بسم الله الرحمن الرحيم

Sudan University of Science and Technology
College of Graduate Studies

Antimicrobial Resistance and Detection of β - Lactamase Genes in Salmonella Isolated from Poultry in Khartoum North

المقاومة للمضادات الحيوية والكشف عن جينات البيتالاكتميز في السالمونيلا المعزولة من الدواجن بمحلية بحري

A Thesis Submitted as a requirement for the degree of M. Sc in Microbiology

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كلية الدراسات العليا

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Declaration

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إقرار

أنا الموقع أدناه أقر بأنني المؤلف الوحيد لرسالة الماجستير المعنونة المقاومة للمضادات الحيوية والكشف عن جينات البيتالاكتميز في السالمونيلا المعزولة من الدواجن بمحلية بحري.

وهى منتج فكري أصيل . وباختياري أعطى حقوق طبع ونشر هذا العمل لكلية الدراسات العليا - جامعه السودان للعلوم والتكنولوجيا، عليه يحق للجامعه نشر هذا العمل للأغراض العلمية .

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Dedication

This work is dedicated to:

My father, mother, brothers and sisters who gave me continuous support and encouragement to continue my studies.

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Abstract

The diseases associated with gastrointestinal tract are wide spread throughout the Sudan affecting both humans and animals. The most endemic types which affect the population health are those related to Salmonella Species.

Towards knowing the extent of presence of these organisms and extent of their resistance to antibiotics both phenotypically and genotypically, two hundred droppings samples were collected from poultry farms throughout Khartoum north. The samples were investigated for the presence of Salmonella. (64) Salmonella isolates were obtained and identified by biochemical tests, these were (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.*

Different Salmonella species showed variable resistance values with different antibiotics, Following are highest and lowest resistance rates of isolated Salmonella sp. Recorded respectively with different antibiotics, Amikacin (60%, 47%) with (*S. choleraesuis , S. pullorum*), Ceftazidine (71%, 16%) with (*S.arizonea, S. typhi*), Chloramphenicol (33%, 14%) with (*S. typhi, S. arizonea*), Aztreonam (61%, 34%) with (*S. gallinarum, S. pullorum*), Tetracycline (90%, 50%) with (*S. choleraesuis, S. gallinarum* and *S typhi*), piperacillin (80%, 33%) with (*S. choleraesuis , S. typhi*), Imipinem (50%, 28) with (*S. gallinarum , S. typhi and S. arizonea*) and Ciprofloxacin (85% , 38%) with (*S. arizonea, S. gallinarum* and *S.typhi*).

Bacterial DNA was extracted from each isolate (*S.pullorum, S.gallinarum*) using boiling method. PCR was used to detect TEM, SHV, and CTX-M genes. The results showed that the genotypic resistance that is mediated by β-lactamases genes in *S.gallinarum* was (100%) for SHV followed by CTX-M and TEM genes both (58%) and in *S. pullorum* was (44%) for CTX-M then TEM (33%) and finally SHV genes (11%).

الـــخـــلاصـــة

تعتبر الأمراض الباطنية ذات الصلة بالجهاز الهضمي أو القناة الهضمية من أكثر الأمراض إنتشاراً في بقاع السودان المختلفة ومن أكثرها شيوعاً وتأثيراً على الصحة العامة تلك الأمراض والأعراض ذات الصلة ببكتريا السالمونيلا.
وللتعرف على مدى إنتشار هذه الأمراض ومدى تجاوبها أو مقاومتها لبعض المضادات الحيوية تم إختيار منطقة الخرطوم بحري لدراسة الوضع حيث جمعت مئتا عينة زرق من عشرة مزارع للدواجن في منطقة الخرطوم بحري وتم إخضاعها للفحص المختبري لدراسة وجود بكتريا السالمونيلا في هذه العينات و إجراء الإختبارات الكيموحيوية. لقد أظهرت أربعة وستون عينة وجود بكتريا السالمونيلا . تم التعرف على عدة أنواع من بكتريا السالمونيلا حيث كانت سبع عزلات من السالمونيلا الأريزونية، عشرة عزلات من سالمونيلا كوليرا الخنازير، ثمانية عشر عزلة من السالمونيلا الدجاجية، وثلاثة وعشرين عزلة من السالمونيلا الفراضية وست عزلات من السالمونيلا التيفية.
تم إختبار حساسية العزلات لمجموعة من المضادات الحيوية لمعرفة نمط إستجابة السالمونيلا للمضادات الحيوية ، أظهرت النتائج إختلاف مقاومة البكتريا للمضادات الحيوية بإختلاف المضاد الحيوي ونوع البكتريا حيت كانت أعلى وأدنى نسب مقاومة لأجناس بكتريا السالمونيلا للمضادات الحيوية كما يلي: للأميكاسين (60%، 47%) مع(سالمونيلا كوليرا الخنازير ، السالمونيلا الفراضية)، للسفتازيدين (71%، 16%) مع (السالمونيلا الأريزونية و السالمونيلا التيفية)، للكلورامفينيكول (33%، 14%)مع (السالمونيلا التيفية، السالمونيلا الأريزونية)، للأستريونام (61%، 34%) مع (السالمونيلا الدجاجية ، السالمونيلا الفراضية)، للتتراسيكلين (90%، 50%) مع (سالمونيلا كوليرا الخنازير، السالمونيلا الدجاجية والسالمونيلا التيفية)، للبايبراسيلين (80%، 33%) مع (سالمونيلا كوليرا الخنازير، السالمونيلا التيفية)، للإمبينيم (50%، 28%) مع (السالمونيلا التيفية، السالمونيلا الدجاجية والسالمونيلا الأريزونية)و للسبروفلوكساسين (58% ،38%) مع (السالمونيلا الأريزونية، السالمونيلا الدجاجية والسالمونيلا التيفية).
تم إستخلاص الحمض النووي الديوكسي رايبوسي بتقنية الغليان من نوعين من السالمونيلا وأستخدمت نظرية التفاعل التسلسلي المتعدد للتعرف على وجود جينات البيتالاكتميزTEM ، CTX-M and SHV وقد أظهرت النتائج وجود هذه الجينات بدرجات متفاوتة حيث كانت في السالمونيلاالدجاجية 58% CTX-Mو TEM و 100% لـSHV.وفي السالمونيلا الفراضية كانت 44% لـCTX-M ، 33 %لـTEM و 11% لـSHV.

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CHAPTER ONE
INTRODUCTION

1.1 General background

Antibiotic drug-resistance is a worldwide problem. Drug resistance is now very wide spread and strains are commonly encountered resistant to more than one drug (Carter, 1985).

Bacteria can acquire resistance to antibiotics as a result of chromosomal mutation, expression of a latent chromosomal gene, by exchange of genetic material through transformation, transduction or conjugation by plasmids (Neu, 1992). The occurrence and establishment of resistant mutants vary with different drugs. Selection of mutants is favored by under dosage, prolonged administration and the presence of a closed focus of infection such as that found in abscesses (Carter, 1985).

Bacteria that resistant to ten or more antimicrobial agents have been reported (Jacoby and Archer, 1991). These highly multi- resistance bacteria are posing important public health problem (Cohn, 1999). *Salmonellae* are Gram negative bacteria that infect human and animals and exert resistance to many antibiotics (Carter, 1985). In the Sudan and other developing countries prescription of antibiotics by physicians and veterinarians based mainly on clinical diagnosis, this may lead to prescription of wrong antibiotics and also may result in emerging of resistant bacterial strains. Moreover, the overuse and misuse of antibiotics by public who can purchase easily antibiotics from pharmacy without prescription make the condition worst. Now many antibiotics are less effective in compacting bacterial infection in the Sudan.

1.2 Problem justification and hypothesis

Several birds in poultry farms in Khartoum North "Bahry" occasionally suffer from symptoms like anorexia, diarrhoea, dehydration, weakness, loss of appetite, drooping wings and somnolence …etc. Salmonella may be incriminated as the causative agent for such symptoms. Antibiotics prescribed to treat such cases are not always effective. It seems that Salmonella organisms have developed some degree of resistance to the antibiotics.

1.3 Objectives

1.3.1 The overall objectives

(i) To detect the presence of drug resistant Salmonella.

1.3.2 Specific objectives

(i) To isolate Salmonella from birds showing typical symptoms of Salmonellosis.

(ii) To determine the antimicrobial susceptibility phenotype of the isolates.

(iii) To detect the specific β - Lactamases resistance genes for Salmonella.

CHAPTER TWO
LITERATURE REVIEW

2.1 Antibiotics

Antibiotics are defined as low molecular weight metabolite which kill or inhibit the growth of susceptible bacteria (Queen, *et al* .2002).

2.1.1 Establishment of bacteria

A bacterium organism protects itself from enemies in various ways. It may produce metabolic waste products, which change the conditions in the medium, such as pH, osmotic pressure and surface tension making the environment unfavorable to the growth of less tolerant organisms. It may elaborate specific toxic substances, which interfere with the metabolism of other organisms to an extent that they are either be killed or prevented from multiplying. These specific toxic substances are called antibiotics, Heritage, *et al* (1996) defined antibiotics as a substance that was produced by microorganisms that is in very low concentration inhibits or kills the growth of another microorganisms.

2.1.2History and discovery of antibiotics

Pasteur and Joubert (1877) noted that a culture of *Anthrax bacilli* was killed if it was contaminated by common air-borne organisms. They realized that such phenomenon might well have therapeutic possibilities (Thomas, 1993).

On the agar plate culture of *Staphylococcus aureus*, Fleming (1929) obtained a mold contaminated that culture and produced a green pigment which prevented bacterial growth some distance around it. He cultivated the mold in a liquid medium and found that a filtrate of the culture had the power, even when greatly diluted to prevent growth of a number of Gram-positive pathogenic bacteria. Since the mold proved to be species of penicillium he

named the antibiotic penicillin; subsequently shown to be more effective against a number of infections than the sulpha drugs. The antibiotic was found to be so nontoxic that amounts beyond the effective curative dose could be safely administrated (Salle, 1971).

In 1940, Chain, *et al* at Oxford University succeeded in obtaining penicillin preparations of high antibacterial activity. These preparations were highly effective in controlling experimental infections in animals. The remarkable clinical potentialities of penicillin were quickly demonstrated (Thomas, 1993).

Dubos (1939) isolated from the soil spore-producing bacillus that was capable of destroying living Gram-positive cocci, the organism was found to be *Bacillus brevis* a large Gram-positive rod similar to *Bacillus subtilis.* He named the antibiotic gramicidin (Salle, 1971).

Most antibiotics are produced by Streptomyces. A few are produced by *Bacillus* spp., actinomycetes and fungi. Several antibiotics are semi-synthetic in origin e.g. cloxacillin and ampicillin are prepared from naturally produced 6-aminopenicillanic acid (Thomas, 1993).

Thousands of antibiotics have been isolated and studied. Some are useful clinically; others are not satisfactory for clinical application but more useful for other purposes (Salle, 1971).

The field of antibiotics offers unlimited possibilities in medicine. Powerful antibiotics, such as penicillin, have proven to be of tremendous importance that an ever-increasing search is going on in the hope that agents superior to those now in use might be isolated (Salle, 1971).

2.1.3 Classification and mechanism of action

Since the antibiotics were first discovered in the 1920s, much knowledge has been acquired on their mode of action and the significance of this action on their relative merits in the therapy of man and animals.

Antibacterial agents can be divided into four groups based on this effects of synthesis of nucleic acid, protein, the formation of cell wall and permeability of cell membrane.

2.1.3.1 Nucleic acid inhibitors

Replication of the nucleic acids of the bacterial cells is prevented directly by nalidixic acid and rifamycin and indirectly by the sulphonamides. Sulphonamides ultimately deprive the cell of nucleic acid and the presence of nalidixic acid prevents its replication.

Sulphonamides were interfered with folic acid pathway by binding the enzyme dihydropteroate synthase. For bacteria to produce precursors that are important for DNA synthesis, they use the folic acid pathway. Sulphonamides target and bind to one of the enzymes dihydropteroate synthase, and disrupt the folic acid pathway (Forbes *et al*, 1998).

Rifampicin was derivative of the rifamycin family of antibiotics and was mainly active against Gram-positive organisms (Thomas, 1993). Rifampin inhibited bacterial growth by binding strongly to the DNA - dependent RNA polymerase of bacteria, thus inhibit bacterial RNA synthesis (Jawetz *et al*, 1989).

Quinolones inhibited bacterial DNA gyrases (topoisomerases) which was catalyzes super coiling of bacterial DNA (Thomas, 1993).

2.1.3.2 Protein synthesis inhibitors

Antibiotic classes that act by inhibiting protein synthesis include aminoglycosides e.g. gentamycin, tobramycin, kanamycin, streptomycin, tetracycline and chloramphenicol macrolides, e.g. erythromycin, azithromycin, and lincosamides e.g. clindamycin (Forbes *et al*, 1998 ; Cheesbrough, 1992).

Aminoglycosides inhibit bacterial protein synthesis by binding to protein receptors on the organism 30s ribosomal subunit. This process interrupts several steps including initial formation of the protein synthesis complex, accurate reading of the mRNA code and disruption of the ribosomal- mRNA complex (Baker and breach, 1980 ; Forbes, *et al*, 1998).

Chloramphenicol inhibits the addition of new amino acids to the growing peptide chain by binding to the 50s ribosomal subunit (Jawetz *et al*, 1989). This antibiotic is highly active against a wide variety of gram-negative and gram-positive bacteria (Forbes, *et al*, 1998).

Tetracyclines inhibit protein synthesis by binding to the 30s ribosomal subunit so that incoming tRNA amino acid complex cannot bind to the ribosome, thus halting peptide chain elongation. Tetracyclines have abroad spectrum of activity that includes gram-positive bacteria, gram-negative bacteria and several intracellular bacteria such as chlamydia and rickettsis (Jawetz et *al*, 1989, Forbes, *et al*, 1998).

Macrolides (erythromycin and azithromycin) bind to the 50s subunit of the ribosomal-RNA and the bindig site was a 23s rRNA (Jawetz *et al*, 1989).

Lincosamides (clindamycin) inhibit protein synthesis by binding to receptors on the bacteria 50s ribosomal subunit and subsequent disruption of the growing peptide chain. Primarily because of uptake associated with gram-negative outer membranes, the macrolides and clindamycin are generally not

effective against most genera of gram-negative bacteria; however, they are effective against gram-positive bacteria (Forbes, *et al*, 1998).

2.1.3.3 Cell wall synthesis inhibitors

The bacterial cell wall composed mainly of peptidoglycan or murein layer plays an essential role in the life of the bacterial cell. The cell wall of the bacteria is tough and rigid and lies external to the cell membrane, giving the whole cell protection from possible osmotic damage (Forbes, *et al*, 1998).Several agents affect cell wall synthesis, the most imports being penicillin's e.g. cloxacillin and amoxyillin, cephalosporines e.g. cepharadine, cefuroxime and ceftazidime and glycol peptides e.g. vancomycin (Cheesbrough, 1992).

Beta- lactam antimicrobial agents are those that contain the four membered, nitrogen- containing beta lactam ring at the core of their structure and mode of action of these drugs that target and inhibit cell wall synthesis by binding the enzymes in the synthesis (Forbes, *et al*, 1998).

Glycopeptides which are the other major class of antibiotic that inhibit bacterial cell wall synthesis e.g. vancomycin. Vancomycin is the most commonly used agent in this class, it inhibits cell wall synthesis by binding to precursors of cell wall synthesis (Forbes, *et al*, 1998).

Vancomycin can not penetrate the outer membrane of most gram-negative bacteria to reach their cell wall precursor targets, because of its relatively large size; therefore, this agent is usually in-effective against gram-negative bacteria (Forbes, *et al*, 1998).

Some fungi of *Cephalosporium* spp yield antimicrobial substances called cephalosporines (Jawetz *et al*, 1989). Cephalosporines consisted of three generations which are cephradine and cephalothin, second was cefuroxime and

third was cefazidime and cefotaxime (Forbes, *et al,* 1998 ; Cheesbrough, 1992).

The mechanism of action of cephalosporines was analogous to that of penicillins to specific Para amino benzoic acid PABA that serves as drug receptors on bacteria inhibiting cell wall synthesis by blocking the transpeptidation of peptidoglycan and activating autolytic enzymes in the cell wall that can produce lesions resulting in bacterial death (Jawetz *et al*, 1989).

2.1.3.4 Cell membrane function inhibitors

Polymyxins (polymyxin B and colistin) are the agents most commonly used that disrupt bacterial cell membrane. Most notably, they are more active against gram-negative bacteria, while activity against gram-positive bacteria tends to be poor (Forbes, *et al,* 1998). Polymyxin becomes family bound to the cytoplasmic membrane and acts by damaging this structure (Thomas, 1993).

2.1.4 Toxicity and side effects of Antimicrobial Agents

Not all antimicrobials, at the concentrations required to be effective completely nontoxic to the cells. Most, however, show sufficient selective toxicity to be of value in the treatment of microbial disease (Cheesbrough, 1992). Most serious side effects of penicillins are due to hypersensitivity. The tetracycline and chloramphenicol produce varying degrees of gastrointestinal upset (nausea, vomiting and diarrhea).

Gentamycin was toxic, particularly in the presence of impaired renal function. Fever, skin rashes and other allergic manifestations may result from hypersensitivity to streptomycin (Jawetz *et al,* 1989).

2.1.5 Antibiotic spectrum

The term broad spectrum is applied to antibacterial agents with activity against a wide range of gram- positive and gram- negative organisms such as

tetracyclines, amino-glycosides, sulphonamides and chloramphenicol (Thomas, 1993 & Cheesbrough, 1992).

2.1.5.1 Narrow spectrum antibiotics

Narrow spectrum antibiotics are those with activity against one or few types of bacteria e.g. vancomycin against Staphylococci.

2.1.6 Type of action

Antibiotics agents are generally described as bacteriostatic when, at usual dosage they prevent the active multiplication of bacteria e.g. chloramphenicol, tetracycline and erythromycin, and are described as bactericidal when, at usual dosage they kill bacteria e.g. penicillins, cephalosporins, glycopeptides and amino glycosides (Cheesbrough, 1992).

Some bacteriostatic agents become bactericidal when used at higher concentration e.g. erythromycin and tetracycline (Thomas, 1993 & Cheesbrough, 1992).

2.1.7 Clinical use of antibiotics

The goal of antibiotic therapy is to cure the patient with the minimum of complications and discomfort. At the same time, it is important to discourage the emergence of drug-resistant organisms. The principles of antibiotic uses should observed. These principles ensure that antibiotics should not be given for trivial infections they should be used for prophylaxis only in special circumstances. Treatment should be based on a clear clinical and bacteriological diagnosis, the choice of antibiotics is essentially a clinical matter, and systemic treatment should be given in full therapeutic dose for an adequate period. In local treatment of superficial infections it is important to use either antiseptics or antibiotics which are rarely or never used systematically, e.g. mupirocin and polymycin, antibiotic solutions and powders

should not be liberated into environment. They can cause hypersensitivity reactions and encourage development of antibiotic-resistant strains (Thomas, 1993).

2.1.8 Combination of antibiotics

Occasionally, a combination of antimicrobials may used to treat mixed infections, to prevent treatment failure and drug resistance from developing, to treat several infections when the organism is not known, or when it necessary obtain a greater antimicrobial effect from two bactericidal drugs acting together (synergistic effect), (Cheesbrough, 1992).

Baker and Breach (1980) stated that when two bacteriostatic antibiotics were given together, the resulting action was synergistic and when a bacteriostatic and bactericidal were given together, the result was antagonistic. Although this is not necessary true with all antibiotics, it is, nevertheless, a good rule to follow, unless tests for combined actions have been performed. It must also be noted that a combination of two β-lactams dose not necessarily produce a synergistic effect (Hugo and Russell 1989).

2.2 drug resistance

If Bactria are repeatedly subcultured in media containing antibiotic the presence of gradually increasing sub inhibitory concentrations of antibiotics are observed. It is usually possible to obtain mutant organisms which will survive and multiply in concentrations which are lethal for the strain (Thomas, 1993).

Antibiotics can be inactivated either by enzymatic cleavage or by chemical modification such that they are no longer interact with the target site or are no longer taken up by the organisms rendering them in activates (Pratt and Taylor, 1990 & Lancini *et al*, 1995).

In 1944 most Staphylococci were susceptible to penicillin, although a few resistant strains had been observed (Jawetz *et al*, 1989). In 1946, about 60% of hospital strains isolated in the U.K were penicillin resistant (Baber & Rozwadowska, 1948). After massive use of penicillin 65-85% of Staphylococci isolated from hospital in 1948 were β-lactamase producer and thus resistance to penicillin (Jawetz *et al*, 1989). The introduction of tetracycline, streptomycin, chloramphenicol and erythromycin for the treatment of infections caused by penicillin resistant Staphylococci was similarly followed by the emergence of strains resistant to these antibiotics (Shanson, 1981).

The degree of resistant and the speed that developed vary with the organism and the drug. Thus the resistance of *Staphylococcus aureus* to penicillin, chloramphenicol and tetracyclines usually develops slowly in multiple small steps and many successive subcultures may be required before a high level of resistance is obtained. In contrast the resistance of various organisms to streptomycin and the *Mycobacterium tuberculosis* to isonized often rises suddenly in one step to very high levels (Thomas, 1993). While it is possible to select antibiotic-resistant mutant of most organisms in vitro, this is really reflect the situation in vivo, thus naturally occurring strains of *Streptococcus pyogenes* have remained fully sensitive to penicillin in spite of wide spread use of the drug and it not has been possible to obtain resistant artificially (Thomas, 1993).

When antibiotic resistant is found in clinically derived strains of bacteria the mechanism of resistance often differs from that obtained by laboratory means. For example penicillin-resistant *Staphylococcus aureus* encountered in hospitals produce (β-lactamase) whereas resistant mutant’s selected in the laboratory have a decreased ability to bind the drug. Likewise, many of the clinically isolated bacteria resistant to amino-glycosides produce plasmid

mediated antibiotic- inactivating enzymes, but resistant strains selected in vitro possess mutant ribosomal binding sites (Thomas, 1993).

The ribosome of *Staphylococci* can become insensitive to erythromycin following specific enzyme modification of rRNA (Davies, 1992). Rifampin resistance was resulted from in-activation of a single target (Spratt, 1994).

A third form of antibiotic resistance is known as "drug tolerance". Penicillin tolerant strains of *Staphylococcus aureus* are inhibited by low concentration of penicillin but are resistant to the lethal (bactericidal) effects of the antibiotic (Thomas, 1993). It has been suggested that tolerant organisms are deficient in autolysis enzyme activity which is necessary for cell lysis and the lethal action of penicillin. Penicillin tolerance is also common among *Streptococci* (Thomas, 1993).

The development of antibiotic-resistant strains during therapy was unlikely to be a serious clinical problem because the fraction of resistant cells in bacterial population was always very small (Davies, 1994). Resistance has increased substantially in recent years and has reduced value of formerly widely prescribed agents such as the sulphonamides and ampicillin (Hugo and Russell, 1989).

2.2.1 Multiple- drug resistance

Multiple- drug resistance has been encountered in *Mycobacterium tuberculosis, Streptococcus pneumoniae, Stapylococcus aureus, Enterococcus spp., Shigella dysentereriae, Escherichia spp, Haemophulus influenza, Klebsiella spp, Proteus spp, Salmonella spp, Serratia spp and Pseudomonas spp* (Cohen, 1992).

Resistant enterobacteriaceae frequently contain multiple plasmids, the larger of which can carry genes for resistance to 10 or more antimicrobial agents (Jacoby and Archer, 1991). These highly resistant bacteria were made

many currently available antimicrobial drugs ineffective and in certain instances were already posing important public health problem (Cohen, 1992). Another aspect of multi resistant was that any one of the antimicrobial agents affected can maintain selection for resistance to the group as was illustrated by the need to restrict the use of kanamycin and tetracycline as well as ampicillin to rid a burn unit of *Pseudomonas aeruginosa* with plasmids encoding linked resistance to carbenicillin, kanamycin and tetracycline (Lowbury and Roe, 1972).

2.2.2 Mechanisms of antibiotics resistance

Successful bacterial resistance to antimicrobial action requires interruption or disturbance of one or more steps that are essential for effective antimicrobial action. These disturbances or resistance mechanisms can come about in various ways, but the end result is partial or complete loss of antibiotic effectiveness. Different aspects concerning anti microbial resistance mechanisms include biologic vs. clinical antimicrobial resistance environmentally mediated antimicrobial resistance, and microorganisms-mediated antimicrobial resistance (Forbes, *et al,* 1998).

2.2.2.1 Biologic vs. clinical resistance

Development of bacterial resistance to antimicrobial agents to which they originally susceptible requires alterations in the cells or structure. Biologic resistance refers to changes that result in the organism being less susceptible to a particular antimicrobial agent that has been previously observed when antimicrobial susceptionlity has been lost to such an extent that the drug is no longer effective for clinical use, the organism has achieved clinical resistance (Forbes, *et al,* 1998).

2.2.2.2 Environmentally mediated antimicrobial resistance

Environmentally mediated antimicrobial resistance is defined as resistance that directly results from physical or chemical characteristics of the environment that either directly alter the antimicrobial agent or alter microorganisms normal physiologic responds to the drug. Example of environmental factors that mediate resistance (Mg ++ and C++) concentrations and thymine-thymidine content (Forbes, *et al,* 1998).

2.2.2.3Microorganisms mediated antimicrobial resistance

Microorganisms mediated antimicrobial resistance refers to antimicrobial resistance that is due to genetically encoded traits of the micro organism and is the type of resistance that is in vitro susceptibility testing methods are targeted to detect (Forbes, *et al,* 1998).

Organisms – based resistance can be divided into two subcategories intrinsic or inherent resistance and acquired resistance.

2.2.2.3.1 Intrinsic resistance

Intrinsic resistance is antimicrobial resistance resulting from the normal genetic structural or physiological state of microorganisms (Forbes, *et al,* 1998). Such resistance is considered to the natural and consistently inherited characteristic that is associated with the vast majority of strains that constitute particular bacterial group, genus or the species. Therefore, this is predictable resistance so that once the identity of the organism is known, it can reflects certain aspects of its antimicrobial resistance profile (Forbes, *et al,* 1998).

2.2.2.3.2 Acquired resistance

Bacteria can acquire the resistance to antibiotics as a result of a chromosomal mutation, expression of a latent chromosomal gene, by exchange of genetic material through transformation (the exchange of DNA),

transduction (bacteriophage) or conjugation (extra chromosomal DNA) (Neu, 1992).

Acquired resistance was usually reversible. However, the ease with which resistance reverts to sensitivity depends on a number of factors such as nature of the organism, nature of the drug, degree of resistance that has been established and weather the resistance has been acquired by genetic or phenotypic adaptation (Salle, 1971). It was antibiotic resistance that results from altered cell physiology and structure caused by changes in microorganism's genetic makeup (Forbes, *et al,* 1998).

The basic mechanism of acquired microbial resistance to antimicrobial agents were generally divided into five categories, the development of an altered drug target, a decrease in the concentration of drug that reaches the receptors by altered rate of entry or removal of the drug , degradation of the antibiotic, synthesis of resistance or alternate metabolic pathways that were no longer susceptible to an antibiotic and failure to metabolize the drug to its active state (Neu,1992, Davies, 1992, Spratt, 1994 &Brody *et al* 1994).

2.2.3 Common path ways for antimicrobial resistance

Weather resistance is intrinsic or acquired; bacteria share similar pathways to strategies to effect to antimicrobial agents. The pathways involve enzymatic destruction or alteration of the antibiotic, decrease intracellular uptake or accumulation of the drug and altered antibiotic target are most common (Davies, 1994& Forbes, *et al*, 1998).

2.2.3.1 Resistance to beta-lactam antibiotic

Extended-spectrum β-lactamases (ESBL) are enzymes produced by some *Salmonella* species (and sometimes other Enterobacteriaceae) that inactivate penicillin, expanded-spectrum Cephalosporins, Monobactams

including older β-lactam antimicrobial agents and are inhibited by clavulanic acids, sulbactam or tazobactam (Bush *et al*, 1995).

The spread of ESBL-producing bacteria occurs rapidly and has been widely reported worldwide. Continuous monitoring systems and effective infection control measures are required to curtail their spread. Prevalence of ESBL producing *salmonella* among clinical isolates varies from country to country; institution to institution and therapeutic choices for infections caused by ESBL-producing bacterial strains remain limited (Pfaller and Jones, 2000).Common ESBL genes coding were TEM (described in the early 1980s from a patient named Teminora from Greece), SHV (for sulphydryl variable, first found in a single strain of *Klebsiella ozaenae* isolated in Germany) and CTX-M [cefotaximase that preferentially hydrolyze cefotaxime], these genes mediated by chromosomes, plasmids or transposons have all been increasingly described worldwide.

Bacterial resistance to beta-lactams may be mediated by enzymatic destruction of the antibiotic, altered antibiotic target or decreased intracellular uptake of the drug (Forbes, *et al*, 1998).

Staphylococci are gram-positive bacteria that most commonly produce β-lactamase, approximately 90% or more of clinical isolates are resistant to penicillin as result of this enzyme production (Forbes, *et al*, 1998). Rare isolates of enterococci also produce β-lactamase.

Gram-negative bacteria including *Enterobacteriaceae, P. aeruginosa, H.influenzae and N.gonorrhoeae* produce dozens of different β-lactamase types that mediated resistance to one or more of the β-lactam antibiotics (Forbes, *et al*, 1998).

Enzymatic inactivation of drugs was common of the most biochemical processes that engender resistance to a variety of antibiotic structural types in

bacteria (Jacoby and Archer, 1991). If a drug acts by inhibiting an enzyme that was critical for the cell growth, then cells that produce greater amounts of the enzyme may be able to produce sufficient amount of the metabolic product to survive in the presence of the drug concentrations that are usually attained in clinical treatment (Pratt and Taylor, 1990).

2.2.3.2 Resistance to glycopeptides

The acquired resistance to vancomycin has been described for enterococci but not for *Staphylococci* or *Streptococci.* The mechanism involves the production of altered cell wall precursors that do not bind vancomycin with sufficient a avidity to allow inhibition of peptidoglycan synthesizing enzymes (Forbes, *et al,* 1998).

Vancomycin is the only cell inhibiting agents for use agent's β-lactams methicillin- resistant *Staphylococci* and ampicillin-resistant *Enterococci* (Forbes, *et al,* 1998).

2.2.3.3 Resistance to amino glycosides

Resistance to the amino-glycosides e.g. gentamicin emerged after some ten years of use and was associated with its extensive use as a topical antibiotic (Nobel and Naidoo, 1978).

Resistance to streptomycin produced by a chromosomal mutation was due to an alteration in the ribosome so that streptomycin could no longer bind to the ribosome (Lacey and Chopra, 1972).

Plasmid-borne resistance to streptomycin was achieved by different mechanisms; it encodes an enzyme which modifies streptomycin so that it can no longer bind to the ribosome (Grinsted and Lacey, 1973).

Analogous to β-lactam resistance, amino-glycoside resistance is accomplished by enzymatic, altered target or decreased uptake pathways.

Several different amino glycoside-modifying enzymes are produced by gram-positive and gram-negative bacteria (Forbes, *et al*, 1998).

Amino-glycosides enter the gram-negative cell by passing through outer membrane poring channels. Therefore, poring alterations may also contribute to amino glycoside resistance among these bacteria (Forbes, *et al*, 1998).

2.2.3.4 Resistance to tetracyclines

Resistance to tetracyclines is mediated with Tet M.gene, a gene that confers tetracyclines resistance, was particularly common, and was identified in *Staphylococci* and *Streptococci* (Roberts, 1990).

2.2.3.5 Resistance to quinolones

Resistance to quinolones is most frequently mediated by either decreased uptake or accumulation or by production of an altered target. Components of the gram-negative cellular envelope can limit quinolones access to the cells interior site of DNA processing (Forbes, *et al*, 1998).

2.2.4 Emergence and dissemination of antimicrobial resistance

The known resistance pathways are not necessarily new mechanisms that have recently evolved among bacteria. By definition, antibiotics originate from microorganisms can kill or inhibit growth of another. Therefore, antibiotic resistance mechanisms always have been parts of the evolution of bacteria as means of survival among antibiotic producing competitors (Forbes, *et al*, 1998).

However, with the introduction of antibiotic into medical practice, clinically relevant bacteria have had to adopt resistance mechanisms as part of their survival strategy. With our use and abuse of antimicrobial agents, a survival of the fittest strategy has been used by bacteria to adapt to the pressure of antimicrobial attack (Forbes, *et al*, 1998).

All bacterial resistance strategies are encoded by one or more genes and these resistance genes are readily shared between strains of the same species, between species of different genera and even between more distantly related bacteria. When a resistance mechanism arises, either by mutation or gene transfer, in a particular bacterial strain or species, there is a propensity of this mechanism to be passed on-to other organism using commonly described paths of genetic communication. Therefore, resistance may spread to a wide variety of clinically important bacteria, and any single organism can acquire multiple genes become resistance to the full spectrum of available antimicrobial agents (Forbes, *et al*, 1998). For example, there already exist strains of *Enterococci* and *Pseudomonas aeruginosa* for which no effective therapy is currently available. Alternatively, multiple resistances may be mediated by a gene that encodes for a single very potent resistance mechanism. One such example is the mecA gene that encodes *Staphylococcal* resistance to methicillin and to all other β-lactams currently available for use against these organisms, leaving vancomycin as the single available and effective cell wall- inhabiting agent (Forbes, *et al*, 1998).

2.3 Mutation

Mutation in chemical terms means alternative in the base pair sequence of the DNA double helix. This may be due to substitution of one base for another, deletion of bases or insertion of new bases (Salle, 1971, Thomas, 1993). Every mutation is due to a change in the structure of a gene (Thomas, 1993).

Smooth –rough(S-R) variation is one of the most obvious and important types of mutation shown by pathogenic bacteria (Thomas, 1993). The variation, involves a change of colonial appearance from smooth to rough, a loss of surface components (somatic and capsular antigens) and loss or

reduction of virulence. It commonly occurs when bacteria are grown for long period on artificial media (Thomas, 1993).

In studies of molecular evolutionary biology, the term mutation rate is applied to estimations of the rate (per generation) of mutation per nucleotide, per locus, or per eventually for the whole genome and selectively favorable, unfavorable, or neutral mutations are considered (Martinez and Baquero, 2000). They stated that the mutation is not simple characteristic of a specific bacterial species- antibiotic association. On the contrary, the probability of the emergence of the antibiotic-resistant mutant is complex phenomenon, as previously recognized by others, in which the physiology, the genetics, the antibiotic-bacterial dynamics, and the historical behavior of bacterial populations, together with the physical structure of the selective medium, play major roles. Martinez and Baquero (2000) assumed that the mutation rate determined under conventional laboratory conditions probably differs greatly from that in vivo at the site of infection.

The mutation rate varies with different properties and different bacteria, but it commonly between 1 in 107 and 1 in 1010. These rates are for a single property. The total number of mutants, involving a variety of properties, is far greater (Thomas, 1993).

Mutagenic agents such as x-rays, ultraviolet light and alkylating agents, cause a general increase in the rates of mutation (Thomas, 1993).

2.3.1 Factors affecting mutations rates

The cell machinery responsible for replication of DNA is extremely efficient, and under normal conditions only rarely a “wrong” base is incorporated into a gene (Salle, 1971). The spontaneous mutation rate can be greatly increased, however, by treating bacteria with mutagenic agents. Induced mutants occur at rates from 10 to 100.000 times more frequently than

spontaneously occurring mutants among bacteria that have been exposed mutagenic agents (Salle, 1971). Mutagenic agents exert their effect by reacting, directly or indirectly, with DNA. Agents which have proved effective mutagens include ultraviolet light, x-rays, carcinogenic chemical such as nitrogen and sulphur mustards, various peroxides and epoxides, and purine and pyrimidine analogous(Salle, 1971). Some mutagens such as nitrous acid and hydroxylamine have been shown to cause mutations in vitro by allowing them to react with purified transforming DNA and then introduced the altered DNA into recipient bacteria (Salle, 1971).

2.3.2 Transformation

Transformation is the insertion of small pieces of DNA from the environment into the bacterial chromosome (Pratt and Taylor, 1990).

In the 1928 Griffith discovered the transformation of rough (R) non-capsulated strain of *Streptococcus pneumoniae* into a smooth (S) capsulated strain. He injected a mouse with a mixture containing a few living rough *pneumococci* derived from type II rough cells (Type II-R) and a large number of heat-killed type III smooth (Type III-S) *pneumococci* could be isolated from it. As later found, this type of transformation resulted from the uptake by the living type II rough (Type II-R) bacteria of DNA released from the heat-killed type III-S *pneumococci*. Drug resistance and other properties can also be transferred by means of free DNA (Salle, 1971).

2.3.3 Transduction

Transduction is the transfer of DNA from a donor cell to a recipient cell by bacteriophage (Salle, 1971, Lacey, 1972, lancini *et al*, 1995). Zinder and Lederberg (1952) stated that two kinds of transduction can be distinguished, a

non-specific transduction which can transfer any part of the host DNA, and specific transduction which is restricted to transfer a specific DNA segments.

In 1972, Lacey stated that transduction was of considerable clinical importance, particularly among the gram-positive bacteria. The great majority of the penicillin-resistant *Staphylococci*, for example, acquired the genes for β-lactamase via phage mediated transduction (Pratt and Taylor, 1990).

Transduction occur when a phage incorporate a part of the genetic material of a host bacterium and carries it to another bacterium (Thomas, 1993). Thus resistance to various antibiotics can be transduced in certain sensitive strains of *Staphylococcus aureus* also certain antigenic and bio chemical properties can be transduced between closely related strains of *Salmonella* (Thomas, 1993).

Transduction may be more important in spreading resistance among gram-positive bacteria than among gram-negative cells (Hugo and Russell 1989) .

2.3.4 Conjugation

Bacterial conjugation was discovered by J. Lederberg in the late 1940s (Hugo and Russell 1989).

Conjugation is transfer of genetic material from cell to cell by direct contact. Sexual conjugation in bacteria was first demonstrated by Lederberg and Tatum in 1946 using mutants of *Escherichia coli* K-12 especially derived for this purpose (Salle, 1971). In bacterial conjugation direct contact occurs between two sexually differentiated cells, and one of these, the donor (male *F1*), directly transfer chromosomal DNA to other, the recipient (female Ḟ) (Salle, 1971).

2.3.5 Plasmids

Plasmids are extra chromosomal DNA elements. The transmission of resistance to antibiotics, of several drugs simultaneously, from one bacterium to another was attributed to (R factors), or plasmids, which were small self-replicating double- stranded DNA circles, which were independent of the bacterial chromosome (Williams, 1979).

The ability to transfer genes that confer drug resistance by cellular conjugation is due to presence in the bacterial cell of DNA elements, known as plasmids that replicate separately from, but usually under the control of, the bacterial chromosome (Hugo and Russell, 1989).

Indeed it is generally accepted that plasmids plays a major role in the mediation and transfer of antimicrobial resistance among the *Staphylococcal* population (Lacey, 1972). Plasmids may be the vectors of the resistance genes, or the genes may be themselves located on discrete movable DNA elements called transposons (Masaudi *et al*, 1990, Neu 1992). A plasmid was recognized in *E.coli* confer resistance to ampicillin, chloramphenicol, kanamycin, streptomycin and sulphonamides (Vila, 1998).

2.3.5.1 Resistance plasmids

Resistance plasmids (R) were large may contain multiple genes and were self-transmissible through conjugation. The R- plasmids were smaller, do not contain the genes for conjugative transfer and usually encoded for resistance to a single antibiotic (Pratt and Taylor, 1990). Some R-plasmids confer resistance to up to eight different antibiotics. The R-plasmids were found in a wide variety of bacteria, but they were particularly important in *Staphylococci* and other gram-positive bacteria, in which they were responsible for most or all the plasmids-mediated drug resistance (Pratt and Taylor, 1990). From an

epidemiological point of view, plasmids resistance was most important since resistance in this form was transmissible and may be associated with other properties that enable microorganisms to colonize and invade therapy, susceptible host (Bordy *et al*, 1994). The wide spread use of antibiotic therapy, especially in hospitals has led to the spread of R-plasmids among pathogenic bacteria.

Not all antibacterial drug resistance of bacteria can be contributed to genes carried by plasmids. Drug resistance genes may also be chromosomally determined, in which case transfer, if it occurs at all, was mediated by a transuding phage or by F+ plasmids- chromosome transfer (Thomas, 1993).

2.3.5.1.1 Extended-Spectrum Beta - lactamase (ESBL) - producing Gram-negative bacteria

Extended-spectrum beta-lactamases (ESBLs) are plasmid-associated beta lactamases that have recently been found in the *Enterobacteriaceae*. ESBLs are capable of hydrolyzing penicillins, many narrow spectrum cephalosporins, many extended-spectrum cephalosporins, oxyimino-cephalosporins (cefotaxime, ceftazidime), and monobactams (aztreonam). Beta-lactamase inhibitors (e.g. clavulanic acid) generally inhibit ESBL producing strains. ESBL producing bacteria are most commonly *Klebsiella sp*, predominantly *Klebsiella pneumoniae, E. coli* and *Salmonella .sp*.

They have been found throughout the *Enterobacteriaeae.* Because ESBL enzymes are plasmid mediated, the genes encoding these enzymes are easily transferable among different bacteria. Most of these plasmids not only contain DNA encoding ESBL enzymes, but also carry genes conferring resistance to several non-ß-Lactam antibiotics. Consequently, most ESBL isolates are

resistant to many classes of antibiotics. The most frequent coresistances found in ESBL-producing organisms are aminoglycosides, fluoroquinolones, tetracyclines and chloramphenicol. Treatment of these multiple drug-resistant organisms is a therapeutic challenge. Now these predominant (ESBLs) include many types of genes: (Morita *et al*, 1998).

2.3.5.1.1.1 TEM

Found in gram negative bacteria, Up to 90% of ampicillin resistance in *E.coli* is due to the production of TEM-1. This enzyme is responsible for penicillin, ampicillin and cephalosporins resistance in increasing numbers. The first derivative of TEM-1 is TEM-2, had a single amino acid substitution from the original ß-Lactamase (Johan *et al*, 2008).

2.3.5.1.1.2 CTX-M

Found in strains of *Salmonella enterica* serovar *Typhimurium* , *E.coli* and other species of Enterobacteriaceae. Hydrolyze cefotaxime, they include CTX-M-1, CTX-M-2,and CTX-M-10 (Gniadkowski, 2001).

2.3.5.1.1.3 SHV

Include more than 100 SHV type, hydrolyze sulphdryl in cephalothin. SHV-1 is a narrow spectrum ß-Lactamase with activity against penicillins, SHV-2 reported in Germany was the first extended spectrum SHV enzyme, and the serine was replaced with glycine compared with SHV-1 at amino acid position 238, which caused resistance to extended spectrum Beta-lactamase (Johan *et al*, 2008).

2.3.5.1.1.4 OXA

Confers resistance against ampicillin, cephalothin, high activity against oxacillin and cloxacillin inhibited by clavulanic acid, this type is responsible for resistance of these antibiotics in Enterobacteriaceae species (Johan *et al*, 2008).

2.3.5.1.1.5 Specific genes associated with Salmonella

Although ESBL genes have been identified in *salmonella* serotypes all over the world yet there is paucity of reports on these genes in despite the phenotypic evidence of resistance to betalactam drugs, (Hohmann, 2001).

The presence of these genes underscores the potential health risk of antibiotics resistance. These genes hydrolyze Oxymino-Cephalosporines and mono bactams but not Cephamycins and sometimes they can be inhibited by Clavulanic acid and they include PER, DHMA, VEB, GES, OXA_2, ACCM, CITM, FOXM, ECBM, MOX and OXA_{10},(Armand, *et al*, 2003).

From these genes the SHV, TEM, CTX, DHMA, OXA_2, and ECBM genes were reported in *Salmonella pulllorum* and *Salmonella gallinarum* (Paraniak *et al,* 2002).

2.3.5.1.2 Risks of extended-spectrum beta-lactamases

Extended-spectrum β-lactamases (ESBLs) producing bacteria are an increasingly important cause of multi-drug resistant infections throughout the world. Bacteria carrying such enzymes genes have long been recognized as a cause of healthcare-associated infection. However, of concern, the incidence of such organisms also appears to be increasing in the community, typically as a cause of urinary tract infection. Infections due to ESBL-producing organisms

can pose a major threat to life; are often difficult and expensive to treat; and can delay discharge from hospital (Ebbing, 1998).

2.3.5.1.3 Methods of detection

Several tests have been developed to confirm the presences of ESBLs include:

2.3.5.1.3.1 Double disk synergy test

In this test disk of third generation Cephalisporines and augmentin are kept 30 mm apart from center to center on inoculated Muller Hinton Agar (MHA). A clear extension of the edge of the inhibition zone of Cephalosprines towards disc 8mm interpreted as positive for ESBL production.

2.3.5.1.3.2 Three dimension test

This test provides the advantage of simultaneous determination of antibiotic susceptibility and β-lactamase substrate profile. Inoculums produced in this method contains between 10^9 and 10^{10} CFU/ml of cells that actively produce β- lactamse. Two types of inocula are prepared one disk diffusion test inoculum (optical density equal to that of 0.5 McFarland standards) and a three dimensional inoculum (contain between 10^9 and10^{10} CFU of cells). Plate is inoculated by disk diffusion procedure. A circular slit is cut on the agar 4mm aside the position at which the antibiotic disks are placed. Conventional (two dimensional) disk diffusion susceptibility test results are measured according to the recommendations of the NCCLS. Distortion or discontinuity in the circular inhibition zone is interpreted as positive for ESBL production (Luzzaro, *et al*, 2006).

2.3.5.1.3.3 Inhibitor potentiated disk diffusion test

Cephalosporin disk is placed on calvulanate containing and without calvulanate containing MHA plates. More than 10mm increase in zone of

inhibition on the calvulanate containing MHA plate indicates ESBL production (luzzaro, *et, al*, 2006).

2.3.5.1.3.4 Disk approximation test

Cefoxitin (inducer) disc is placed at a distance of 2.5cm from cephalosporin disk. Production of inducible β- lactamase is indicated by flattening of the zone of inhibition of the cephalosporin disk towards inducer disk by >1mm (luzzaro, *et al*, 2006).

2.3.5.1.3.5 MIC reduction test

An 8 fold reduction in the MIC of cephalosporin in the presence of calvulanic acid indicates production of ESBL (luzzaro, *et al*, 2006).

2.3.5.1.3.6 Vitek ESBL test

Four wells containing cards are inoculated. A predetermined reduction in growth of Cephalosporin well containing calvulanic, where compared with the level of growth in well with Cephalosporin alone indicates presence of ESBL (luzzaro, *et al*, 2006).

2.3.5.1.3.7 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).

2.3.5.1.3.8 Molecular Detection of ESBL

The epidemiology of ESBL-producing *Salmonella* is complex and varies among institutions (Edelstein *et al*, 2003).

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).

2.4 Salmonella

Salmonella is a genus of rod-shaped, Gram-negative, non-spore-forming, predominantly motile enterobacteria with diameters around 0.7 to 1.5 µm, lengths from 2 to 5 µm. The flagella grade in all directions (i.e. peritrichous). They are chemoorganotrophs, obtaining their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes. Most species produce hydrogen sulfide, which can readily be detected by growing them on media containing ferrous sulfate, such as TSI (Clark and Barret, 1987).

2.4.1 Scientific classification

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Salmonella* (Clark and Barret, 1987).

2.4.2 History

The genus *Salmonella* was named after Daniel Elmer Salmon, an American veterinary pathologist. While Theobald Smith was the actual discoverer of the type bacterium (*Salmonella enterica* var. *choleraesuis*) in 1885, Dr. Salmon was the administrator of the USDA research program, and thus the organism was named after him. Smith and Salmon had been searching for the cause of common hog cholera and proposed this organism as the causal agent. Later research, however, showed that this organism (now known as *Salmonella enterica*) rarely causes enteric symptoms in pigs, and was thus not the agent they were seeking (which was eventually shown to be a virus).

However, related bacteria in the genus *Salmonella* were eventually shown to cause other important infectious diseases (Kauffmann, 1941).The bacteria were first isolated by Theo bald Smith in 1885 from pigs.

2.4.3 *Salmonella* nomenclature

Salmonella nomenclature is complicated. Initially, each *Salmonella* species was named according to clinical considerations, e.g., *Salmonella typhi-murium* (mouse typhoid fever), *S. cholerae-suis* (hog cholera), (Kauffmann, 1941).

Then after it was recognized that host specificity did not exist for many species, new strains (or serovar, short for serological variants) received species names according to the location at which the new strain was isolated (Minor, 1987).

Salmonella composed of bacteria related to each other both phenotypically and genotypically. *Salmonella* DNA base composition is 50-52 mol% G+C similar to that of *Escherichia, Shigella* and *Citrobacter*.

Names derived from the geographical origin of the first isolated strain of the newly discovered serovars were next chosen, e.g. *S. London, S. panama,* and *S.stanleyville* (Okamura *et al*, 2001).

2.4.4 Habitats

The principal habitat of *Salmonella* is the intestinal tract of humans and animals. *Salmonella* are disseminated in the natural environment (water, soil, Sometimes plants used as food) through human or animal excretion. Humans and animals (either wild or domesticated) can excrete *Salmonella* either when clinically diseased or after having had salmonellosis if they remain carriers. *Salmonella* organisms do not seem to multiply significantly in the natural environment (out of digestive tracts), but they can survive several weeks in

water and several years in soil if conditions of temperature, humidity, and pH are favorable (Mermin *et al*, 1997).

2.4.5 Sources of infection

Salmonella bacteria can survive several weeks in a dry environment and several months in water thus, they are frequently found in polluted water, contamination from the excrement of carrier animals being particularly important. Aquatic vertebrates, notably birds and reptiles, are important vectors of *Salmonella.* Poultry, cattle, and sheep frequently being agents of contamination. Salmonella can be found in food, especially in milk, meat and sometimes in eggs which have cracks. Sources of infection include:

- Infected food, often gaining an unusual look or smell, then is introduced into the stream of commerce.
- Poor kitchen hygiene, especially problematic in institutional kitchens and restaurants because this can lead to a significant outbreak.
- Excretions from either sick or infected but apparently clinically healthy people or animals (especially endangered are caregivers and animals).
- Polluted surface water and standing water (such as in shower hoses or unused water dispensers).
- Unhygienically thawed fowl (the melt water contains many bacteria).
- An association with reptiles is well described (Gantois *et al*, 2009).

2.4.6 *Salmonella* as disease-causing agents

Salmonella infections are zoonotic, they can be transmitted by humans to animals and vice versa. Infection via food is also possible.

2.4.6.1 Salmonellosis

Is a gastroenteritis characterized by nausea, vomiting, and diarrhea. It is the most common disease caused by the organisms e.g. *Salmonella typhimurium*. Abdominal cramping also may occur. Salmonellosis thus produces the symptoms that are commonly referred to as food poisoning. Although food poisoning is usually a mild disease, nausea, vomiting and diarrhea can lead to dehydration and even death (about 500 per year in the United States), (Wills and Simpson, 1994).

2.4.6.2 *Salmonella enteritidis*

Found in the intestines of cattle, rodents, ducks, poultry (and their eggs) and human, causes calf paratyphoid fever and acute gastroenteritis in humans (Stiles and McMullen, 1999).

2.4.6.3 *Salmonella paratyphi A*

Solely a human pathogen causes paratyphoid fever, transmission by contact and infected food or water (Joffe and Schlesinger, 2002).

2.4.6.4*Salmonella paratyphi B*

In central Europe usually a human pathogen, causes paratyphoid fever, transmission by contact and infected food, water or fly excrement (Herboid, 2000).

2.4.6.5 *Salmonella dublin*

Is the one of the pathogens causing cattle salmonellosis, transmission by contact and infected foods (Herboid, 2000).

2.4.6.6 *Salmonella brandenburg*

Is responsible for abortion and enteritis in sheep and cattle (Herboid, 2000).

2.4.6.7 *Salmonella arizonae*

Salmonella arizonae, the cause of Arizonosis, is biochemically different from other *Salmonella* serotypes. *S.* Arizonae causes disease in poultry and humans. *Salmonella arizonae* infections are of particular economic significance in turkeys in North America. The disease causes Chicks and poults to show weakness, anorexia and shivering, reduced egg production and hatchability, Outbreaks in turkeys and chickens can have up to 60% mortality. Transmission is by Fecal-oral route, some transmission through eggs, infected birds can become long-term intestinal carriers. *S.* arizonae is less hardy than most *Salmonella*. The bacteria can still survive for months in soil, feed and water (Hand *et al*, 2000).

2.4.6.8 *Salmonella choleraesuis*

It mainly causes gastroenteritis. Infection is caused by consuming contaminated food or drinks (Baron *et al*, 1994).

2.4.6.9 *Salmonella typhi*

Infection of *S. typhi* leads to the development of typhoid, or enteric fever. This disease is characterized by the sudden onset of a sustained and systemic fever, severe headache, nausea, and loss of appetite. Other symptoms include constipation or diarrhoea, enlargement of the spleen, possible development of meningitis, and/or general malaise. Untreated typhoid fever

cases result in mortality rates ranging from 12-30% while treated cases allow for 99% survival (Nabbut, 1993).

2.4.6.10 *Salmonella gallinarum*

Salmonella gallinarum infection primarily causes fowl typhoid disease in chickens and turkeys, but ducks, pheasants, guinea-fowl, peafowl, grouse, and quail can also be affected. It is one of a group of diseases in poultry caused by Salmonella species which include Pullorum Disease, Arizona infection and Paratyphoid infection. Due to extensive testing and control by the poultry producers, Fowl Typhoid is rare in countries with a modern poultry industry. The disease has gained incidence in South America and other countries throughout Africa and Asia in recent year. Canada and the United States are presently free of the disease. This bacterium limits itself to avian species and is not known to cause disease in humans (Nabbut, 1993).

Outbreaks are characterized by increased mortality, anorexia, and a drop in egg production. A watery to mucoid yellow diarrhoea is characteristic, and the birds are depressed with rapid breathing. Sub acute outbreaks can occur, and egg transmission may lead to increased dead or weak chicks. Recovered birds may be carriers (Erbeck *et al*, 1993).

Control is related to blood testing for *S. Pullorum,* as the common antigenic structure of the bacteria leads to cross reaction. Elimination of pullorum positive reactors will effectively decrease *S. gallinarum* infection. National programs in many countries have virtually eliminated this and Pullorum disease (Majid *et al*, 1991).

2.4.6.11 *Salmonella pullorum*

This serotype causes pullorum disease, also called bacillary white diarrhea. This serotype is a poultry-adapted serotype that does not cause disease in humans. Infections still occur worldwide in non-commercial poultry but are rare in most commercial systems now. It affects mostly chickens, but other production birds such as turkeys, game birds, guinea fowls and ostriches. Between 10-80% of the flocks may be sick, and deaths may be up to 100% in sick stressed and immune depressed flocks (Salem *et al*, 1992). Transmission of bacteria may be associated with cannibalism. Signs of disease are white diarrhea, lack of appetite, depression, ruffled feathers, closed eyes, loud chirping, vent pasting, gasping and lameness. Flocks infected with *Salmonella pullorum* are usually stamped out. Other *Salmonella* serotypes are capable of causing disease in young chickens, turkeys and ducks, such as Derby, Newport, Montevideo, Anatum and Bredeney. Many serotypes may be present in poultry without causing any signs of disease. Even if they do not cause clinical disease, their presence may be undesirable for public health reasons. Between 0-90% of the flock may be infected. The route of infection is fecal-oral, and may be transmitted through shell egg contamination. Certain serotypes may become resident in hatcheries or flocks. The bacteria are often persistent in the environment (Cummings *et al*, 2010).

2.5 Importance of Poultry in human Lives

Poultry are domesticated birds kept by humans for the purpose of producing eggs, meat, and/or feathers. These most typically are members of the super order Galloanserae (fowl), especially the order Galliformes (which includes chickens, quails and turkeys) and the family Anatidae (in order Anseriformes), commonly known as "waterfowl" (e.g. domestic ducks and domestic geese). Poultry also includes other birds which are killed for their meat, such as pigeons or doves or birds considered to be game, like pheasants. Poultry comes from the French/Norman word, poule, itself derived from the Latin word Pullus, which means small animal. Poultry is the second most widely eaten meat in the world, accounting for about 30% of meat production worldwide, after pork at 38% (Raloff, 2003).

Poultry have been on the earth for over 150 million years, dating back to the original wild jungle fowl Now we include ducks, geese, turkeys, pheasants, pigeons, peafowl, guinea fowl and chickens in the list of species under the general term poultry. Poultry provide humans with companionship, food and fiber in the form of eggs, meat and feathers. The poultry industry is a large business. It generated in excess of 20 billion dollars in 1991, in the United States. It is worth an estimated 150 million dollars to Connecticut (Scanes, 2007).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection of samples

A total of 200 droppings samples were collected in sterile clean containers from poultry farms located in Khartoum North. The samples were immediately submitted to the laboratory under ice for bacterial diagnosis.

3.2 Media for isolation and identification

3.2.1 Nutrient Broth (Oxoid, CM1)

The medium contains (per liter) meat 5.0 g of peptone, 3.0 g of meat extract and final pH adjusted to 7.0± 0.2. It was prepared by dissolving 28.0 grams of powder (Oxoid) in 1 liter of distilled water, heated to boiling to dissolve completely. Sterilized by autoclaving at 121°C, for 15 minutes. Mixed well and poured into sterile containers under aseptic conditions.

3.2.2 Selenite Cystine Broth (Oxoid, CM395)

The medium contains (per liter) 5.0 g of meat peptone, 4.0 g of lactose, 10.0 g of Disodium phosphate, 0.01 g of L- Cystine and final pH adjusted to 7.0± 0.2.

Four grams of sodium biselenite were dissolved in 1 liter distilled water and then 19 g of Selenite Cystine Broth base were added. The ingredients were warmed to dissolve and they were dispensed into containers. Sterilization was carried by placing in free flowing steam for 15 minutes.

3.2.3 Salmonella Shigella Agar S.S Agar (Oxoid, CM533)

The medium composed (per liter) 5.0 g of Peptone, 10.0 g of Lactose, 8.5 g of Bile salts, 10.0 g of Sodium citrate, 8.5 g of Sodium thiosulphate, 1.0 g of Ferric citrate, 0.00033 g of Brilliant green, 0.025 g of Neutral red, 15.0 g of Agar and pH adjusted to 7.0 ± 0.2.

The medium was prepared by suspending of 63.1 g from dehydrated medium in 1 liter distilled water, then heated to boiling to dissolve the medium completely for 2minutes. Cooled to 50°C and pour into sterile Petri dishes.

3.2.4 Brilliant Green Agar (Oxoid, CM329)

The medium contains (per liter) 8.259g of Peptic digest of animal tissue, 1.900 g of Lactose sugar, 0.205 g of Sodium sulphite, 0.0295 g of Ferric chloride, 0.0153 g of Mono potassium phosphate, 0.0649 g of Evioglarcing, 0.0776 g of Basic fuchsine, 0.00295 g of Oxgall, 0.0000295 g of Brilliant green, 10.15 g of Agar and final pH at 25°C adjusted to 6.9±0.2.

The medium was prepared by suspending 20.7 grams in 1 liter water, heated to boiling to dissolve completely, sterilized by autoclaving at 15 Ibs pressure at 121°C for 15 minutes.

3.2.5 Bismuth Sulphite Agar (Oxoid, CM201)

Composed (per liter) 1.6 g of Bismuth sulphite $Bi_2(SO_3)_3$,1.0g of Pancreatic digest of casein, 1.0g of Pancreatic digest of animal tissue, 1.0g of Beef extract, 1.0 g of Glucose, 0.8g of Dibasic sodium phosphate, 0.06g of Ferrous sulphate.$7H_2O$ and final pH adjusted to 7.7 at 25°C.

The medium was prepared by dissolving 47.5 grams of media in 1liter of distilled water, heated to boiling to dissolve completely. This medium is filter-sterilized, not autoclaved, mixed well and then poured into sterile containers using aseptic conditions.

3.2.6 Xylose Lysine Deoxycholate Agar (X.L.D) (Oxoid, CM469)

The medium contains (per liter) 3.0g of Yeast extract, 5.0g of L-Lysine HCl, 3.75g of Xylose, 7.5g of Lactose, 7.5g of Sucrose, 1.0g of Sodium desoxycholate, 5.0g of Sodium chloride, 6.8g of Sodium thiosulphate, 0.8g of Ferric ammonium citrate, 0.08g of Phenol red, 12.5g of Agar and final pH adjusted to7.4 ± 0.2 at 25°C.

The medium was prepared by dissolving 53 grams in 1 liter distilled water, heated to boiling to dissolve the medium completely for 2minutes, sterilized by autoclaving. Then the medium was cooled to 50°C and poured into sterile Petri dishes.

3.2.7 MacConkey's Agar (Oxoid, CM7b)

The medium contains (per liter) 20.0g of Peptic digest of animal tissue, 10.00g of Lactose, 5.00g of Bile salts , 0.075g of Neutral red, 12.00g of Agar and Final pH adjusted to 7.4±0.2 at 25°C.

The medium was prepared by suspending 47.0 grams in 1 liter distilled water and the desired amount of carbohydrate either individually or in combinations was added, heated to boiling with gentle stirring to dissolve the agar completely. Sterilized by autoclaving at 121°C for 15 minutes.

3.2.8 Mueller Hinton Agar (Oxoid, CM337)

The medium contains (per liter) 300.00g of Beef- infusion, 17.50g of Cosein acid hydrolysate, 1.50g of Starch, 17.00g of Agar and pH adjusted to 7.4±0.2.

The medium was prepared by suspending 38.0 grams in 100 ml distilled water, then heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 121°C for 15 minutes the medium was mixed well before pouring.

3.2.9 Kligler Iron Agar (KIA) (Oxoid, CM33)

The medium contains (per liter) 20.0g of Polypeptide, 10.0g of Lactose, 1.0 g of Dextrose, 5.0 g of Sodium Chloride, 0.5 g of Ferric Ammonium Citrate, 0.3 g of Sodium Thiosulfate, 15.0 g of Agar, 0.025 g of Phenol Red, adjusted Final pH 7.4 ± 0.2.

Prepared by dissolving 36.15 grams of media in 1 liter distilled water, heated to boiling to dissolve completely, sterilized by autoclaving at 121°C for 15 minutes. The medium was set as slope in test tubes to ensure that the slant is over a butt about 3cm deep. The **Trible Sugar Iron agar (TSI)** (Oxoid, CM277) medium, containing Sucrose 1% in addition to lactose and glucose.

3.2.10 Hugh and Leifson's medium

This medium contains (per liter) 2g of Peptone, 5g of NaCl, 3g of agar, 0.3 g of KH_2PO_4 , 3ml of bromothymol blue (1% aqueous) and Final pH was7.1 ± 0.2 at 25°C.

Prepared by dissolving 11 grams of ingredients in 1liter, autoclaved 15 min at 121°C. The medium was then cooled to 50°C, added 100 ml/liter of a 10 % filter-sterilized solution of D (+) glucose, lactose, sucrose or other carbohydrates were added, mixed well. The medium then dispensed into tubes to give a depth of approx. 5 cm, immediately after cooling overlay half of the tubes with a 1 cm layer of sterile paraffin oil (paraffin viscous). The prepared culture medium is dark-green to blue-green in color.

3.2.11 Christensen's Urea medium (Oxoid, CM0053)

The medium contains (per liter) 1.0g of Peptone, 1.0 g of Glucose, 5.0 g of Sodium chloride, 1.2 g of Disodium phosphate, 0.8g of Potassium dihydrogen phosphate, 0.012 g of Phenol red, 15.0 g of Agar, pH 6.8 ± 0.2 at 25°C.

Prepared by dissolving 2.4 grams of media in 1 liter distilled water, heated to boiling to dissolve completely, sterilized by autoclaving at 121°C for 15 minutes. The medium cooled to 50°C and aseptically 5ml of sterile 40% urea solution was added. Mixed well, distribute 10ml amounts into sterile containers and allowed to set in slope position.

3.2.12 Simmons Citrate Agar (Oxoid, CM155)

The medium contains (per liter) 0.2g of Magnesium Sulfate, 1.0 g of Ammonium Phosphate dihydrogen, 1.0 g of Potassium Phosphate, 2.0g of dibasic Sodium Citrate, 5.0 g of Sodium Chloride, 0.08 g of Bromothymol Blue, 1.5 g of Agar, pH adjusted to 6.9 ± 0.2 at 25°C.

Prepared by dissolving 24.2 grams of ingredients in 1liter of purified water, heated to boiling with frequent agitation for1 minute. Sterilized by autoclaving at 121°C for 15 minutes. The medium then cooled to 45-50°C, mixed well thou roughly and then dispensed into sterile Petri dishes or into sterile culture tubes.

3.2.13 Tryptone Bile Agar (Oxoid, CM595)

The medium contains (per liter) 10.0g of Tryptone, 5.0g of Dextrose, 10.04g of Bromocresol purple, 12.0g of Agar, final pH adjusted to 6.9 ± 0.2 at 25°C.

Prepared by dissolving 27 grams of media in 1 liter of distilled water, heated to boiling to dissolve completely, sterilization was carried by autoclaving at 121°C for15 minutes. The medium was mixed well and pour into sterile containers under aseptic conditions.

3.2.14 Peptone water (Oxoid, CM9)

The medium contains (per liter) 10g of Peptone, 5g of Sodium chloride and final pH adjusted to 7.2±0.2 at 25°C.

Prepared by dissolving 10 grams of media in 1 liter of distilled water, heated to boiling to dissolve completely. Sterilization was done by autoclaving at 121°C, 15 Ibs for15 minutes. The medium was mixed well and poured into sterile containers under aseptic conditions.

3.2.15 Arginine dihydrolase broth (Oxoid, CM1)

The medium contains (per liter) 1.0g of peptone, 5.0 g of NaCl , 0.30g of K_2HPO_4, 3.0 g of Agar, 0.01 g of phenol red, 10.0 g of DL-Arginine HCl, and final pH adjusted to 7.2 at 25°C.

Prepared by dissolving 10 grams of media in 1 liter of distilled water, heated to boiling to dissolve completely. Sterilization was done by autoclaving at 121°C for 15 minutes. The medium was then poured into sterile containers under aseptic conditions.

Certain bacteria contain the enzymes to hydrolyze arginine. This hydrolysis results in an alkaline change in the media results in a color change in the media. This test can be used for differentiated different bacteria.

3.2.16 Bile Esculin agar (Oxoid, CM0888)

The medium contains (per liter) 5.00g of peptone Pancreatic digest of gelatin, 3.00 g of Beef extract, 20.0 g of Ox gall, 0.50 g of Ferric citrate, 1.00 g of Esculin, 14.0g of Agar and final pH adjusted to 7.0.

Prepared by dissolving 5 grams of the dehydrated media in 1 liter of distilled water, heated to boiling to dissolve completely. Sterilization by autoclaving at 121°C for15 minutes. The medium was mixed well and poured into sterile containers under aseptic conditions. Final pH was adjusted to 6.8 at 25°C.

3.2.17 Glucose phosphate medium MR -VP medium (Oxoid, CM43)

The medium contains (per liter) 7g of peptone, 5g of Glucose, 5g of K_2HPO_4 and final pH adjusted to 6.9 at 25°C.

Prepared by dissolving 5 grams of media in 1 liter of distilled water, heated to boiling to dissolve completely. Sterilization by autoclaving at 121°C for 15 minutes. Mixed well and poured into sterile containers under aseptic conditions.

3.3 Methods of isolation

An enrichment technique was used for the isolation. About 1 gram of each drooping sample was put into test tube containing **Selenite cystine broth** and incubated at 37°C for 24 hours. Then a loopfull was streaked onto each four selective culture media, namely; **S.S agar, Bismuth sulfite agar, Brilliant green agar and XLD agar**. Incubated at 37°C for 24 hours. Presence of salmonella was indicated by the appearance of circular smooth transparent, may be black centered colonies on S.S agar. On brilliant green agar Salmonella colonies were circular pink colonies with changing the color of the surrounding medium into pink. Black metallic sheen discrete colonies were seen on bismuth sulfite agar and black colonies appeared on XLD agar.

3.3.1 Primary tests

-All tests were performed according to Barrow and Fellthan (1993).

3.3.1.1 Preparation of smears

A small portion of bacterial colony was emulsified in a drop of sterile normal saline and spread on clean slide. Smears were allowed to dry in air and then fixed by gentle heating

3.3.1.2 Gram stain

This was done as described by Barrow and Fellthan (1993). After gram staining of smears, stained smears were examined microscopically to see the shape, arrangement, and the color of the bacteria**.**

3.3.1.3 Catalase test

Using a sterile wooden stick a colony of test organism was taken and immersed in 3% H_2O_2. Active bubbling indicated positive test due to formation of H_2O and O_2 from H_2O_2 breakdown.

3.3.1.4 Oxidase test

A portion of the tested organism was smeared on the oxidase paper disk that impregnated with tetra methyl para-phenylene diamine dihydrochloride. Production of a purple color immediately indicated positive test.

3.3.1.5 Motility test

Using a straight wire the tested organism was stabbed in a semisolid media. Motile bacteria were spread away from the inoculation line.

3.3.1.6 O/F test

Using a straight wire the tested organism was dipped in a Hugh and Leifson's medium in two test tubes, paraffin oil was added to one of the tubes to provide anaerobic conditions. The degradation was allowed to take place while the medium was exposed to air or under exclusion of air when paraffin added.

3.3.1.7 Glucose fermentation test

Bijou bottle containing glucose medium and inverted Durham's tube was inoculated with the tested organism and incubated aerobically at 37°C for up to 72 hours.

Positive reaction was indicated by changes of color to yellow due to acid production. Durham's tube was also examined for the presence of gas production.

3.3.2 Secondary test

-All tests were performed according to Barrow and Fellthan (1993).

3.3.2.1 Citrate utilization test

Using a bacteriological loop the tested organism was streaked onto a Simmon's Citrate agar. Change of color to blue within seven days indicated positive result.

3.3.2.2 Indole test

Tryptone medium was inoculated with the tested organism, then incubated at 37°C for 48 hours.0.5 ml Kovac's reagent was added, and the mixture was shaken thoroughly. Development of a red ring between indicates a positive reaction.

3.3.2.3 Lactose fermentation test

This test was performed by culturing of Salmonella organism onto MacConkey's agar and incubated aerobically for 36 hours at 37 °. Obtaining of pink colonies means the organism is lactose fermenter.

3.3.2.4 Kligler Iron Agar "KIA" test

This test is based on fermentation of lactose, glucose, production of H_2S. Using sterile wire pick a small part of Salmonella isolated colony and inoculate into the butt and slope of KIA media. The results were observed by looking for the color of butt and slope after incubation period of 24 hours at 37ċ as below.

Butt	**Slope**	**H2S**	**Gas**	**Sugar utilized**
Yellow	Red	±	±	Fermentation of glucose only
Yellow	Yellow	±	±	Lactose and glucose
Red	Red	±	±	No glucose and no lactose utilized
H_2S production results are noticed by the blackening of the medium.				
Gas production results are noticed by the cracking of the medium.				

3.3.2.5 Lactose/Glucose/Sucrose fermentation test on TSI

This test is based on fermentation of lactose, glucose, sucrose production of H_2S. Using sterile wire a small part of Salmonella colony was piked and inoculated into the butt and slope of TSI media. The results observed by looking for the color of butt and slope after incubation period of 24 hours at 37°C as below.

Butt	Slope	H2S	Gas	Sugar utilized
Yellow	Pink	±	±	Fermentation of glucose only
Pink	Yellow	±	±	Sucrose
Yellow	Yellow	±	±	Lactose only
Pink	Pink	±	±	No sugar utilized
H_2S production results are noticed by the blackening of the medium.				
Gas production results are noticed by the cracking of the medium.				

3.3.2.6 Urease test

This test is based on production of urease enzyme and break down of urea into CO_2 and NH_3. This test was performed by streaking of the examined organisms onto slant surface of Christensen's medium and incubated at 37°C for 24 hours. The test is positive when the color changed to pink.

3.3.2.7 Methyl Red (MR) and Voges Proskauer (VP) tests

These tests are based on sugars degradation and production of acids. To perform MR. test, the glucose phosphate broth medium was inoculated with the tested organism and after incubation two to three drops of 0.02% methyl red

indicator was added. Positive reaction was indicated by the development of red color (pH 4.5 or less).

After completion of M.R. test, to 5 ml of glucose phosphate broth culture 0.6 ml 5% α-naphthol was added, followed by 0.2ml of 40% KOH. The tube was thoroughly mixed and sloped, Development of a deep rose red color in the culture 15 minutes following the addition of reagents is indicative of the positive VP test result.

3.3.2.8 Esculin hydrolysis

This test is based on esculin hydrolysis and production of esculetin and dextrose. Using a straight needle the test organism was dipped in a bile esculin agar, incubated at 37°C for 7days. Blackening of the medium indicates positive result.

3.3.2.9 Arginine hydrolysis

Arginine broth medium was inoculated with the tested organism, incubated aerobically at 37°C up to 48 hours. 0.5ml of misselov solution was added and the mixture was shaken. Positive reaction was indicated by the development of deep brown color immediately.

3.3.2.10 Sugars fermentation

The tested organism was inoculated into peptone water containing the BTB indicator and the following sugars: Glucose, Arabinose, Lactose, Sucrose, Mannitol, Xylose, Mannose, Trehalose, Salicin, Maltose, Rhaminose, Raffinose, Melibiose and Cellobiose and incubated at 37°C for up to 30 days. Production of yellow color indicates positive result.

3.3.2.11 Determination of antibiotic susceptibility

Sensitivity of **64** Salmonella isolates to **8** antibiotics discs was determined using Kirby-Bauer disc diffusion assay, the following antibiotics were used: **Amikacin, Ceftazidine, Chloramphenicol, Aztreonam, Tetracycline, Piperacillin, Imipenem** and **Ciprofloxacin.**

Pure culture colonies (3-5) of isolated bacteria were suspended in a test tube containing **Nutrient broth** and incubated at 37°C for 24 hours. Two ml of each culture were spreaded over Muller-Hinton agar, the plate was left to dry for 15 minutes and the excessive fluid was aspirated. The commercially prepared antibiotic disks were placed on the agar surface using sterile forceps and pressed gently to ensure full contact with the surface of the culture medium. The plates were then incubated at 37°C for 24 hours. The area showing no visible growth was taken as the zone of growth inhibition and as measured in millimeters from the underside of the plate.

3.4 Molecular methods

3.4.1 Preparation of reagents

3.4.1.1 Primers

Primers for PCR were used to amplify *bla* TEM, *bla* SHV and *bla* CTX-M specific product as in table (1).

Table.1. Primer sets used in characterizing β-lactamases.

Name	Sequence (5' -------- 3')	Product size	Annealing	References
CTX-R	5- ACC GCG ATA TCG TTG GT - 3¯	550 bp	50 °C	(Naas, *et al*, 2005)
CTX- F	5- CGC TTT GCG ATG TGC AG - 3¯			
SHV-R	5- TGC TTT GTT ATT CGG GCC -3¯	753 bp	60 °C	(Bradford, 2001)
SHV- F	5- ATG CGT TAT ATT CTG TG - 3¯			
TEM-R	5- AGC GAT CTG TCT AT - 3¯	752 bp	45 °C	(Pitout *et al*, 2003).
TEM-F	5- AAA CGC TGG TGA AAG TA - 3¯			

The working solutions of primers were prepared according to the following formula:

TEM-F: for 100µl 268µl of DW were added.

TEM-R: for 100µl 377µl of DW were added.

CTX-M (5`- 3`) (CGC): for 100µl 319µl of DW were added.

CTX-M (5`- 3`) (ACC): for 100µl 312µl of DW were added.

SHV- F: for 100µl 272µl of DW were added.

SHV-R: for 100µl 272µl of DW were added.

DW ≡ distilled water.

3.4.1.2 Preparation of 10x TBE buffer

Amount of 108g Tris base were weighted and added to 55g of boric acid and 9.3g EDTA then dissolved into one liter of distilled water.

3.4.1.3 Preparation of 1x TBE buffer

10ml of 10x TBE buffer was added to 90ml of distilled water and heated until completely dissolved.

3.4.1.4 Preparation of Agarose gel

One gram of agarose was dissolved into 100 ml 1x TBE then 5µl of Ethidium Bromide was added before pouring the liquid agarose gel into the gel casting tray which was equipped with suitable com and its open sides were closed and left to solidify, the comb was gently removed and the closure from the opened sides were removed.

3.4.1.5 Preparation of Ethidium Bromide

Five milligrams of Ethidium Bromide powder dissolved into 500µl distilled water and kept into a brown bottle.

3.4.1.6 Preparation of loading dye

Three ml of glycerol were added to seven ml of distilled water and 25g of bromophenol blue was dissolved into 100ml distilled water, the mixture was used as loading dye.

3.4.2 DNA extraction

Boiling method was used for bacterial DNA extraction in this study and the method was as follows:

3.4.2.1 Preparation of bacterial strains

Five loop fulls were transferred to 1ml of sterile physiological normal saline in eppendorf tube (1.5ml capacity) and then centrifuged at 2000 rpm for 5minutes. The later step was repeated three times (washing step). The

supernatant was discarded and the pellets were emulsified into 50µl of physiological normal saline.

3.4.2.2 Extraction procedure

Amount of 100µl of ice cold 5mM sodium phosphate pH 8 was added to 50µl of emulsified *Salmonella sp* in eppendorf tube. And spin at 2000rpm for 5minutes. This step was repeated two times for washing. The supernatant was discarded and then 100µl of di-ionized water added to the pellet then transferred to water path (Scott-Science-UK) at 100°C for boiling for 10 minutes then centrifuged at 12000rpm for 10 minutes. The supernatant was transferred into a new eppendorf tube (1.5) ml and then kept into refrigerator at (-20°C).

3.4.2.3 Detection of DNA

Five micro liters of DNA extract were mixed with 0.5µl of loading dye by using automatic pipette (0.5-10µl) then transferred to the wells of the gel. This conducted into electrophoresis tank after1% of agarose gel was prepared. Then the gel was poured after fixed the spacer and comb, let it to solidify, few buffers were added to facilitate the removal of the comb. Gel electrophoresis system and tank was filled with electrophoresis buffer, separation of DNA was carried out at a constant voltages of 75 volts for 30 minutes. After running, the gel was photographed by using UV light.

3.4.2.4 Measurement of DNA Concentration

The concentration of extracted DNA was read using the spectrophotometer, (Bioeppendorf), Spectrophotometric analysis is based on the principles that nucleic acids absorb ultraviolet light in a specific pattern. In the case of DNA and RNA, a sample that is exposed to ultraviolet light at a wavelength of 260 nanometres (nm) will absorb that ultraviolet light.

3.5 Polymerase Chain Reaction “PCR” techniques

3.5.1 Preparation of master mix

The primers were prepared. The desired master mix consists of buffer, MgCL2, dNTBs, and Tag polymerase was prepared for 25 reactions, and this minimizes reagent loss and enables accurate pipetting. As 10µl of dNTBs(0.4µl), 6.25µl of Tag polymerase(0.25µl), 37.5µl of MgCL2 (1.5µl),62.5µl buffer (2.5µl), 333,75µl of distilled water (13.35µl), 12.5µl of primers forward(0.5µl), 12.5µl of primers reverse(0.5µl). The total volume was 21µl, then completed up to 25µl by 4µl of sample (template). Master Mix without template DNA was used as negative control. All these reactions performed into PCR tubes (0.2ml capacity).

3.5.2 PCR amplification

A-protocol used for amplification of CTX-M gene:

The amplification was done using Convergys® td peltier thermo cycle (Germany).Initial denaturation at 94 °C for 5 minutes, followed by 32 cycles of denaturation at 94 °C for 40 seconds, primer annealing at 50 °C for 35 seconds and elongation at 72 °C for 50 seconds. Final extension at 72 °C for 7 minutes, (Naas, *et al*, 2005).

B-protocol used for amplification of SHV gene:

The PCR program consisted of template denaturation for 5minutes at 95 °C followed by 35 cycles of an initial denaturation step at 94 °C for 30 seconds, primer annealing 60 °C for 60 seconds, elongation at 72 °C for 1 minutes, final extension at 72 °C for 7minutes, (Bradford, 2001).

C-protocol used for amplification of TEM gene:

The PCR program consisted of an initial denaturation step at 96 °C for 15 seconds followed by 24 cycles of DNA denaturation at 96 °C for 15 seconds, primer annealing at 50 °C for 15 seconds and primer extension at 72

°C for 2 minutes. After the last cycle the products were stored at 4 °C, (Pitout *et al*, 2003).

3.5.3 Visualization of PCR products

The gel casting tray was put into the electrophoresis tank flooded with 1xTBE buffer just to cover the gel surface, 5µl of PCR products from each samples was mixed with 0.5µl of loading dye and then a gel of electrophoresis was runed. 5µl of DNA ladder (Marker) was mixed with 0.5µl loading dye and were added to the well in each run. The gel electrophoresis apparatus was connected to power supply (Prime, 125v, 500µA, UK). The electrophoresis was carried at 75 V for 30 minutes, after electrophoresis period, the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized by UV Trans illuminator (Uvitee- UK).

CHAPTER FOUR
RESULTS

4.1 Isolation of Salmonella:

Out of 200 dropping samples from poultry farms in Khartoum North (Khartoum State) , 64 (32%) samples were found positive to *Salmonella sp* , growth on special media like MacConkey's, S.S agar, Bismuth sulfite agar, Brilliant green agar and XLD agar was observed.

4.1.1 Identification of Salmonella:

Conventional methods for identification of *Salmonella sp* isolated using gram's stain, morphology, motility test and other biochemical and sugar fermentation tests revealed five Salmonella species namely *Salmonella arizonae* 7(10.9%), *Salmonella choleraesuis* 10 (15.6%), *Salmonella gallinarum* 18(28.1%), *Salmonella pullorum* 23(35.9%) *and Salmonella typhi* 6(9.3%). Percentages of different Salmonella species isolated were shown in (fig.1).

4.2 Drug sensitivity of the Salmonella:

Overall resistance of Salmonella isolates to different antibiotics is shown in (tab.3) and (fig.2).

Individual resistance of each Salmonella species to eight antibiotics is shown in (tab.4) and (fig.3).

4.3 PCR:

Detection of DNA bands from antimicrobial resistant Salmonellae was done using gel electrophoresis (fig.4)

4.3.1 Determination of CTX-M genes

Amplification of thirty DNA extracts from *Salmonella gallinarum* and *Salmonella pullorum* isolates with CTX-M specific primers and Marker (M) are shown in figures (5 and 6).

4.3.2 Determination of SHV genes

Amplification of thirty DNA extracts from *Salmonella gallinarum* and *Salmonella pullorum* isolates with SHV specific primers and Marker (M) are shown in figures (7-11).

4.3.3 Determination of TEM genes

Amplification of thirty DNA extracts from *Salmonella gallinarum* and *Salmonella pullorum* isolates with TEM specific primers and Marker (M) are shown in figures (12 and 13).

Polymerase Chain Reaction (PCR) showed the presence of CTX-M, SHV and TEM genes in different rates. In *Salmonella gallinarum* the predominant gene was SHV genes (100%), followed by CTX-M and TEM genes (58%) for each. For *Salmonella pullorum* the presence of CTX-M, SHV and TEM genes was (44%), (11%) and (33%) respectively (tab .5) and (fig. 14).

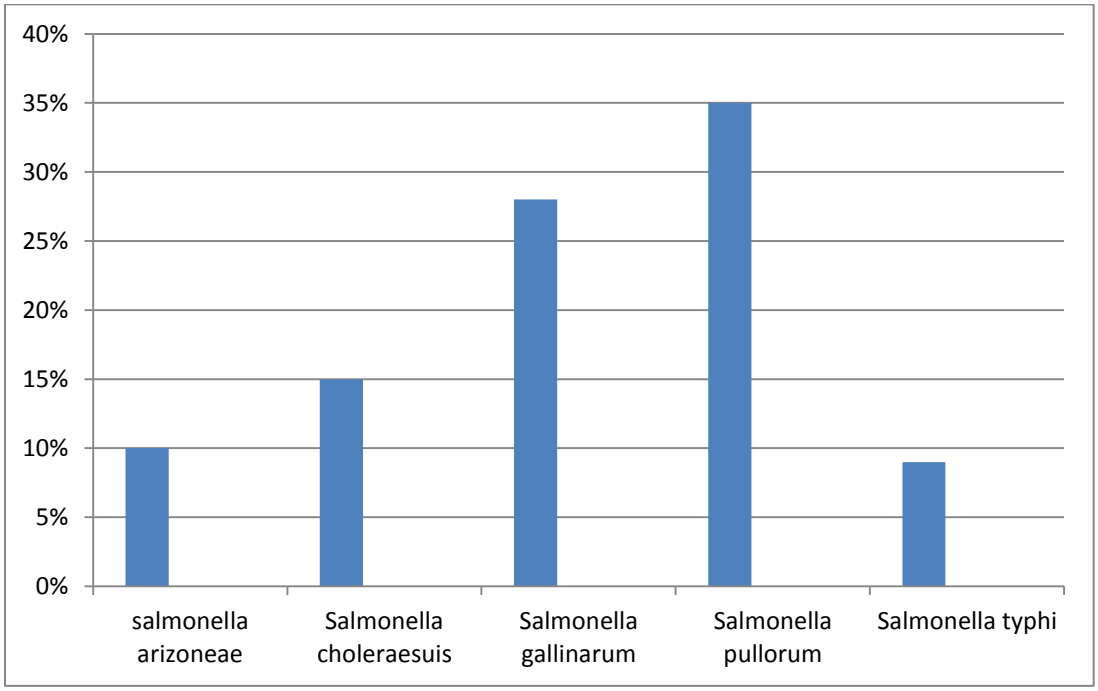

Figure.1. Percentage of isolated Salmonella species.

Table .2. Characteristics of Salmonella spp. to biochemical tests.

Salmonella sp	***S. arizonae*** (7 isolates)	***S. choleraesuis*** (10 isolates)	***S.gallinarum*** (18 isolates)	***S. pullorum*** (23 isolates)	***S.typhi*** (6 isolates)
Tests					
Gram stain	-	-	-	-	-
Shape	Rod	Rod	Rod	Rod	Rod
Motility test	-	+	-	-	+
Growth in air	+	+	+	+	+
Catalase test	+	+	+	+	+
Oxidase test	-	-	-	-	-
Acid from glucose	-	-	+	+	+
O/F test	Fermentative	Fermentative	Fermentative	Fermentative	Fermentative
Citrate utilization	+	-	-	-	-
Gas from glucose	+	+	-	+	-
Indole test	-	-	-	-	-
H2S in TSI	+	-	+	+	+
Arginine hydrolysis	+	+	-	-	+
Asculine hydrolysis	-	-	-	-	-
Methyl red	+	+	+	+	+

Sugars fermentation					
Glucose	+	+	+	+	+
Arabinose	+	+	+	+	-
Lactose	+	-	-	-	-
Sucrose	-	-	-	-	-
Mannitol	+	+	+	+	+
Xylose	+	-	+	+	+
Mannose	+	+	+	+	+
Trehalose	+	+	+	+	+
Salicin	+	+	-	-	-
Maltose	+	-	-	-	+
Rhaminose	-	-	+	+	-
Raffinose	-	-	+	+	-
Mellibiose	+	-	-	-	+
Cellobiose	-	-	-	-	-

Table.3. Resistance of Salmonella isolates to eight antibiotics in terms of percentage:

Antibiotics	No .of Resistant Isolates	% of Resistant isolates
Amikacin	34/64	53%
Ceftazidine	23/64	35%
Chloramphenicol	14/64	21%
Aztreonam	34/64	53%
Tetracycline	37/64	57%
Piperacillin	37/64	57%
Imipenem	27/64	42%
Ciprofloxacin	32/64	50%

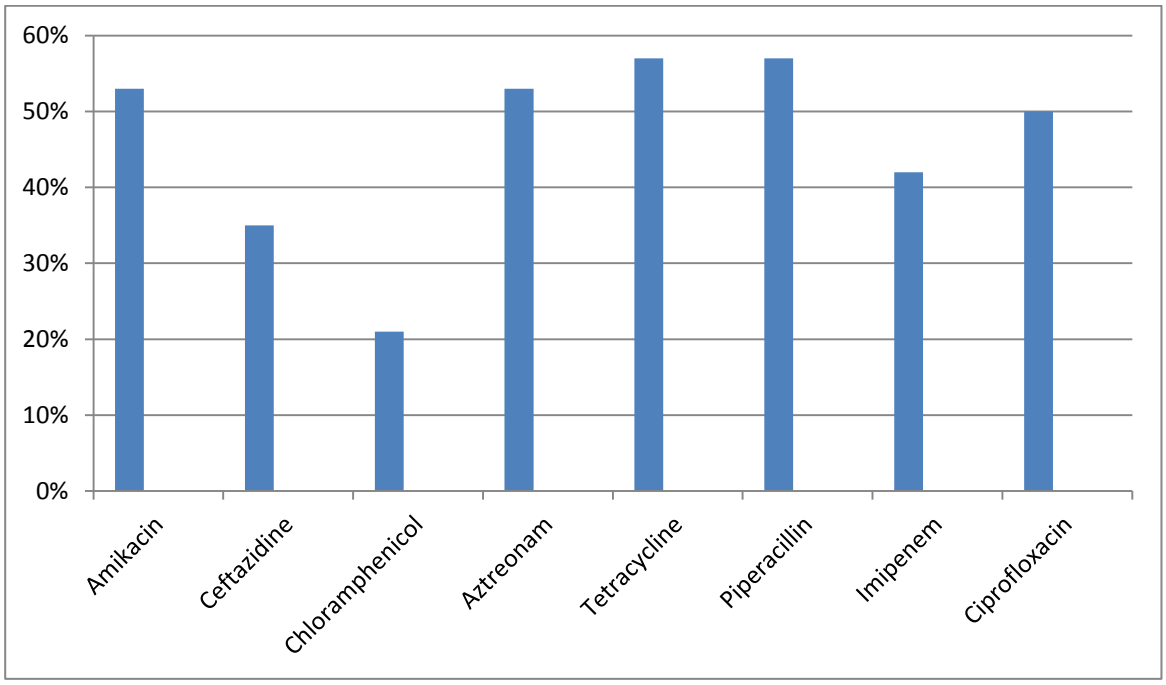

Figure.2. Levels of Salmonella resistance to different antibiotics.

Table.4. Resistance of each Salmonella species to eight antibiotics in terms of percentage.

Antibiotic	*S .arizonae* (n=7)		*S. choleraesuis* (n=10)		*S. gallinarum* (n=18)		*S. pullorum* (n=23)		*S .typhi* (n=6)	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Amikacin	4	57	6	60	10	55	11	47	3	50
Ceftazidine	5	71	5	50	6	33	6	26	1	16
Chloramphenicol	1	14	3	30	3	16	5	21	2	33
Aztreonam	4	57	6	60	11	61	8	34	5	38
Tetracycline	4	57	9	90	9	50	12	52	3	50
Piperacillin	4	57	8	80	8	44	15	65	2	33
Imipenem	2	28	4	40	9	50	10	43	3	50
Ciprofloxacin	6	85	4	40	7	38	10	43	5	38

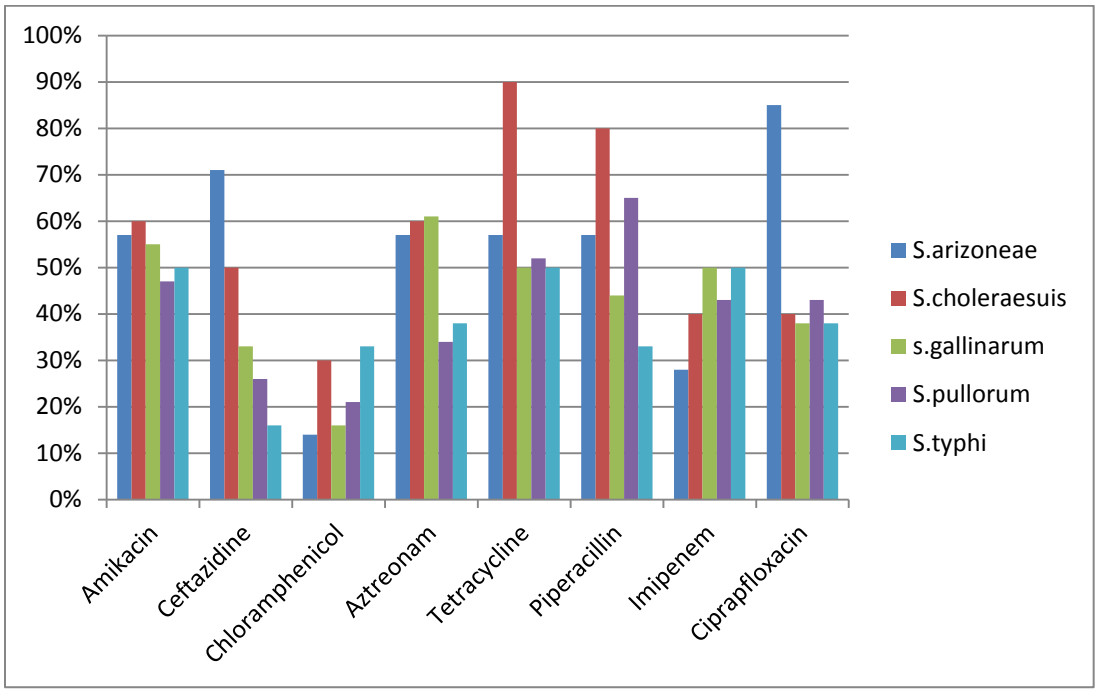

Figure.3. Levels of Salmonella species resistance to eight antibiotics.

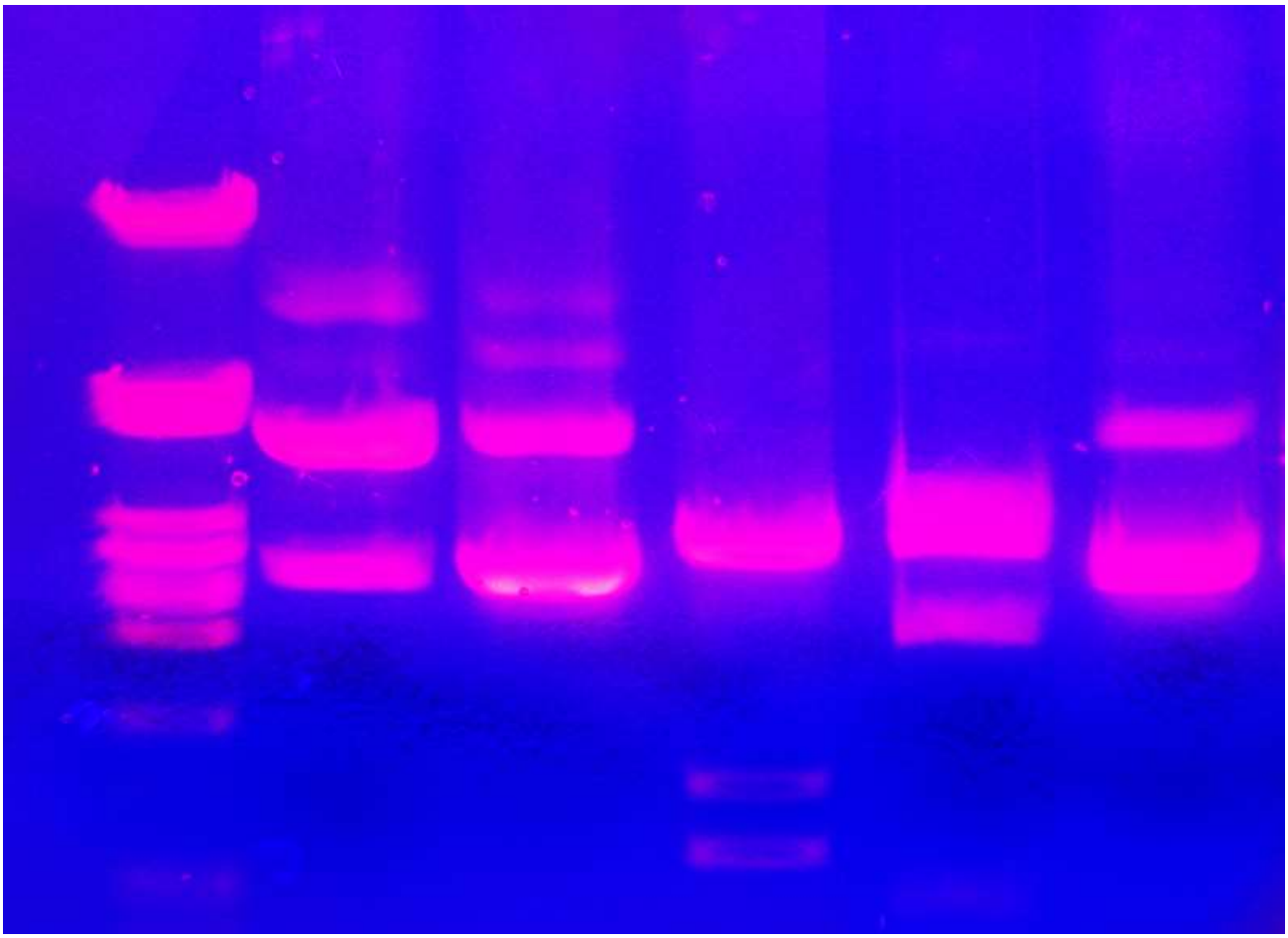

Figure.4. DNA bands separated by gel electrophoresis.

- Measured DNA concentration by Spectrophotometer was 330µg/ml.

- **CTX-M genes**

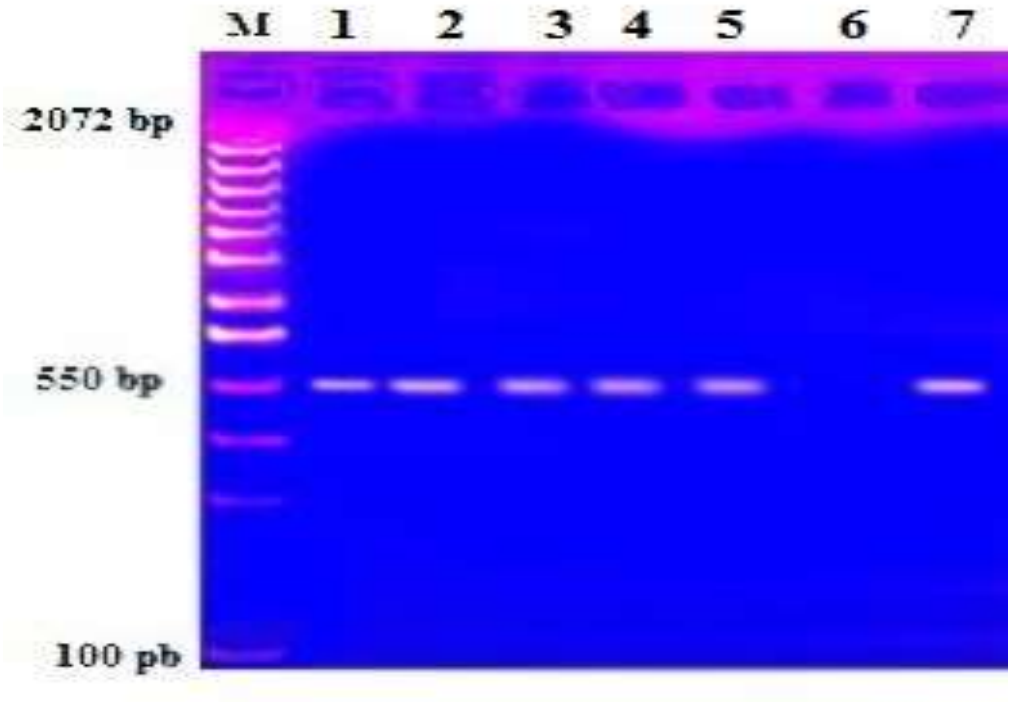

Figure.5. Electrophoretic pattern for the plasmid profile of 2-7 templates

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella gallinarum* isolates with CTX-M specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp) , lane 1 is positive control lanes 2,3,4,5and 7 were positive to the CTX-M genes lane 6 was negative.

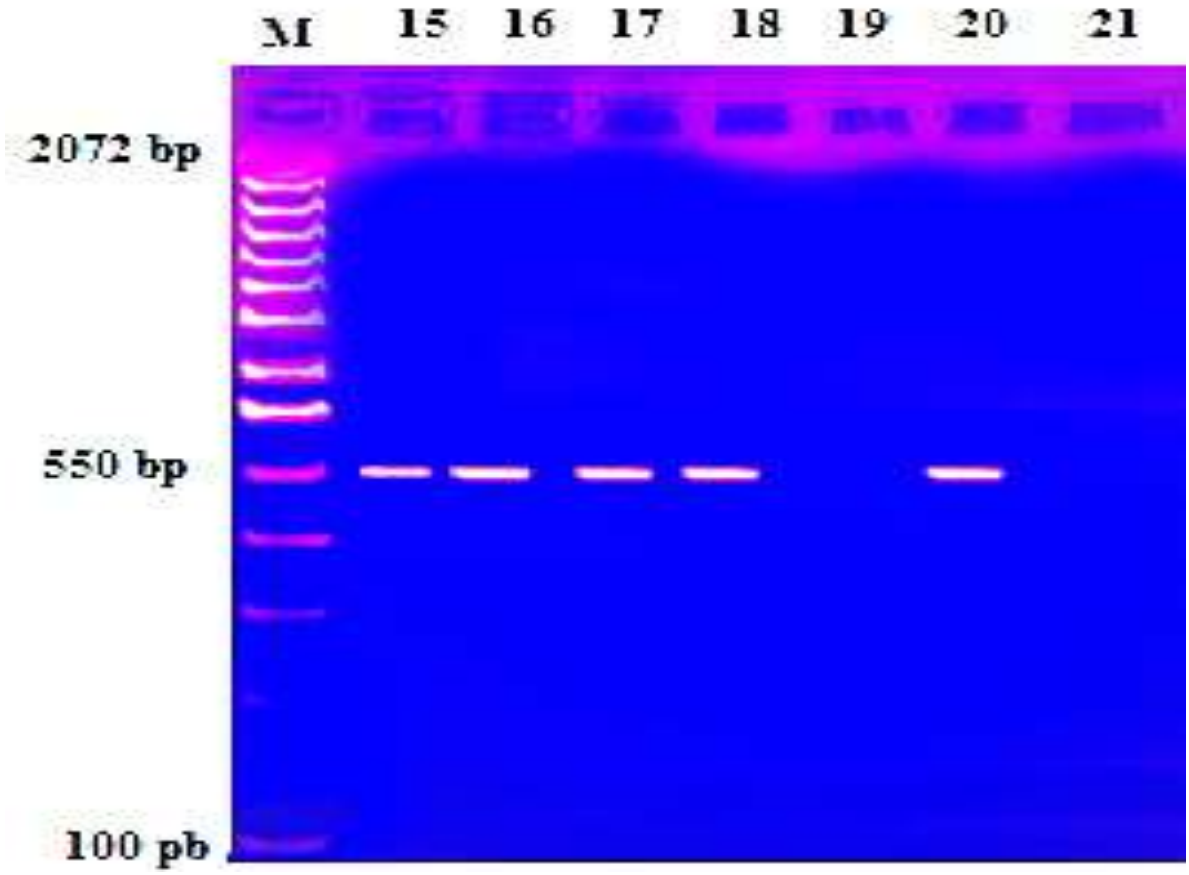

Figure.6. Electrophoretic pattern for the plasmid profile of 16-21templates

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella pullorum* isolates with CTX-M specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp) , lane 1 is positive control lanes 2,3,4 and 6were positive to the CTX-M genes lanes 5and 7 were negative.

- **SHV genes**

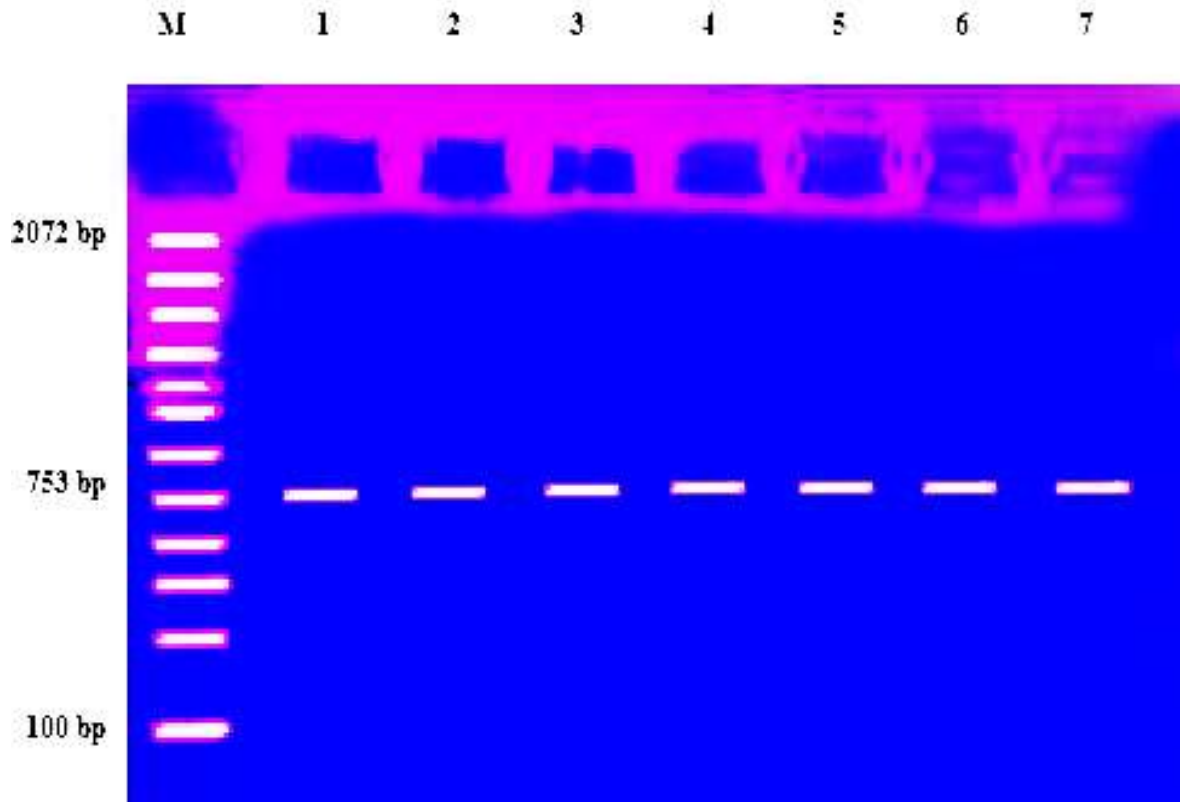

Figure.7. Electrophoretic pattern for the plasmid profile of 2-7 templates

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella gallinarum* isolates with SHV specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp), lane 1 is positive control lanes 2, 3,4,5,6 and 7were positive to the SHV genes.

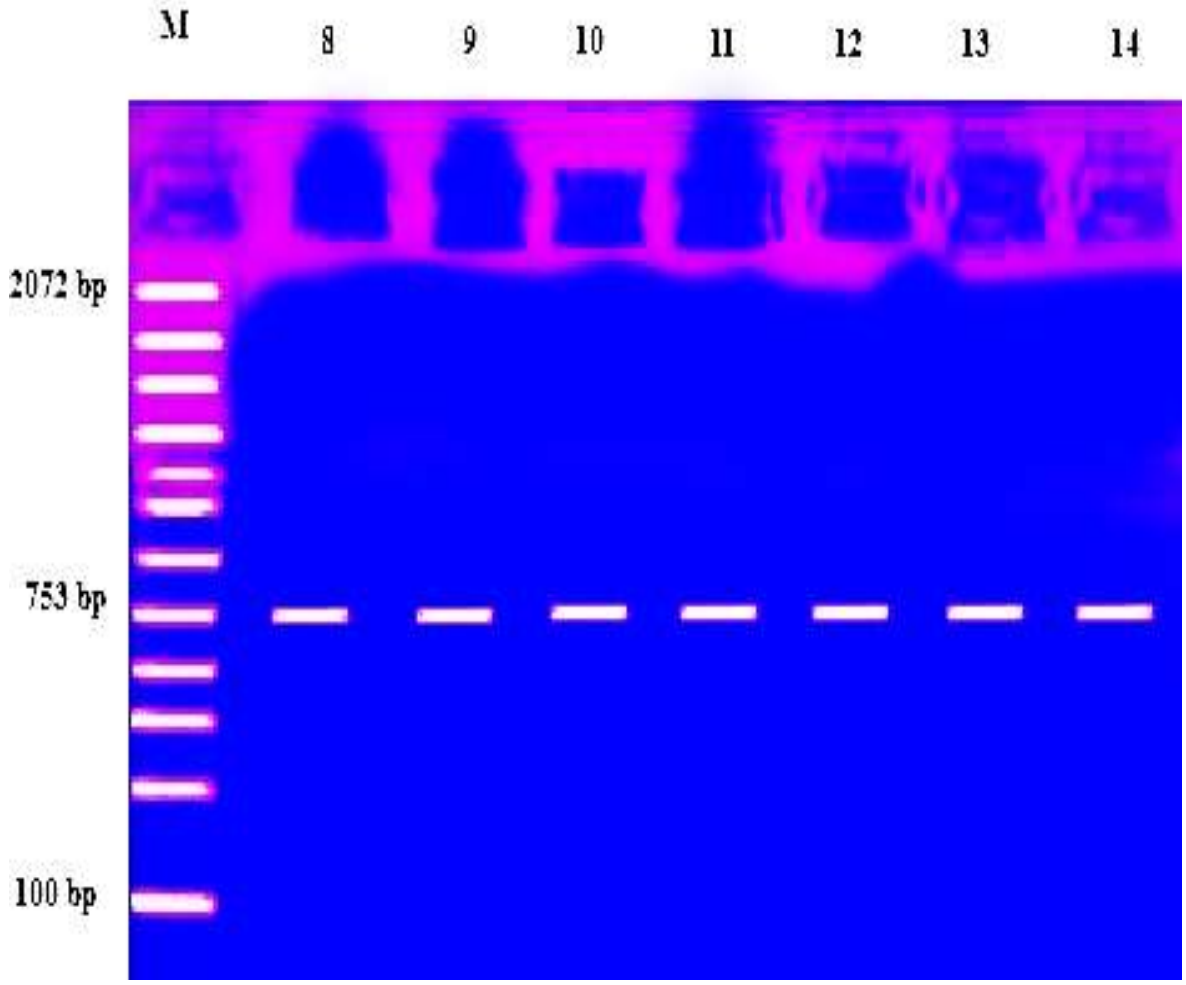

Figure.8. Electrophoretic pattern for the plasmid profile of 9-14templates

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella gallinarum* isolates with SHV specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp), lane 1 is positive control lanes 2, 3,4,5,6 and 7were positive to the SHV genes.

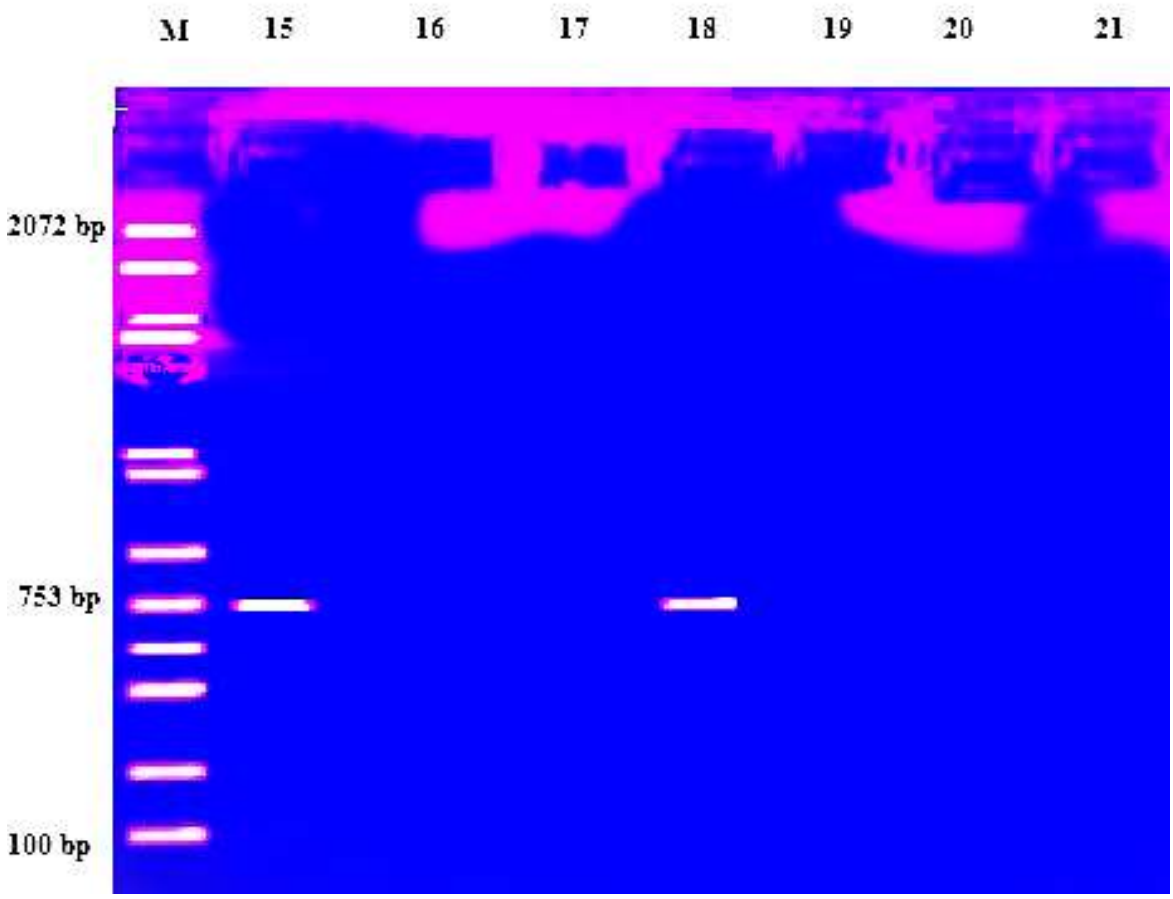

Figure.9. Electrophoretic pattern for the plasmid profile of 16-21 templates

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella pullorum* isolates with SHV specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp), lane 1 is positive control lane 4 was positive to the SHV genes, lanes 2,3,5,6 and 7 were negative.

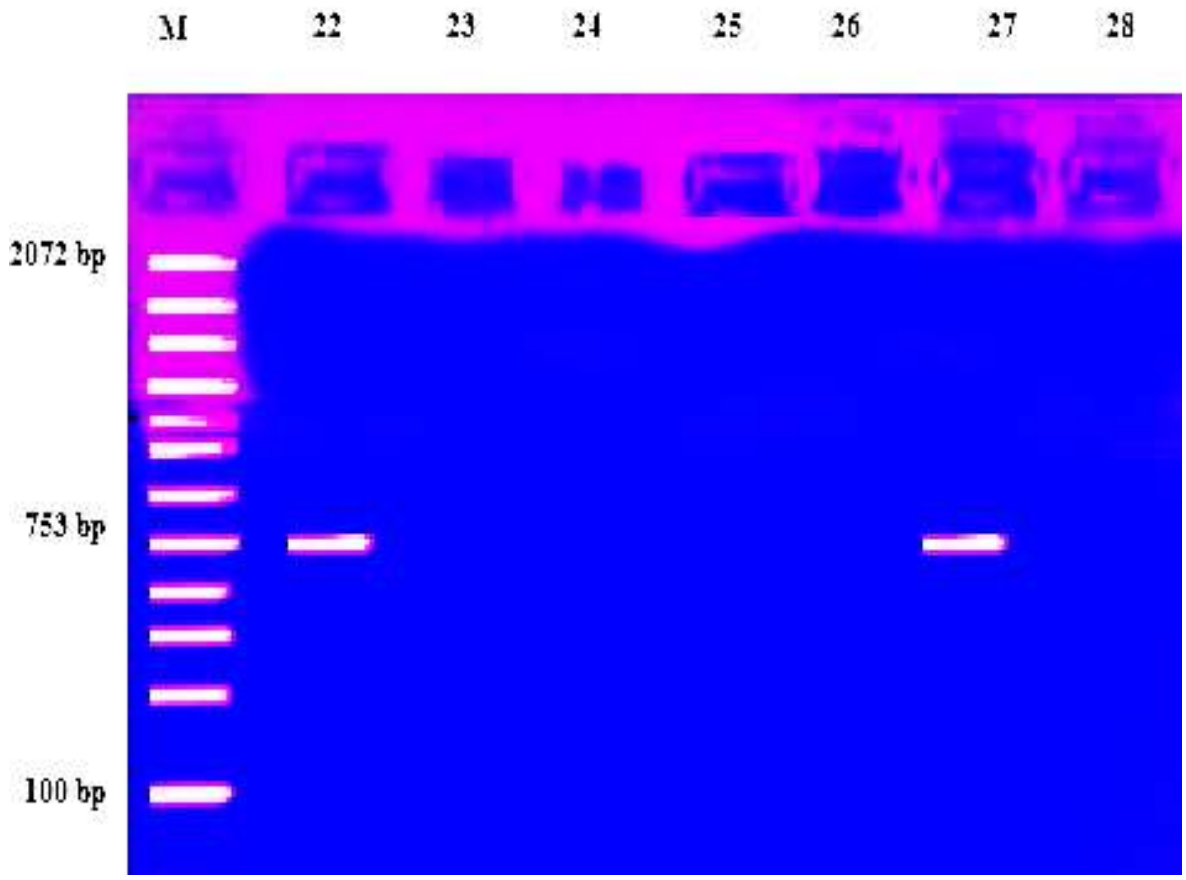

Figure.10. Electrophoretic pattern for the plasmid profile of 23-28 templates

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella pullorum* isolates with SHV specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp), lane 1 is positive control lane 6 was positive to the SHV genes, lanes 2,3,4,5 and 7 were negative.

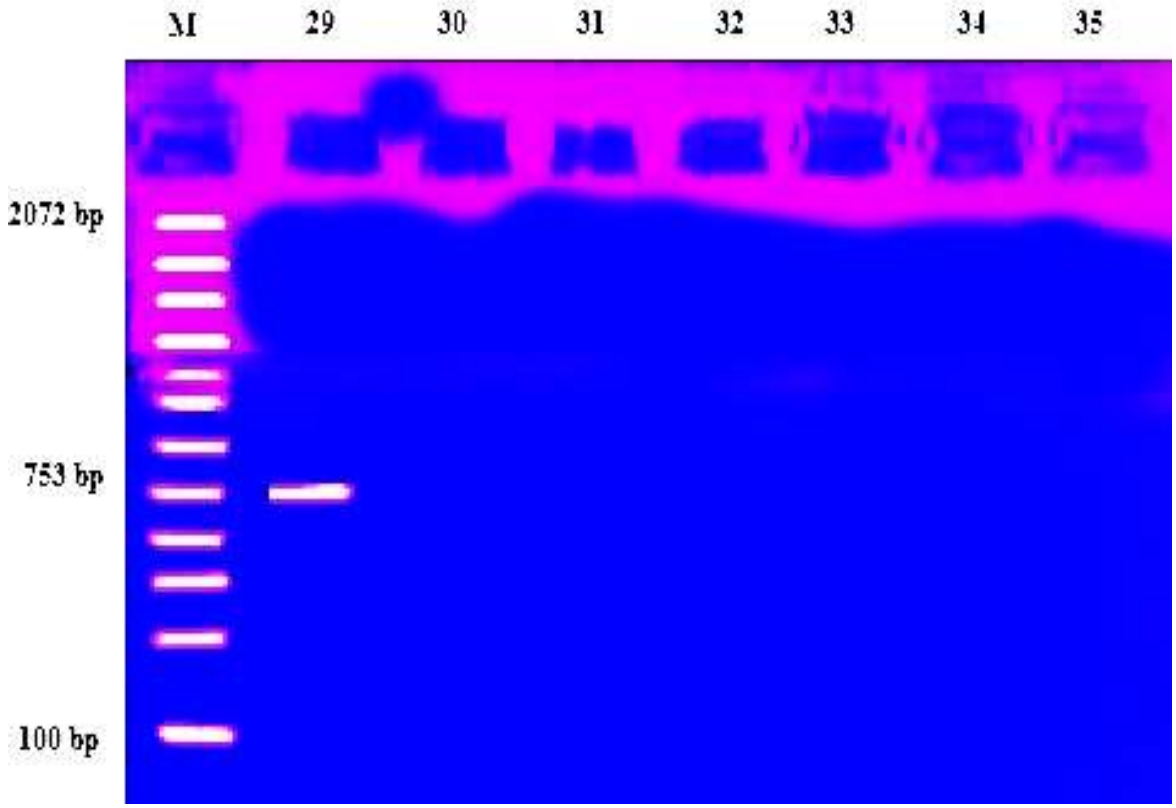

Figure.11. Electrophoretic pattern for the plasmid profile of 30-35 templates

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella pullorum* isolates with SHV specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp), lane 1 is positive control lanes 2,3,4,5,6 and 7 were negative to the SHV genes.

- **TEM genes**

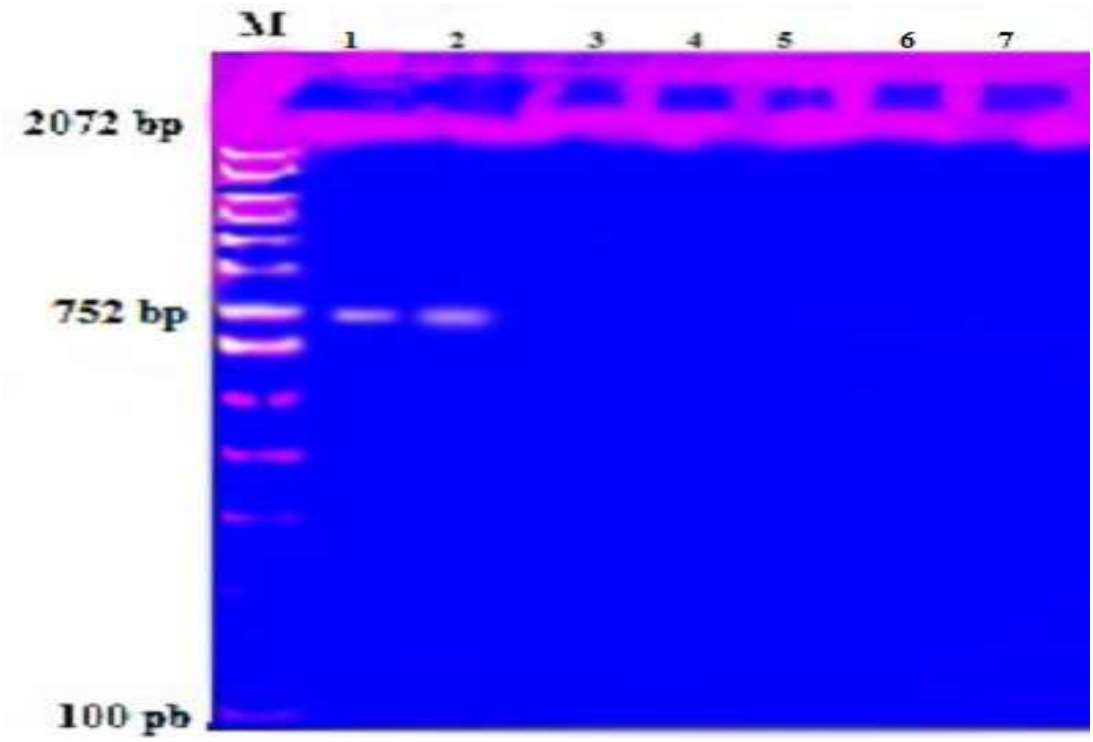

Figure.12. Electrophoretic pattern for the plasmid profile of 2- 7 templates

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella gallinarum* isolates with TEM specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp), lane 1 was positive control, lane 2 was positive to the TEM genes lanes 3,4,5,6 and 7 were negative.

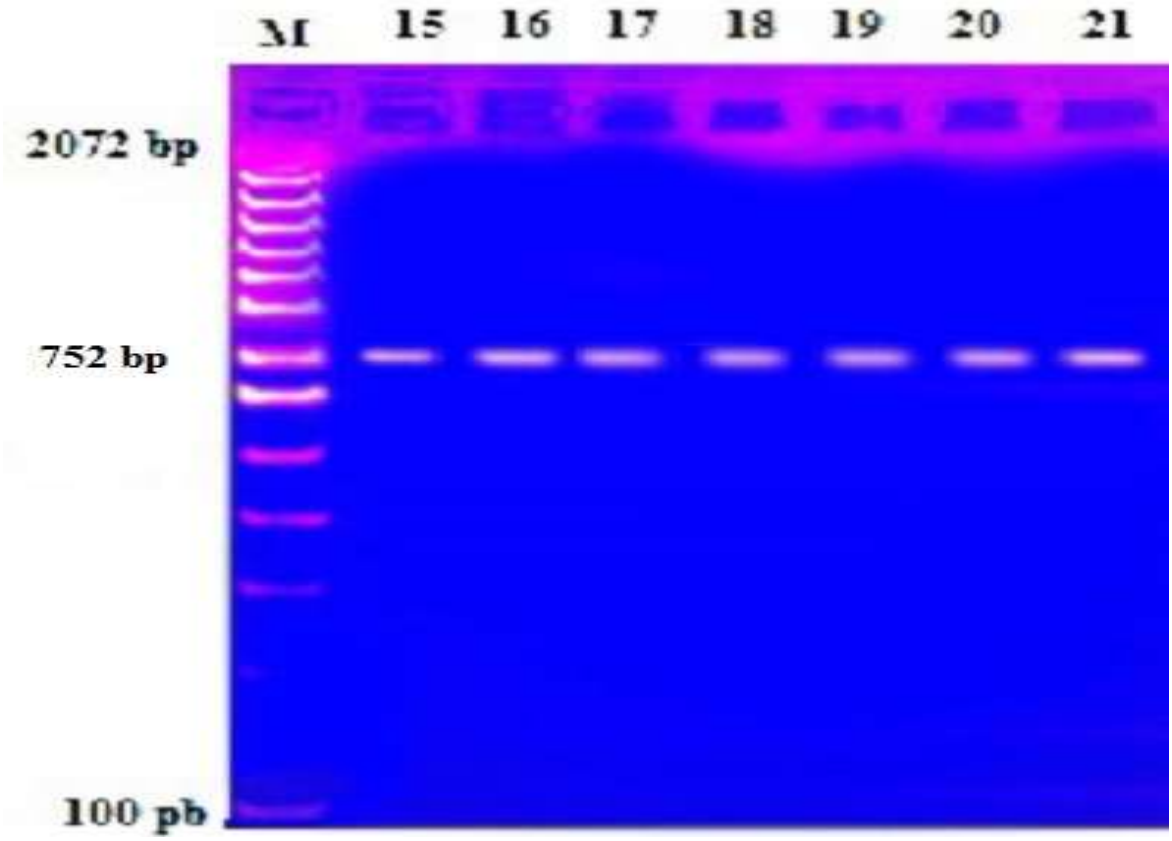

Figure.13. Electrophoretic pattern for the plasmid profile of 16-21 templates

Agarose gel (1%) used for separation of PCR products. Amplification of six DNA extracts of *Salmonella gallinarum* isolates with TEM specific primers and Marker (M) with different bands (scale from 100 bp. up to 2072 bp), lane 1 is positive control, lanes 2, 3,4,5,6 and 7 were positive to the TEM genes.

Table.5. Analyses of PCR outputs

Genes	*S. gallinarum*	*S.pullorum*
CTX-M	7(58%)	8(44%)
SHV	12(100%)	2(11%)
TEM	7(58%)	6(33%)

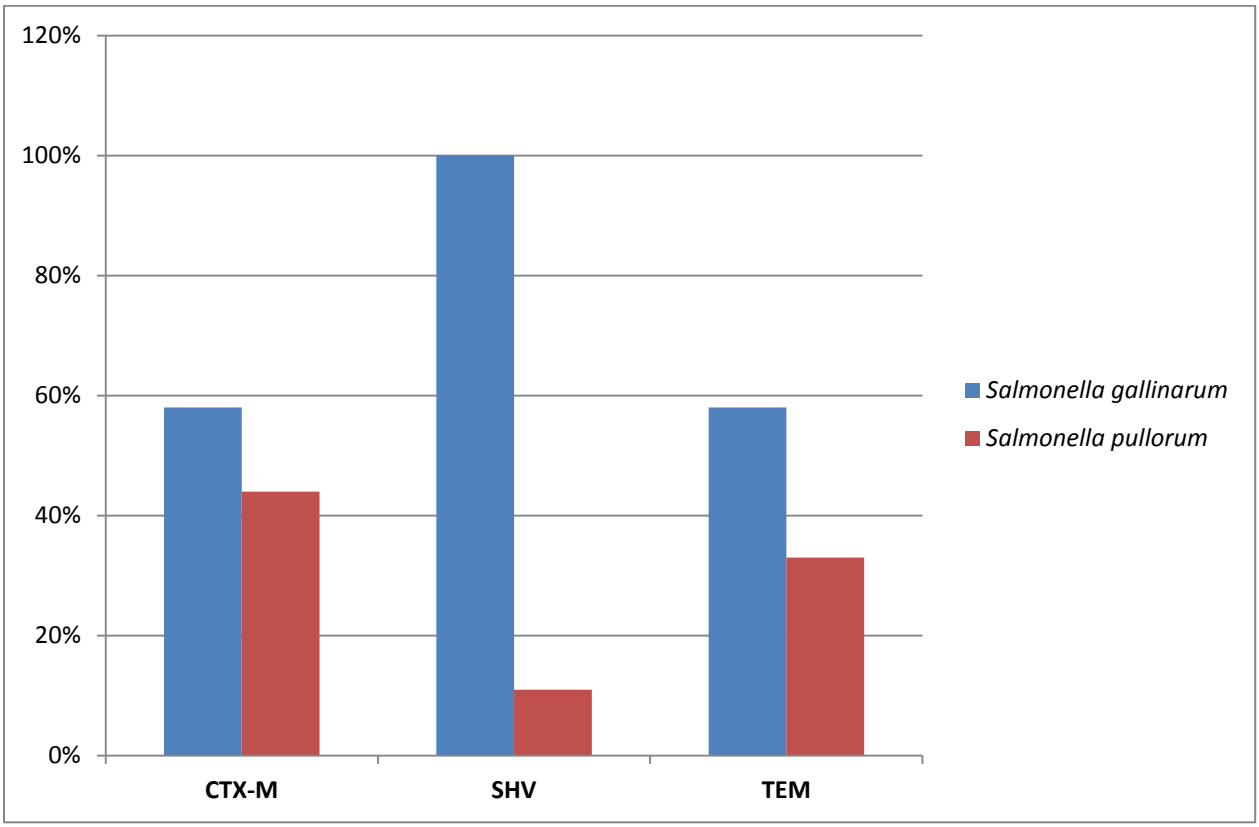

Figure.14. Genotypic resistance pattern of Salmonella isolates

CHAPTER FIVE
DISCUSSION

5.1 Discussion

This study was conducted to detect TEM, SHV, and CTX-M genes in extended spectrum beta-lactamases producing *S. gallinarum* and *S. pullorum* isolates, DNA was extracted by boiling method which is rapid and inexpensive procedure (Dario *et al*, 2003).

Results of the sensitivity tests showed variety levels of Salmonella response to different antibiotics and this may be due to antibiotic misuse.

The antibiotics to which Salmonella showed high resistance percentage were Tetracycline (57%) of total isolates, (50 %) of *S. gallinarum*, (52%) of *S.pullorum*, resistance to Piperacillin was (57%) from total Salmonella isolates (44 %) of *S. gallinarum*, (65%) of *S. pullorum* and this may be attributed to in discriminate antibiotics usage. Although Tetracycline is not commonly prescribed to treat Salmonella cases. This study was showed similar results of other studies in the investigation of phenotypic drug resistance pattern (Holmberg *et al*, 1984).

Chloramphenicol cannot be used unless it is prescribed by physicians or veterinarians because of its side effects. However (16 %) of *S. gallinarum*, (21%) of *S. pullorum* were found resistant to chloramphenicol.

Since 1996, the National Antimicrobial Resistance Monitoring System (NARMS) has identified increasing numbers of Salmonella isolates resistant to Chloramphenicol and Tetracycline. These isolates also have decreased

susceptibility or resistance to Ceftriaxone, an antimicrobial used to treat serious infections (NARMS, 2006). In 2004, Martin et al. reported in Canada several Salmonella with Multi Drug Resistant (MDR), which also resist many types of antibiotics.

Ciprofloxacin is recommended for treatment of Salmonellosis, however in this study Ciprofloxacin resistance occured in (50%) from the total Salmonella isolates, (38 %) of *S. gallinarum*, (43%) of *S. pullorum* isolates. In the United States a study showed that (10%) of Salmonella isolates were likely resistant to ciprofloxacin (Bauer, 2006). The high resistance percentage to Ciprofloxacin could be attributed to its misuse by poultry farmers and this lead recently quinolones in poultry to be banned by the National Medicines and Poisons Board in Sudan.

Several studies have been published focusing on the severe health consequences from multidrug-resistant Salmonella infections. Antibiotic-resistant Salmonella have been isolated from various food products. In 1998, (20%) of ground meat samples were positive for Salmonella, and (84%) of these were resistant to at least one antibiotic in Washington, D.C. area .From 1999 to 2003, (18%) of Salmonella isolates from various food products tested by the FDA were resistant to two or more antimicrobials(White *et al*, 2001and Kiessling *et al* 2007) .

Twelve DNA extracts from *S. gallinarum* and eighteen extracts from *S.pullorum* isolates were investigated for the presence of SHV, CTX-M and TEM genes. Polymerase Chain Reaction (PCR) showed the presence of the above examined genes with different rates. In *S.gallinarum* the predominant was SHV genes (100%), followed by CTX-M and TEM genes both of (58%),

however in the isolates of *S. pullorum* the presence of CTX-M (44%) then TEM (33%) and finally SHV genes (11%).

The results of this study are similar to results recorded in Germany among 679 Salmonella isolates. Investigate ESBL prevalence was still low (10%) with predominant TEM gene (Rodriguez *et al*, 2004).

Similar study in Nigeria showed that the predominance of SHV and TEM genes and absence of CTX-M genes among *S. pullorum* (Akinlabi *et al*, 2001).

The TEM was the second gene among all Salmonella isolates, this is in accordance with Osama (2010) in Sudan. This study revealed that SHV gene of the lowest occurrence in *S. pullorum*. However David *et al*, (2003) reported the absence of SHV genes in Salmonella isolates.

A similar activity has been reported among *Salmonella* species in other countries like Turkey, Nepal and South Africa (Irajian *et al*., 2009). The enzyme has been reported to bring about resistance to Piperacillin, Ceftazidime and aztreonam as it is coded on conjugative plasmids, transposons or integrons, genetic materials which can be spread readily (Irajian *et al*., 2009).

There is paucity in reports about *S. gallinarum* and *S. pullorum* especially in drug resistance pattern field, however in Nigeria Akinlabi (2001) reported the presence of the TEM, SHV, OXA_2, DHMA and ECBM genes among *S. gallinarum* and *S. pullorum* isolates

Should these resistance be transferred from food animals to man, it can jeopardize success of effective treatment constituting a potential grave public health hazard.

This work thus provides an initial database for genes responsible for resistance of beta- lactam drugs in Salmonella strains isolated from poultry farms in Khartoum State.

5.2 Conclusions

The study could be concluded in the followings:

- This study showed high prevalence of Salmonellosis (32%) among poultry. The sensitivity and resistance to antibiotics is not constant.
- Bacterial DNA extracted from two species of Salmonella reflected appearance of CTX-M, SHV and TEM genes which were responsible of resistance to betalactam antibiotics.
- There is a widespread Beta-lactamase activity in and around the poultry, causing antibiotic resistance to *Salmonella*.
- There is a real need for the control of indiscriminate antibiotic use in poultry, which encourages antibiotic resistance, and thus exacerbating an existing global problem of antibiotic resistance.

5.3 Recommendations

1. Salmonella infections need to be controlled through sanitary and treatment programs.
2. Raising awareness of proper and correct sanitary behavior is to be adopted.
3. Increase of hygienic measures among workers in the field of food and water resources.
4. Prescription and administration of antibiotics must be achieved by authorized person.
5. Sensitive antibiotics with recommended dose must be used in treatment of avian Salmonellosis.
6. Updating of antibiotic sensitivity test against bacteria incriminated in the causation of disease condition.
7. Molecular detection and identification of β-lactamase should be put in mind for further research.

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APPENDIX

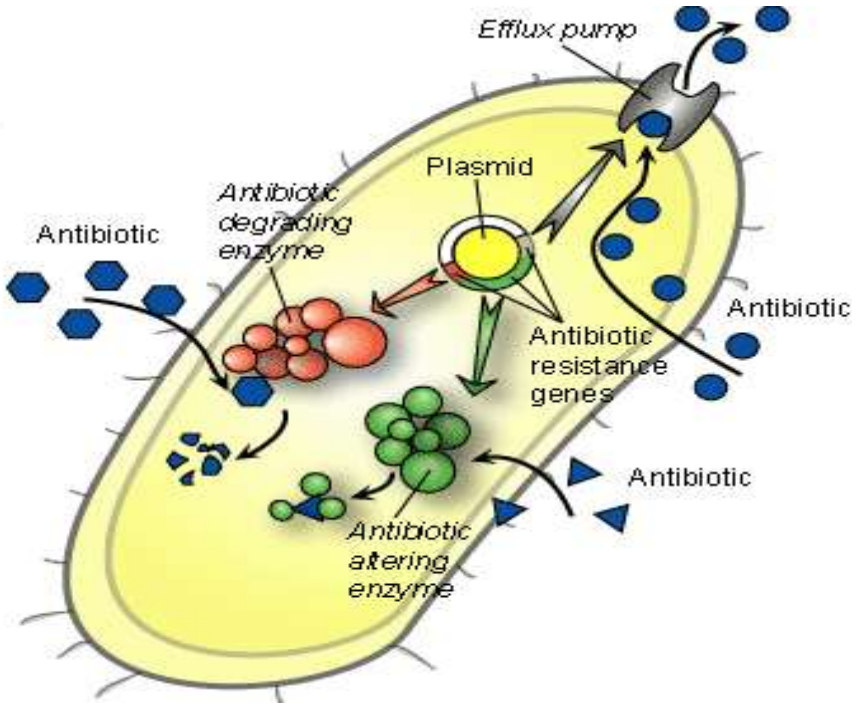

Figure . 15. Mechanisms of drug resistance

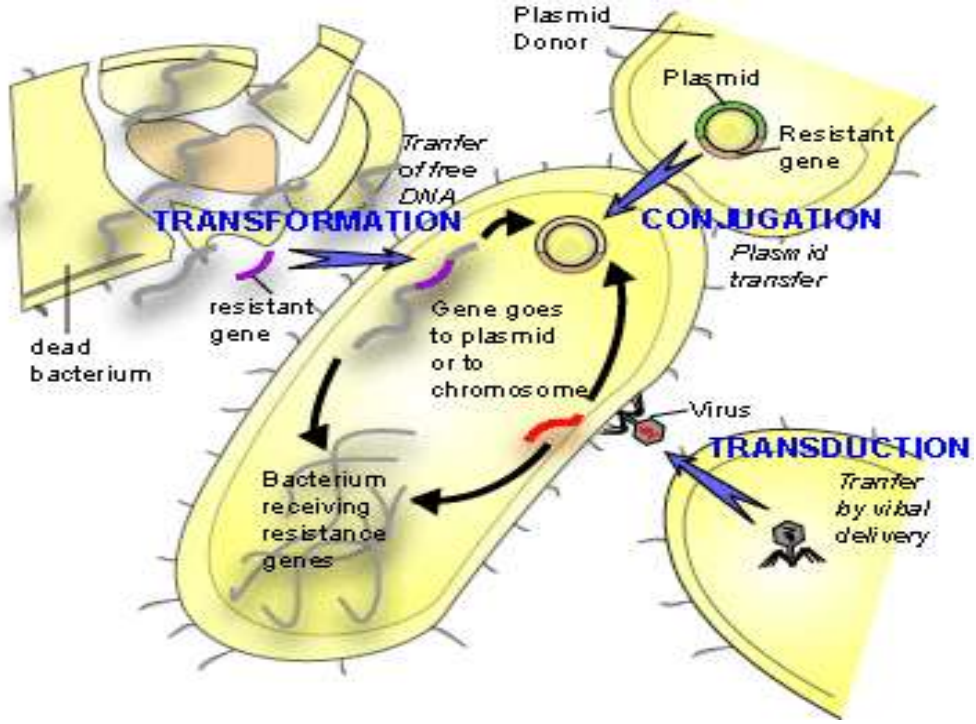

Figure .16. Mechanisms of drug resistance" genetic transfer"

➢ Reagents

- **Kovac's reagent**

This reagent contains 5g of para dimethyl aminabenzaldehyde, amyl alcohol 75ml and HCL Conc 25ml. The aldehyde was first dissolved in the alcohol at 50-55°C then cooled and the acid was added carefully. The reagent was stored at 4°C to be used for indole test.

- **Oxidase reagent**

1%-naphthol in 95% ethanol.

Tetramethyl-p-phenylenediamine dihydrochloride was prepared freshly each time by adding a loopfull of it to 3 ml of D.W.

- **Methyl red reagent**

Methyl red powder 0.04g, ethanol 40 ml, and distilled water 100ml. Dissolve the methyl red in the ethanol and dilute to volume with the distilled water. The reagent was stored at 40°C for later use for methyl red test.

- **Bromothymol blue**

Bromothymol sulfonephthalein 1%, BTB indicator in acidic medium (yellow), neutral (green), and alkaline (blue) according to pH concentration. Dissolve 0.10g in 8.0 cm^3 N/50 NaOH and dilute with water to 250 cm^3.

➢ Stains

- **Gram stain**
- **Crystal violet**

Crystal violet dye 10g, absolute methanol 500ml, dissolves the dye gently and stored at room temperature in screw caped bottle.

- **Safranines**

Safranine dye 10g, dissolved in 1000 ml – D.W.

- **Lugol's iodine**

The 5% solution of lugol's iodine consists of 5% (wt/v) iodine (I_2) and 10% (wt/v) potassium iodide (KI) mixed in distilled water. It is used as a mordant.

- **Ethanol**

Used as a decolorized with absolute concentrations.

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