Sudan University of Science and Technology
College of Graduate Studies

Evaluation of Environmental impact on Antimicrobial Resistance of *Salmonella species* isolated from poultry farms in Khartoum State.

تقييم الأثر البيئي علي مقاومة مضادات الميكروبات لأنواع بكتيريا السالمونيلا المعزولة من مزارع الدواجن بولاية الخرطوم.

A Thesis Submitted in the fulfillment of the requirement for the degree of Doctor of Philosophy in Microbiology

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قَالَ تَعَالَىٰ:

﴿ وَإِذَا مَرِضْتُ فَهُوَ يَشْفِينِ ﴾

سورة الشعراء (الاية 80)

Dedication

I dedicate this work to:

The soul of my father Yousif

My mother Daralsalam

My extended family

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Abstract

This study is targeted the prevalence of *Salmonella species* in Khartoum State chicken broiler farms environment, and to detect the resistance pattern to different antibiotics by using phenotyping method and detecting beta lactams resistant genes.

Four hundred and sixty five samples were collected randomly from 44 farms in Khartoum, Omdurman, and Bahry Localities, they included water source, drinkers, feed source, feeders, faeces, Litter, Cloacal swabs, hand swabs from workers, dust swabs, carcass swabs, carcass meat, and Knife swabs. The samples were analyzed by using ISO 6579: (2002), then confirmed by using API 20E strips. Twenty nine *Salmonella species* (6.2%) were identified as 21(72.4%) *Salmonella arizonae* and 8 (27.6%) *Salmonella choleraesuis* collected from 21 farms in Khartoum State. The isolates were from water source 1 (3.4%), drinkers 4 (13.8%), feeders 4 (13.8%), faeces 3 (10.3%), litter 10 (34.5%), cloacal swabs 1 (3.4%), hand swabs from workers 1 (3.4%), dust swabs 4 (13.8%), and Knife swabs 1 (3.4%). There was no *Salmonellae* isolated from feed source, carcass swab, and carcass meat.

Isolates showed high resistance to amoxicillin 26 (89.7%), followed by tetracycline 16 (55.2%), nalidixic acid14 (48.3%), ampicillin 13 (44.8%), co-trimoxazole 10 (34.5%), streptomycin 8 (27.6%), colisitin and chloramphenicol 3 (10.3%), and gentamicin 2 (6.9%), however, all isolates showed high sensitivity to amikacin, ciprofloxacin, cefotaxime, cefixime, cefalexin, and ceftriaxone.

Salmonella arizonae showed multidrug resistance to nine antibiotics, amoxicillin 19 (90.5%), tetracycline 12(57.1%), nalidixic acid 11(52.4%), ampicillin10 (47.6%), co trimoxazole 6 (28.6%), streptomycin 5 (23.8%), chloramphenicol and colistin 3(14.3%), and gentamycin 2(9.5%), however all isolates showed high sensitivity to amikacin, ciprofloxacin, cefotaxime, cefixime, cefalexin, and ceftriaxone.

Salmonella choleraesuis isolates showed multidrug resistance to six antibiotics, amoxicillin 7 (87.5%), co-trimoxazole and tetracycline 4 (50%), ampicillin, streptomycin, and nalidixic acid 3 (37.5%), however all isolates showed high sensitivity to amikacin, ciprofloxacin, cefotaxime, cefixime, cefalexin, ceftriaxone, gentamicin, chloramphenicol, and colistin.

A double-disc synergy test (DDST) was used to detect beta lactamase-producing isolates. The DDST was positive for 18 (62.1%) out of the 29 isolates of which13 (72.2%) were *Salmonella arizonae,* and 5 (27.7%) were *Salmonella choleraesuis.*

To detect genotyping resistance, boiling method was carried out to extract bacterial DNA plasmid from positive beta lactamase-producing isolates (18 isolates). The primers were used to amplify the genes encoding CTX-M, SHV, and TEM. The results showed detection of CTX-M in 8(44.4%), SHV in 7(38.9%), and TEM in 5(27.8%) from 18 isolates.

The results showed that the genotypic resistance that is mediated by β-lactamases genes in *Salmonella arizonae* was 6 (85.7%) for SHV followed by CTX-M 5 (62.5%), and TEM 4 (80%), and in *Salmonella choleraesuis* was 3 (37.5%) for CTX-M, 1 (14.3%) for SHV, and 1 (20.0%) for TEM.

On conclusion *Salmonella choleraesuis* and *Salmonella arizonae* were isolated from poultry farm in Khartoum State 6.2%. They are sensitive to amikacin, ciprofloxacin, cefotaxime, cefixime, cefalexin, and ceftriaxone. Resistance was found to amoxicillin, tetracycline, nalidixic acid, ampicillin, co-trimoxazole, streptomycin, colisitin, chlaramphenicol, and gentamicin. Beta lactamase was found in (62.1%) isolates, and the gene CTX-M was 44.4% SHV was 38.9%, and TEM was 27.8%.

الخلاصة

استهدفت هذه الدراسة التعرف علي معدل انتشار بكتيريا السالمونيلا في بيئة مزارع الدجاج اللاحم بولاية الخرطوم، والتعرف علي نمط استجابة بكتيريا السالمونيلا للمضادات الحيوية عن طريق استخدام النمط المظهري والتعرف علي جينات البيتالاكتميز .

تم جمع 465 عينة من 44 مزرعة بمحليات الخرطوم، أم درمان، وبحري عبارة عن عينات من مصدر المياه، ومياه الشرابات،علف من المصدر، علف من الأكالات، زرق، الفرشة، مسحات من المذرق، مسحات من أيدي العمال، مسحات غبار، مسحات من الذبيح ، عينات من لحم الذبيح، ومسحات من سكين الذبح. تم تحليل هذه العينات باستخدام طريقة الهيئة العالمية للمقاييس 6579(2002)، وتم إجراء الفحوصات التأكيدية باستخدام شرائط دليل ملف تحليلي.

أظهرت الدراسة وجود 29(6.2 %) عزلة من بكتيريا السالمونيلا وتم التعرف علي 21 (72.4%) من السالمونيلا الأريزونية و 8(27.6%) من سالمونيلا كوليرا الخنازير تم جمعها من 21 مزرعة بولاية الخرطوم. تم عزل عينة سالمونيلا واحدة(3.4%) من مياه الشرب، 4(13.8%) من مياه الشرابات، 4(13.8%) من علف الأكالات، 3(10.3%) من الزرق، 10(34.5%) من الفرشة، 1(3.4%) من المذرق، 4 (13.8%) من مسحات أيدي العمال، 4 (13.8%) من مسحات الغبار، 1(3.4%) من مسحات سكين الذبح. ولم يتم عزل بكتيريا السالمونيلا من مصدر العلف، مسحات الذبيح، وعينات لحم الذبيح.

أظهرت العزلات مقاومة عالية للمضاد الحيوي اموكسيسيلين26 (89.7 %) يليه تتراساكلين 16(2.55 %)، ناليديكسيك اسيد 14(48.3 %)،امبيسيلين 13(44.8 %)، كوترايموكساسول 10(34.5 %)، استربتوميسين 8(27.6%)، كوليسيتين وكلورامفينيكول 3(10.3%)، و جنتاميسين 2 (6.9%). بينما أظهرت جميع العزلات حساسية عالية للأميكاسين، سبروفلوكساسين، سيفوتاكسيم ، سيفاليكسين، وسيفترياكسون.

أظهرت السالمونيلا الأريزونية مقاومة متعددة لتسعة مضادات حيوية: اموكسيسيلين19 (90.5 %)، تتراساكلين 12(57.1 %)، ناليديكسيك اسيد 11(52.4 %)، امبيسيلين 10(47.6 %)، كوترايموكساسول 6(28.6 %)، استربتوميسين 5(23.8%)، كلورامفينيكول وكوليسيتين 3(14.3%)، و جنتاميسين 2(9.5%). بينما أظهرت جميع العزلات حساسية عالية للأميكاسين، سبروفلوكساسين، سيفوتاكسيم، سيفيكسيم، ، سيفاليكسين، وسيفترياكسون.

أظهرت السالمونيلا كوليرا الخنازير مقاومة متعددة لستة مضادات حيوية: اموكسيسيلين7 (87.5 %)، كوترايموكساسول و تتراسيكلين4 (50.0 %) ، امبيسيلين، استربتوميسين ، ونااليديكسيك اسيد 3(37.5 %).

بينما أظهرت جميع العزلات حساسية عالية للأميكاسين ،سبروفلوكساسين، سيفوتاكسيم، سيفيكسيم، ، سيفاليكسين، سيفترياكسون، جنتاميسين، كلورامفينيكول، وكوليسيتين.

تم استخدام طريقة التآزر باللأقراص المزدوجة للتأكد من وجود العزلات المنتجة لإنزيمات البيتالاكتام حيث كانت 18(62.1%) إيجابية للإختبار منها 13(72.2%) من السالمونيلا الأريزونية و 5 (27.7%) من سالمونيلا كوليرا الخنازير.

للتعرف علي جينات المقاومة تم استخلاص الحمض النووي الديوكسي رايبوسي من العينات الإيجابية لإنزيمات البيتالاكتام18(62.1%) بتقنية الغليان واستخدمت نظرية التفاعل التسلسلي المتعدد للتعرف علي وجود الجينات سي تي اكس أم، شيف، وتيم. وأظهرت النتائج تواجدها بنسب 8(44.4%)، 7(38.9%)، و5(27.8%)علي التوالي.

السالمونيلا الأريزونية أظهرت 6 (85.7 %) شيف، يليها 5 (62.5 %) سي تي اكس ام، و4 (80.0%) تيم.

وفي السالمونيلا كوليرا الخنازير3(37.5%) سي تي اكس ام، 1(14.3%) شيف و 1 (20.0%) تيم.

تم عزل السالمونيلا كوليرا الخنازير والسالمونيلا الأريزونية بنسبة 6.2% من مزارع الدجاج اللاحم.وأظهرت حساسية عالية للأميكاسين، سبروفلوكساسين، سيفوتاكسيم، ، سيفاليكسين، وسيفترياكسون. وأظهرت الدراسة مقاومة للمضادات الحيوية اموكسيسيلين، تتراسايكلين ، ناليديكسيك اسيد ،امبيسيلين ، كوترايموكساسول ، استربتوميسين ، كوليسيتين، كلورامفينيكول ، و جنتاميسين .

وأوضحت الدراسة أن البيتالاكتاميز وجدت بنسبة 62.2% من العزلات. و وجود الجينات سي تي اكس أم(44.%)، شيف 38.9%، وتيم 27.8%.

Table of contents

List of tables

List of figures

CHAPTER ONE

Introduction

1. 1. General background

One of the most important threats to modern medicine is the development of bacterial resistance to antibiotics, making bacterial infections treatment more difficult or even impossible. The human use of antibiotics is the largest contributor to antibiotic resistance. The use of antibiotics to prevent or treat common production diseases in intensive farming led to the emergence of antibiotic-resistant bacteria such as *Salmonella*, that colonize farm animals and can be transmitted to people in food or through the environment. When these bacteria cause illnesses in people they are more difficult to treat and the resistant bacteria spread further by being transmitted between people. In addition, the genes for resistance can be passed from resistant bacteria to other bacteria that are also potentially disease-causing in people (WHO, 2011).

Microorganisms may be naturally born resistant, achieve resistance by mutation or have resistance by transfer of plasmids and other mobile genetic elements. In recent years problems related to *Salmonella* have increased significantly, both in terms of the incidence and severity of cases of human Salmonellosis. Since the beginning of the 1990s, strains of *Salmonella* which are resistant to a range of antimicrobials including the first choice agents for treatment of humans have emerged and are threatening to become a serious public health problem. Drug resistant *Salmonella* emerge in response to antimicrobial usage in humans and in food animals and selective pressure from the use of antimicrobials is a major driving force behind the emergence of resistance. Multi-drug resistance to critically important antimicrobials is compounding the problem (WHO, 2005).

The anticipation and fear is that many serovars of *Salmonellae* are more likely to evolve to become more pathogenic, resistant to multiple drugs and be involved in a

number of outbreaks and sporadic cases. The pathogen lives primarily in the intestinal tract of birds, insects, mice, farm animals, other animals and sometimes in eggs (Coburn *et al*., 2007). Farm pertaining samples and their environmental conditions including faeces, soil, crevices, dusts, manure, litter, feeders and drinkers harbour Salmonellae and lead to increase the rate of contamination (Wales *et al*., 2006). However, environmental sampling has been reported to be a good indicator for the presence of *Salmonella* in poultry flocks (Davies and Breslin, 2001).

1.2. Problem justification and hypothesis

Until recently, research on antibiotic use has been mainly directed toward their beneficial and adverse effects on the end user, human and animal. However, there have been relatively few studies on the effect of these antibiotics on the environment. Continuous usage and accumulation of antibiotics in the environment has resulted in the increase of antibiotic resistant bacteria .

The levels and types of resistance in zoonotic bacteria in Sudan and other countries gives cause of concern. The detection, invention, and global uses of antibiotics and antimicrobial agents in human and veterinary medicine, agriculture and aquaculture have initiated a 'Darwinian' experiment bringing about the survival of resistant microorganisms coupled with the elimination of susceptible ones in antibiotic-containing environments. The transfer of bacteria that are resistant to antimicrobial agents or resistance genes from animals to humans via the food chain is increasingly environmental problem. Therefore it is important to determine the species and the numbers of bacteria involved in this phenomenon and evaluation of the levels of antibiotic-resistant bacteria in the environment.

A number of factors have contributed to the spread of *Salmonella* in poultry. Among these are stocking densities of poultry farms, poultry feeds, farming activities, Lesser concerned area is the association between *Salmonellae*, poultry house environments, feeds, and the significant role they may play to integrate other factors in contributing

to the spread of *Salmonella* in poultry. Furthermore, techniques for isolating and identifying *Salmonella species* in poultry house environments are crucial for reliable reporting purposes to reduce the spread of *Salmonella* by poultry .

1.3. Objectives

The overall objective was to evaluate the environmental impact on antimicrobial resistance of *Salmonella species* isolated from poultry farms in Khartoum State.

Specific objectives were to:

1- Determine the prevalence of *Salmonella spp* in the environment of the broiler chicken farms.

2- Determine resistance pattern of the isolates by using disc diffusion method.

3- Detect beta lactams resistance by using phenotyping method (Double disk synergy test (DDST).

4-Detect genes related to β-lactams resistance(SHV, TEM, and CTX-M) genes, in *Salmonella* isolates .

CHAPTER TWO

Literature Review

2.1 Poultry

2.1.1 Global Poultry industry

The modern poultry industry can produce market ready broiler chickens in less than six weeks. This accomplishment is done through genetic selection, improved feeding and keen health management practices involving usage of antibiotics as therapeutic agents to treat bacterial diseases in intensive farming systems (Hemen *et al.*, 2012). Global broiler meat production rose to 84.6 million tons in 2013. The largest producers were the United States (20%), China (16.6%), Brazil (15.1%) and the European Union (11.3%) (United States Department of Agriculture(USDA), 2014).

2.1.2 Poultry industry in Khartoum

The Sudanese poultry industry is located in Khartoum State-Sudan, which is located between latitudes 15.08° and 16.39c° North and longitudes 31.36° and 34c°East and divided in to three major localities (Khartoum, Bahri, and Omdurman). The weather is hot and dry with rain in summer, and cool and dry in winter, the annual rainfall ranges from 75 to 160mm, falling mainly in July and August. Generally the dry period extends for 8-10 months. The data from the daily average minimum temperature is 21.6C°; the maximum temperature in summer is exceeds 40C°while the minimum temperature in winter is 5C°(El.siddig *et al*., 2006).

The annual production of broiler meat has been steadily rising from 16000 metric tons in the 1980 to about 30 000 metric tons in 2002, with an annual growth rate of 3% (FAO, 2005).

The growing population together with the GDP income per capita has increased the demand for meat. In 2008, about 3640 Sudanese poultry producers were registered, although many smallholders are presumed not to be listed (Freiji, 2008). In 2002, the

Sudan had a net import of 490 000 live chicks and the number of birds in the country was estimated to 45, 3 million (Ahmed, 2009). Ninety six percent of the commercial poultry production is located in the Khartoum state as a result of the continuous urbanization from rural areas to the cities, implying a future rising market demand in this area. However, Sudan remains the Arabic country with the lowest intake of poultry meat per capita a year. In 2005, the intake of commercially bread poultry was 0, 77 kg of meat per capita which can be compared to Egypt with 9 kilos per year or Saudi Arabia with a yearly intake of 39 kg of poultry meat per capita (Freiji, 2008). The low intake of poultry in Sudan can be explained by the price of meat. Traditionally, the price of red meat from sheep and cattle has been low, but during the last decade, a rise has been noted. In the past, poultry meat production has been dependent on the importation of production inputs such as feed, vaccines and parent stock (Freiji, 2008). As the industry is growing and the agribusinesses establish themselves, the agribusinesses tend to be able to produce chicken more efficiently. For Sudanese poultry producers, the cost of environmental regulation systems and feed are the two major expenditures affecting the producers' final profit. Feed cost itself stands for 50-70% of the producers' total costs. Depending on the production system used, air condition can be the second largest expense (Emam and Hassan, 2010).

Poultry producers can be divided in to three main groups ;household poultry keeping, traditional open house poultry farming and modern intensive poultry farming. Open house systems are still the most common way of producing poultry for a commercial market (Emam and Hassan, 2010). This system consists of a metal-roofed enclosure fenced with simple chicken wire. The open house systems have long been the standard for 95% of poultry production farms in the Khartoum state (Emam and Hassan, 2010).

Semi- closed systems are open house systems with air conditioning that manage to lower the temperature of the houses, usually 5-10 degrees. On a hot summer day in Khartoum this could still mean that the birds growth environment lies over 35 degrees which is about 10 degrees over an ideal growth environment (Emam and Hassan, 2010).

The modern closed system is the most efficient form of poultry production (Emam and Hassan, 2010). These systems provide secure optimal circumstances for the birds regarding protection from cold, rain, wind, and hot sun (Alhusain, 2005). The closed systems are mainly used by the large poultry producers since they are expensive and require a high degree of technical inputs such as air conditioning and fans.

The number of farms in Khartoum State as stated by Sirdar (2010) are 527 of which 517 are open system farms divided as 316, 171, and 30 represented Khartoum North(Bahri) and Omdurman respectively while 10 are intensive closed systems divided as 2, 6 , and 2 represented the same localities respectively. The two most important constrains to household poultry and traditional open house producers are inadequate health care and inappropriate housing (Khalafallah *et al.,* 2001). There is a substantial drop in supply during the hot season mainly due to the infavourable circumstances for broilers in open housing systems which causes poor growth results and high mortality. The drop of supply affects the price of poultry meat during the hot season. Heat stress begins when the ambient temperature climbs above 27C° and is readily apparent above 30C° (Sirdar, 2010).

In the middle of the 1970s the Sudanese Kuwaiti Company established a poultry farm South of Khartoum as an agri-business. The modern intensive broiler operations in an evaporated cooled housing system which enables them to produce all year round. Only the largest broiler producers have an integrated operation including parent stock, hatchery, and slaughter house (Sirdar, 2010). However, Most poultry

farms suffer from New castle disease and bursal disease in broilers which markedly decreased the productivity on these farms (Khalafallah *et al.,* 2001).

The problems associated with environmental contamination in the open system in Khartoum are posing real threat to this industry. Most owners of these farms have no veterinary information to deal with chicken diseases and their laborers are mostly illiterate or weakly educated (Selma and Tawfig, 2011).

2.2 Salmonellosis

Many serotypes of *Salmonella* including *S.Typhimurium, S. Enteriditis, and S. Infantis* do not have host specificity and cause disease in all kinds of animals and humans. *S. Typhi, S. Paratyphi* and *S. Choleraesuis* are highly adapted to humans and cause severe diseases (Murray *et al*., 2009). *Salmonella enterica* serovar *Enteritidis* is a major cause of food borne disease and during last decade it has been isolated worldwide in increasing numbers. Furthermore *S. enterica* serovar *Typhimurium* is the most frequently isolated serovar worldwide (Madadgar *et al*., 2008). Since 1987, *Salmonella enteritidis* has been the main cause of *Salmonella* poisoning in humans from poultry products (Doyle and Beuchat, 2007).

2.2.1 Routes of transmission

The spread of salmonellosis is associated with the consumption of contaminated food products from pigs, poultry, ruminants, contaminated drinking water, overseas travel, and direct contact with domestic and wild animal faeces via environmental and occupational exposure (Mullner *et al.*, 2009). Foods of animal origin, especially poultry and poultry products, have been consistently implicated in sporadic cases and outbreaks of human salmonellosis. Handling of raw poultry carcasses and products, together with the consumption of undercooked poultry meat has been reported as the commonest causes of *salmonella* infection in humans (Panisello *et al.,* 2000).

2.2.2 Salmonellosis treatment

The most widely used antibiotics for treatment of Salmonellosis in humans is a group of fluoroquinolones and third-generation cephalosporins. The earlier drugs chloramphenicol, ampicillin, amoxicillin and trimethoprim-sulfamethoxazole are occasionally used as alternatives (WHO, 2005), while in poultry, the most commonly used antimicrobial agents for either chemoprophylaxis or therapy for control of bacterial diseases includes sulfadiazine, tetracycline, gentamycin, amoxicillin, neomycin, enrofloxacin, colistin, flumequine , spectinomycin, ampicillin, , tylosin, and trimethoprim, (Sirdar, 2010).

2.2.3 Salmonellosis and risk factors for flock colonization

A number of risk factors for horizontal transmission have been identified including inadequate cleaning and disinfection of broiler rearing houses leading to contamination of the following flock , a poor level of hygiene , and contamination of feed . Other factors are: the size of the farm , rearing of flocks in the autumn , and the presence of litter-beetle in the house and rodents on the farm. Contamination of *Salmonella* negative flocks during transport to and processing at the slaughter plant has been observed with contaminated crates and plant contamination as apparent sources (Herman *et al* ., 2003). *Salmonella* contamination on the broiler grow-out farms is complex and can come from multiple sources in the environment such as feed, feed ingredients, water, litter and from breeding stock (Maciorowski *et al*., 2006).

2.2.3.1 *Salmonella* in feed

Feed has been implicated as an important source of *Salmonella* to poultry (Jones, and Richardson, 2004.). It has been suggested that occurrence of *Salmonella* contamination in feeds produced in feed mills may be due to transfer of *Salmonella* from birds, rodents or other pests (Alvarez *et al.*, 2003). Estimated survival time of *Salmonella* in poultry feed is more than 98 days and it is found that viability of *S.*

typhimurium in feed, at room temperature, is 71 weeks and in litter, 78 weeks. Furthermore the organism may survive up to 79 weeks in feed. More than 80C° temperature is required for the elimination of *Salmonella* from feed during steam conditioning (Maqsood, 2012).

Animal feed is a recognized source of pathogenic microorganisms for farm livestock (Davies and Hinton, 2000). Feed containing ingredients of animal origin is a potential source of *Salmonella* infection to herds, and the ingredients of vegetable origin can also be a source of *Salmonella*-contaminated feed.

2.2.3.2 *Salmonella* and Hygienic status

Management required that all farm staff understand the importance of personal hygiene and are aware of the means by which infection can be spread on hands, clothing and equipment. Adequate toilet and washing facilities (including soap) should be available and work boots and overalls should be provided for use only on the farm. It is preferable to provide separate boots and, if possible, protective clothing for each house. Staff should not keep or have contact with any other poultry and should avoid working with other livestock. Where this is not possible, cleaning and disinfection on entry and on leaving the poultry unit is most important, in addition to using clothing dedicated for use on the unit and kept there. Those who enter poultry buildings should wear disposable overalls or overalls which are capable of being laundered and boots which can be cleaned and disinfected. When they leave the poultry house they should wash their hands with soap, or use a hand disinfectant spray, and disinfect their boots. Visitors (such as fieldsmen, maintenance personnel, delivery and collection staff, veterinarians, officials, etc.) are a potential means of introducing infection, especially if they visit other poultry farms. Catching and cleaning gangs and their vehicles are a particular hazard especially during thinning as infection can be introduced during this process. Operators should be encouraged to

use the same high hygiene standards as farm staff. Non-essential visitors to the farm should be discouraged (www.thepoultrysite.com).

Flocks with two persons or less taking care of the birds and with no visitor entering the poultry house during rearing are at lesser risk of *Salmonella* colonization. The portable material that visitors might bring could also be a potential risk factor for the horizontal transmission of *Salmonella* (Heyndrickx *et al*., 2002). Equipment used for catching and transporting birds poses a high risk of introducing *Salmonella* onto a site, particularly crates which are a well known hazard. It is best to avoid sharing equipment with other farms. If this is unavoidable any equipment transferred from other sites should be cleaned and disinfected before transport and again before use on the site. Facilities for spray disinfection of the exterior of cleaning and catching team vehicles and equipment before entry to the poultry houses are advisable. Flocks should be checked on a daily basis and any dead birds and culled birds should be removed and placed in a closed leak proof and pest proof container at the perimeter of the site ready for disposal. Under the Animal By-Products Order 1999 disposal must be by incineration, rendering or removal to a knacker's yard. Only in exceptional circumstances may carcasses be burnt (other than in an incinerator) or buried on-site. Composting is not a permitted disposal option. Equipment used for the storage and disposal of dead birds should be subjected to a documented hygiene protocol (www.thepoultrysite.com). Dead animals were considered as possible sources of contamination, and that boots of animal caretakers were also found positive for *Salmonella* indicating that a particular attention should be given to improve the disinfection of boots to avoid the dissemination of *Salmonella*(Amass *et al.*, 2000).

2.2.3.3 *Salmonella* in litter

Chicken litter is a mixture of faeces, wasted feeds, bedding materials, and feathers (Kim *et al*., 2012). Sampling litter, drag swabs, and foot covers are some of the

methods used for testing on the farm (Hiett *et al.,* 2007). Some research indicates that using foot covers is a low cost and effective way to determine *Salmonella* presence (McCrea *et al.,* 2005). Manure should not be spread on land whilst other livestock have access to it. Where possible litter should be stacked for at least four weeks before spreading. Where facilities exist, the incineration of used litter from flocks infected with *Salmonella* is preferred. Animals should not be grazed on land on which poultry litter has been spread for at least five weeks. Vehicles and equipment should be cleaned and disinfected after being used for removal of litter. They should not be used for carrying feedstuffs or new litter but if this is unavoidable, for example on small farms, the items should be cleaned and disinfected immediately after litter removal, left to dry completely then re disinfected and dried before use for feedstuffs or new litter. (www.thepoultrysite.com)

2.2.3.4 *Salmonella* in faeces

The large number of competing bacteria is one of the major limiting factors in the isolation of *Salmonella* from faeces and other environmental samples. Faeces (especially if fresh) provide an indication of current infection of flocks, (EFSA, 2007), whereas contaminated dust may also indicate previous infection compared with faeces (Haysom and Sharp, 2003). Cloacal swabs are relatively insensitive due to other reasons, a typically low prevalence of infection in individual birds, combined with intermittent shedding and the relatively low number of organisms excreted by infected birds in many cases. Moreover, since cloacal swabs only obtain a small amount of faeces and *Salmonella* maybe present in low numbers or be non-uniformly distributed in the faeces, this method is likely to be relatively insensitive compared with the culture of more voluminous faecal material (Kotton *et al*., 2006). They should be taken as aseptically as possible, avoiding cross-contamination of the swab from the integument of the birds. Any previous antibiotic treatment may mask the success of the isolation.

2.2.3.5 *Salmonella* in dust

Dust is a more sensitive type of sample for detecting *Salmonella* in poultry flocks (EFSA, 2007). This is likely to be due to the comparative advantage of *Salmonella* in this type of matrix compared to other Enterobacteriaceae, which do not tend to survive as well in dry conditions (Haysom and Sharp, 2003).

2.2.3.6 *Salmonella* in water

The drinking water plays an important role in the transmission of many pathogenic agents, and there have been many reports about water contamination with *Salmonella spp.* Diseases that can be transmitted to the bird flock through the drinking water may originate from water contamination by faeces and secretions of sick birds in the same flock or from the utilization of water already contaminated by pathogenic organisms originating from other animal species and the man (Jafari *et al.,* 2006).

2.2.3.7 *Salmonella* in Processing plant

Slaughter and dressing operations do not have a treatment capable of destroying all pathogens. FSIS, (2010) expected plants to have food safety systems designed to ensure birds are processed in a manner that reduces possible contamination during slaughter and dressing and expects plants to have treatments in place to reduce the level of incoming contamination on the exterior of the birds throughout the operation. The procedures and treatments that are used to reduce contamination should be documented as part of their food safety systems (FSIS, 2010).

Cross contamination of both birds and cages is frequently made worse when the birds are moved to the plants. There can be a 20-40% increase in *Salmonella* both inside and outside the birds during movement. Moving the birds causes them to pass more fecal material. If the birds have *Salmonella,* the cages have *Salmonella* as well (FSIS, 2010).

More research suggests that a two-step process that first cleans and disinfects the cages is effective at reducing *Salmonella*. Pre-cleaning the cages prior to immersing in hot water for 30 seconds at 60 ºC or higher or immersing for 30 seconds in a solution of sodium hypochlorite at 750 ppm or higher appears to reduce *Salmonella* on transport cages (Ramesh, *et al*., 2004).

2.3 *Salmonella*

2.3.1 General characteristics of *Salmonella*

Salmonella is a facultative anaerobe, Gram-negative rodshaped,2 – 3 x 0.4 – 0.6 μm in size and motile by peritrichous flagella except for *S. gallinarum* and *S. pullorum* which are immotile. They are urease and Voges-Proskauer negative and citrate utilizing .Optimum temperature for growth is in the range of 35 – 37C° but some can grow at temperatures as high as 54C° and as low as 2C°. Salmonellae are typically non-lactose, non-sucrose fermenting but are able to ferment glucose, maltose and mannitol with the production of acid only as in the case of *S*. typhi and acid with H2S in the case of *S. paratyphi* and for most other *Salmonella* serovars. *Salmonella* grow in a pH range of 4 - 9 with the optimum being 6.5 – 7.5. They require high water activity for growth (> 0.94) but can survive at aw of < 0.2 such as in dried foods. Inhibition of growth occurs at temperatures < 7ºC, pH < 3.8 or aw < 0.94(El Hussein *et al*., 2012)

2.3.2 Incidence of *Salmonella* in poultry farms in Sudan

Mohammed *et al.* (2009) isolated four *Salmonella* (not serotyped) from three litter samples of AL-Halfaya farm (layer), and from one water sample of Shambat farm (broiler) out of eighty samples included poultry feed from feeders, litter, drinking water and from drinkers in Al-Halfaya, Shambat, Hillat Kuku and Al-Zakiab areas.

Mohammed (2009) examined 733 samples from clinical, food, poultry, and water at Khartoum, he found 36 *Salmonella* represented 4.9% and the detected serovars were *S. Anatum, S. Allerton, S. Enteritidis, S.kentucky, S. Albertslund, S. Abortus bovis, S.*

Tchad, *S. okerara*, *S. harrisonburg*,and *S. maiduguri*. El Hussein *et al.* (2010) isolated 92 *Salmonella enterica* subspecies *enterica* out of 996 represented raw and cooked food items, fish, chlorinated drinking water, domestic livestock meat and poultry meat, livestock feces and human fecal samples for restaurant workers. representing 30 different serovars of which 19 serovars were reported for the first time in Sudan.

Abdalla *et al*.(2012) reported that from 81 swab samples collected randomly from 27 carcasses from chicken carcasses slaughtered at modern poultry abattoir in Khartoum State from 9 Critical Control Points (CCPs), namely; after bleeding with feathers, after scalding, after defeathering, after evisceration, after spray wash, after chilling and packing, workers' hand, knives and scalding water. The results showed one isolate after spray washing.

Gasm Alseed (2014) reported that (64) *Salmonella* isolates out of 200 faeces samples collected from poultry farms of Khartoum North Locality were obtained and identified as: (7) isolates of *Salmonella arizonea*, (10) isolates of *Salmonella choleraesuis*, (18) isolates of *Salmonella gallinarum*, (23) isolates of *Salmonella pullorum* and (6) isolates of *Salmonella typhi*.

2.3.3 Detection of *Salmonella*

2.3.3.1 Culturing methods

2.3.3.1.1 Traditional Methods

Preharvest estimates of *S. enterica* prevalence are most commonly based on traditional culture methods despite their lack of sensitivity and expediency. However, there is no single method for culture of *S. enterica*. In fact, there are probably more techniques and methods for culturing *S. enterica* than for any other bacterium. This fact was clearly illustrated in a nationwide survey of methods used for culture of *S. enterica* from poultry samples. Studies performed to determine the sensitivity of various culture methods on different substrates have shown that certain methods

perform better with specific substrates. Thus, the use of culture methods appropriate for recovery of *Salmonella* from food, feed, or environmental sources may not be optimal for recovery from feces.(Rybolt *et al.,* 2005). Analysis of studies that determine the prevalence of *Salmonella* infection, based on cultural methods, can then be accomplished with a greater assurance that the diagnostic sensitivity of the method used did not affect the perceived prevalence rate(Love and Rostagno, 2008).

2.3.3.1.2 ISO 6579 for *Salmonella* isolation

There is a current International Organization for standardization horizontal method, ISO 6579 (2002), for the detection *Salmonella spp*. in food and animal feed. The method was amended in 2007 to include testing of animal faeces and environmental samples from primary production. Similar standard methods have been published elsewhere by other bodies, notably in the USFDA Bacteriological Analytical Manual (BAM). The first stage in traditional detection methods for most food samples is usually a pre-enrichment culture in a non-selective liquid medium such as buffered peptone water, incubated at 37ºC for 18 hours. Modified pre-enrichment methods may be necessary for samples containing inhibitory compounds. The pre-enrichment culture is then typically subcultured into two different selective enrichment media, such as Rappaport Vasiliadis Soy broth (RVS) and Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn) broth, and incubated for a further 24 hours at 41.5ºC (RVS) or 37ºC (MKTTn). The selective enrichment culture is usually inoculated onto at least two selective agar media and incubated at 37ºC for 24 hours. The ISO method specifies the XLD agar and one optional selective medium. A variety of alternatives are available, including Bismuth Sulphite agar, Brilliant Green agar and Hektoen Enteric agar. A number of selective chromogenic agar media specifically designed for the differentiation of *Salmonella* colonies are commercially available. Typical *Salmonella* colonies on selective agar are subcultured onto non-selective media prior to confirmatory testing.(Love and Rostagno2008).

2.3.3.1.3 Confirmation and Identification of *Salmonella* isolates

2.3.3.1.3.1 Biochemical detection: Analytical Profile Index(API)

The Analytical Profile Index (API20E strips) is used as a biochemical system for identification of *Salmonella*. The API20E strip consists of 20 microtubes containing dehydrated substrates. These strips are incubated in bacterial suspensions for 18 to 24 h at 37°C. During the incubation period, metabolism produces changes that are either spontaneous or revealed by the addition of reagents. The standard is scored according to a reading table and the identification is obtained by referring to the API catalogue (Zaki *et al.*, 2009)

2.3.3. 2 Rapid Methods

Rapid screening methods have been developed to produce results more quickly for food and environmental samples since traditional methods take at least three to five days to obtain a result. Many of these are available commercially and have been successfully validated by the AOAC and/or AFNOR. The AOAC database of performance tested methods contains more than 40 products for the rapid detection of *Salmonella*.

Salmonella rapid test and screening kits utilize several different technologies, including novel culture techniques, immunomagnetic separation, EIA- and ELISA-based assays incorporating fluorescent or colorimetric detection, simple lateral flow assays incorporating immunochromatographic technology, and molecular techniques such as DNA hybridisation and PCR-based assays, many of which now include real-time detection. Some methods can be automated to screen large numbers of samples. Almost all rapid test protocols include a selective enrichment stage, and then apply concentration and/or rapid detection techniques to replace culture on selective agars and further confirmatory tests. Most can claim to produce a result in approximately 48 hours or less, depending on the enrichment protocol (Love and Rostagno, 2008).

2. 3.3.3 Serotyping of *Salmonella*

Salmonella express flagellar, polysaccharide and capsular antigens which determine strain pathogenicity and therefore variation of these antigens has formed the basis for *Salmonella* serotyping. The genus *Salmonella* is divided into two species based on differences in 16S rRNA sequence data : *Salmonella enterica* which is further divided into six subspecies: subspecies *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and subspecies *indica* ,the other species is *Salmonella bongori* (Popoff and Minor, 2001).

The Kauffmann-White scheme was first published in 1929, divides *Salmonella* into more than 2500 serotypes according to their antigenic formulae (Mortimer *et al.*, 2004) . Kauffmann-White scheme recognizes 2610 *Salmonella* serovars, the majority (2587) belongs to *S. enterica* while the remaining (23 serovars) are assigned to *S. bongori* (Guibourdenche *et al.,* 2010). The Kauffmann-White scheme classifies members of *Salmonella* species according to three major antigenic determinants composed of somatic (O-antigens), flagellar (H-) and virulence (K-) antigens (Scherer and miller, 2001).

2.3.3.3.1 Antigens for serotyping

2.3 3.3.1.1 O antigen

Agglutination by antibodies specific for the various O-antigens, groups the salmonellae into six serogroups: A, B, C1, C2, D and E. Rarely cross reactivity between O antigens of *Salmonella* and other genera of Enterobacteriaceae do occur. Therefore further classification of serotypes is based on the highly specific H-antigens (Scherer and miller, 2001).

2.3.4.3.1.2 H-antigenes

Most serotypes are diphasic, (they express two flagella antigens), and a minor part are monophasic, (express one flagella antigen). *Salmonella gallinarum* is the only

serotype in the Kauffmann-White scheme that does not express any flagella antigen and is therefore non-motile (Sonne-Hansen *et al.,* 2005).

2.3.3.3.1.3 K-antigen

K-antigens are produced by serovars that are characterized by extracellular polysaccharide capsules (Hu and Kopecko, 2003).

2.3.3.3.2 Conventional serotyping

Conventional serotyping of *Salmonella* is the most commonly used method to differentiate strains, which are epidemiologically the smallest bacterial unit from which isolates share the same phenotypic and genotypic traits (Yan *et al.,* 2003). In most clinical studies, initial serotyping is done using polyvalent O antisera to allow *Salmonella* isolates to be grouped into different O groups designated in capitalized letters. Many *Salmonella* show diphasic production of flagellar antigens and each strain can spontaneously and reversibly vary between these two phases with different sets of H antigens. In phase 1 or the specific phase, the different antigens are designated by small letters, and in phase 2 or the group phase, the antigens first discovered are numbered. In a single cell, usually only one antigen is expressed at a time (Yan *et al.,* 2003).

2.3 3.4 Molecular characterization

Since all molecular techniques are based on variability of microbial chromosomes or plasmids, then DNA sequencing would appear to be the best approach for differentiating subtypes (Liebana *et al.,* 2001).

2.4 *Salmonella* antimicrobial resistance

There are reports of high prevalence of resistance in *Salmonella* isolates from countries such as Taiwan (Lauderdale *et al.,* 2006), India (Mandal *et al.,* 2006), resistant isolates from France (Weill *et al.,* 2006), Canada (Poppe *et al.,* 2006), Ethiopia (Molla *et al.,* 2003), and Sudan (Ahmed *et al.,* 2014 a). Similarly, there are various reports of multi-drug resistant *Salmonella* organisms isolated from chickens

in India. One of the studies indicated a rise in the antibiotic resistance in *Salmonella typhi* (Gautam *et al.*, 2002).

2.4.1 Developing resistance to antibiotics

Resistance to antibiotics has been described as 'the best-known example of rapid adaptation of bacteria to a new ecosystem (Carattoli , 2003). Horizontal transmission of resistance genes is now recognized as a major cause of increasing antibiotic resistance. It occurs through natural processes of gene transfer between cells, often via mobile segments of DNA known as transposons (jumping genes) and plasmids (circles of DNA that can replicate themselves). Plasmids can carry several resistance genes giving resistance to several different antibiotics at once (Carattoli , 2003).

2.4.2 Transmission of antibiotic resistance in *Salmonella spp*

2.4.2.1Bacterial plasmids

Plasmid mediated resistance is the transfer of antimicrobial resistant genes carried on plasmids and can be transferred between prokaryotes through horizontal gene transfer. They are important because they affect replication, metabolism, fertility as well as resistance to antibiotics, toxins (bacteriocins) and bacteriophages. The spread of multiple antimicrobial resistance has been enhanced by selective pressure from human and veterinary medicine (Carattoli, 2003). A single R-plasmid can code for resistance of up to 10 different antibiotics simultaneously. Plasmids are able to replicate within a cell and are subject to mutations involving either the loss or gain of genes. Furthermore, they are capable of combining with other plasmids, thus conferring resistance to several antibiotics that can reside on one plasmid. Most importantly, bacteria are capable of transferring plasmids from one cell to another through a process termed conjugation, which is a mechanism of horizontal gene transfer and allows the transfer of plasmids coding for antibiotic resistance among an entire colony of bacterial cells (Levy, 2002).

2.4.2.2Transposons

It is possible for resistance genes to reside on small pieces of DNA called transposons. In general, these pieces of DNA contain terminal regions that participate in recombination and express a protein(s) (e.g. transposase or recombinase) that facilitates incorporation into and movement from specific genomic regions. These pieces of DNA have the capability to 'jump' from one region of the chromosome, to another and vice versa. This way, a resistant gene can be directly incorporated into host chromosomal DNA and not be dependent on plasmid transfer for spread (Levy, 2002).

2.4.2.3 Integrons

These are two component gene capture and dissemination system, initially discovered in relation to antibiotic resistance and are found in plasmids, chromosomes and transposons. The elements are divided in to three classes based on the general gene arrangement that they encode and IntI integrase. Before 2001, there were only four classes of integrons which had been known, mainly from clinical isolates. These are integrons class-1, class-2, class-3 and class-4 (Collis *et al.*, 2002). The development of multidrug resistance depends on the capacity of integrons to cluster the gene cassettes and to express antimicrobial resistance genes. Mostly integrons are not mobile by themselves, but may be integrated into transposable elements, such as Tn*1696* or located on plasmids, and then can be spread with these elements (Abatcha *et al* ., 2014). Antunes *et al*. (2006) have reported the presence of class 1 and 2 integrons in *Salmonella* serotypes Typhimurium, Enteritidis, Muenhen, Rissen, Derby, Saintpaul, Heidelberg, Bredeney, Brandenburg, and Brikama isolated from humans, food products and environment.

2.4.2.4 Bacteriophages

Bacteriophages are bacterial viruses discovered independently by Twort in 1915 in England and by d'Herelle at the Pasteur Institute in Paris in 1917. Like viruses,

bacteriophages are incapable of independent growth in artificial media and are obligate intracellular parasites. Bacteriophages in the aquatic environment are very diverse and most of them have been studied elaborately and include the viruses that infect the enteric group such as *E. coli* and *S. typhimurium*. Those most commonly found in nature have double strand (ds) DNA genome though there are others that have single strand(ss) RNA, ds RNA and ss DNA genomes. Two types of viral life cycles, namely virulent and temperate, exist. Virulent, also known as lytic phages, which lyse or kill the host after infection while the temperate or lysogenic as they are otherwise referred to, achieve a state where they get integrated into the genome and replicate along with the host genome without killing them. They provide a mechanism to transfer antibiotic resistance genes through lysogenic cycle, the process being referred to as phage mediated transduction. When these viruses enter new hosts, they are able to integrate their DNA as well as the antibiotic resistance genes picked up from the previous host into the chromosome of new host (Levy, 2002).

2.4.3 Resistance of *Salmonella* to various groups of antibiotics

2. 4.3.1 Resistance to aminoglycosides

There are three mechanisms of aminoglycoside resistance in bacteria; reduced uptake or decreased cell permeability, alteration at the ribosomal binding sites and production of aminoglycoside modifying enzymes leading to the enzymatic detoxification of drugs (Abatcha *et al.*, 2014).

Integron-borne gene cassettes conferring resistance to aminoglycosides are also very diffused in Gram-negative bacteria, and integrons have frequently been associated with the widely distributed transposon Tn*21* (Liebert *et al* ., 1999). The Tn*21* transposon encodes genes and sites required for transposition (including *tnpA*, *tnpR*, *tnpM*, *res*, and inverted repeats), and integrons are located in the left arm, adjacent to the *tnpM* gene. The Tn*21*-associated integrons often carry

the *aadA1* gene cassette, known to confer resistance to streptomycin and spectinomycin (Liebert *et al* **.,**1999).

2. 4.3.2 Resistance to β-lactam antibiotics

Most β-lactam antibiotics work by inhibiting cell wall biosynthesis in the bacterial organism and are the most widely used group of antibiotics. Resistance to β-lactam antibiotics is mainly due to inactivation by β-lactamase enzymes and also due to decreased ability to bind penicillin binding protein (PBPs) and other mechanism such as permeability (Abatcha *et al.,* 2014). In *Salmonella*, the secretion of a beta-lactamase is the common mechanism of resistance to beta-lactamases. These enzyme acts by hydrolyzing the structural rings of the Blactam, by producing beta amino acids with no antimicrobial activity. In *Salmonella* encoding genes are found or carried on the plasmid (Mascaretti, 2003).

2. 4.3.3 Resistance to quinolones/fluoroquinolones

There are many generations of quinolones, which are more effective against bacterial infection. However, their mode of action varies, the early and late generation of quinolones target DNA gyrase and DNA topoisomerase IV (Mascaretti, 2003). Fluoroquinolones are usually the antimicrobials of choice for treatment of severe or systemic human salmonellosis (Rotimi *et al.,* 2008). An increase in isolation of *Salmonella* strains with reduced susceptibility to fluoroquinolones such as ciprofloxacin is a public health problem, since it may cause failure of clinical treatment, limiting the therapeutic options for treatment(Aznar *et al.,* 2007).

2. 4.3.4 Resistance to sulphonamides

Sulfonamides, the first class of antimicrobial agents, were discovered in 1932 and put into clinical use in 1935 (Skold, 2000). The *sul1* gene is normally found linked to other resistance genes in class 1 integrons, while *sul2* is usually located on small non conjugative plasmids or large transmissible multiresistance plasmids (Enne *et al*.,

2001). The recently described *sul3* has been detected together with *sul1* on a large multiresistance plasmid from *S. choleraesuis* (Chiu *et al*., 2005).

2. 4.3.5 Resistance to tetracyclines

Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of Gram positive and Gram-negative bacteria, and are currently used for therapy and prophylaxis for human infections and for the prevention and control of bacterial infections in veterinary medicine (Roberts, 1996). The most frequent types of *tet* genes belong to classes A, B, C, D, and G (Chopra and Roberts, 2001). The *tet*(G) gene has been identified in Salmonella genomic island 1, located within the *S. enterica* serotype Typhimurium DT104 chromosome (Cloeckaert and Schwarz, 2001).

2. 4.3.6 Resistance to trimethoprim

Trimethoprim compound is a diaminopyrimidine that selectively inhibits bacterial dihydrofolate reductase (DHFR) by preventing the reduction of dihydrofolate to tetrahydrofolate. The most common trimethoprim resistance mechanism is the supplementation of a trimethoprim sensitive dihydrofolate reductase with a trimethoprim resistant dihydrofolate reductase, resulting in high level trimethoprim resistance in various bacteria. A total of 13 *dfrA* genes were detected in various *Salmonella* serovars with most of these located in class 1 and class 2 integrons (Peirano *et al*., 2006) . The association of these genes with mobile genetic elements such as transposons or plasmids has often been observed, and the presence of these genes in cassettes as part of integrons is also common (Peirano *et al*., 2006).

2.5 β-lactam antimicrobials

2.5.1 β-lactam antimicrobials used in veterinary medicine and food animal production

Ampicillin and amoxicillin are regarded as the drugs of choice in avian medicine in many continents. These drugs are used in most European countries, with the

exception of Finland, Denmark and Sweden. In Spain, amoxicillin-clavulanic acid is also allowed for use (Schwarz and Chaslus-Dancla, 2001). Third-generation cephalosporins are rarely used in poultry and only under very limited conditions for treatment of valuable poultry stocks (Guardibassi *et al.*, 2008). In Europe, cephalosporins are not allowed for use in poultry (Smet *et al*., 2008).

2.5.2 β-lactams used in veterinary and human medicine

They are mentioned by Guardabassi *et al.* (2008); Hammerum and Heuer (2009), and Hornish and Kotarski(2002) as follows:

2.5. 2.1 Penicillins

They are mainly active against Gram-positive bacteria. Aminopenicillins are also active against Gram-negative bacteria. Ampicillin, amoxicillin, benzylpenicillin, cloxacillin, hetacillin are used in veterinary medicine, while penicillin, ampicillin, amoxicillin are used in human medicine.

2.5.2.2 Penicillin-β-lactamase inhibitor combinations

These exhibit negligible antimicrobial activity. Their sole purpose is to prevent the inactivation of β-lactam antibiotics and, as such, they are co-administrated mostly with penicillins. Amoxicillin-clavulanate is used in veterinary medicine, while amoxicillin-clavulanate, piperacillin tazobactam are used in human medicine.

2.5.2.3 First generation cephalosporins

They are moderate spectrum agents. Effective alternatives for treating staphylococcal and streptococcal infections. Cepadroxil, cefapirin, cephalexin are used in veterinary medicine, while cefalozin is used in human medicine.

2.5.2.4 Second generation cephalosporins

This is a greater Gram-negative spectrum while retaining some activity against Gram-positive bacteria . Cefaclor, cefamandole, cefonicid, ceforanide, cefuroxime are used in veterinary medicine, while cefuroxime, cefoxitin are used in human medicine.

2.5. 2.5 Third generation cephalosporins

They have a broad spectrum of activity and further increased activity against Gram-negative organisms . Cefovecin, cefpodoxim, ceftiofur are used in veterinary medicine, while ceftriaxone, cefotaxime, ceftazidime are used in human medicine .

2.5. 2.6 Fourth generation cephalosporins

They have the broadest activity both against Gram-negative and Gram-positive bacteria. Cefquinome is used in veterinary medicine, while cefepime is used in human medicine.

2.5. 2.7 Monobactams

They have a strong activity against susceptible Gram-negative bacteria, but no useful activity against Gram-positive bacteria or anaerobes. They are not in use in veterinary medicine, and aztreonam is used in human medicine.

2.5.2.8 Carbapenems

They have a broad spectrum of activity against aerobic and anaerobic Gram-positive and Gram-negative bacteria . Imipenem, meropenem are used both in veterinary medicine and human medicine.

2.5.3 Use of β-lactams in poultry treatment according to clinical indication

Benzylpenicillins are used for dysbacteriosis, Ampicillin and amoxicillin are used in collibacilosis, *Ornithobacterium rhinotracheale* infection , fowl cholera , and *Riemerella anatipestifer* infections. while penicillins are used for erysipelas. (Guardabassi *et al.*, 2008).

2.5.4 β-lactam resistance mechanisms

Bacterial resistance to β-lactams can be due to at least three mechanisms. The first mechanism consists of mutations in genes encoding PBPS, the acquisition of alternative PBPS or the creation of mosaic PBPS. All these altered PBPS have a reduced affinity for β-lactams and as such can keep their function in maintaining the cell wall. The second mechanism consists of a change in the permeability of the cell

wall. This may be due to alterations in the expression of porins or active efflux. The third mechanism, and by far the most common one, is the inactivation of the drug by β-lactamases (Batchelor *et al.*, 2005) β-lactamases inactivate β-lactams by hydrolysing their four-membered β-lactam ring. They break a bond in the β-lactam ring to disable the molecule (Shah *et al.*, 2004).

2. 6 Extended-spectrum beta lactamases (ESBL)

More than 400 β-lactamases have been reported and new β-lactamases continue to emerge worldwide (Jacoby, 2009).

2..6.1 TEM-type β-lactamases

More than 150 TEM-type β-lactamases have been found, and all of them are derivatives of TEM-1 or TEM-2 by point mutations. TEM-1 was first demonstrated in 1965 in an *Escherichia coli* isolate from a patient in Athens, Greece, named Temoneira (designation TEM) (Datta and Kontomichalou, 1965). In contrast to the majority of TEM β-lactamases, TEM-1, TEM-2 and TEM-13 are not ESBLs and are only able to hydrolyse penicillins. Some TEM derivatives have been found to have a reduced affinity for β-lactamase inhibitors and are called inhibitor resistant TEM (IRT). However, mutants of the TEM derivatives (called CMT-1, CMT-2, CMT-3 and CMT-4) have been identified that have the ability to hydrolyse both third-generation cephalosporins and β-lactamase inhibitors (Neuwirth *et al.*, 2001).

2.6.2 SHV-type β-lactamases

Another family of β-lactamases are the SHV (sulfydryl variable) enzymes. The progenitor of the SHV enzymes, SHV-1, was first described in *Klebsiella pneumoniae*. SHV-1 confers resistance to broad-spectrum penicillins. In 1983, a *Klebsiella ozaenae* strain was isolated in Germany possessing a SHV-2 enzyme which efficiently hydrolyzed cefotaxime and, to a lesser extent, ceftazidime. More than 50 SHV derivatives are known, all being derivatives of SHV-1 or SHV-2. Like the TEM-type enzymes, the majority of the SHV enzymes are ESBLs (Gupta, 2007).

2. 6.3 CTX-M-type β-lactamases

A third family consists of the CTX-M enzymes, which are also ESBLs and were first isolated in Munich. The designation CTX-M reflects the hydrolytic activity of these β-lactamases against cefotaxime. It appears that the CTX-M-type β-lactamases are closely related to β-lactamases of *Kluyvera* spp. CTX-M enzymes have 40% or less identity with TEM and SHV-type ESBLs. So far, more than 70 CTX-M enzymes have been isolated. They are divided into 5 clusters on the basis of the amino acid sequence: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Gupta, 2007).

2. 6.4 OXA-type β-lactamases

Most OXA-type β-lactamases, so named because of their oxacillin-hydrolizing capabilities, do not hydrolyse extended-spectrum cephalosporins and are not regarded as ESBLs. The exceptions to this rule are OXA-10 and OXA-13 to OXA-19 (Toleman *et al.*, 2006). The evolution of ESBL OXA-type β-lactamases from parent enzymes with narrow spectra has many parallels with the evolution of TEM- and SHV-type ESBLs.

There are other examples of ESBLs include PER (*Pseudomonas* extended-resistant), VEB (Vietnam extended-spectrum β-lactamase), BES (Brazil extended-spectrum), GES (Guiana extended-spectrum), TLA (named after Tlahuicas Indians), SFO (*Serratia fonticola*) and IBC (integron-borne cephalosporinase) are other examples of ESBLs that have been discovered (Gupta, 2007). These enzymes are not so common among *Enterobacteriaceae* as the ESBLs described above.

2. 7 Classification of broad-spectrum β-lactamases

The first classification system, devised by Bush *et al.* (1995) is based on the activity of the ß-lactamases against different ß-lactam antimicrobials (substrate specificity). It contains a wide variety of subgroups. Three major groups of enzymes can be defined: Cephalosporinases that are not greatly inhibited by clavulanic acid, and penicilinases,

cephalosporinases and broad-spectrum β-lactamases that are inhibited by β-lactamase inhibitors, and metallo-β-lactamases (carbapenamases) that hydrolyze penicillins, cephalosporins and carbapenems with the exception of aztreonam (Bush *et al.*, 1995). The second , and most widely used classification scheme for β-lactamases is the Ambler system, which divides β-lactamases into four classes (A, B, C and D) on the basis of their amino acid sequences. At first, Ambler described two classes: class A β-lactamases (TEM, SHV and CTX-M enzymes), which have their active site at a serine residue, and class B enzymes (metallo-β-lactamases), which utilize a bivalent metal ion (zinc ion) to attack the β-lactam ring (Ambler, 1980). Later on, when new insights were acquired, a novel class of serine β-lactamases, class C (AmpC β-lactamases), was defined. These showed little sequence similarity to the class A enzymes. Finally, another new class of serine β-lactamases, known as the OXA β-lactamases (class D), was identified (Ambler, 1980).

2. 8 Methods for detection ESBL

2. 8.1 Combine disk test (Inhibitor potentiated disk test)

Cephalosporins disks (cefotaxime 30µg, ceftazidime 30 µg, Cefpodoxime 30µg) with and without 10µg clavulanic acid are placed on Muller Hinton agar inoculated with test organisms . An increase in the inhibition zone diameter of ≥ 5mm in cephalosporins disk combined with clavulanic acid, compared to cephalosporins alone, indicates ESBL production(Carter *et al*,, 2000).

2.8.2 Double disk synergy test

The disc that contains oxyimino β lactam (30µg) is placed 30mm apart (center - centre) from amoxicillin- clavulanate disk (20/10µg) clear extension of the edge of the inhibition zone towards amoxicillin- clavulanate disk is interpreted as positive ESBL production(CLSI, 2006) .

2.8. 3 Three dimension test

The standard inoculum of test organisms is inoculated on Muller Hinton agar plate, a slit is cut on agar plate in which a broth suspension of tested organism is placed; antibiotic disc is placed 3-4mm from the slit . Distortion of circular inhibition zone is interpreted as positive ESBL production (Menon *et al*., 2006).

2. 8.4 MIC reduction test

An 8 fold reduction in the MIC of cephalosporin in presence of clavulanic acid, using E Test or broth micro/macro dilution indicates ESBL (Leverstein *et al* ., 2002) .

2. 8.5 Vitek ESBL test

Wells containing cards are inoculated, the reduction in growth of cephalosporins well contains clavulanic acid; when compared to with level of growth in well with cephalosporin alone indicates presence of ESBL production. (Leverstein *et al* ., 2002).

2. 8.6 E test

The **E** test strip carries two gradients, on the one end ceftazidime and on the opposite end ceftazidime plus calvulanic acid. MIC is interpreted as the point of intersection of the inhibition ellipse with the E test strip edge. Ratio of ceftazidime MIC and ceftazidime calvulanic aid MIC equal to or greater than 8 indicates the presence of ESBL (luzzaro *et al.,* 2006).

2.8.7 BD Phoenix Automated Microbiology system

The phoenix ESBL test uses the growth response to cefpodoxime, ceftazidime and cefotaxime to detect ESBL production (Leverstein *et al* ., 2002).

2.8.8 Molecular Detection of ESBL

These include DNA probes, PCR, oligotyping, PCR-RFLPs and nucleotide sequencing. Molecular methods can detect different variants of ESBL but they can be labor intensive and expensive to be adopted as routine methods (Bonnet, 2004).

Several clinical microbiology tests that presumptively identify the presence of an ESBL has been reported, but the task of identifying which specific ESBL is present in a clinical isolate is more complicated. Although the presence of ESBL in clinical isolates of *Salmonella*. *spp* have phenotypically been previously detected and reported in Trinidad and Tobago, yet none of the different types of ESBL genes have been described. The molecular detection of the several ESBL gene types prevailing in clinical isolates of *Salmonella* was performed (Bradford, 2001).

CHAPTER THREE

Materials and Methods

3.1 Sampling design and strategy

This research was conducted at Khartoum State the capital of Sudan including Khartoum, Bahri, and Omdurman Localities between May, 2013 and November, 2016. Isolation of *Salmonella species* and antimicrobial resistance was done at the Microbiology laboratory, Faculty of Medical laboratory Science, Al-Neelain University. Genotyping method was done in the Central Laboratory, Ministry of Higher Education, Khartoum.

3.2 Study area

A total of 465 samples were collected from different 44 chicken farms comprising the three localities of Khartoum State as follows;

3.2.1 Khartoum locality: A total of 162 samples representing 18 different farms were collected from different sources of the chicken farms of Arak Salih, Tayba-Hassanab, El-Shegailab ,El- Dekhainat, El- Ghadesya ,El- Fetaih, and Wad El-Agaly which represented the southern of Khartoum State, besides 39 samples collected during slaughtering.

3.2.2 Bahri Locality: A total of 162 samples representing 18 different farms were collected from different sources of the chicken farms of Elkabbashi ,El-Kadaro , El-Droshab, El-Halfaya, and Shambat which represent the northern of Khartoum State, addition to Hillat Kuku, and Om-doum which represented eastern of Khartoum State, besides 30 samples collected during the slaughtering .

3.2.3 Omdurman Locality: A total of 72 samples were collected from 5 farms in the north of Kilo 11, Karary, El-Heraizab, and 3 farms in the west (El-Muwaileh , Dar-elsalam, and Libia).

3.3 Sampling

A number of 9 samples were taken from each farm included water (source and drinkers), feed (poultry feed and feeders), dust, litter, cloacal swabs, faeces and hand swabs from poultry handlers), whereas samples collected during slaughtering included knives, carcass swabbing, and carcass meat.

Isolation and identification of *Salmonella* contamination in the environment and processing plant were done, and the isolates were tested for antimicrobial resistance to find out the resistance pattern of the organism. The antimicrobials for resistance testing were selected based on the present therapeutic use of those antimicrobials in human and commercial poultry production; ciprofloxacin, chloramphenicol, amoxicillin, ampicillin, tetracycline, gentamycin, streptomycin, co-trimoxazole, nalidixic acid, colistin, amikacin, cefotaxime, cefixime, ceftazidime, cefalexin, and ceftriaxone .

3.3.1 Collection of samples and culturing methods

3.3.1.1 Hand swabs

A number of 44 hand swabs were collected from poultry handlers. Sterile swabs were moistened in sterile buffered peptone water (BPW), rolled all over the hand and then immersed into test tubes containing 9 ml BPW **(**Mohamed *et al*., 2004).

3.3.1.2 Poultry cloacal swabs

A number of 44 sterile swabs moistened in sterile BPW were inserted into the cloaca and then withdrawn. The swabs were directly immersed into tubes containing 9 ml BPW **(**Mohamed *et al*., 2004**)**.

3.3.1.3 Poultry feed samples

A number of 44 samples of feed were collected from each the source and from feeders. After mixing, about 100 g. feed samples collected by sterile spoon and placed in sterile ISO bags, then transferred to the lab where 25g were taken from each

sample in a sterile aluminum foil, then aseptically added to 225 ml BPW and mixed well **(**Mohamed *et al*., 2004).

3.3.1.4 Litter samples

After mixing, about 100 g. litter samples (44 samples) were collected by sterile spoon and placed in sterile ISO bags, then transferred to the lab where the sample was mixed, 25g were taken from each sample in a sterile aluminum foil, then aseptically added to 225 ml BPW and mixed well (ISO 6579: 2002) **.**

3.3.1.5 Water samples

A number of 44 samples were collected from the source water (tap water) in 250 ml sterile glass bottles after cleaning the tap water using ethanol 70% and let the water pass thoroughly for 15minutes (World Health Organization **(**WHO),1998), then 25 ml of each thoroughly mixed sample was added to 225 ml BPW(ISO 6579, 2002). Also 44 samples were collected from drinkers by using sterile syringes. Twenty five ml of each mixed sample was added to 225 ml BPW(ISO 6579: 2002).

3.3.1.6 Dust samples

A number of 44 sterile swabs were moistened in sterile BPW, rolled all over the fans, and walls and then immersed into test tubes containing 9 ml BPW.

3.3.1.7 Faecal samples

After thorough mixing, about 100 g faeces samples(44 samples) were collected by sterile spoon and placed in sterile ISO bags, then transferred to the lab. In the lab., the sample was thoroughly mixed and 25g were taken from each sample in a sterile aluminum foil, then aseptically added to 225 ml BPW and mixed well **(**ISO 6579, 2002).

3.3.1.8 Knives samples

A number of 23 sterile swabs were moistened in sterile BPW, The knife blade was swabbed from its tip to its base twice((Botteldoorn *et al* .,2003)) and then immersed into test tubes containing 9 ml BPW **.**

3.3.1.9 Poultry samples

A number of 23 sterile swabs were moistened in sterile BPW, rolled all over the carcass and then immersed into test tubes containing 9 ml BPW. Also 23 samples of a whole chicken carcass were purchased after the slaughtering and transported to the laboratory in cool boxes. A weight of 25 g of chicken breast was cut into fine pieces(Dahal, 2007) and transferred into 225 ml of buffered peptone water. The sample mixture was shaken approximately for 2 minutes and the samples were incubated at 37 ^{o}C ± 1 ^{o}C for 18 ± 2 hours (ISO6579:2002).

3.3.2 Preparation of culture media

3.3.2.1 Buffered peptone water. (HIMEDIA M614)

Twenty grams were suspended in 1000 ml distilled water, then dispensed in 225 ml amount and 9 ml amount and sterilized by autoclaving at 15 lbs pressure (121C°) for 15 minutes.

3.3.2.2 Rappaport vasiliadis broth. (MICROMEDI MN 0070)

A weight of 26.8 gms were suspended in 1000 ml distilled water, mixed and heated until dissolved. Then autoclaved at 115 C°for 15 minutes.

3.3.2.3 Muller-Kuffman Tetrathionate Novobiocin Broth Base. (HIMEDIA M 14961)

A weight of 89.42 grams of dehydrated medium was suspended in 1000 ml distilled water and heated just to boiling, then cooled to 45C°-50C°.Before used aseptically 20 ml of iodine solution(20 g iodine and 25 g potassium iodide in 100 ml sterile distilled water) were added along with rehydrated contents of one vial of MKTT novobiocin supplement(FD 203), then mixed and dispensed in the sterile tubes to disperse Calcium carbonate uniformly.

3.3.2.4 Xylose Lysin Deoxycholate agar. (HIMEDIA M 031)

A weight of 56.68 grams were suspended in 1000 ml distilled water, then heated with frequent agitation until the medium boiled (autoclaving and overheating were avoided), then transferred immediately to a water bath at 50 C° and poured after cooling in to sterile petri dishes.

3.3.2.5 Salmonella-Shigella agar. (HIMEDIA M 108)

A weight of 63.02 grams were suspended in 1000 ml distilled water and heated to boiling with frequent agitation to dissolve the medium completely. Autoclaving and overheating were avoided to avoid destruction of the selectivity of the medium. Then cooled to about 50 C° and poured in to sterile petri dishes .

3.3.2.6 Nutrient agar. (HIMEDIA M001)

A weight of 28.0 gram was suspended in 1000 ml distilled water, then heated to boiling to dissolve the medium completely, and sterilized by autoclaving at 15lbs pressure (121C°) for 15 minutes, mixed well and poured in to sterile petri plates.

3.3.2.7 Glucose Phosphate broth. (HIMEDIA M070)

A weight of 17.0 gms was suspended in 1000 ml distilled water, heated to dissolve completely, distributed in test tubes, and sterilized by autoclaving at 15lbs pressure (121C°) for 15 minutes.

3.3.2.8 Lysine Decarboxylase Broth.(HIMEDIA M376)

A weight of 14 gms was suspended in 1000 ml distilled water, heated to dissolve completely, then dispensed in 5 ml amount in to screw capped test tubes and sterilized by autoclaving at 15Ibs pressure (121C°)for 15 minutes, cooled in an upright position, inoculated and overlaid with 2-3ml of sterile mineral oil.

3.3.2.9 Triple Sugar Iron agar .(SHARLAU-01-192)

A weight of 64.6 gms of powder was suspended in 1000 ml of distilled water , then boiled and dispensed in to tubes and sterilized at 121C° for 15 minutes, and left to solidify with short slant and good butts.

3.3.2.10 Urea agar base (christensen).(HIMEDIA M 112)

A weight of 24.01 gms was suspended in 950 ml of distilled water, heated to dissolve completely, then sterilized by autoclaving at 115 C° for 20 minutes. Then cooled to 50C°and 50 ml of sterile 40% of urea solution was added aseptically.

3.3.2.11 Peptone water phosphate buffered(SHARLAU 02-568)

A weight of 20 gms of powder was suspended in 1000 ml of distilled water and distributed in to test tubes , then sterilized in the autoclave at 121C°for 15 minutes.

3.3.2.12 Muller Hinton Agar(HIMEDIA M1084)

A weight of 38 gms of powder was suspended in 1000 ml distilled water, mixed well and heated to boiling, then sterilized by autoclaving at 121C°for 15 minutes.

3.4 API20E (Ref 20 100)

The kit consists of 25 API 20 E strips, 25 incubation boxes, 25 result sheets, 1 clip seal, and 1 package insert. The strip consists of 20 microtubes containing dehydrated substrates which are inoculated with a bacterial suspension that reconstitutes the media , and are used for the detection of beta- galactosidase, Arginine dihydrolase, Lysine decarboxylase, Ornithine decarboxylase), Tri sodium citrate utilization, H_2s production, Urease, Tryptophane deaminase, Indole production, Acetoin production, Gelatinase, besides sugar fermentation or oxidation(D- glucose, D- manitol, Inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, Amygdalin ,L-Arabiose.

3.5 Antibiotics used for Kirbey-Bauer disk diffusion method

Antibiotics used in this study were obtained from HIMEDIA: ampicillin (AMP 10mcg/disc SD002-ICT), amoxicillin (AMX 10 mcg/ disc. SD00-ICT),colistin(CL 10 mcg/disc), chloramphenicol(C30 mcg/disc. SD006-ICT), streptomycin (S10 mcg/disc.SD031ICT),ciprofloxacin(CIP5mcg/disc.SD0060ICT),tetracycline(TE30mcg/disc.SD037-ICT),coTrimoxazole(Sulpha/Ttimethoprim25mcg/disc)SDO10 ICT,gentamicin(GEN10mcg/disc.SD016-ICT),nalidixicacid(NA30mcg/disc),

cefixime, (CFM 5 mcg/disc), cefotaxime(CTX 30mcg/disc), cetriaxone (CTR 30 mcg/disc), ceftazidime (CAZ 30mcg/disc), cefalexin (CN 30 mcg/disc), amikacin (AK 30 mcg/disc), and Amoxyclav (AMC 30 mcg/ disc).

3.6 Analysis methods

3.6.1 *Salmonella* isolation procedure

The study was conducted utilizing the conventional methods for the detection of *Salmonella* following the standard guide lines from ISO 6579:2002 (Microbiology of food and animal feeding stuffs horizontal method for the detection of *Salmonella spp*.)

3.6.1.1 Non-selective pre-enrichment

A weight of 25 grams of a solid sample , and a volume of 25 ml of a liquid sample was transferred into 225 ml of buffered peptone water and swabs samples were transferred in to 9 ml of buffered peptone water (HIMEDIA) and incubated at 37 ^{o}C ± 1 ^{o}C for 18 ± 2 hours.

3.6.1.2 Selective enrichment

The pre-enrichment broth after incubation was mixed and 0.1 ml of the broth was transferred into a tube containing 10 ml of Rappaport-Vassiliadis broth (MICROMEDIA). Another 1 ml of the pre-enrichment broth was transferred into a tube containing 10 ml of Muller-Kauffmann tetrathionate novobiocin broth (MKTTn broth-HIMEDIA). The inoculated RV broth was incubated at 41.5 ^{o}C±1 ^{o}C for 24 ± 3 hours and the inoculated MKTTn broth at 37 ^{o}C±1 ^{o}C for 24 ± 3 hours.

3.6.1.3 Plating out and identification

After incubation for 24 ± 3 hours, a loop-full of material from the RV broth(incubated at 41.5 ^{o}C±1 ^{o}C) and MKTTn (incubated at 37 ^{o}C±1 ^{o}C) was transferred and streaked onto the surface of Xylose lysine deoxycholate agar (XLD

agar-HIMEDIA) and Salmonella Shigella agar (S.S agar-HIMEDIA) separately. The plates were incubated at 37 ^{o}C±1 ^{o}C for 24 ± 3 hours. The plates were incubated in an inverted position and after incubation; the plates were checked for growth of typical *Salmonella* colonies.

3.6.2 Confirmation

Five typical colonies per plate grown on the XLD agar and S.S agar were transferred and inoculated on triple sugar iron agar (TSI), incubated at 37 ^{o}C ±1 ^{o}C for 24 ± 3 hours. In fewer than five typical or suspected colonies per petri dish observed, all suspected colonies were streaked on the surface of pre-dried nutrient agar plates, to get single colonies The inoculated plates were incubated at 37^{o}C±1^{o}C for 24 ± 3 hours. Thus the pure culture obtained was used for microscopic, biochemical, and genotyping confirmation.

3.6.2.1 Microscopic examination

3.6.2.1.1 Gram- staining technique

This technique was performed according to the method described by Cheesbrough (1991).A dried smear was fixed and then covered with crystal violet stain for 60 seconds, the washed off with clean water, then the smear was covered with lugol's iodine for 60 seconds then washed off with clean water, acetone-alcohol was added rapidly and washed immediately with clean water, the smear was covered with safranin for 2 minutes and washed with clean water, and left to dry, then was examined microscopically with the oil immersion objective. Salmonellae appear as red bacilli.

3.6.2.1.2 Motility

The motility test was performed according to the method described by Cheesbrough (1991) to differentiate motile bacteria from the non-motile one. Before performing the test, a pure culture of the organism was allowed to grow in nutrient broth. One

drop of cultured broth was placed in the centre of a slide and covered with a cover glass(bubbles were avoided),a drop of molten Vaseline was placed on each corner of the cover glass, then the end of the slide was reheated to seal the preparation . Motility slide was then examined carefully under 40-power .

3.6.2.2 Biochemical confirmation

It was carried out as recommended by ISO 6579 (2002):

3.6.2.2.1 Oxidase test

By using wooden sticks a single colony of the culture was emulsified on oxidase disc (HIMEDIA, DD018). Yellow colour indicates negative result, while changing the colour into violet indicates positive result.

3.6.2.2.2 Triple sugar iron agar (TSI agar)

The agar slant surface was streaked and the butt stabbed and incubated at 37^{o}C± 1 for 24± 3 hours. Typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and formation of hydrogen sulfide (blackening of the agar).

3.6.2.2.3 Urea agar

The agar slant surface was streaked and incubated at 37^{o}C± 1^{o}C for 24± 3 hours and examination was done at intervals for 24± 3 hours.

3.6.2.2.4 L-lysine decarboxylation medium

Inoculated liquid medium was incubated at 37^{o}C ±1^{o}C for 24± 3 hours. Turbidity and a purple colour after incubation indicate a positive reaction. A yellow color indicates a negative reaction.

3.6.2.2.5 Indole reaction

Inoculated tube with the tested organisms containing 5 ml of peptone water phosphate buffered medium was incubated at 37 ^{o}C±1^{o}C for 24 ± 3 hours. After

incubation, one ml of the Kovacs reagent was added. The formation of a red ring indicates a positive reaction. A yellow-brown ring indicates a negative reaction.

3.6.2.2.6 Voges-Prauskauer (V-P) reaction

Two ml of sterile glucose phosphate peptone water was inoculated with the tested organisms and incubated at 37 ^{o}C for 48 hours, a small amount of creatine was added and mixed, then 3 ml of sodium hydroxide was added and shaked, the bottle cap was removed and was left for 1 hour at room temperature . Slow development of a pink red colour was recorded as positive reaction.

3.6. 3 API20E

3.6. 3.1 Preparation of the strip

Five ml of distilled water (without additives) was distributed in to the wells of the tray to create a humidity atmosphere.

3.6. 3.2 Preparation of the inoculums

Sterile pipette was used to remove a single young colony from an isolation plate in to a tube of 5 ml sterile saline and emulsified carefully to achieve a homogenous bacterial suspension and this was used immediately after preparation.

3.6. 3.3 Inoculation of the strip

The bacterial suspension was distributed into the tubes of the strip by using the same pipette and avoiding formation of bubbles.

3.6. 3.4 Reading and interpretation of the results

The strip was read after incubation. All the spontaneous reaction was recorded on the result sheet and also additions of reagents(A drop of TDA reagent was added to the TDA test, a reddish brown colour indicates positive result, also JAMES reagent was added to IND test, a pink colour in the whole cupule indicates a positive reaction. VP1 and VP2 reagents were added for at least 10 minutes to VP test ,a pink or red

colour indicates positive reaction). Identification was obtained with the numerical profile.

3.6.4 Antibiotics susceptibility test

3.6.4.1 Kirbey-Bauer Method

The Clinical and Laboratory Standards Institute (CLSI), disc-diffusion method was used for antibiotic sensitivity testing (CLSI, 2006). Turbidity of the isolates was compared with 0. 5 McFarland standard and each of the isolates was inoculated onto the surface of a sterile Muller and Hinton plates using a sterile swab in order to ensure even distribution of the inoculums, the plates were allowed to dry and antibiotic discs with concentrations were placed on the surface of the plates. After 30 minutes of applying the disc, the plates were inverted and incubated for 24 hours at 37 C°. The clear zone that developed around each disc was measured as the zones of inhibition from underneath each plate with the aid of a ruler in centimeter and converted to millimeter (mm) and calculated on the basis of CLSI guidelines .

3.6.5 Double disc synergy test

The double disk synergy test (DDST) was performed by placing a disk of co-amoxiclav on the inoculated Mueller-Hinton agar plate at a 20 mm distance from the indicator drugs (ceftazidime, cefexime, ceftriaxone, and cefotaxime). Extended spectrum beta lactamase (ESBL) production was considered positive when the clavulanate mediated, enhancement of the activity of an indicator drug produced a keyhole effect(Ejaz *et al*., 2011).

3.7 Molecular methods

3.7.1 DNA Extraction

DNA was extracted from pure cultures of isolates by boiling. Two to 3 colonies of a pure culture of isolates were suspended in 50 µL of deionized water in a 1.5 mL eppendorf tube and vortexed to ensure a homogenous suspension. The suspension is

then incubated at 100^0C for 10 minutes, quick chilled on liquid nitrogen and repeated three times then centrifugated at 13,000 rpm for5 minutes. 5 µL of the supernatant was stored in -20^0C till used.

3.7.2PCR amplification and Visualization of PCR products

3.7.2.1 Polymerase chain reaction (PCR) for detection TEM

PCR reaction included 25 µl final reaction volume of 5 µl master mix (intron biotechnology, korea) consisted of (taq polymeras ,Mgcl, buffer and DNTPs),1 µl of each 10 P mol forward primer 5- AGC GAT CTG TCT AT - 3 and reverse primer5- AAA CGC TGG TGA AAG TA - 3specific for TEM gene from conserve region((Pitout *et al.*, 2003) ,5 µl from DNA template and 13 µl from deionized water) to complete the volume to 25 µl. The PCR program (techne, UK) initial denaturation94$C^O$for 5 min, followed by 35 cycles 94C^O for 30sec anneling 50 C^O for 30 sec and extension at 72 C^O for 30 sec final extension for 5 min. The amplicons were resolved and screened using 1.5% agarose gel electrophoresis method. All PCR reactions were performed with appropriate negative and positive controls which are size band 752 bp to avoid any false negative and positive results.

3.7.2.2 Polymerase chain reaction (PCR) for detection CTX-M

PCR reaction included 25 µl final reaction 5 µl master mix (intron biotechnology, korea) consisted of (taq polymeras ,Mgcl, buffer and DNTPs), 1 µl of each 10 P mol forward primer 5- ACC GCG ATA TCG TTG GT – 3and reverse primer5- CGC TTT GCG ATG TGC AG – 3specific for CTX-M gene from conserve region (Naas *et al*, 2005) ,5 µl from DNA template and 13 µl from deionized water) to complete the volume to 25 µl. The PCR program (techne, UK) initial denaturation 95C^O for 5 min, followed by 35 cycles 95C^O for 60 sec anneling 50 C^O for 60 sec and extension at 72 C^O for 60 sec final extension for 5 min. The amplicons were resolved and screened using 1.5% agarose gel electrophoresis

method. All PCR reactions were performed with appropriate negative and positive controls which are size band 550 bp to avoid any false negative and positive results.

3.7.3.3 Polymerase chain reaction (PCR) for detection SHV gene

PCR reaction included 25 µl final reaction 5 µl master mix (intron biotechnology, korea) consisted of (taq polymeras ,Mgcl, buffer and DNTPs), 1 µl of each 10 P mol forward primer 5- TGC TTT GTT ATT CGG GCC -and reverse primer 5- ATG CGT TAT ATT CTG TG - 3specific for SHV gene from conserve region (Bradford, 2001), 5 µl from DNA template and 13 µl from deionized water) to complete the volume to 25 µl. The PCR program (techne, UK) initial denaturation 95C^O for 5 min, followed by 35 cycles 95C^O for 60 sec annealing 57 C^O for 60 sec and extension at 72 C^O for 60 sec final extension for 7 min. The amplicons were resolved and screened using 1.5% agarose gel electrophoresis. All PCR reactions were performed with appropriate negative and positive controls which are size band 753 bp to avoid any false negative and positive results.

CHAPTER FOUR

Results

4.1 Laboratory analysis

4.1.1 Isolation of *Salmonella*

Out of 465 samples collected from water source, drinkers, feed source, feeders, litter, dust, hand swabs, cloacal swabs, faeces, knives, carcass meat, and carcass swabs 29 (6.2%) samples were found positive to *Salmonella spp* using the method described by ISO 6579:2002, identified by using API-20E strips as *Salmonella arizonae* 21 (72.4%), and *Salmonella choleraesuis* 8 (27.6%).

4.1.2 Distribution of Salmonella isolates

Salmonella arizonae showed higher prevalent 21(72.4%) out of the total isolates distributed as 11(52.4%) and 10 (47.6%) isolated from Bahri Locality and Khartoum Locality respectively. *Salmonella choleraesuis* revealed 8 isolates (27.6%) detected only from Bahri Locality (Figure 1).

One *Salmonella arizonae* isolate (3.4%) was isolated from water source in Bahri Locality. Also 4 isolates (13.8%) were isolated from drinkers distributed as 1 *Salmonella arizonae* (3.4%) collected from each Bahri and Khartoum Localities, besides 2 *Salmonella choleraesuis* (6.9%) isolated from Bahri Locality. There was no *Salmonellae* isolated from feed source. Furthermore 4 *Salmonella arizonae* (13.8%) were isolated from feeders as 1(3.4%) and 3(10.3%) in Khartoum Locality and Bahri Locality respectively. Also 1 *Salmonella choleraesuis* (3.4%) was isolated from hand swab of a worker in Bahri Locality. Also 4 *Salmonella arizonae* (13.8%) were isolated and distributed equally as 2(6.9%) for each Bahri and Khartoum Localities. Litter showed the highest contamination by *Salmonella species* 10(34.5%) distributed as 4(13.8%) and 2(6.9%) *Salmonella arizonae* isolated from Khartoum Locality and Bahri Locality respectively, besides 4 *Salmonella choleraesuis* (13.8%) isolated from

Bahri Locality. Also 1 *Salmonella arizonae* (3.4%) was isolated from a cloacal swab in Khartoum Locality. This study also showed that 3 *Salmonella spp* (10.3%) were found in faeces distributed as *Salmonella arizonae* in both Khartoum Locality and Bahri Locality, besides 1 *Samonella choleraesuis* (3.4%) isolated from Bahri Locality. Detection during slaughtering showed isolation of *Salmonella arizonae* from one knife in Bahri Locality. This study also showed negative isolation of *salmonella* from both carcass swabs and carcass meat. (Table 1 and Figure 2).

4.2 Results of the Prevalence of *Salmonella species* from broiler chicken farms in Khartoum State

A total of 396 environmental samples were collected during rearing from 44 different farms located in Bahri, Khartoum, and Omdurman Localities divided as 162,162, and 72 respectively, this showed that the overall prevalence of *Salmonella species* in the environment of Khartoum State broiler chicken farms was 28 (7.1%). The highest prevalence of *Salmonella* was recorded in Bahri Locality 18 (11.1%), followed by Khartoum Locality10(6.2%), while there was no detected *Salmonellae* in Omdurman Locality. Geographical distribution of the investigated poultry chicken farms and prevalence of isolated *Salmonella spp* are shown in figure (3).

Also, a number of 23 (52.3%)out of 44 detected farms were also investigated for the presence of *Salmonella spp* during slaughtering, 69samples were collected directly from10(43.5%) farms in Bahri Locality and 13(56.5%) farms in Khartoum Locality during slaughtering. These were collected from knives, carcass meat, and carcass swabbing, only one (4.3%) *Salmonella arizonae* was detected from a knife in Bahri Locality (Figure 4).

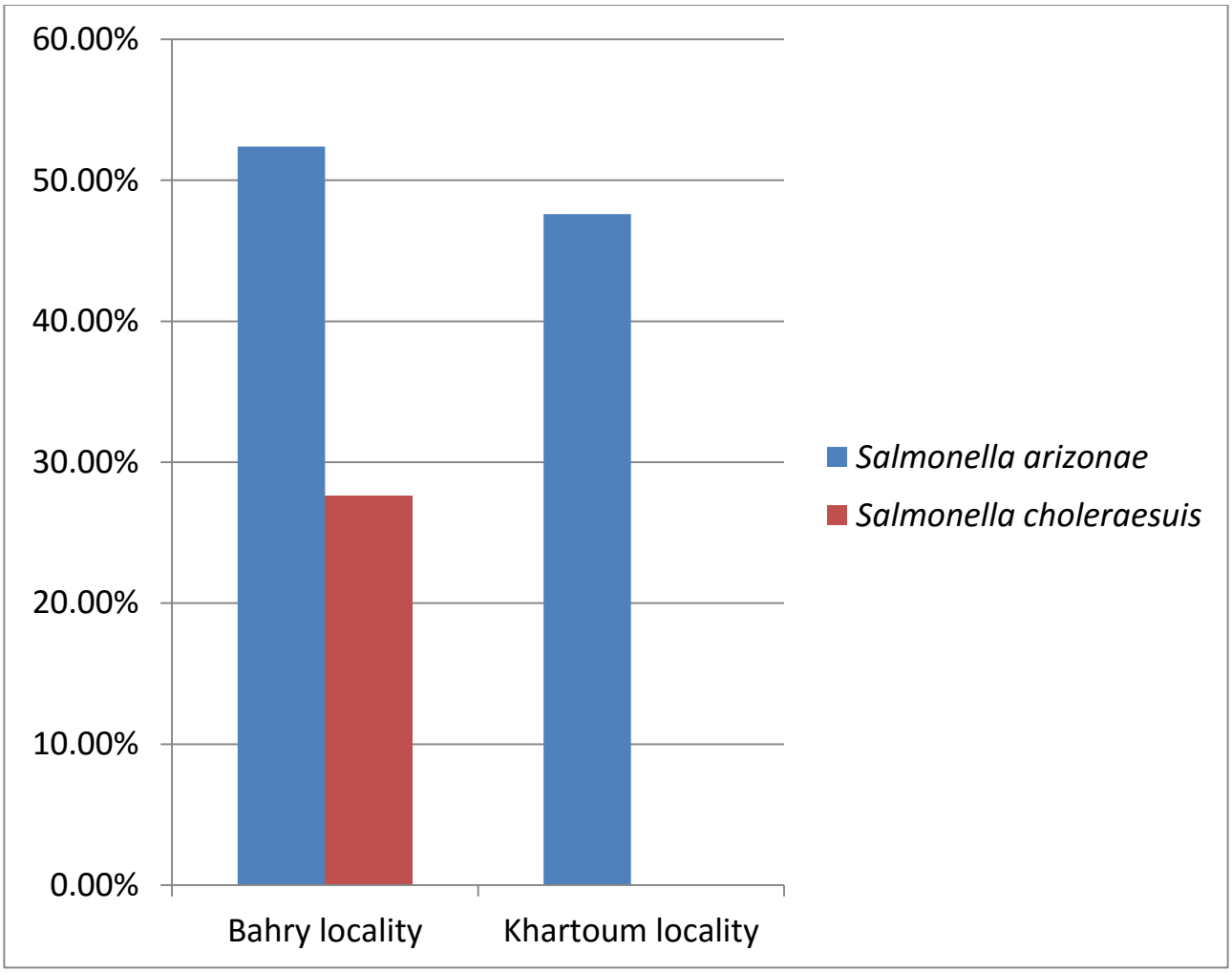

Figure. (1) Percentage of *Salmonella* isolates from broiler chicken farms in Khartoum State

Table 1. Distribution of *Salmonella* isolates in Khartoum State chicken farms

	Isolated organisms		*Salmonella arizonae*		*Salmonella choleraesuis*	
Source	**No of samples**	**No of positive**	**Khartoum Locality**	**Bahri Locality**	**Khartoum Locality**	**Bahri Locality**
Water source	44	1	0(0.0%)	1(3.4%)	0(0.0%)	0(0.0%)
Drinkers	44	4	1(3.4%)	1(3.4%)	0(0.0%)	2(6.9%)
Feed source	44	0	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
Feeders	44	4	1(3.4%)	3 (10.3%)	0(0.0%)	0(0.0%)
Hand swabs	44	1	0(0.0%)	0(0.0%)	0(0.0%)	1(3.4%)
Dust	44	4	2(6.9%)	2(6.9%)	0(0.0%)	0(0.0%)
Litter	44	10	4(13.8%)	2(6.9%)	0(0.0%)	4(13.8%)
Cloacal swabs	44	1	1(3.4%)	0(0.0%)	0(0.0%)	0(0.0%)
Faeces	44	3	1(3.4%)	1(3.4%)	0(0.0%)	1(3.4%)
Knives	23	1	0(0.0%)	1(3.4%)	0(0.0%)	0(0.0%)
Carcass swabs	23	0	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
Carcass meat	23	0	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
Total	465	29	10(34.5%)	11(38.0%)	0(0.0%)	8(27.5%)

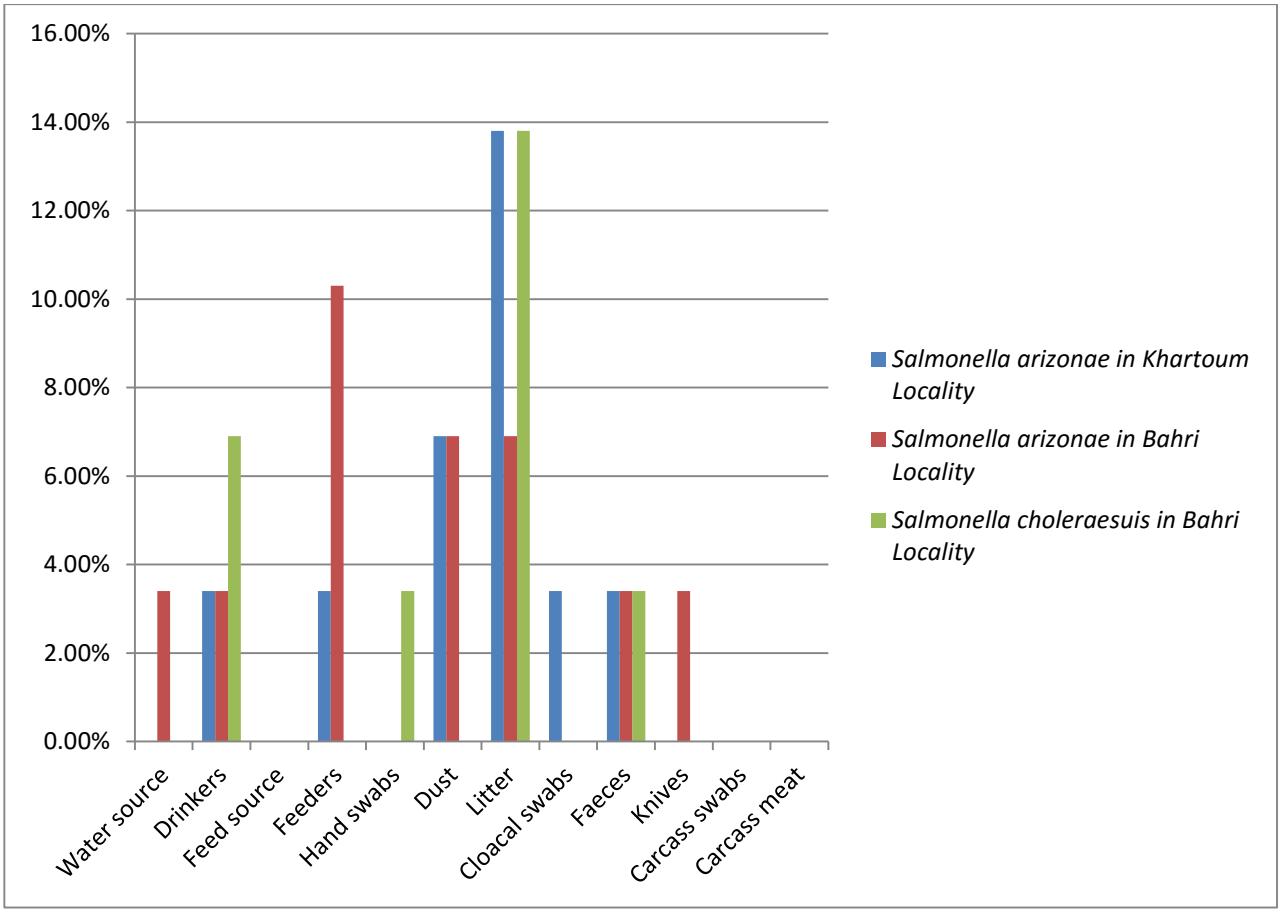

Figure.(2) Distribution of *Salmonella* isolates from different sources in Khartoum State farms

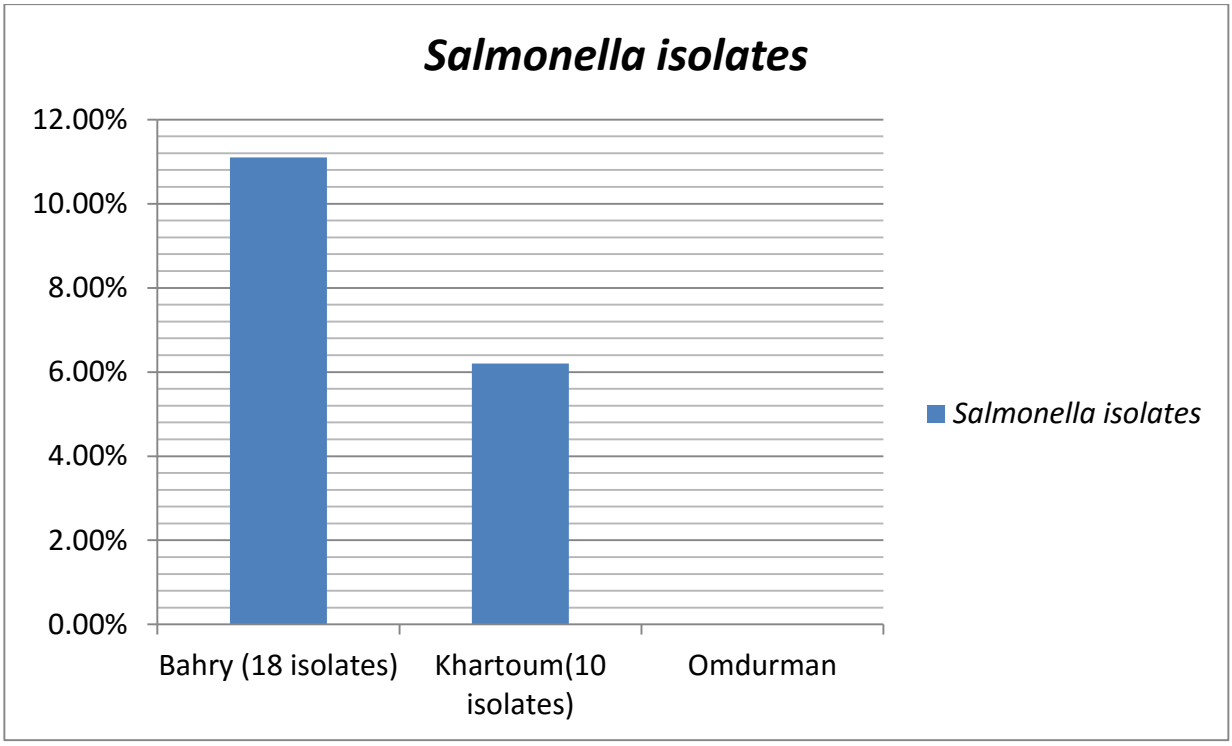

Figure. (3) Percentage of *Salmonella spp* isolation according to geographical distribution

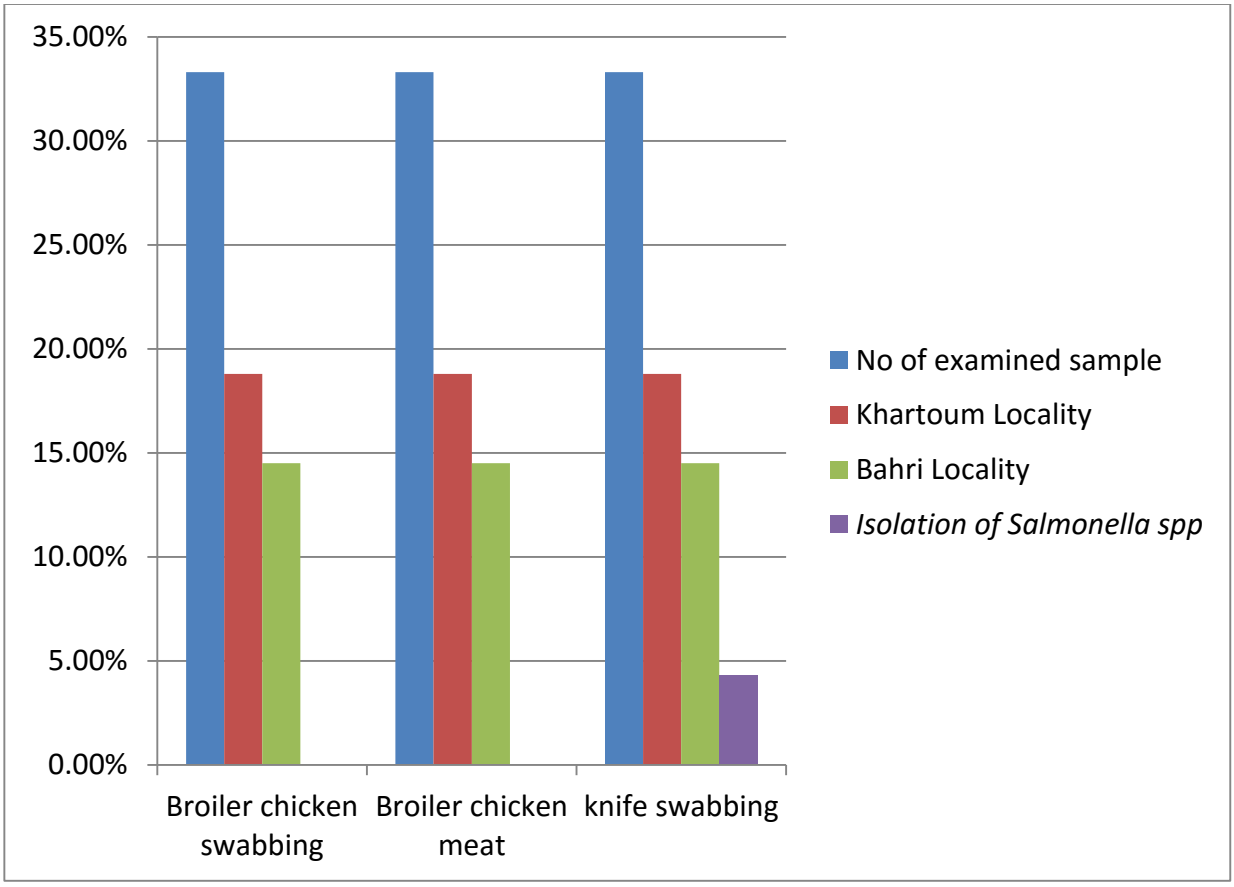

Figure. (4) Prevalence of *Salmonella spp* isolated during slaughtering in Khartoum State

4.2.1Prevalence of *Salmonella spp* in the environment of broiler chicken farms of Bahri Locality

The results showed that 18(11.1%) out of 162 collected samples were positive for *Salmonella spp,* these were recovered from 13(72.2%) farms and identified as *Salmonella arizonae* 9(50.0%) and *Salmonella choleraesuis* 9(50.0%) distributed as1(5.6%) *Salmonella arizonae* isolated from water source in Om-doum, 3(16.7%) were isolated from drinkers in El-Droshab identified as *Salmonella arizonae*, and El-Kadaro, and El-Kabbashi identified as *Salmonella choleraesuis*. Also 3(16.7%) *Salmonella arizonae* were isolated from feeders in Om-doum, Hillat Kuku, and El-Drosahab. Furthermore 6 *Salmonella* isolates (33.3%) were isolated from litter distributed as 2 *Salmonella arizonae* (11.1%) collected from El- Halfaya, and El-Droshab, and 4 (22.2%) *Salmonella choleraesuis* in El-Kadaro, Hillat Kuku and El-Kabbashi. Also 2 *Salmonella arizonae* (11.1%) were detected from dust in Hillat Kuku, and El- Kadaro. Also 1 *Salmonella choleraesuis* (5.6%) was isolated from hand swab of a worker in Shambat, besides 2(11.1%) *Salmonella choleraesuis* and *Salmonella arizonae* were detected from faeces in El-Halfaya, and El-Kadaro respectively. However, there was no *Salmonellae* in samples collected from cloacal swabs and feed source (Table 2, and Figure 5).

Table 2. Isolation of *Salmonella spp* collected from broiler chicken farms environment in Bahri locality

Source of sample	No of examined samples	No of positive samples	*Salmonella choleraesuis*	*Salmonella arizonae*
Water source	18	1	0(0.0%)	1(5.6%)
Drinkers	18	3	2(11.1)	1(5.6%)
Poultry feed	18	0	0(0.0%)	0(0.0%)
Feeders	18	3	0(0.0%)	3(16.7%)
Litter	18	6	4(22.2%)	2(11.1)
Dust	18	2	1(5.6%)	1(5.6%)
Hand swabs	18	1	1(5.6%)	0(0.0%)
Cloacal swabs	18	0	0(0.0%)	0(0.0%)
Faeces	18	2	1(5.6%)	1(5.6%)
Total	162	18	9(50.0%)	9(50.0%)

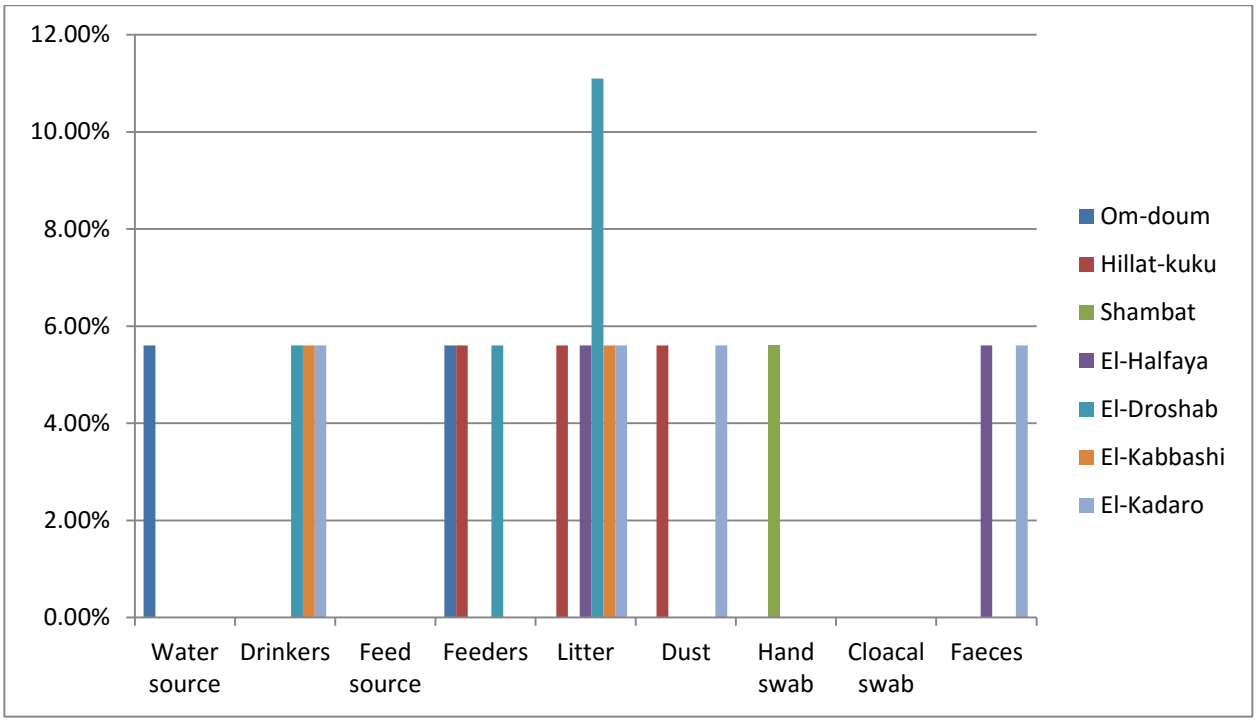

Figure.(5) Distribution of isolated *Salmonella spp* from different areas of Bahri farms environment

4.2.2Prevalence of *Salmonella spp* in the environment of broiler ckicken farms in Khartoum Locality

All samples were collected from Jabal Awliya Locality broiler chicken farms. The results showed that 10 (6.2%) out of 162 collected samples were positive for *Salmonella arizonae*. Eight (44.4%) out of 18 farms were contaminated with *Salmonella arizonae.* There was no *Salmonellae* isolated from water source, feed source and hand swabs of workers. One isolate (10.0 %) was isolated from a drinker in El Fetaih, and from a feeder in Tayba El-Hassanab. Furthermore 3(30.0%) were isolated from litter in El-Fetaih, Tayba El-Hassanab, and Arak salih; and 3(30.0%) were detected from dust in El- Shegailab, El-Fetaih, and Tayba El-Hassanab. *Salmonella* were collected from faeces1 (10%) and cloacal swabs 1(10%) from wad El-Agali, and Tayba El-Hassanab respectively (Table 3, and figure 6).

4.2.3 Prevalence of *Salmonella spp* in the environment of chicken farms in Omdurman Locality

The results showed that there was no *Salmonellae* isolated from water source, drinkers, feed source, feeders, litter, dust, hand swabs, cloacal swabs, and faeces in 72 samples collected from the eight different farms of Omdurman Locality represented as one farm located in each of Karrary , Kilo 11, El-Muwaileh, DarElsalam, and Libia , besides three farms located in El Heraizab .

Table 3. Isolation rate of *Salmonella arizonae* collected from broiler chicken farms in Khartoum Locality.

Source of sample	No of examined samples	No of positive samples	% of positive samples
Water source	18	0	0.0
Drinkers	18	1	10.0
Poultry feed	18	0	0.0
Feeders	18	1	10.0
Litter	18	3	30.0
Dust	18	3	30.0
Hand swabs	18	0	0.0
Cloacal swab	18	1	10.0
Faeces	18	1	10.0
Total	162	10	6.2

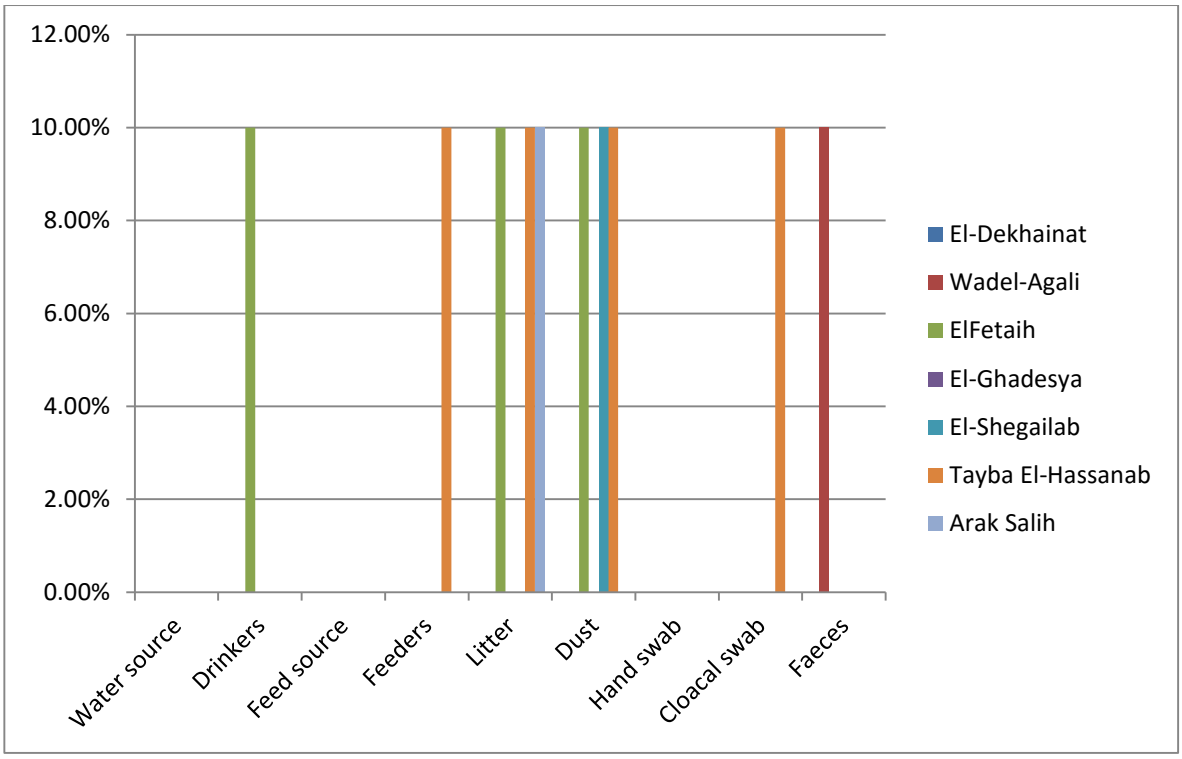

Figure.(6) Distribution of *Salmonella arizonae* isolated from different areas of Khartoum Localit

4.3. Antibiotics susceptibility of isolated *Salmonella* from different sources in Khartoum State

This study showed that all the isolates were sensitive to amikacin, ciprofloxacin, cefixime, ceftriaxone, cefalexin, cftazidime, and cefotaxime followed by, chloramphenicol (89.7%), colistin (89.7%), ceftazidime(89.7%) gentamicin (82.8%), streptomycin (65.5%), co-trimoxazole (65.5%), nalidixic acid (48.3%) ampicillin (44.8%), tetracycline (44.8%), and amoxicillin (3.4%). Also the isolates showed intermediate resistance to, ampicillin, ceftazidime, and gentamycin (10.3%), amoxicillin and streptomycin (6.9%) for each, nalidixic acid(3.4%) while they showed resistance to amoxicillin (89.7%),%), tetracycline (55.2%), nalidixic acid (48.3%), ampicillin(44.8%), co trimoxazole (34.5%), streptomycin (27.6%) colistin, and chloramphenicol (10.3%), (Table 4). Resistance pattern of isolates were shown in Figure (7).

4.3.1 Antibiotics susceptibility of isolated *Salmonella* from broiler chicken farms in Bahri locality

This study showed that all the isolates were sensitive to ciprofloxacin, amikacin, cefixime, ceftriaxone, cefalexin and cefotaxime followed by, gentamicin (94.4%), chloramphenicol (88.9%), colistin (83.3), streptomycin (66.7%), co-trimoxazole (66.7%), nalidixic acid (61.1%) ampicillin (55.6%), tetracycline (55.6%), and amoxicillin (5.6%). Also the isolates showed intermediate resistance to amoxicillin, ampicillin, gentamycin, nalidixic acid, and streptomycin (5.6%) for each, and ceftazidime(10.5%) while they showed resistance to amoxicillin (88.9%),%), tetracycline (44.4%), ampicillin(38.9), co trimoxazole (33.3%), nalidixic acid (33.3),streptomycin (27.8%) colistin (16.7), and chloramphenicol(11.1%)(Table 5). Figure (8) shows resistance pattern of 19 isolates against sixteen antibiotics.

Table 4. Antibiotics susceptibility of 29 isolated *Salmonella spp* from broiler chicken farms in Khartoum State

Antimicrobial agent	Code	Concentration (mcg/disc)	Sensitive %	Intermediate %	Resistant %
Amoxicillin	AMX	10	1(3.4%)	2(6.9%)	26(89.7%)
Ampicillin	AMP	10	13(44.8%)	3(10.3%)	13(44.8%)
Ciprofloxacin	CIP	5	29(100)	0(0.0%)	0(0.0%)
Gentamycin	GEN	10	24(82.8%)	3(10.3%)	2(6.9%)
Chloramphenicol	C	30	26(89.7%)	0(0.0%)	3(10.3%)
Streptomycin	S	10	19(65.5%)	2(6.9%)	8(27.6%)
Co-Tri moxazole	COT	25	19(65.5%)	0(0.0%)	10(34.5%)
Tetracycline	TE	30	13(44.8%)	0(0.0%)	16(55.2%)
Nalidixic acid	NA	30	14(48.3%)	1(3.4%)	14(48.3%)
Cefixime	CFM	5	29(100%)	0(0.0%)	0(0.0%)
Cefotaxime	CTX	30	29(100%)	0(0.0%)	0(0.0%)
Ceftriaxone	CTR	30	29(100%)	0(0.0%)	0(0.0%)
Cefalexin	CN	30	29(100%)	0(0.0%)	0(0.0%)
Ceftazidime	CAZ	30	26(89.7%)	3(10.3%)	0(0.0%)
Amikacin	AK	30	29(100%)	0(0.0%)	0(0.0%)
Colistin	CL	10	26(89.7%)	0(0.0%)	3(10.3%)

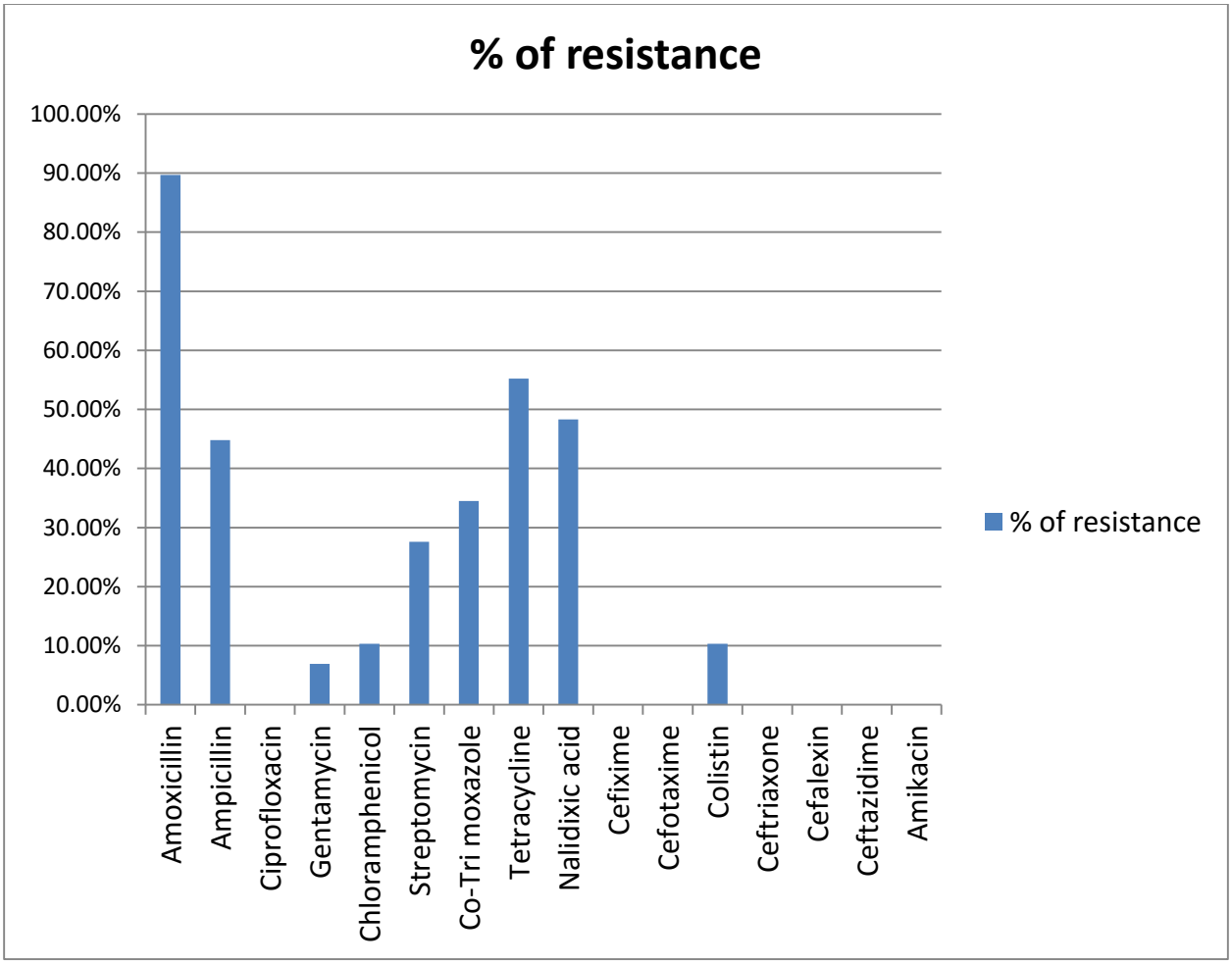

Figure. (7) Percentage of resistance of 29 isolated *Salmonella species* to Sixteen antibiotics in Khartoum State

Table 5. Antibiotics resistance of *Salmonella* isolated from broiler farms of Bahri Locality

Antimicrobial agent	Code	Concentration (mcg/disc)	Sensitive %	Intermediate %	Resistant %
Amoxicillin	AMX	10	1(5.3%)	1(5.3%)	17(89.4%)
Ampicillin	AMP	10	10(52.6%)	1(5.3%)	8(42.1%)
Ciprofloxacin	CIP	5	19(100%)	0(0.0%)	0(0.0%)
Gentamycin	GEN	10	17(89.4%)	1(5.3%)	1(5.3%)
Chloramphenicol	C	30	17(89.4%)	0(0.0%)	2(10.5%)
Streptomycin	S	10	12(63.1)	1(5.3%)	6(31.6%)
Co-Tri moxazole	COT	25	12(63.1%)	0(0.0%)	7(36.8%)
Tetracycline	TE	30	10(52.6%)	0(0.0%)	9(47.4%)
Nalidixic acid	NA	30	11(38.0%)	1(5.3%)	7(36.8%)
Cefixime	CFM	5	19(100%)	0(0.0%)	0(0.0%)
Cefotaxime	CTX	30	19(100%)	0(0.0%)	0(0.0%)
Ceftriaxone	CTR	30	19(100%)	0(0.0%)	0(0.0%)
Cefalexin	CN	30	19(100%)	0(0.0%)	0(0.0%)
Ceftazidime	CAZ	30	17(89.4%)	2(10.5%)	0(0.0%)
Amikacin	AK	30	19(100%)	0(0.0%)	0(0.0%)
Colistin	CL	10	16(84.2%)	0(0.0%)	3(15.8%)

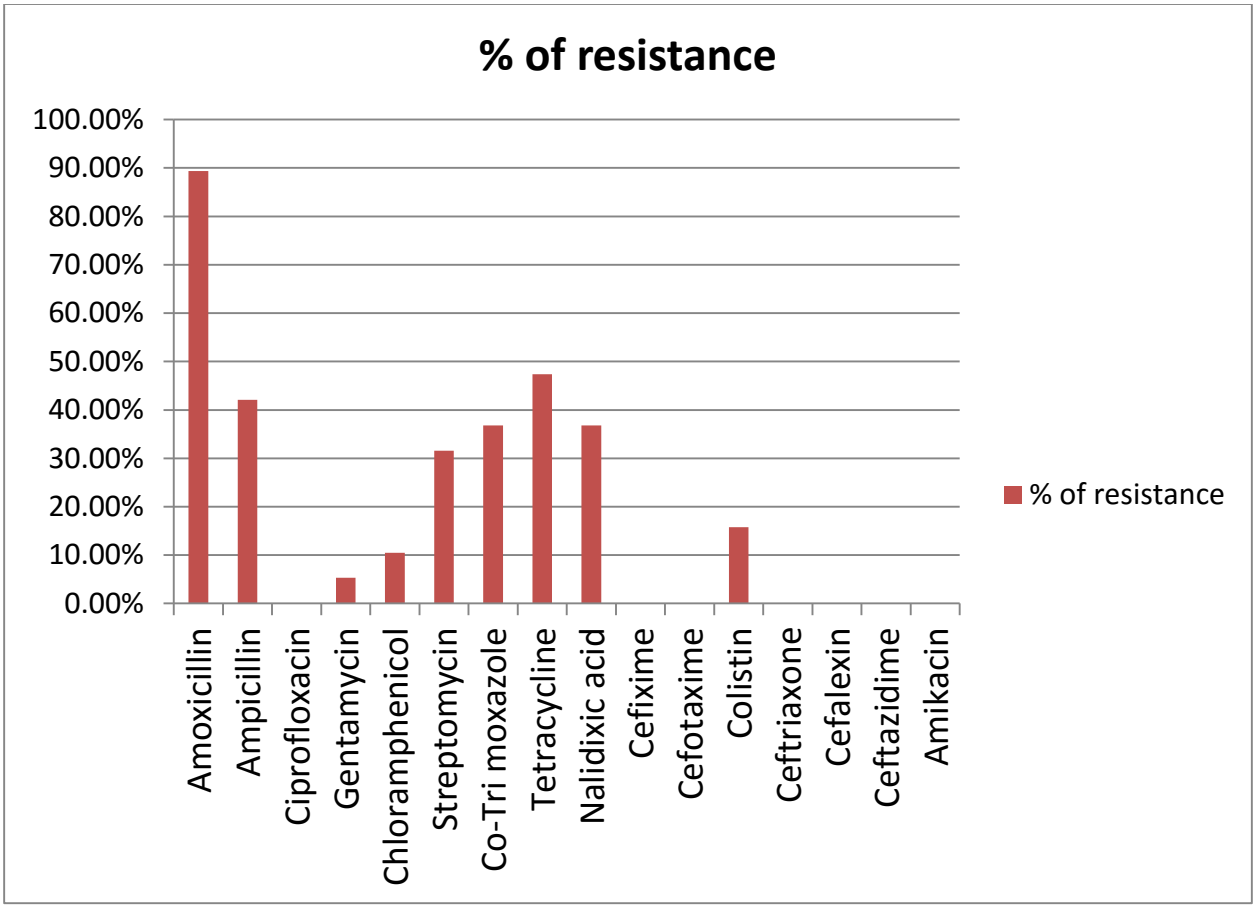

Figure.(8) Resistance of isolated 19 *Salmonella species* to sixteen antibiotics in Bahri Locality

4.3.2 Antibiotics susceptibility of isolated *Salmonella arizonae* from broiler chicken farms in Khartoum locality

This study showed that all the isolates were sensitive to ciprofloxacin, amikacin, cefixime, cefotaxime, ceftriaxone, cefalexin, and colistin, followed by, chloramphenicol, and ceftazidime (90.0%), co-trimoxazole (70.0%), streptomycin (70.0%), gentamicin (70.0%), ampicillin (30.0%), tetracycline (30.0%), nalidixic acid (30.0%), however, amoxicillin showed highly resistance. Also the isolates showed intermediate resistance to gentamycin (20.0%), ampicillin (20.0%), amoxicillin (10.0%), and streptomycin (10.0%), ceftazidime(10%) while they showed resistance to amoxicillin (90.0%), tetracycline (70%) nalidixic acid (70.0%), ampicillin (50%) cotrimoxazole (30.0%), streptomycin (20.0%),gentamicin (10.0%), and chloramphenicol (10%.0) (Table 6). Resistance pattern is shown in Figure(9).

4.3.3 Multidrug resistance among *Salmonella* isolates

This study showed multidrug resistance to *Salmonella arizonae* , and *Salmonella choleraesuis* as is shown in table (7).

4.3.4 Resistance of isolates against tested antibiotics

Salmonella arizonae showed resistance to nine antibiotics used in this study whereas *Salmonella choleraesuis* isolates were resistant to six antibiotics revealing sensitivity to gentamicin, chloramphenicol, and colistin. Resistance is shown in Figure(10).

Table 6. Antibiotics susceptibility of *Salmonella arizonae* isolated from Khartoum Locality farms

Antimicrobial agent code	Concentration on (µg/ml)	S %	I %	R %
Amoxicillin	10	0(0.0%)	1(10%)	9(90%)
Ampicillin	10	3(30%)	2(20%)	5(50%)
Ciprofloxacin	5	10(100%)	0(0.0%)	0(0.0%)
Gentamycin	10	7(70%)	2(20%)	1(10%)
Chloramphenicol	30	9(90%)	0(0.0%)	1(10%)
Streptomycin	10	7(70%)	1(10%)	2(20%)
Co-Tri moxazole	25	7(70%)	0(0.0%)	3(30%)
Tetracycline	30	3(30%)	0(0.0%)	7(70%)
Nalidixic acid	30	3(30%)	0(0.0%)	7(70%)
Cefixime	5	10(100%)	0(0.0%)	0(0.0%)
Cefotaxime	30	10(10.0%)	0(0.0%)	0(0.0%)
Ceftriaxone	30	10(100%)	0(0.0%)	0(0.0%)
Cefalixin	30	10(100%)	0(0.0%)	0(0.0%)
Ceftazidime	30	9(90%)	1(10%)	0(0.0%)
Amikacin	30	10(100%)	0(0.0%)	0(0.0%)
Colistin	10	10(100%)	0(0.0%)	0(0.0%)

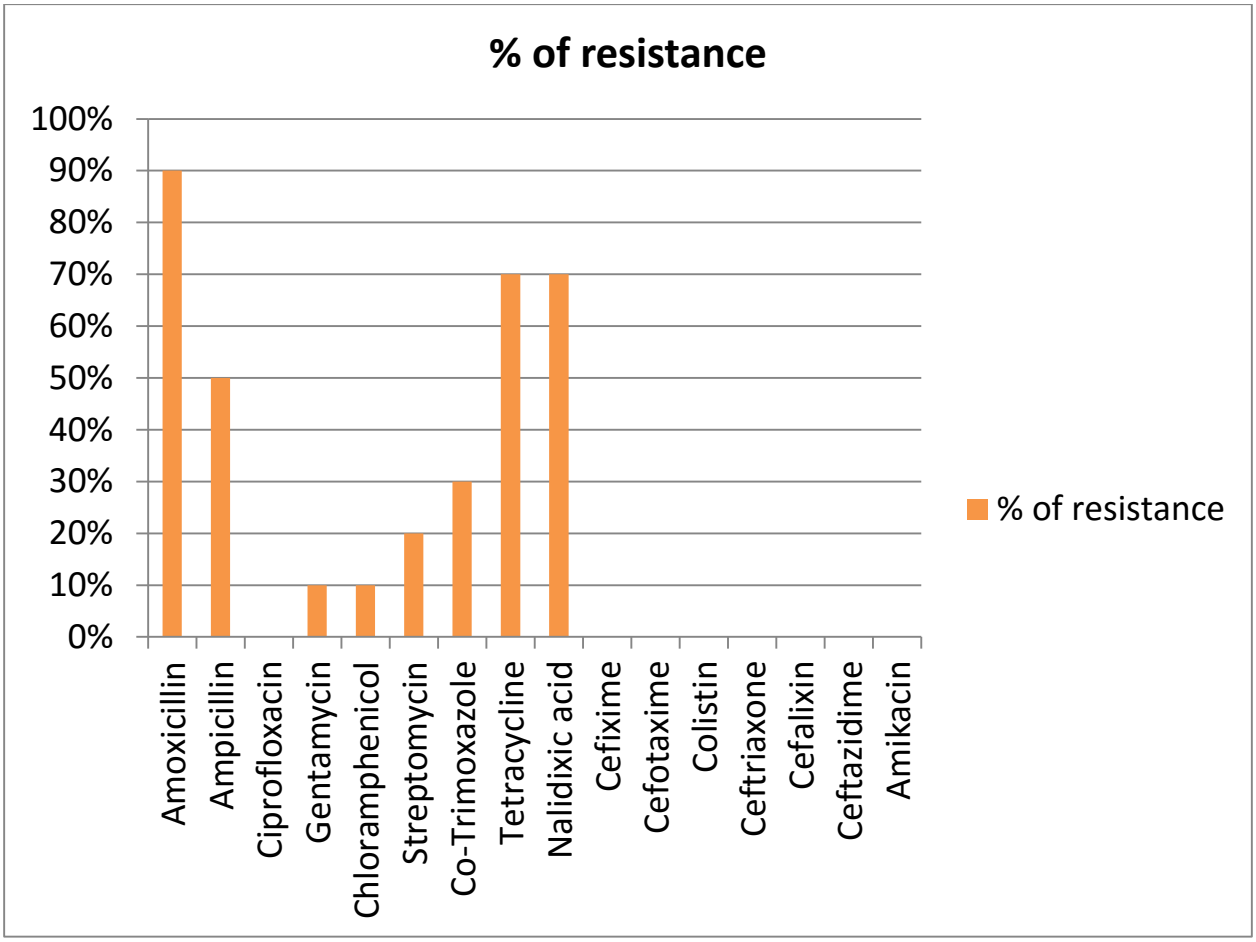

Figure .(9) Resistance of isolated 10 *Salmonella arizonae* to sixteen antibiotics in Khartoum Locality

Table 7. Resistance of *Salmonella arizonae* and *Salmonella Choleraesuis* to tested antibiotics

	Salmonella arizonae **(n=21)**		***Salmonella choleraesuis*** **(n=8)**	
Antibiotic	**No**	**Resistance%**	**No**	**Resistance%**
Amoxicillin	19	90.5	7	87.5
Ampicillin	10	47.6	3	37.5
Ciprofloxacin	0	0	0	0
Gentamycin	2	9.5	0	0
Chloramphenicol	3	14.3	0	0
Streptomycin	5	23.8	3	37.5
Sulpha-tri methoprim	6	28.6	4	50
Tetracycline	12	57.1	4	50
Nalidixic acid	11	52.4	3	37.5
Cefixime	0	0	0	0
Cefotaxime	0	0	0	0
Ceftriaxone	0	0	0	0
Cefalexin	0	0	0	0
Ceftazidime	0	0	0	0
Amikacin	0	0	0	0
Colistin	3	14.3	0	0

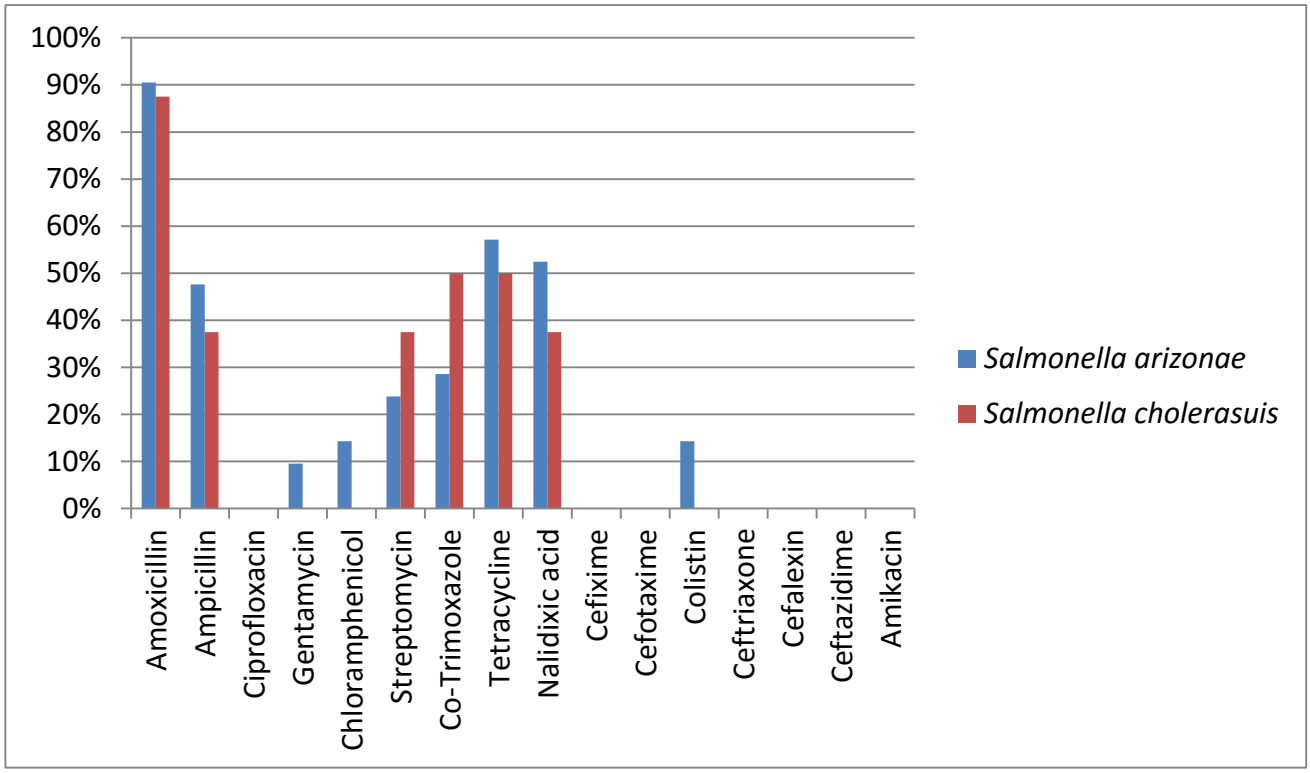

Figure.(10) Resistance of *Salmonella arizonae* and *Salmonella choleraesuis* to sixteen antibitics

4.4 Results of Double Disk synergy test

The present study revealed that 18(62.1%) out of 29 isolates were ESBL producers of which 13(72.2%) were *Salmonella arizonae,* whereas 5 (27.7%) were *Salmonella choleraesuis*. Eleven (37.9%) were not ESBLs producers of which 8(44.4%) *Salmonella arizonae*, and 3(27.3%) *Salmonella choleraesuis* (Figure 11).

4.5 Results of genes related to β-lactams resistance (blaSHV, blaTEM, and blaCTX-M)

A number of 20 genes were detected from 12 *Salmonella spp* isolates (66.7%) out of 18 ESBL producers. CTX-M gene was detected in 8 isolates distributed as 5 *Salmonella arizonae* (62.5%) and 3 *Salmonella choleraesuis* (37.5%). SHV gene was detected in 7 isolates distributed as 6 *Salmonella arizonae* (85.7%) and 1 *Salmonella choleraesuis* (14.3%). TEM gene was detected in 5 isolates distributed as 4 *Salmonella arizonae* (80%) and 1 *Salmonella choleraesuis* (20%) (Table 8). Genotypic resistance pattern of *Salmonella* isolates was shown in (Figure 12).

Distribution of the detected 20 genes was as follow; Only one isolate *Salmonella arizonae* (8.3%) out of 12 showed positive detection for the three resistance genes (feeder sample), 4 isolates (33.3%) showed detection of both CTX-M and SHV distributed as 3 *Salmonella arizonae* and 1 *Salmonella choleraesuis*, 2 isolates (16.7%) were positive for both CTX-M and TEM detected in 1 *Salmonella arizonae* and 1 *Salmonella choleraesuis*, 3 isolates (25.0%) were positive for both SHV and TEM detected in *Salmonella arizonae*, whereas 6 isolates (33.3%) out of 18 ESBL producers showed negative detection for the three genes (4 *Salmonella arizonae* detected in litter, faeces, and dust samples, and 2 for *Salmonella choleraesuis* detected in litter samples)(Table 9 and Figure 13). Figures(14, 15, and 16) showed PCR results for TEM, CTX-M, and SHV.

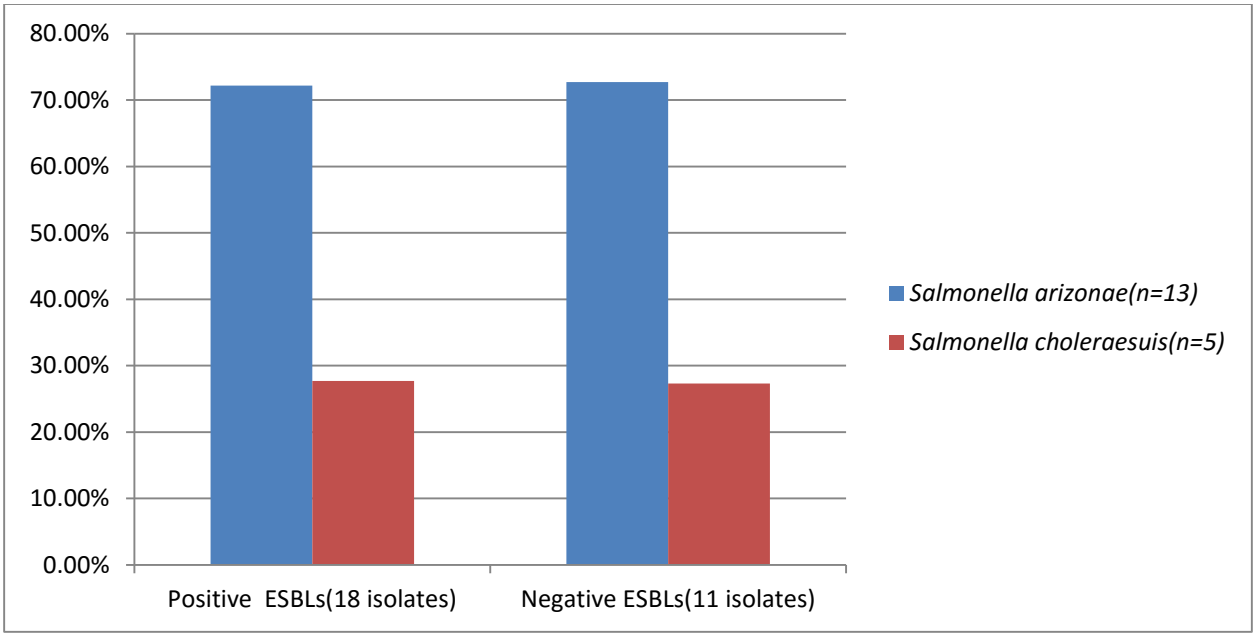

Figure. (11) Beta lactams resistance by using phenotyping method to *Salmonella* isolates

Table 8. Analysis of β-lactams resistance (blaSHV, blaTEM, and blaCTX-M)

Genes	*Salmonella arizonae(n=13)*	*Salmonella choleraesuis(n=5)*	Total of detected genes
CTX-M	5(62.5%)	3 (37.5%)	8(100%)
SHV	6(85.7%)	1(14.3%)	7(100%)
TEM	4(80.0%)	1(20.0%)	5(100%)

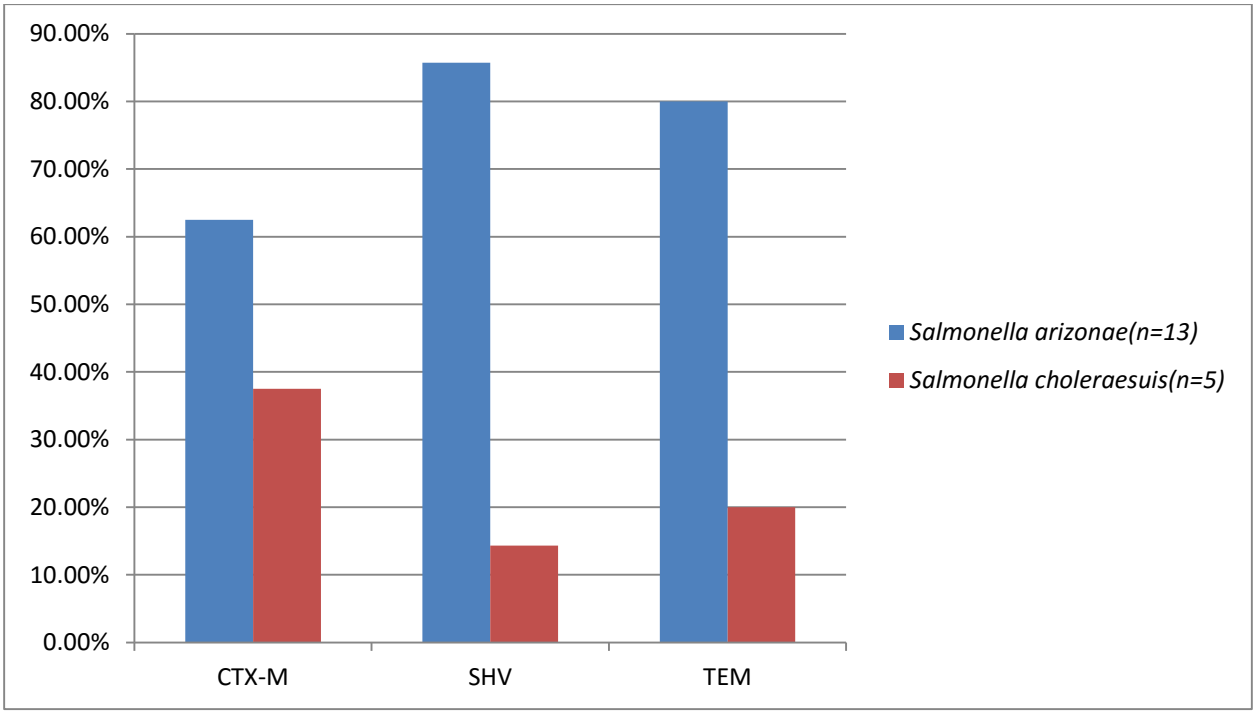

Figure.(12) Genotypic resistance pattern of *Salmonella* isolates

Table 9. Detection of CTX-M, TEM, and SHV in ***Salmonella arizonae*** **and** ***Salmonella Choleraesuis***

	Salmonella arizonae**(n=13)**	***Salmonella choleraesuis*****(n=5)**
Positive detection to all selected resistant genes	1(8.3%)	0(0.0%)
Positive detection to CTX-M gene	5 (62.5%)	3(37.5)
Positive detection to SHV gene	6 (85.7%)	1(14.3%)
Positive detection to TEM gene	4(80.0%)	1(20.0%)
Negative isolation to all genes	4(66.7%)	2(33.3%)

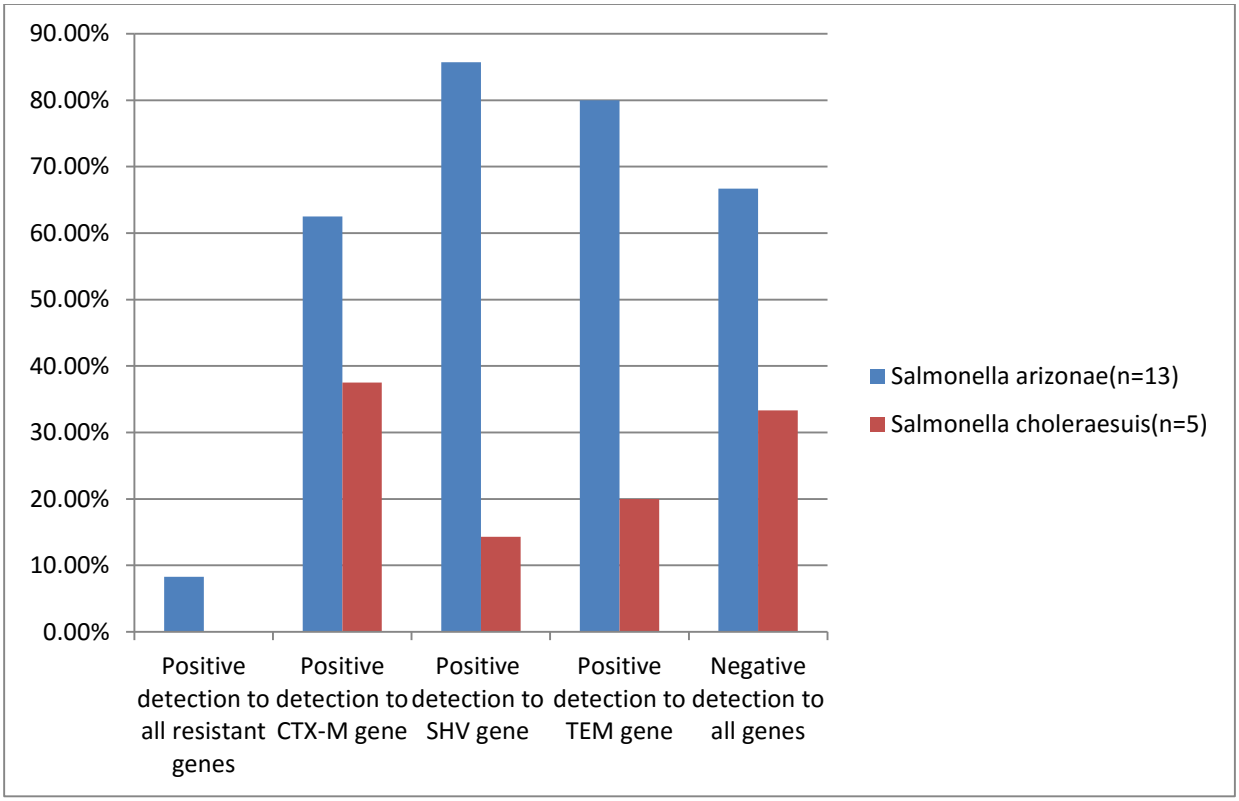

Figure.13 Detection Pattern of CTX-M, SHV, and TEM from *Salmonella* isolates

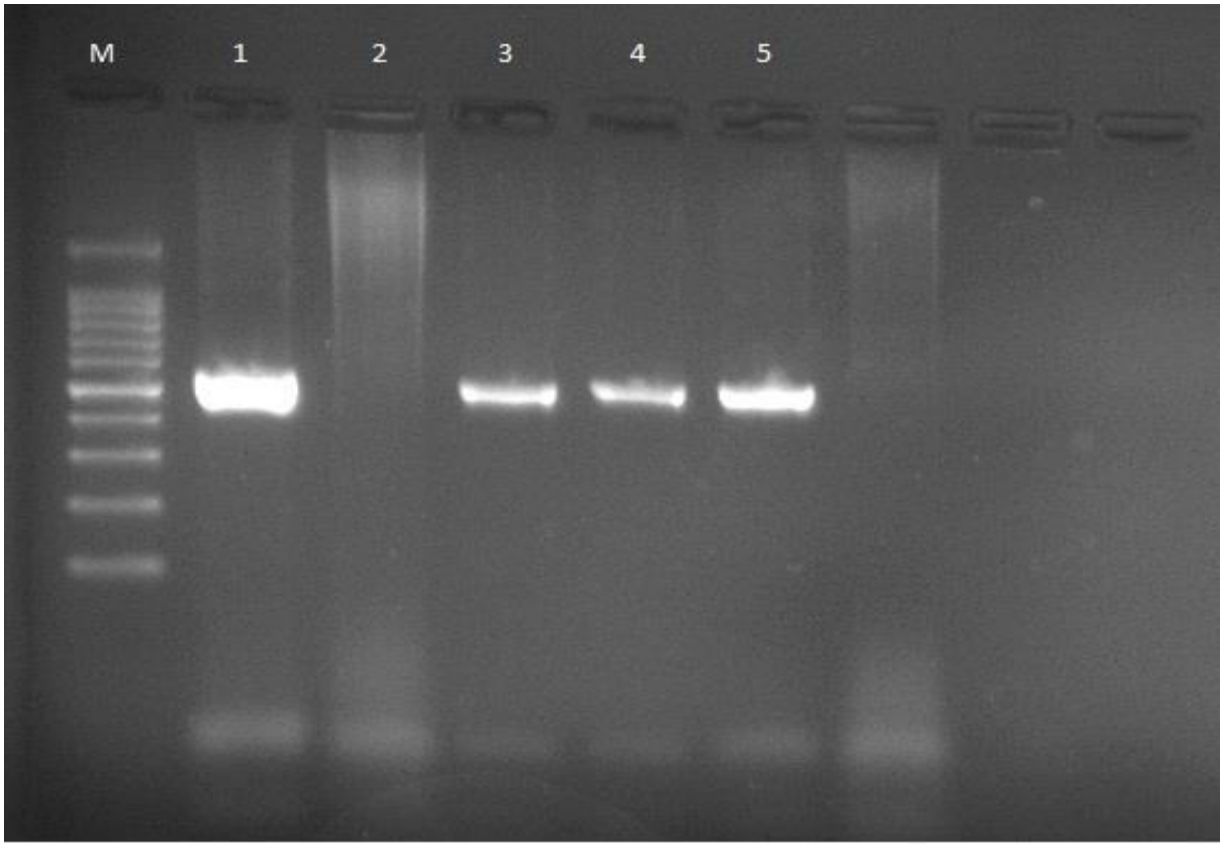

Figure.14 M marker (100bp Vivants. Malysia), Lane 1, positive control , lane 2, negative control; lane 3 and 4 positive *Salmonella arizonae*, and lane 5 positive *Salmonella choleraesuis* to CTX .

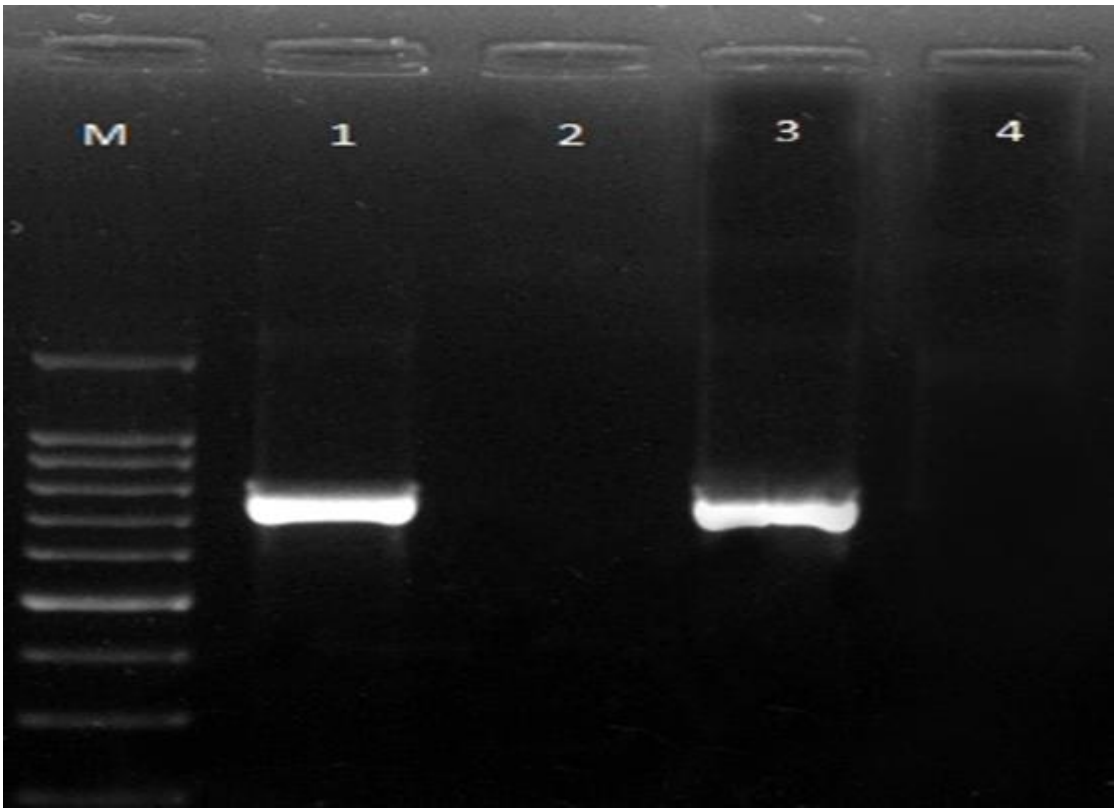

Figure.15 M marker(100bp Vivants. Malysia), Lane 1, positive control , lane 2, negative *Salmonella arizonae* , and lane 3, positive *Salmonella choleraesuis* to TEM , lane 4 negative control.

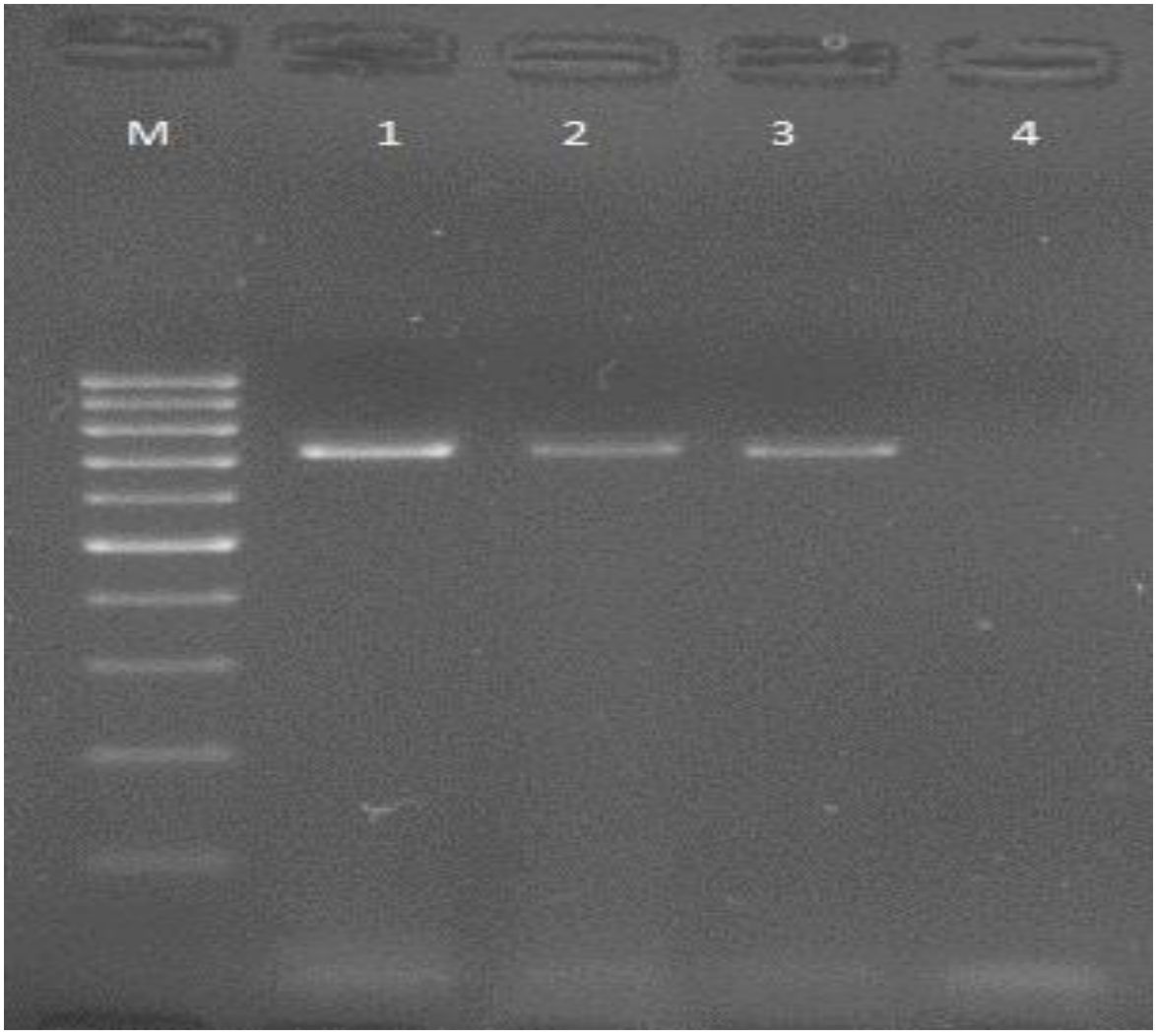

Figure. 16 M marker(100bp Vivants. Malysia), Lane 1, positive control , lane 2, positive *Salmonella arizonae* , and lane 3, positive *Salmonella choleraesuis* to SHV , lane 4 negative control.

CHAPTER FIVE
Discussion

5.0 Discussion

Out of 465 studied samples, 29 were *Salmonella spp* positive. This result is higher than that were obtained by Mohammed *et al.* (2009), Mohammed (2009), Al-Zenki *et al.* (2007), and Curtello *et al*. (2013). However, it is lower than the results of Roy *et al*.(2002), Akhtar *et al*.(2010), Henry *et al*. (2012), and Akond *et al*. (2012). *Salmonella species* were not isolated from Omdurman chicken farms in the present study, this may be attributed to the low number of collected samples(72 samples compare to 168 for each Khartoum and Bahri), and good hygienic status, besides the variation of seasons. Poultry chicken farms of Bahri Locality showed higher isolation of *Salmonella species*18(11.1%) than Khartoum Locality 10(6.2%), this may be attributed to the differences in applied hygienic measures.

In this study *Samonella arizonae* showed higher prevalence 21(72.4%) out of 29 isolates, whereas *Salmonella choleraesuis* has lower prevalence 8(27.6%) detected in Bahri broiler chicken farms, whereas Khartoum Locality farms showed negative isolation.

The drinking water plays an important role in the transmission of many pathogenic agents, and there have been many reports about water contamination with *Salmonella spp* (Jafari *et al.*, 2006). In this study 1(3.4%) and 4(13.8%) *Salmonella spp* were isolated from drinking water and drinkers respectively, which confirm that *Salmonellae* may originate either from faeces and secretions of sick birds in the same flock or from water already contaminated by pathogenic organisms. This percentage disagrees with El Hussein *et al*. (2010) who isolated 7(7.23%) out of 97 from drinking water by using the same method, this may be attributed to the variation in the numbers of collected samples, and Nayak *et al.* (2003) who

reported isolation rate of 10%. Also this result is higher than that of Yagoub and Ahmed (2009) since they showed less contamination of drinking water with *Salmonella* in Khartoum State tap water and reported that 0.5% *Salmonella* was isolated from tap water, this may be attributed to the difference in the isolation methods used. Also the study showed higher isolation than Mohammed *et al*. (2009) who reported that 1(3.8%) *Salmonella spp* were isolated from 26 collected samples from drinking water and drinkers from broiler in Shambat. However, Alali *et al.* (2010), and Curtello *et al*. (2013) were un able to isolate *Salmonella spp* from poultry farms which is similar to the results of Omdurman poultry farms. The present results showed lower percentage than that of Zaman *et al.* (2012) who revealed that *Salmonella typhi* represents 28% from isolated *Salmonella spp* collected from 50 samples from tanks and drinkers in Iran.

This study showed that 10 *Salmonella spp* (34.5%) were isolated from litter. *Salmonella* from litter can lead to heavy contamination of the bird's feathers and feet which increases the probability to recover the organism from carcasses in poultry processing plants due to fecal shedding onto the litter (Trampel *et al.*, 2000). The number of isolates that were obtained from litter are higher than those obtained locally by Mohammed *et al.* (2009), and by Soliman *et al*.(2009) from Eygypt, yet it is lower than the results obtained by Ibrahim *et al.* (2013) .This higher percentage obtained in this study may be attributed to lack of biosecurity measures of the investigated farms of which 36 (81.8%) are open system, whereas 3(6.8%) semi closed system and 5(11.4%) are closed, besides 50% of the investigated farms has other animals besides chicken. This study revealed that the predominant environmental media for the recovery of *Salmonella spp* isolates was poultry litter .

Cloacal swabs method is likely to be relatively insensitive compared to the culture of more voluminous faecal material (Kotton *et al*., 2006).This study showed

isolation of 1 *Salmonellae* 3.4%.This percentage of *Salmonella species* isolates that were obtained is different from those of Saad *et al*. (2007), Soliman *et al*.(2009), Akond *et al*.(2012),and Parvej *et al*.(2016), whereas Curtello *et al*. (2013) showed negative isolation of *Salmonella* from cloacal swab. Ahmed *et al. (*2014 b) who detected 12% from 100 cloacal swabs in Egypt.This may be attributed to the differences of the strains and the number of the chickens, their susceptibility to be infected with *Salmonella*, and the system of the management of the farms from cloacal swabs which confirm that cloacal swabs are relatively insensitive due to relatively low prevalence of infection in individual birds, and low number of organisms excreted by infected birds in many cases. Moreover, cloacal swabs obtained a small amount of faeces and *Salmonella* maybe present in low numbers or be non uniformly distributed in the faeces, this method is likely to be relatively insensitive compared with the culture of more voluminous faecal material (Kotton *et al*., 2006).This study also revealed that 3(10.3%) fresh faeces samples were positive for *Salmonella spp* which provide an indication of current infection of flocks, however Dione *et al*. (2009) reported isolation of 35.1% and Alali *et al.* (2010) reported that 10(5.6%) out of 180 were positive for *Salmonella* in faeces from organic broiler farms, whereas 93(38.8%) out of 240 were isolated from conventional broiler farms. Also Umeh and Enwuru, (2014) reported that 105 (52.5%) out of 200 chicken faeces were isolated.

Dust in the poultry houses in large amount may also be a hazard, since dust has been recognized as a vehicle of transmission of *Salmonella* when large numbers of organisms are present (Harbaugh *et al*., 2006). Contaminated dust may also indicate previous infection compared with faeces. Dust is however a more sensitive type of sample for detecting *Salmonella* in poultry flocks (EFSA, 2007). This is likely to be due to the comparative advantage of *Salmonella* in this type of matrix compared to other *Enterobacteriaceae*, which do not tend to survive as well in dry

conditions (Haysom and Sharp, 2003). The present study showed that 4 isolates 13.8% were positive for *Salmonella* in dust, this percentage is higher than Nayak *et al.* (2003) who isolated 5% from environmental swab. Complications of isolating *Salmonella* from feed not only has been suggested to stem from the non-uniform distribution of the organism within the samples, but also from the effect of stress on the organisms from processing treatments used in feed mills. (Zdragas *et al.*, 2000). In addition, the treatment of feed with formaldehyde can interfere with detection methods and give a false negative result (Carrique-Mas and Davies, 2008). Poultry feeds can be sources of *Salmonella* and consequently serve as an indirect cause of human infection to people consuming poultry meat and meat products. Feeds are contaminated either from feed mills or on farms during feed formulation, feeding or handling and subsequently spread to poultry mostly through ingestion. *Salmonella* have the ability to survive under prolong periods in dry conditions like feeds and may be recycled in all production stages in commercial feed preparation (Whyte *et al.*, 2003). This study also revealed that there was a negative detection for *Salmonella spp* from feed source, which agrees with Curtello *et al*. (2013). Also the study revealed that 4(13.8%) *Salmonella spp* were isolated from feeders which disagrees with Nayak *et al.* (2003) who isolated 3% and 1% from feed and feeder samples respectively.

This study also revealed that 1 hand swab 3.4% was positive for *Salmonella* which confirm the role of poultry in contaminating the hands of poultry handlers with *Salmonella* which disagrees with Chotinun *et al.* (2014) who showed no isolation of *Salmonella spp* from workers hands.

Also one isolate of *Salmonella* was isolated from a knife, and this could be due to the manual work during slaughtering, moreover, the practice of using the same cutting knives for the uninfected and infected carcass, results in a further chance of cross-contamination.

Also this study revealed that chicken meat and chicken skin were free from *Salmonella spp* which disagrees with Schlossera *et al*.(2000) who showed isolation of 639 (79.57%) out 803 chicken carcass, Dione *et al*. (2009) who detected 40.4% out of 285 from chicken and 38.6% *Salmonella* from carcass skin in Senegal. Dallal *et al*. (2009) mentioned isolation of 62.7% *Salmonella* in Tehran retail chicken . Akhtar *et al*.(2010) who revealed 26 (30%) out of 85 *Salmonella* were isolated from meat, This result indicates that chicken in Khartoum is fit for consumption.

The present study showed that amoxicillin resistance was most common among the various patterns observed, whereas all isolates were susceptible to ciprofloxacin, amikacin, cefixime, ceftriaxone, cefalexin and cefotaxime. The predominant resistance patterns among *Salmonella* isolates were amoxicillin 89.7%, tetracycline 55.2%, nalidixic acid 48.3% ,ampicillin 44.8%, cotrimoxazole 34.5%, streptomycin 27.6%, chloramphenicol, colistin 10.3%, and Gentamycin 6.9%.

This study showed that one isolate 3.4% was sensitive to all antimicrobials used in this study, 8(27.6%) isolates were resistant to one antibiotic, 2(6.9%) were resistant to two antibiotics, 6(20.7%) were resistant to three antibiotics, 3 (10.3%)were resistant to four antibiotics, 2(6.9%) were resistant to five antibiotics,6(20.7%) were resistant to six antibiotics, and 1(3.4%) was resistant to seven antibiotics. so, the study revealed that 28(96.6%) from the 29 isolates were resistant to one or more of the antibiotics used , and multiple antimicrobial resistance (resistance to more than one antimicrobial) was found in 20(68.7%). Intermediate resistance to amoxicillin, ampicillin, gentamycin, streptomycin,cetazidime, and nalidixic acid were found. Habrun *et al*.(2012) mentioned that 66 (41.7%) isolates were sensitive to all antimicrobials, 68 (43%) were resistant to one antimicrobial, 20 (12.7%) to two antimicrobials and 4 (2.6%) to three tested antimicrobials. Al-Bahry *et al*.

(2007) showed 15 (1.2%) Of 1242 isolates of *Salmonella* from food handlers, were resistant to one or more antibiotic of which 515 isolates from chicken and 432 from sewage water showed resistance to one or more antibiotic (23.7% and 14.1% respectively). Maloo *et al.* (2014) mentioned that multiple resistance to more than two antibiotics, occurred in 3 (42.8%) out of 7 of *Salmonella spp*.

Chotinun *et al*.(2014) mentioned that 68.4% (54/79) of the pathogens were resistant to at least one antimicrobial, while 50.6% (40/79) of the pathogens were multidrug resistant. Abdel-Maksoud *et al*.(2015) mentioned that Multidrug resistance was detected in 82% (64/78) of the isolates, Also Yhiler and Bassey (2015) explained that 68.8% of *Salmonella* isolates exhibited resistance against more than one type of antibiotics hence revealing a high rate of multidrug resistant *Salmonella* strains. Elmadiena *et al*.(2013) mentioned that the most common pattern of multiple drug resistance included resistance to ampicillin and cephalexin.

The present study showed high sensitivity of the isolated *Salmonella* to the selected cephalosporins (cefotaxime, cefixime, cefalexin, ceftriaxone), ciprofloxacin, and amikacin antibiotics which could be related to less or non usage of these drugs for therapeutic purposes in poultry farms, therefore reducing the chance for resistance to develop.

This study showed that all isolates were susceptible to ciprofloxacin which agrees with Hanson *et al*. (2002), Larkin *et al*.(2004), Al-Bahry *et al*. (2007), Mohammed (2009), Kinney (2009), Petkov *et al*. (2010), Alali *et al*.(2010), Hammad *et al*. (2011), Fadlalla *et al*.(2012), Musa *et al*. (2014), Chotinun *et al*.(2014), Ibrahim *et al*. (2013), Indrajith *et al*. (2015), Yhiler and Bassey, (2015), whereas the result disagrees with Firoozeh *et al*.(2011), Akond *et al*. (2012), Onyenwe *et al*. (2012), and Barua *et al*. (2012) .Habrun *et al*. (2012) revealed that 135 (85.4%) were sensitive to ciprofloxacin, Rahmani *et al*. (2013) showed

resistance of 34 (94%) out of 36 isolates , and Gasm Al seed (2014) who showed 50% resistance to ciprofloxacin.

Also the present study showed that all isolates were sensitive to amikacin which agrees with Kinney (2009), Ammari *et al*. (2009**),** Petkov *et al*. (2010), and Hleba *et al*.(2011).

This study also mentioned that all isolates were sensitive to cefotaxime which agrees with Hleba *et al*. (2011), Maloo *et al*. (2014), and Chotinun *et al*.(2014), whereas Habrun *et al*. (2012) showed that 2 (1.3%) out of 158 were resistant to cefotaxim, Firoozeh *et al.* (2011) showed resistance of (3.4%), and Onyenwe *et al*. (2012) showed resistance of 6 (12%) out of 50 isolates, Ibrahim *et al*. (2013) showed resistance of 80%.

The present study also mentioned that no resistance was shown to cefixime, which disagrees with Firoozeh *et al*.(2011).

Also the present study showed that all isolates were sensitive to ceftriaxone which agrees with Kinney (2009), Hleba *et al*.(2011), Hammad *et al*. (2011), whereas the result disagerees with Onyenwe *et al*. (2012).

This study showed no resistance to ceftazidime which disagrees with Onyenwe *et al*. (2012) who showed resistance of 21 (42%%) out of 50 isolates . This study also showed no resistance to cefalexin which disagrees with Enabulele *et al*.(2010) who mentioned that cefalexin resistance was 9% .

In this study *Salmonella* resistance to tetracycline was 55.2% which is dissimilar to the studies carried by Habrun *et al*. (2012) , Petkov *et al*.(2010), and Eja *et al*. (2012.).

The results of gentamycin varies from those obtained by Firoozeh *et al*. (2011), and Mohammed (2009),

This study also showed that 3 (10.3%) isolates were resistant to colistin, whereas , Suresh *et al*. (2006) and Kavitha *et al*. (2008) observed complete resistance of *Salmonella* strains to colistin.

This study also revealed that 8 (27.6%) were resistant to streptomycin which disagrees with Alali *et al*.(2010), Enabulele *et al*. (2010), Firoozeh *et al*.(2011), Fatma *et al*. (2012), Akhtar *et al*. (2010), and Onyenwe *et al*. (2012), whereas Akond *et al*. (2012) showed no resistance.

The present study showed that (34.5%) were resistant to co-trimoxazole, which differs from Al-Bahri *et al*. (2007), Onyenwe *et al*. (2012), Eja *et al*. (2012), and Adetunji and Odetokun, (2012), whereas Maloo *et al*. (2014) showed no resistance.

This study also revealed that 48.3% were resistant to nalidixic acid which is not similar to Hanson *et al*.(2002), and Ammari *et al*. (2009**).**

The present study showed 3(10.3%) isolates were resistant to chloramphenicol , whereas Al-Bahry *et al*. (2007), Hammad *et al*. (2011), and Firoozeh *et al*. (2011) have different results.

The present study showed that 89.7% isolates were resistant to amoxycillin however, Ammari *et al*. (2009),Oliveira *et al*. (2006), Onyenwe *et al*. (2012), and Eja *et al*. (2012) have also different results.

Also this study showed that 44.8% isolates were resistant to ampicillin, which differs from Hanson *et al*. (2002), Al-Bahry *et al*. (2007), Firoozeh *et al*. (2011), Habrun *et al*. (2012, and Enabulele *et al.(* 2010) .

Extended-spectrum β-lactamases(ESBLs)are enzymes hydrolyzing most penicillins and cephalosporins, The present study revealed that 18(62.1%) *Salmonella* isolates were ESBLs producers. ESBLs has been detected by Gasm Alseed (2014), and Abdel-Maksoud *et al*. (2015).

This study showed detection of Cefotaximases (CTX-M) in 8 (44.4%) isolates. They have been also detected by Riano *et al*.(2006), Wittum *et al*. (2012), and Gasm Alseed (2014), while Elumalai *et al*.(2104) revealed negative detection for the bla CTX-M gene.

In the present study TEM gene was detected in 5 isolates (27.8%) . They have been detected also by Olesen, *et al*.(2004), Riano *et al*.(2006), Gasm Alseed (2014), Elumalai *et al*. (2104), and Abdel-Maksoud *et al*. (2015).

This study revealed that SHV was present in 7(38.9%) isolates. Detection of SHV genes have been also detected by Riano *et al*. (2006), Gasm Alseed (2014), and Abdel-Maksoud *et al*. (2015), whereas Elumalai *et al*.(2104) revealed negative isolation for the blaSHV gene.

5.1. Conclusions

This study demonstrated the contamination of chicken broiler farms environment of Khartoum State with *Salmonella arizonae* and *Salmonella choleraesius* with prevalence of 6.2%.

This study revealed the presence of an alarming number of multidrug-resistant *Salmonella* isolates 20(68.7%) out of 29 which suggest an emerging problem that could impact negatively on efforts to prevent and treat poultry and poultry-transmitted human diseases, and confirmed the role of poultry environment as a reservoir of multidrug resistant *Salmonella* .

This study showed that cephalosporins were most effective against *Salmonella arizonae* and *Salmonella choleraesuis,* which could be attributed to that cephalosporins are not used in poultry in Sudan as well as in Europe, where they are not allowed for use in poultry beside ciprofloxacin which lead them to be an effective cure for human diseases.

This study showed high resistance to amoxicillin and ampicillin, which are largely used in veterinary medicine and human medicine in Sudan. This is an alarm for clinicians that consumption and prescription of these antibiotics must be changed.

This study revealed that Phenotypic detection of beta lactams showed that 18 (62.1%) out of 29 isolates were positive of which13 (72.2%) isolates were *Salmonella arizonae* , and 5 (27.7%) isolates were *Salmonella choleraesuis* which attributes high penicillins resistance.

This study revealed that molecular characterization of β-lactamases resistance genes showed presence of TEM gene in 5(27.8%) isolates, SHV in 7(38.9%) isolates, and CTX-M in 8 (44.4%) isolates.

5.2 Recommendations

- The study calls for more research to be done to determine the prevalence and antibiotic resistance patterns of *Salmonella* contaminated poultry,poultry environment, meat, and its products. Also effort is needed to control Salmonellosis in poultry flocks to reduce the threat of this organism to public health.
- Effort is needed to adopt measures to control the spread of multidrug resistant pathogens to humans. Care must be taken in the use of antibiotics in farm animals to reduce the selection of multidrug resistant strains.
- Serotypes should be utilized in identification and be included in a national surveillance database to allow comparisons with findings within Sudan and from other countries in the region.
- In order to control *Salmonella* infection of poultry in Khartoum State detailed epidemiological investigation and strain identification are prerequisites.
- Monitoring of ESBL production and antimicrobial susceptibility testing are necessary to avoid treatment failure in poultry farms.
- Bacteriological monitoring of broiler flocks and separation of infected flocks from food production together with introduction of good manufacturing practices and hygiene control must be implemented.
- Further investigations with bigger sample size are needed to confirm occurrence of *Salmonella* in chicken meat and chicken carcass.

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Appendix

Figure 17. Traditinal open house farming system

Figure 18. Manual slaughtering for chicken

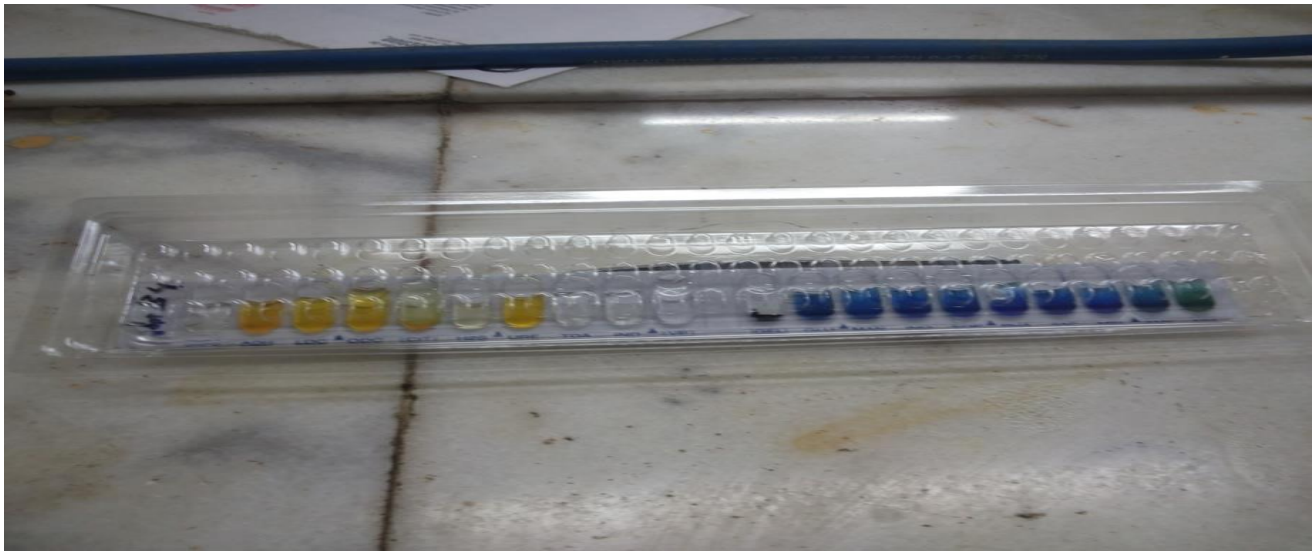

Figure.(19) Negative reactions of biochemical tests of *Salmonella arizonae* on API20 E strips .

Figure.(20) Positive reaction of biochemical tests of *Salmonella arizonae* on API20 E strips.

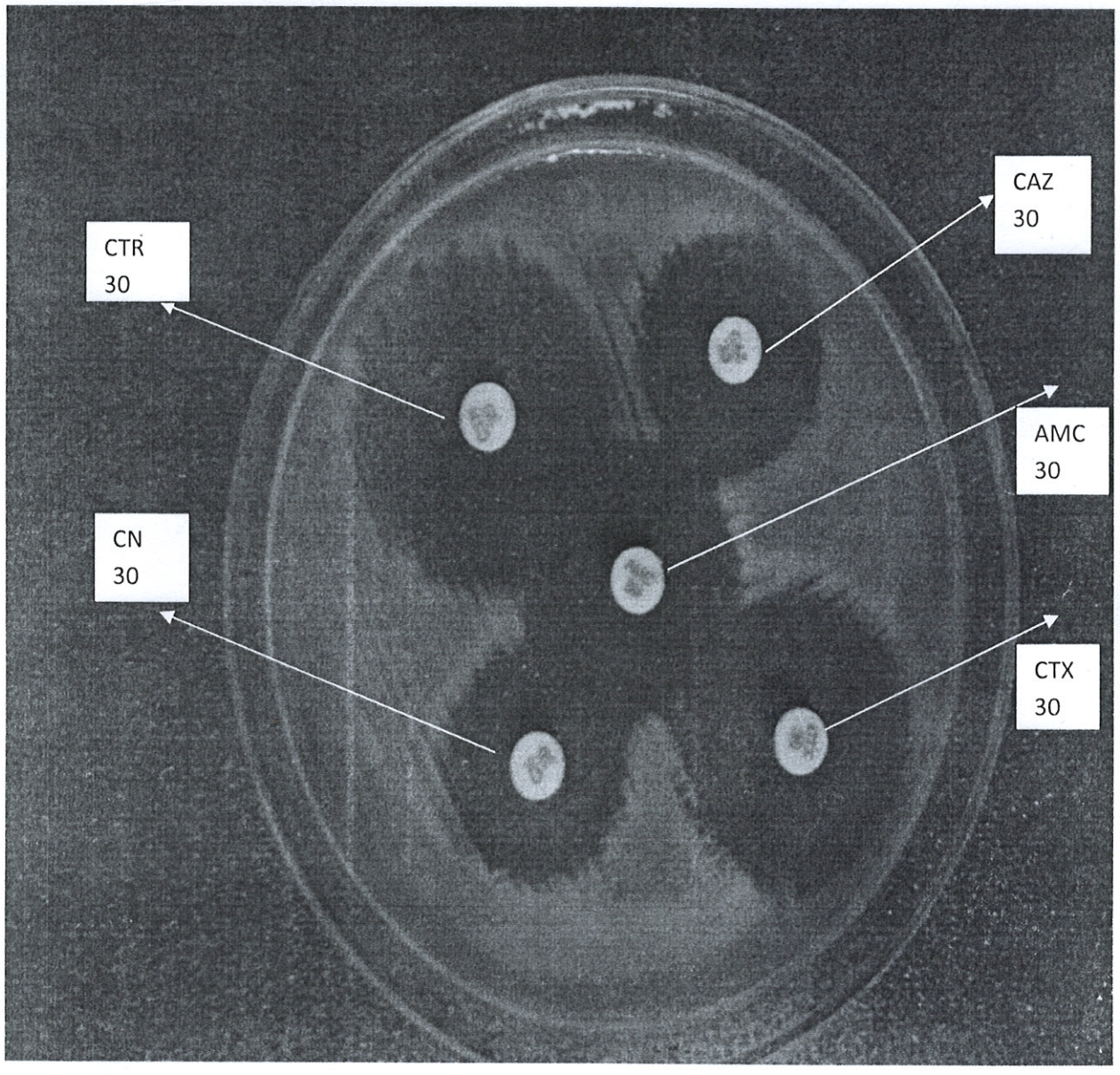

Figure. 21Positive double disc synergy test for ESBL showing synergy between amoxicillin clavulanic acid and indicator antibiotics (cefotaxim, ceftazidime, ceftriaxone ,and cefixime

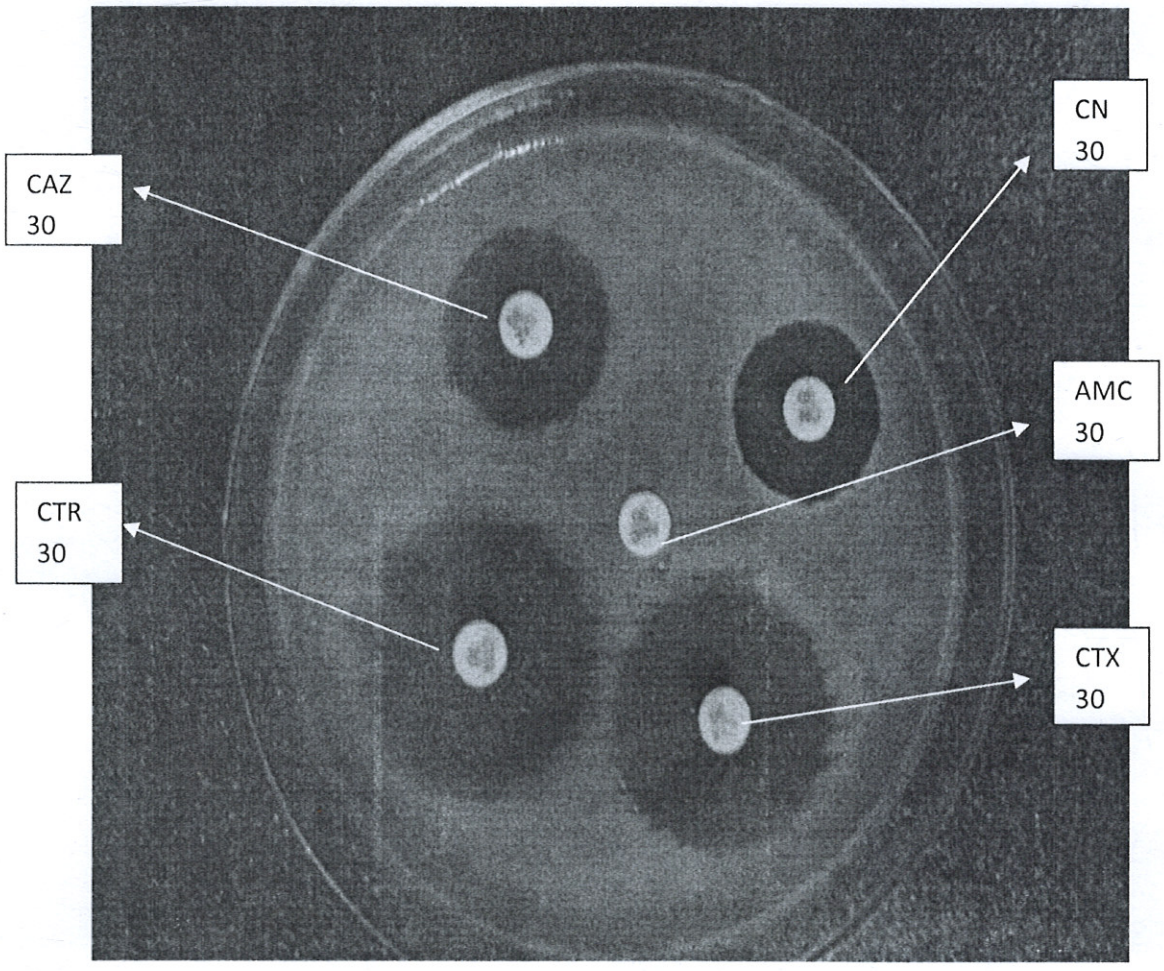

Figure. 22 Negative double disc synergy test for ESBL using amoxicillin clavulanic acid and indicator antibiotics (cefotaxim, ceftazidime, ceftriaxone ,and cefixime(No synergy)