et al.* (1985) and Saif *et al* .(2003). The high prevalence of *E. coli* isolation from chicks that died of YSI observed in the current study may suggest that it can be the main etiologic agent of YSI. However, the mechanism on how this micro-organism causes the signs and lesions associated with YSI is yet to be determined.

Omphalitis observed in this study has been the subject of research by several workers who indicated several reasons for increased incidence of navel deformity or 'Black button' navels among which include prolonged storage of eggs, improper temperatures and humidity during incubation (Jordan, 1990; Anjum 1997, Leeson *et al*., 1978).

Too high or low incubation temperature during the final days of incubation will produce poorly closed navels. When eggs are stored for prolonged periods prior to incubation, more chicks with black scab navels are observed, indicating unhealed navels at the moment of hatching. Too high humidity during incubation results in insufficient weight loss. As a result, the residual yolk sac becomes enlarged, which prevents the navel from closing properly. Conversely, when humidity is too low, the yolk sac dehydrates and becomes hard, which can damage sensitive tissue around the navel Sainsbury, (1992) Sarma *et al.* (1985) and Saif *et al.* (2003).

The higher susceptibility of bacterial isolates obtained from YSI to antimicrobials such as Gentamicin, Chloramphenicol, Amikacine and Kanamycine in in-vitro drug sensitivity test were in agreement with the previous reports (Salehi *et al*, 2006; Sharada *et al*., 2010). The resistance of *E. coli* isolates to ciprofloxacin, amoxicillin, Ampicillin, tetracycline, Norfloxacin, sulphamethoxazole and that of *Salmonella* to tetracycline, ciprofloxacin, penicillin, Amoxicillin, and Norfloxacin. *Staphylococcus aureus* to penicillin, sulphamethoxazole and Tetracycline supports the reports of previous studies (Khan *et al.,*

2002; Lee *et al.,* 2005; Nasrin *et al.,* 2012; Abadi *et al.,*2013). This may probably suggest the wide use and commercial availability of these antimicrobials for treatment of bacterial infections in both animals and humans as these bacteria are environmental contaminants from clinical cases.

6. CONCLUSION AND RECOMMENDATIONS

The results of the present study in Bishoftu poultry farms from cases of omphalitis in chicken show that *E. coli,* Salmonella species and *Staphylococcus aureus* were the predominant bacteria species isolated from yolk sac samples indicating that these bacteria are the major cause of yolk sac infection. Although antimicrobials such as Gentamicin, Chloramphenicol, Kanamycine and Amikacine may be potentially effective for treatment of yolk sac infection in chicks. The isolation of multi-drug resistant strains of *E. coli, Staphylococcus aureus* and Salmonella species from cases of chicks suffering from omphalitis from all hatcheries studied is alarming as this resistance may spread to microbes infecting man and animals. Thus based on the findings of the present study and the above conclusions the following recommendations are forwarded:

- Research should be conducted in order to determine the extent of the problem in different poultry farms, role and pathogenicity of each bacterial species involved in yolk sac infection.
- Efficient strategies should be established for the prevention and control of bacterial omphalitis through rational use of antimicrobials and careful improvements of sanitary measures in the hatcheries.
- Aim to produce day old chicks without navel deformities by optimising incubation conditions.
- Existing predisposing factors should be explore for yolk sac infection and yolk retention in poultry.
- Awareness should be created among the poultry farms for the implementation of better control and subsequent reduction of yolk sac infection in the study farm in particular and in Ethiopia in general.

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8. ANNEXES

Annex I : Laboratory procedure for Biochemical testes

Indole production

1. A drop from a 24-hour broth culture inoculated in tryptophan broth and Incubated at 35° C in ambient air for 48 hours.
2. 0.5 mL of Kovac's reagent added to the broth culture.
3. 1 mL of xylene added to the culture and shakes vigorously to extract the Indole.
4. 0.5 mL of Ehrlich's reagent added down the side of the tube

Interpretation Positive: Pink- to wine-colored ring, Negative: No color change

Methyl Red/Voges-Proskauer (MRVP) tests

PRINCIPLE

This test is used to determine the ability of an organism to produce and maintain stable acid end products from glucose fermentation, to overcome the buffering capacity of the system, and to determine the ability of some organisms to produce neutral end products (e.g., acetyl-methylcarbinol or acetoin) from glucose fermentation.

1. MRVP broth Inoculated with 1 drop from a 24-hour broth culture
2. Incubated at 37° C for 48 hours in ambient air.
3. The broth spited into aliquots for MR test and VP test.

A. MR (methyl red) test

1. 6 drops of methyl red reagent added per 5 mL of broth.

Interpretation: Positive: Bright red color, Negative: Yellow color

B. VP (Voges-Proskauer) test (Barritt's method)

1. Add 0.6 mL (6 drops) of solution A and 0.2 mL (2 drops) of solution B (KOH) added to 1mL of MRVP broth.
2. After vigorously Shaking

Interpretation: Positive: Red color , Negative: Yellow color

Triple sugar iron agar

1. With a straight inoculation needle, the top of a well-isolated colony touched
2. Inoculated on TSI by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant.
3. The cap on loosely and incubated the tube at 35° C in ambient air for 18 to 24 hours.

Interpretation: Acidic (yellow) indicates glucose is utilized by a fermentative organism, Blacken of the medium indicate H_2S formation.

Procedure for catalase test

1. Place a drop of 3% H_2O_2 on a glass slide.
2. Touch a sterile loop to a culture of the organism to be tested and pick up a visible mass of cells (colony).
3. Mix the organism in the drop of hydrogen peroxide.
4. Observe for immediate and vigorous bubbling.

Interpretation: Bubbling indicates a positive test and no bubbling indicates a negative test.

Annex II : Procedures in *in vitro* Antimicrobial susceptibility test

0.5 McFarland turbidity standard.

Solution A (0.048M $Bacl_2$)

1.175 g $Bacl_2$, 2 H_2O

Make up to 100 ml with distilled water

Solution B (0.8M H_2SO_4)

1.0 ml H2SO4 (1.84) make up to 100 ml with distilled water

For standard

0.5 ml solution A added to 99.5 ml solution B

Shake vigorously and dispensed in to 4-6 ml sealed tubes and placed in dark place until preparation of media

Before disk placement, the plate surface was inoculated using a swab that has been submerged in a bacterial suspension standardized to match the turbidity of the 0.5 McFarland turbidity standard (i.e., 1.5 × 108 CFU/mL). The surface of the plate was swabbed in three directions to ensure an even and complete distribution of the inoculum over the entire plate. Within 15 minutes of inoculation, the antimicrobial agent disks were applied and the plates were inverted for incubation to avoid accumulation of moisture on the agar surface that can interfere with interpretation of test results. incubated at 35° C in air for 16-18 hrs and recorded the result the entire clear zone in millimeter.

Interpretation : clear zone indicates susceptible, no clear zone indicates resistance