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Antibacterial and Antimutagenic Effects of Synthetic Histidine

A Thesis

Submitted to the College of Science/Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

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Summary

This study was accomplished to determine the Histidine behaviors toward two pathogenic bacteria (*Staphylococcus aureus* and *Escherichia coli*) toward exposed to various conditions, as well as to test their susceptibility toward related antibiotics. The method of His measurement in colostrum and natural Iraqi breast milk was modified by transferring the separation column of Amino Acid Analyzer (AAA) to a High Performance Liquid Chromatography (HPLC) that contains a fluorescent detector. Results of analysis showed high concentrations rate of free His which reached two folds (222 mg/l) in the colostrum compared with the combined (94 mg /l).No noticeable differences were detected between the concentration rates of the free and combined His in milk; they were 51 and 88 mg/l, respectively. It was found that the free His in the colostrum was about four times than that of the free histidine in natural milk.Results of determining the effect of exposure to ultraviolet light at (254 nm) and high frequencies of microwave on the structure of histidine by using the FTIR analysis have not shown any effect on the structure of histidine and its active groups. Where polymerization and condensation were not available in these methods. Results showed that the His used as an antibacterial agent, sensitivity of *S. aureus* and *E. coli* was not effected at concentrations up to 10 mg/ml. It was found that presence of His in the solidified medium led to a significant change in the sensitivity of the isolates toward the antibiotic used. Histidine led to increase the sensitivity of the isolates against the Rifampicin(Rp) and reducing it toward Levofloxacine (Lev), Ciprofloxacin (Cp), Gentamicin(Gm) without change toward Streptomycin (S), Metronidazole (Mz), Cloxacillin (Cx), Tetracycline (T), Nalidixic acid (Na), Penicillin (P) Clarithromycin (Cla), Vancomycine (Va) and Chloramphenicol (C). Results of the genomic profile indicated the presence of small plasmids in *E. coli* but absent in *Staph. aureus*. The use of chemical and physical curing agents caused

changes in the sensitivity of *S. aureus* against the three selected antibiotics (Rp, Lev and Cx) regard less to the presence or absence of Histidine in the plasmids. The change also includes *E. coli* at different levels. Histidine reflected a large heterogeneous behaviors, after mixing with the suspension of isolates and exposed to the physical mutagenesis (UV250 nm),as compared with the three selected antibiotics. These interactions were restricted between the histidine and the microorganism. The results also showed that *E. coli* isolate was turned into a sensitive against Rp, Cx and resistant against Lev. While *S. aureus* was turned to be more sensitive against Rp and Cx, but did not change against the Lev. The role of Histidine was investigated by examing the sensitive and resistant isolates of *Mycobacterium tuberculosis* (TB) against Rp was studied, results exhibited that presence of Histidine in the concentrations 5 and 10 mg/ml which turned the resistant TB isolates against Rp (40 µg / ml) into sensitive. These results had a significant effect to avoid patients from exposure subsequent levels of high risk antibiotics.

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List of abbreviations

Abbreviations	Full name
D.W	Distilled water
DNA	Deoxyribonucleic acid
E. coli	*Escherichia. coli*
EDTA	Ethylene demine tetra acetic acid
EMB	Eosin Methylene Blue
FTIR	Fourier Transform Infrared spectroscopy
His	Histidine
HPLC	High Performance Liquid Chromatography
M. tuberculosis	*Mycobacterium. Tuberculosis*
OPA	O- pthaldehyde
RNA	Ribonucleic acid
SDS	Sodium Dodecyl Sulfate
S. aureus	*Staphylococcus. Aureus*
TBE	Tris- borate EDTA
TE buffer	Tris- EDTA
Topo IV	Topoisomerase IV
UV	Ultra violate
RP	Rifampicin

Chapter One

Introduction and Literature Review

1-1 Introduction

L-Histidine, abbreviated His, is one of the essential basal amino acids which is important in protein synthesis and a source able to turn into glucose (Glycogenic amino acids). Studies consider this acid as an essential amino acid, especially for infants. Human and animal has the ability to synthesis this amino acid in very small amount. So, it must be provided with food and feed to meet the need. Normal flora found in the digestive system plays an important role in the synthesis and supply of this amino acid. Plants and microorganisms are a major source of His synthesis supplying in almost similar pathways(Yongsong *et al*., 2013).

Free and combined His are found in colostrum and natural milk of the breastfeeding mother. The free His is more concentrated than the combined in the colostrum stage and the combined is associated with the immune proteins. Histidine inters in the synthesis of transportation systems across the cell membrane and in the composition of immunological agents such as Lactoferrin, an important factor in immune protection for the newborn infants.This acid has a great significance since it contains imidazole group that has positive charge in the biological systems as a natural state and its function in synthesis of anti-fungal, anti-mutagenic and anti-cancerous tumors and other therapeutic drugs. The side chain group has the ability to link metalloprotein enzymes and in the active sites. Unprotonated imidazole is nucleophile and can serve as a general base, while the protonated form can serve as general acid (Kim, 2015).

One of the important characteristics of the His is its ability to gain and loss of protons in acidic and alkaline conditions where it present in the biological liquid at relative normal pH. The carboxyl group (COO^-) of the acid is unprotonated status while the amine group acquisition proton (NH^{+3}) in biological systems(Kopple and Swendseid, 1975). All of these ion cases lead to

the addition of new characteristics and different behaviors in its interaction with various compounds in acidic or alkaline conditions.

Polyhistidine is manufactured by condensation and polymerization methods and used in the pharmaceutical industry and as a carrier of drugs. There are a lot of the characteristics of acid that are not fully investigated. The potential of exploiting the acid as antibiotic is further needs more experimental work. Many characteristics of acid needs to investigate especially its behaviors if present with antibiotics and mutagenic agents or with microorganisms under the effect of these materials.

According, The following objectives were deigned.

1- Samples collection from lactating woman , starting from Colostrum stage and to end of first month breastfeeding .The presented samples collection will be the followed . Maternity hospitals and childcare centers are the location of samples collection .
2- Evolution of the contamination of samples with microbes .
3- Study the concentrations differences of both free and combined Histidine within peptides among collection periods .
4- Study the ability and simple characteristics of producing the polymers of histidine using chemical modification .
5- Induced the reveres mutant in the pathogenic bacteria and flora with the suitable mutagenic source .
6- Study the antimicrobial effect of the histidine and their polymers against pathogenic and flora microbes under normal and mutagenic cases in laboratory media .
7- Study the effect of addition the histidine and their polymers to the re-constitute milk infected with pathogenic and flora microbes under normal and mutagenic cases in comparison with control re-constitute milk.

1-2 Literature review

1-2-1 Histidine

The amino acid L-histidine (His) was discovered independently by Kossel and Hedin in 1896 (Robert, 2011). It is an essential amino acid, has a side chain of positively charged imidazole functional group. The imidazole group makes it a common participant in enzyme catalyzed reactions. Unprotonated imidazole is nucleophile and can serve as a general base, while the protonated form can serve as general acid. The residue can also serve a role in stabilizing the folded structures of proteins **http//** **www.**biology.arizona.edu.(2003).

Histidine is only produced in very small amounts by the body, it must predominantly be taken in through the diet. Infants in particular need an additional source of L-histidine, either through breast milk, special supplements, or formula milk, as a deficiency can lead to growth problems and other conditions. Given its wide application in medicine, biology, pharmaceutical and food industry, due to their biological properties, such as antiviral, antibacterial, antioxidant and immune modulatory activities. (Yamawaki *et al*., 2005).

Histidine is a precursor to histamine, a chemical that increases the body's inflammatory responses of the skin and mucous membranes (Paul *et al*., 2000).

1-2-2 Chemical and Physical properties

Amino acids are organic molecules characterized by carboxylic and amino groups with the general formula NH_2-CH(R)-COOH. In aqueous solution, for most pH values, and in the solid state, the amino acids take a zwitterionic form, i.e., NH^{3+}-CH(R)-COO. **His** has a variety of functionalities found in biological systems and because the zwitterion displays two tautomeric forms occurring in equilibrium at biological pH. **His** is moreover characterized by an imidazole ring, formed by two nitrogen and three carbon atoms, as depicted in the inset of (Figure 1.1). In solution, histidine presents five pH-

dependent different protonation states and shows six distinct geometrical conformations ((De Sousa *et al*., 2012).

As a consequence, histidine can act as a donor or as an acceptor for the hydrogen bond (influencing the secondary and tertiary structure of proteins and participating in several enzyme reactions . In particular, the imidazole of histidine participates in the tuning of the electronic properties of the copper ion, which is involved in catalysis (Jarzęcki , 2009)

Histidine one of the twenty standard amino acids, the imidazole side group of His has a pKa of approximately 6, allowing it to alternate between the protonated and unprotonated states under physiological conditions (Figure 1.1).

This property allows His to participate in general acid-base catalysis (Fersht, 1999), and consequently it is present in the active sites of many enzymes. the unprotonated imidazole group of His plays important roles as a nucleophile in phosphoryl transfer, and in the co-ordination of metal ions in a range of metalloproteins, perhaps best known in the zinc finger motif (Harding, 2004)

The melting point of **His** is 287c^{o} and its soluble in water and ethanol (David, 2000).

1-2-3 Histidine biosynthesis

The genes necessary for histidine biosynthesis have been identified in many bacteria, fungi, plants, and archaea. The pathway in all of these organisms is identical, with small differences in some of the enzymes used. Histidine is an essential amino acid, and is not synthesized by mammals (Robert *et al*., 2014).

Histidine biosynthesis seems to be conserved in all organisms including archaea (Lee *et al*., 2008), Gram-positive bacteria (Chapman and Nester, 1969), lower eukaryotes (Fink,1964), and plants (Stepansky and Leustek, 2006).

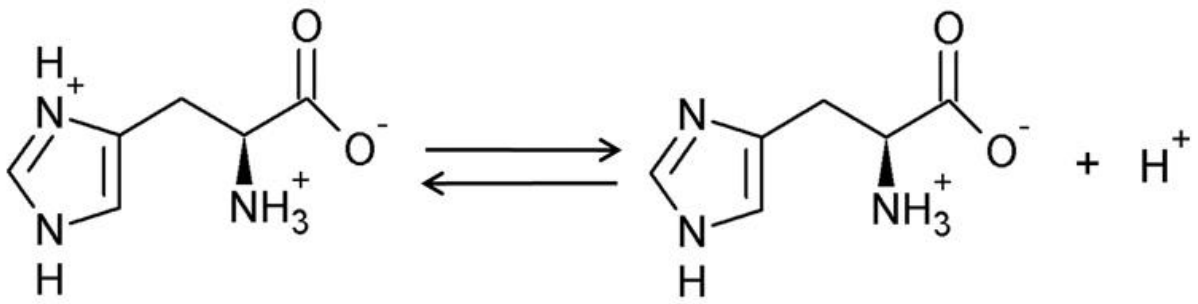


Figure (1-1): Structure of L-histidine. The imidazole side group is a weak acid with a pK_a of approximately 6, allowing it to switch between the protonated and unprotonated states under cellular conditions (Harding,2004).

Figures (1-2) shows the His biosynthetic pathway according to (Robert, 2011). Abbreviations used for enzyme names are indicated in parentheses, and the corresponding Arabidopsis gene names and AGI codes are shown in blue. Allosteric inhibition of ATP-phosphoribosyltransferase (ATP-PRT) activity by L-His is indicated in red. Abbreviations used for intermediates are: PRPP (5′-phosphoribosyl-1-pyrophosphate), PRATP (*N*′-5′-phosphoribosyl-ATP), PRAMP (*N*′-5′-phosphoribosyl-AMP), ProFAR (*N*′-[(5′-phosphoribosyl) formimino]-5-aminoimidazole-4-carboxamide) ribonucleotide, PRFAR (*N*′-[(5-phosphoribulosyl)formimino]-5-aminoimidazole-4-carboxamide)ribonucleotide, IGP (imidazole glycerol-phosphate), IAP (imidazole acetol-phosphate), AICAR (5′-phosphoribosyl-4-carboximide-5-aminoimidazole) and 2-OG (2-oxoglutarate). Hyperlinks to chemical structures and TAIR locus pages are provided.

1-2-4 Benefits and biochemistry of Histidine

Histidine is an essential amino acid (children should obtain it from food) needed in humans for growth and tissue repairing. Histidine is important for

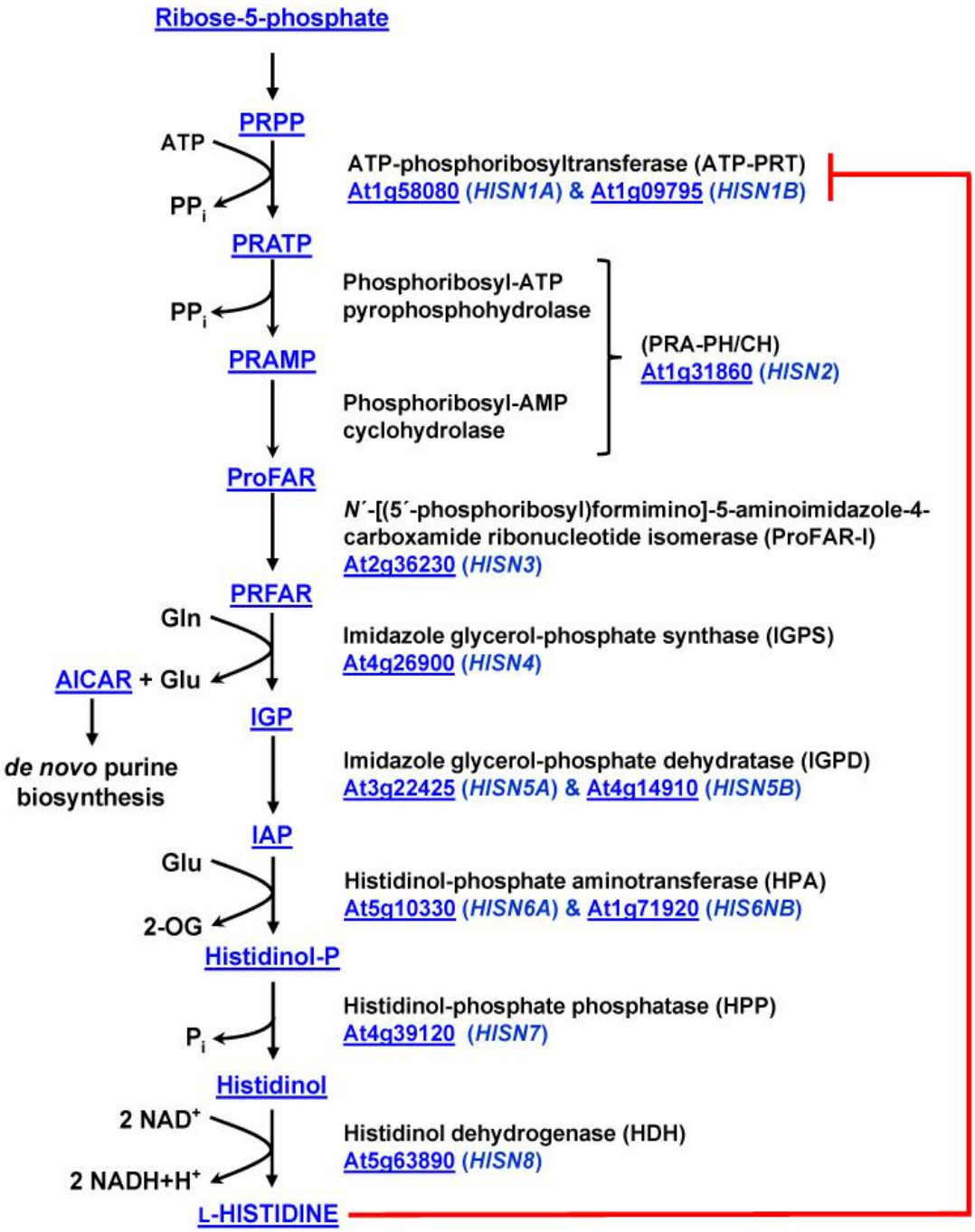
maintenance of myelin sheaths that protect nerve cells and metabolized to the neurotransmitter histamine. Histamine plays many roles in immunity, gastric secretion, and sexual functions. It also required for blood cell manufacture and protects tissues against damage caused by radiation and heavy metals (NCIT).

Low free histidine has been found in the serum of some rheumatoid arthritis patients. Serum concentrations of other amino acids have been found to be normal in these patients (Adlesic *et al*., 2007)

L-histidine is an excellent chelating agent for such metals as copper, iron and zinc. Copper and iron participate in a reaction (Fenton reaction) that generates potent reactive oxygen species t Suppressor T cells have H2 receptors, and histamine activates them. Promotion of suppressor T cell activity could be beneficial in rheumatoid arthritis. Further, histamine has been shown to down-regulate the production of reactive oxygen species in phagocytic cells, such as monocytes, by binding to the H2 receptors on these cells (Austin and Taylor, 2010).

That could be destructive to tissues, including joints (Jesús *et al*., 2011; Lodyga and Grace, 2011) .

This latter mechanism is the rationale for the use of histamine itself in several clinical trials studying histamine for the treatment of certain types of cancer and viral diseases. In these trials, down-regulation by histamine of reactive oxygen species formation appears to inhibit the suppression of natural killer (NK) cells and cytotoxic T lymphocytes, allowing these cells to be more effective in attacking cancer cells and virally infected cells (Knox *et al*., 2011).



Figure(1-2): Histidine biosynthesis pathway (Robert, 2011).

Lactoferrin, are being investigated as novel therapeutic agents. A dynamic, bioactive fluid, human milk changes in composition from colostrum to

late lactation, and varies within feeds, diurnally, and between mothers (Ballard and Morrow. 2012).

Histamine is a biogenic amine, which is involved in a variety of biological processes comprising inflammation, allergic responses, neurotransmission and regulation of gastric acid secretion. The key enzyme for the generation of histamine is histidine decarboxylase (HDC), which converts the amino acid **His** to histamine(Hocker *et al*., 1996)

Histamine is a neurotransmitter released throughout the body that regulates multiple physiological responses. Primarily histamine is acknowledged for its role in inflammatory reactions to foreign pathogens that enter the body. Aside from inflammatory responses, histamine expression and synthesis has been detected in various cancer cell lines and multiple malignancies (Mihan *et al*., 2011; Kennedy *et al*., 2012;).

Metals such as zinc, copper and nickel are transported by binding with His, and such binding appears essential for rapid excretion of excess metal. Toxic metals such as mercury, lead, cadmium, and threatening excesses of essential minerals zinc and copper stimulate the rapid formation of metallothionein inside cells of the brain, liver, and kidneys. Metallothionein is a molecule designed to store metals in such a way as to prevent uncontrolled oxidation reactions - protecting the normal workings of the cell (Vickery, 2007).

1-2-5 Histidine in human milk

1-2-5-1 Combined His

Combined Histidine as mentioned in by (Darrel and Gaoeoyao,1994) is the His interacted with amino acids through the peptide bond to form protein or derivatives , so its effect differs as in free form.

Lactoferrin is one of the important protein in the ripe milk consist of The N-and C-terminal halves form two separate globular lobes, connected by a short a-helix, and carry one iron-binding site each. Each lobe has the same folding, based on two domains of similar supere condary structure, with their onsite at the domain interface. Each iron atom is coordinated by four protein ligands: two tyrosine, one His, and one aspartate (Knox et al., 2011).

1-2-5-2 Free His

Free His level in the ripe milk change significantly with increasing the lactation and different according to the geographical distribution (Zhang *et al*., 2013).

Free His converted to histamine by histidine decarboxylase. Pasteurization at 62.5 30 ºC min did not affect the levels of polyamines in human milk which are growth factors and protective substances. There was a significant increase on histamine levels as lactation progressed (Baldeon *et al*., 2014).

1-2-6 Antimicrobial activity of histidine

Histidine contains an imidazole side chain. The high therapeutic properties of the imidazole related therapies have been used in the medicinal chemists to synthesize a large number of new chemotherapeutics. anti-inflammatory, antibacterial, antifungal, antiviral, antitubercular, antidiabetic and antimalarial (Katritzky, 1984).

This group presents in azoles antifungal which inhibit the accumulation of methylated sterols destroy the composition of the lipid bilayer of membranes. Some imidazole drugs, at high concentrations, could exert direct inhibitory action on membranes, without interference with sterols and sterol esters (Ujjinamatada *et al*., 2007 ; Kumari *et al*., 2010).

Infectious microbial disease causes worldwide problem, because microbes have resisted prophylaxis or therapy longer than any other form of life. In recent decades, problems of multidrug-resistant microorganisms have reached an alarming level in many countries around the world. Resistance of anti-microbial agents such as β-lactam antibiotics, macrolides, quinolones and vancomycin etc. and different species of bacteria causes increased important global problem (Shingalapur *et al*., 2009).

Imidazole and its derivatives are reported to be physiologically and pharmacologically active and find applications in the treatment of several diseases. many compound contain His has antibacterial activity like Lactoferrin provides antibacterial activity to human infants. Lactoferrin interacts with DNA and RNA, polysaccharides and heparin, and shows some of its biological functions in complexes with these ligands (Levin *et al*., 2006).

Histidine-rich glycoprotein (HRGP), an abundant heparin-binding protein found in plasma and thrombocytes, exerts antibacterial effects against Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) (Rydengård, 2007).

1-2-7 Pathogenic bacteria

1-2-7-1 *Escherichia. coli*

It was first described by the German pediatrician Dr. Theodor Escherich in 1885 as *Bacterium coli* that had been isolated from the feces of a healthy infant (Sussman 1985; Bettelheim and Goldwater, 2014)

In clinical microbiology, *E. coli* has been the most commonly isolated facultative anaerobic Gram-negative rod in feces, and a common cause for intestinal and extra-intestinal infections. Some kinds of *E. coli* can cause

diarrhea, while others cause urinary tract infections, respiratory illness and pneumonia, and are also the main cause of neonatal men-ingitis in human and animals (Kaper *et al*. 2004).

Some straine of *E.coli* cause haemorrhagic colitis, and life-threatening complications like haemolytic uremic syndrome and thrombotic thrombo cyto penicpurpura may occur in haemorragic colitis patients (Rydengård *et al*., 2007)

1-2-7-1-1 Mechanism of action of antibiotics against *E. coli*

In this study was used three types of antibiotic according to the changing in response of *E.coli* in presence of His**.** The Levofloxacin belong to Fluoroquinolones groupe , class of antimicrobials interferes with the *E. coli* DNA gyrase, which relieves DNA supercoiling (Chambers, 1997) .

Maintenance of chromosomal topology by targeting DNA gyrase (topoisomerase II) and topoisomerase IV (topoIV), these enzymes at the DNA cleavage stage and preventing strand rejoining Despite the general functional similarities between topoIV and gyrase, the susceptibility of these targets to quinolone antibiotics varies across bacterial species. gyrase is the primary target and topo the secondary target of these drugs in Gram-negative bacteria (Kohanski *et al*.,2007).

The second type of antibiotic Rifampicin which belong to Rifampin groupe RP inhibits bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerase (Duhon *et al*, 2015) .

It binds to RNA polymerase at a site adjacent to the RNA polymerase active center and blocks RNA synthesis by physically blocking the formation of the phosphordiester bond in the RNA backbone, preventing extension of RNA products beyond a length of 2-3 nucleotides ("steric-occlusion" mechanism) (Campbell *et al*., 2001; Feklistov *et al*., 2008)

Rifampicine are considered bactericidal against Gram-positive bacteria and bacteriostatic against Gram- negative bacteria, a difference that has been attributed to drug uptake and not β subunit affinity.

1-2-7-2 *Staphylococcus. aureus*

S. aureus are spherical, approximately 1μ m in diameter, arranged in grape like clusters. They may be found singly, in pairs or in short chains especially in liquid culture. They are non-motile and non-sporing and some strains possess microscopically visible capsules (Anathanarayan 2002).

S. aureus is an important bacterial pathogen in the hospital and community settings, especially *Staph. aureus* clones that exhibit methicillin-resistance (MRSA). Many strains of Staph. aureus are utilized in the laboratory, underscoring the genetic differences inherent in clinical isolates. S. aureus grows quickly at 37°C with aeration in rich media (e.g. BHI) and exhibits a preference for glycolytic carbon sources. Furthermore, *S. aureus* has a gold pigmentation, exhibits β-hemolysis, and is catalase and coagulase positive. (Nicholas and Anthony, 2013)

S. aureus is the almost-universal cause off uruncles, carbuncles, and skin abscesses and world-wide is the most commonly identified agent responsible for skin and soft tissue infections. *S. aureus* skin and soft tissue infections frequently begin as minor boils or abscesses and may progress to severe infections involving muscle or bone and may disseminate to the lungs or heart valves (i.e., endocarditis) (McCaig *et al*., 2006)

1-2-7-2-1 Mechanism of action of antibiotics against *Staph. aureus*

In this research was used three types of antibiotic according to the changing in response Of *Staph. aureus* in presence of His.

The first type of antibiotics Levofloxacin belong to the Fluoroquinolones have sufficient activity against staphylococci to be considered for the treatment of serious infections by these organisms. The primary target of fluoroquinolones in Staphylococi is topoisomerase IV, which separates concatenated DNA strands(Chambers, 1997).

these class affected by interferes with the maintenance of chromosomal topology by targeting DNA gyrase (topoisomerase II) and topoisomerase IV (topoIV), these enzymes at the DNA cleavage stage and preventing strand rejoining Despite the general functional similarities between topoIV and gyrase, the susceptibility of these targets to quinolone antibiotics varies across bacterial species shown that topoIV is the primary target of quinolones in Gram-positive bacteria(Kohanski *et al*.,2007).

The second type of antibiotics Rifampicin which belong to Rifampin group has a potent bactericidal anti staphylococcal agent with MICs of 0.05 µg/ml or less. It blocks protein synthesis by inhibiting RNA polymerase .RP penetrates well into tissues and abscesses, which are poorly penetrated by most other anti staphylococcal agents. RP inhibits bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerase (Duhon, 2015) .

Rifampicin binds to RNA polymerase at a site adjacent to the RNA polymerase active center and blocks RNA synthesis by physically blocking the formation of the phosphordiester bond in the RNA backbone, preventing extension of RNA products beyond a length of 2-3 nucleotides ("steric-occlusion" mechanism) (Campbell *et al*., 2001 ; Feklistov*et al*., 2008).

Rifampicin are considered bactericidal against Gram-positive bacteria and bacteriostatic against Gram- negative bacteria, a difference that has been attributed to drug uptake and not β subunit affinity (Kohanski *et al*.,2007).

1-2-7-3 *Mycobacterium. tuberculosis*

Mycobacterium. tuberculosis is an obligate pathogenic bacterial species in the family Mycobacteriaceae and the causative agent of most cases of tuberculosis (Martinez *et al*., 1999).

First discovered in 1882 by Robert Koch, *M. tuberculosis* has an unusual, waxy coating on its cell surface (primarily due to the presence of mycolic acid), which makes the cells impervious to Gram staining; M. tuberculosis can appear Gram-negative and Gram-positive in clinical settings (Fu and Fu-Lin, 2002)

The Ziehl-Neelsen stain, or acid-fast stain, is used instead. The physiology of *M. tuberculosis* is highly aerobic and requires high levels of oxygen. Primarily a pathogen of the mammalian respiratory system, it infects the lungs. The most frequently used diagnostic methods for tuberculosis are the tuberculin skin test, acid-fast stain, and chest radiographs (Talip *et al*., 2013)

M. tuberculosis is an obligate aerobe. For this reason, in the classic case of tuberculosis, MTB complexes are always found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, a physiological characteristic that may contribute to its virulence.(WHO, 2004)

Two media are used to grow *M. tuberculosis* Middle brook's medium which is an agar based medium and Lowenstein-Jensen medium which is an egg based medium.

M. tuberculosis colonies are small and buff colored when grown on either medium. Both types of media contain inhibitors to keep contaminants from out-growing MT. It takes 4-6 weeks to get visual colonies on either type of media.(WHO,2004). *M. tuberculosis* are also called acid-fast bacilli . it is

resistant to drying and survive long periods in dried sputum; they keep their viability for five days at +4 ºc and for years at – 80 ºc (Belay *et al*., 2011).

For initial empiric treatment of TB, start patients on isoniazid, rifampin which used for the first four month of treatment. The mode of action of rifampicin in *M.tuberculosis* is by binding to the β-subunit of the RNA polymerase, inhibiting the elongation of messenger RNA The majority of rifampicin-resistant clinical isolates of M. tuberculosis harbor mutations in the rpoB gene that codes for the β-subunit of the RNA polymerase. As a result of this, conformational changes occur that decrease the affinity for the drug and results in the development of resistance

In about 96% of *M.tuberculosis* isolates resistant to rifampicin, there are mutations in the so-called―hot-spot region‖ of 81-bp spanning codons 507–533 of the rpoB gene. This region is also known as the rifampicin resistance-determining region. (Palomino and Martin, 2014)

monoresistance to rifampicin is quite rare and almost all rifampicin-resistant strains are also resistant to other drugs, especially to isoniazid. This is the reason why rifampicin resistance is considered as a surrogate marker for MDR-TB (Traore *et al*., 2000).

1-2-8 Plasmid curing

Plasmid is an extra chromosomal genetic material that occurs in most of bacterial strains. They are usually found in bacteria. Plasmid sizes vary from 1 to over 1,000kbp .Plasmid host-to-host transfer requires direct, mechanical transfer by conjugation or changes in host gene expression allowing the intentional uptake of the genetic material by transformation. One way of classify plasmids is by their ability to transfer to other bacteria. Another way to classify plasmids is by function. There are five main classes: FertilityF-plasmids,

Resistance (R) plasmids, Col plasmids, Degradative plasmids, Virulence plasmid (Pemberton and Schmidt,2001).

1-2-8-1 Physical agent

As mentioned by (Carlton and Brown, 1981) Physical agent such as elevated growth temperature is commonly used in bacteria plasmid curing. The mode of action of elevated growth temperature is through complete or partial deletions of strain's plasmid DNA. Elevated incubation temperature (5–7◦C) above the optimal growth temperature can be used as a curing method

1-2-8-2 Chemical agent

As mentioned by (Devi *et al*.,2009)Sodium Dodecyl Sulfate (SDS) is an anionic detergent that is used as a chemical curing agent in bacteria. Plasmid containing cells are possibly more sensitive to SDS because of plasmid-specified pili on cell surface. The chemical acts in dislodging the indigenous plasmid from its site of attachment

1-2-9 Mutagenesis of the bacteria

Is a process by which the genetic information of an organism is changed in a stable manner resulting in a mutation. It may occur spontaneously in nature or as a result of exposure to mutagens. It can also be achieved experimentally using laboratory procedures. Artificial UV/chemical mutagenesis can be used to introduce changes. This mutation method mimics the natural process of evolution, but the library sizes are relatively small number of changes is generally small (Paul and Leemor, 2007).

1-2-9-1 Physical mutagens

Any agent that damages DNA can in principle lead either to the death of that organism or amongst the survivors to mutation. This is true of irradiation as

well as chemical agents. Many types of physical mutagen have been used to generate mutation. The higher energy rays such as X-rays however require expensive apparatus and safety equipment and are not really suitable for routine use in microbiology laboratory. In addition, they produce an excessive amount of chromosomal damage that is not easily repaired by the microorganism. Ultraviolet irradiation on the other hand is easily controlled (although eye and skin protection is necessary and requires only comparatively in expensive equipment (Mitra, 1996).

The principle effect of UV irradiation is the production of pyrimidine dimmers (commonly referred to thymine dimmers). Where two pyrimidine residues are adjacent on the same DNA strand, the result of UV irradiation is the creation of covalent links between them. These pyrimidine dimmers cannot be replicated and are therefore lethal to cell unless it is able to repair the damage. It is the attempts to repair the damage caused by ultraviolet irradiation that can led to mutagenic effects. Although most repair mechanism are reasonably accurate (error- free repair), in the event of these mechanism being unable to cope with damage an additional defense comes to play (Jeremy and simon 2004).

1-2-9-2 Chemical mutagens

The natural rate of spontaneous mutation is much too low for convenient isolation of most types of mutants (a part from a handful of easily selected mutations such as antibiotic resistance). Ways must be found for enhancing that frequency. It is often possible to use in vitro mutagenesis or transposon mutagenesis, but there are still many situations where chemical or physical procedures are preferred or essential. Many different chemical agent interact with DNA or the replication machinery as to produce alterations in the DNA sequence. Of these simplest to understand are those agent that act by chemically modifying abase on the DNA so that it resembles a different base

(Abd-Allah *et al*., 2010). Alkaliting agent such as Acridine orange which is florescent dye used to diffrentation between DNA and RNA (Ultzur and Weiser, 1981), and it used as chemical mutagens acting by removing or adding nucleotide to the DNA these due to change the codons of nucleic acid and these due to change the amino acid which due to change the structure of protein and its function (Arshad *et al*., 2006).

Acridine orange was used recently for treatment of cancer because of its ability to stopped the replication of nucleic acid(Onyenwe *et al*., 2011)

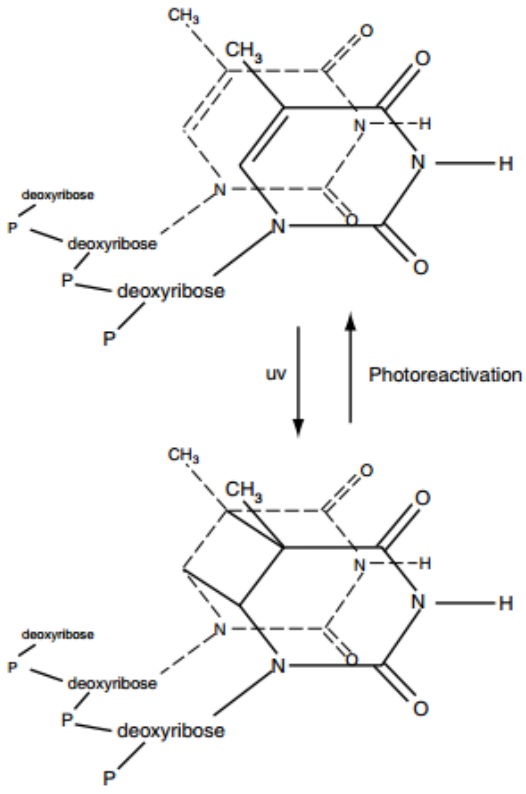


Figure (1.3): structure of thymidine dimmers (Jeremy and Simon, 2004)

Chapter Two

Materials and Methods

2- Materials and Methods

2-1 Materials

2-1-1 Instruments and tools

Equipment and apparatus used in this study as follow:

Apparatus	**Company**	**Origin**
Amino acid analyzer	Agilent	Germany
Autoclave	Express	Germany
Balance (analytical)	Sartorius	Germany
Balance (sensitive)	Ohaus	Germany
Camera **(**digital**)**	Sony	Japan
Centrifuge (cooling)	Harrier	UK
Compound light Microscope	Olympus	Japan
Freezer (chest)	GFL	Germany
FTIR	Bruker	Germany
Gel Molecular Imager	Bio Rad	USA
High Speed Cooling Centrifuge	Eppendrof	Germany
Hood	ESCO	USA
HPLC	Agilent	Germany
Incubator (cooling)	Sanyo	Japan
Magnetic stirrers	SCO tech	India
Nanodrop	Agilent	USA
pH meter	Hana	Italy
Refrigerator	Concord	Lebanon
UV - Transilluminator	Ultraviolet	USA
UV-Visible Spectrophotometer	BUCK	USA

Vortex	Bohemia	Czech
Water bath	Memmert	Germany
Water distillatory	GFL	Germany

2-1-2 Chemicals

Chemicals	**Company**	**Origin**
Acridine orange dye	Fluka	Switzerland
Ammonium chloride (NH4Cl)		
Calcium chloride ($CaCl_2$)		
Hydrochloric acid (HCl)		
L-Histidine hydrochloride (His)	Fluka	
L-Asparagine	Fluka	
Magnesium citrate ($C_6H_6MgO_7$)		
Magnesium sulfate ($MgSO_4$)		
O-pthaldialdehyde (OPA)	Agilent	Germany
Perchloric acid ($HClO_4$)	Scharlau	Spain
Potassium phosphate (KH_2PO_4)		
Potassium carbonate (K_2CO_3)	Scharlau	
Sodium Phosphate (Na_2HPO_4)	BDH	England
Sodium chloride (NaCl)		
Sodium nitrate ($NaNO_3$)	Fluka	

2-1-3 Cultural media

2-1-3-1 Ready to use media

These media were prepared and autoclaved at 121 ^{o}C for 15 min as referred by manufactured companies:

Medium	Company	Origin
Agar agar	Hi-media	India
Eosin methylene blue agar (EMB)		
Mannitol salt agar (MSA)		
Mueller hintone agar (MHA)	Lab	England
MacConkey agar (MA)		
Nutrient agar (NA)	Difco	USA
Nutrient broth(NB)		

2-1-3-2 Laboratory prepared media

a- Minimal medium (M9) media: (Davis *et al*.,1986)This media composed of:

1- M9 salts: composed of the following ingredients:

Ingredients	Weight (g/l)
Na_2HPO_4	5.8
KH_2PO_4	3g
NaCl	20
NH_4Cl	0.5
D.W	Complete to 1L

2-

Ingredient	**Molarity %**	**Per 1L**
Glucose	40%	10ml
$CaCl_2$	1mM	1ml
Histidine	0.3%	5ml
$MgSO_4$	1M	1ml
Yeast extract	1%	10ml
Agar agar	2%	

The ingredients (1) mixed with the ingredients (2) and then autoclaved. Glucose and His were sterilized separately by Millipore filter (0.22 µm), then added to the medium after cooling to 50 °C. The medium was poured in sterile Petridishes .

B- Lowenstein-Jensen medium (LJ media)(WHO,2004)

This medium was used for cultivation of *M. tuberculosis*, which is composed of the following ingredients :

Ingredient	**Weight (gm)**
MgSO4	0.12
$C_6H_6MgO_7$	0.3
KH_2PO_4	1.2
L-Asparagine	1.8
Glycerol	6

The above ingredients were dissolved in 300 ml D.W and autoclaved. After cooling, the following ingredients two were added:

Malachite green 2% in DW.	10ml
Fresh egg components mixed by a sterilized mixer then filtered through a sterile piece of cloth and the volume of the filtrate was measured by sterile graduated cylinder	500ml

Five ml from the complete medium was distributed into sterilized screw cupped tube, then put in oven at 82 ^{o}C for 2 and half hours.

2-1-4 Kits

Kit	**Company**	**Origin**
DNA extraction Kit	Promega	USA
Gram stain kit	CDH	India
Maxwell 16 Tissue DNA purification kit	Promega	
Rapid anti -*M. tuberculosis* test kit	INTEC	China

2.1.5 Antibiotics Discs (MAST company, UK).

Antibiotic (µg/disc)		
Amoxicillin A(10)	Erythromycin E (15)	Tetracycline T(30)
Ciprofloxacin CIP(5)	Levofloxacin LEV(5)	Rifampicin Rp(5)
Clarithromycin CLA(15)	Metronidazole MZ(5)	Cloxacilline Cx(5)
Streptomycin S (10)	Penicilline P(10)	Chloramphenicol C(30)
Vancomycine Va (30)	Gentamycin Gm(120)	Nalidixic acid Na(30)

2-1-6 Solutions and buffers

2-1-6-1 Standard solutions and buffers

- **Physiological normal saline:** (Collee *et al*., 1996)

It was prepared by dissolving 0.85 g of NaCl in 100 ml D.W. and sterilized by autoclaving.

- **McFarland standard solution($OD_{600\ nm}$):**(Benson, 2001)

Solution	**Quantity/ Volume**
Solution A	
Barium chloride ($BaCl_2$)	1g
D.W	100 ml
Solution B	
Concentrated sulphuric acid(98%)	1ml
D.W	Completed to100
Macfarland No. 0.5	
Solution A	0.05 ml
Solution B	9.95 ml

Solutions A and B were mixed by vortex for 2 min and stored in dark bottle until used.

- **Tris-EDTA buffer (TE buffer):** (Sambrook and Russell, 2001)

It was prepared by disolving Tris-OH(0.05M) and EDTA(0.001M) in 1L D.W . After adjusting pH to 8 the buffer was NaOH solution and sterilized by autoclaving and stored at 4˚C until used.

- **Acridine orange:** (Bettenbork *et al*.,1999)

It was prepared by dissolving 17 g of dye in 1L D.W.

2-1-6-2Electrophoresis solutions and buffers

- **Tris-Borate-EDTA buffer 10X (TBE buffer 10X):** (Sambrook and Russell, 2001)

Tris-OH	0.089 M
Boric acid	0.089 M
EDTA	0.02

The pH of buffer was adjusted to 8 before autoclaving .

- **Ethidium bromide solution (10mg/ml):** (Sambrook and Russell, 2001)

It was prepared by dissolving 0.2 g of ethidium bromide in 20 ml distilled water and mixed by magnetic stirrers for 4 hrs to disolve ethidium bromide, then it was filtrated, and stored in to a dark bottle at 4 °C, until use.

2-1-7 Stains

Gram stain: (Atlas *et al*., 1995)

All the bacterial isolates were examined after stained with Gram stain to examine cells shape, grouping, gram reaction microscopically. Ready to use kit.

2-1-8 Sterilization techniques

- Autoclave was use to autoclaving the media, physiological solutions and others at 121ºC (15Ib/inch^2)for 15 min. unless otherwise stated.
- Electrical oven was used to sterilize glassware at 80ºC for 3hr.
- Filtration sterilization technique by using sterilized Millipore filter (0.22 µm to filtrated the His and 0.45 µm to filtrated the sugars.

2-1-9 Storage strategies

2-1-9-1 Short term

The bacterial cultures were stored for days to few weeks in the refrigerators by taking single pure and active colony of each and growing in the recommended medium at 37ºC for 24hr before maintaining in agar plate or broth at 4ºC the cultures were renewed periodically.

2-1-9-2 Mid term

Tubes that contained 8 ml of sterile nutrient agar in slants position were inoculated with the bacterial isolate, and then incubated at 37 Cº overnight, before stored at 4 Cº.

2-1-9-3 Long term storage

A test tube contained 10 ml of sterile brain heart infusion broth inoculated with a single colony of *Klebsiella* and incubated at 37 Cº for 24 hrs., then 8.5 ml of the cell suspension was mixed with 1.5 ml of glycerol (15 %), and stored for long time at -20 C°.

2-2 Methods

2-2-1 Collection of milk samples

Twenty five colostrum samples were collected from mothers during the first week after childbirth from Al-Alwyia Public Hospital Maternity / Baghdad. It was also collected 13 samples of natural milk-feeding were collected during the first six months of breastfeeding from the children health care centers

deployed in Baghdad. Part of milk samples were stored in the chest freezer at -40 ^{o}C, while those in use were freeze - dried.

2-2-2 Detection of free and combined Histidine (Darrel and Gaoeoyao, 1994)

2-2-2-1 Sample preparation

The procedure was conducted in the Environmental and Water Treatment Center/ Ministry of Sciences and Technology, as followe:

a - Free His

- Five ml of milk sample was centrifuged at 3000 rpm for 10 min to remove fat and mononuclear cells.

- One ml of defatted milk was deproteinized for 5 min with 1.5 mol /l $HClO_4$.

- The supernatant was neutralized with 0.5 ml of 2 mol /l K_2CO_3.

- The neutralized supernatant was diluted 20 times with HPLC – water grade and immediately used in the determination free His .

b- Combined His

-The precipitate (protein pellet) from above step was washed 3 times with 10 ml deionized water and dried in atmosphere.

- The dried precipitate was dissolved in 5 ml of 5 mol /lHCl at 110^{o}C for 24 hr under nitrogen.

-The hydrolyzed protein was diluted to 0.2 ml with deionized water, then diluted to 2 ml with HPLC – grade water .

2-2-2-2 HPLC analysis

The fluorometric HPLC method was used which involved pre-column derivatization with O – phthaldialdehyde (OPA) as follow:

- Separation process was performed on a suplco 3- µ m reversed – phase C18 column(4.6 x 150 mm) graduated by a suplco 40 µm reversed phaseC18 column (4.6 x 50 mm) and quantified with aid of amino acid standards (Ajilant chemicals)
- Ethanolamine was used as the internal standard for protein hydrolysis.
- External standard calibration method was used for the analysis of free amino acid because Ethanolamine present in milk.
- His concentration was calculated in mg/l.

2-2-3 Physical treatments of Hisidine

2-2-3-1- UV radiation

A portion of 10 mg of His was disolved in 1ml D.W and exposed to the UV radiation using UV – Transilluminator (Ultraviolet) at 256_{nm} for periods of (5 min,10 min, 30 min, 1hr and 1.5hr) separately in aseptic cabinet. The samples were analyzed by Fourier Transform Infrared spectroscopy (FTIR) in Chemistry Department., College of Science , Al-Nahrian university.

2-2-3-2 Microwave radiation

A portion of 10 mg of His was disolved in 1ml D.W and exposed to the microwave radiation for 1 and 2 min separately **.** The samples were analyzed by FTIR in Chemistry Department ., College of Science , Al-Nahrian university.

2-2-4 Activation of bacterial isolates

Escherichia. coli and *Staphylococcus. aureus* isolates were obtained from Isolates Bank of College of applied biotechnology / Al-Nahrian University (*E. coli* from UTI infection and *S. aureus* from the burn infection).The isolate were activated in enrichment and selective media (Nutrient agar, MacConky agar, Eosin methylene blue agar and mannitol salt agar).These isolates were maintained in and

plates and slants of nutrient agar for short and mid-term storage and were reactivated periodically in nutrient broth.

2-2-5 Preparation of bacterial suspension

Bacterial suspension was prepared by centrifugation of NB activated cultures at 5000 rpm for 15 min. The supernatant was discarded while the precipitate was resuspended in normal saline and washed twice before adjusting the optical density of suspension was to 0.5 by using spectrophotometer at 600_{nm}.

2-2-6 Histidine medium

This medium was prepared by adding the His solution and by using two techniques as follow:

a- A volume of 25 ml of molten Mueller Hinton agar was inoculated with 1ml of isolate cells suspension and poured into plates. The wells of 0.6 mm diameter were made by crock borer. Serial concentrations of His (0.0-1 mg/ml and 0-10 mg/ml were added to the wells and incubated at 37^{o}C for 24hr .

b- Serial concentrations of His 0-1 mg/ml and 0-10 mg/ml of Mueller hinton agar were prepared and distributed in 25 ml in universal screw cupped tubes. The media were autoclaved at and poured in plates. The plate were inoculated with 0.1 ml of cells suspension of the isolates by spreading technique and incubated at the same conditions**.**

2-2-7 Antimicrobial sensitivity test (Barry, 1976) :

Many antimicrobial agents were selected for each isolates according to their resistance and sensitivity. Table (2.1) illustrates the antimicrobial agents discs used in the experiments. *E.coli* and *S. aureus* isolates were subjected to the antibiotics listed in table (2.1) depending the isolates resistancy or sensitivity against these antibiotics.

Table 2.1: Antibiotics used against *E. coli* and *S. aureus* isolates (Clsi, 2014).

Bacterial isolate	**Antibiotics**	
	Bacterial resistance	**Bacterial sensitive**
E. coli	Vancomycine Streptomycin Metronidazole Cloxacilline Rifampicin Penicillin	Tetracycline Levofloxacin Ciprofloxacin Nalidixic acid
Staph. aureus	Metronidazole Cloxacilline Clarithromycin Rifampicin Tetracycline	Vancomycine Gentamycin Levofloxacin Ciprofloxacin Chloramphenicol

The antibiotics test were conduct as follow:

a- Petri dish contained 25ml of Mueller-Hinton agar were inoculated with isolate cell suspension by swabbing , then left 10 min. Antibiotic discs were dispersed over the agar surface and incubated at the same conditions.

b- Histidine media (0 -10 mg/ml) as mentioned in 2-2-4,b were inoculated with 0.1ml of cells suspension by spreading then dispersed antibiotic discs according to the table(2.1) for each isolates.

2-2-8 Genomic profile

2-2-8-1 Extraction of DNA and electrophoresing (Total genomic profile)

Maxwell ® 16 Tissue DNA Purification Kit and Maxwell device for genomic DNA extraction were used. Active and pure colonies of bacterial

isolates were selected from nutrient agar culture and the DNA was extracted in Maxwell device according to the directions of the manufactured company. The purity of DNA extract was determined by nanodrop device.

Gel electrophoresis of DNA extracts was conducted as follow (Maniatis *et al.*, 1982):

- Preparation of Agarose l (2%): It was prepared by dissolving 2gm of agarose in 100 ml of TBE buffer (1X) in a conical flask by heating on hot plate magnetic stirrers. After the solution was cooled to 60ºC, 2µl of ethidium bromides was added from the stock solution and mixed thoroughly.
- The clean glass tank (17×12×4 cm) was set on a horizontal section of the bench. The comb was set in position 0.5-1.0 mm above the surface of the tank so that a complete well was formed when agarose was added.
- The molten agarose was poured into the tank.
- After the gel was completely set (20-30 min at room temperature), the comp was carefully removed and the gel mounted in the electrophoresis tank which contain previously small amount of 1X TBE buffer.
- A volume of 600 ml of 1X TBE was added to cover the gel to a depth of about 1 mm.
- A volume of 10 µl of the DNA was added slowly into the slots of the submerged gel using an automatic micropipette.
- A volume of 5 µl of DNA marker was mixed with 2 µl of loading buffer. The mixture was set slowly into the slots of the submerged gel using an automatic micropipette.
- The gel was subjected to electrophoresis at 70 volts until the bromophenol blue tracking dye, migrated at least two-thirds of the way down the gel.
- The gel was examined by UV transilluminator then photographed.
- DNA bands were visualized using the molecular imager device.

2-2-9 Plasmid Curing (Ahmed *et al.*, 2011)

Curing experiments were conducted by two methods:

Method one- Physical treatment :Thermal treatment was conducted as follow:

- One loopfull from active culture of bacteria was inoculated 5 ml of nutrient broth and incubated for 18h at 37 ^{o}C. After incubation , cultures were exposed to the thermal treatment at 55 ^{o}C for 10 min in water bath.
- 0.1ml of the cultures was spreading on nutrient agar and incubated at the same conditions.
- Sensitivity test was conducted by dispersing three types of antibiotic discs selected previously from the experiments for each isolate on Mueller Hintone agar.4- 0.1 ml of the thermal treated cultures was spreading on Mueller Hintone agar contains His (10 mg/ml)and sensitivity tests performed with selected antibiotics discs as above .All cultures incubated at the same conditions.
- DNA extraction and profile were conducted for thermal treated cultures as mentioned in (2-2-8).

Method two- Chemical treatment with SDS

Nutrient broth was mixed with SDS 1.5% and autoclaved at 121 ^{o}C for 15 min. After cooling, it was inoculated with 1% of active culture from each isolate and incubated at the same conditions. Subsequent steps were conducted as mentioned in 2-2-7.

2-2-10 Mutation experiments

2-2-10-1 Mutation with physical mutagens (Kidambi *et al*.,1996)

Ultra violet radiation at 254_{nm} was used to radiate 5 ml of cells suspension of normal and cured isolates in normal saline for 30 and 60 sec at constant distance of 11 cm. These steps were repeated with the cells suspension

contains 10 mg/ml of His. 0.1ml of treated suspension was spreading on Mueller Hintone agar. Selected antibiotic discs for each isolate were distributed and incubated under same conditions.

2-2-10-2 Mutation with chemical mutagen (Bttenbork *et al*., 1999)

Acridine orange solution was used to conduct the chemical mutation on the cells suspension of the isolates (normal and cured) in presence and absence the His. 0.1 ml of acridine solution was added to 5 ml suspension and incubated for (0, 30 and 60) min at 37°C., 0.1 ml from the treated suspension was spreaded on Mueller Hinton agar and the selected antibiotics were dispersed and incubated under same conditions.

2-2-10-3 Histidine biosynthesis

After physical and chemical mutation experiments(2-2-10-1 and 2-2-10-2) , the suspension of isolates were washed and centrifuged (5000 rpm for 10 min) twice with normal saline to remove the His and debris. 0.1 ml of the washed suspension was spreaded on M9 agar medium in presence and absence of His. The plates were lift to stand for 15 min and incubated under the same conditions.

2-2-11 Response of *M. tuberculosis* (TB) to the Histidine

Responsing of TB to His was conducted in TB institute , Ministry of Health of Iraqi. The following test were performed by using the standard steps recommended by the World Health Organization (WHO,2004):
Colony morphology, pigment production, acid fast stain , rate of growth, niacin test, nitrate reduction test.

2-2-11-1 Culture medium

The ideal medium for isolation of tubercle bacilli is the Lowenstein-Jensen medium (LJ media). Two concentrations of His (5 and 10 mg / ml) of

the recommended LJ-medium: LJ now was used to examine the sensitivity of TB isolates that previously identified as sensitive and resistance to the RP (Rifampicin)in comparison with the control treatment (without His but contains RP).

2-2-11-2 Inoculum preparation

A representative sample of approximately 4-5 mg was taken from the primary culture by a loop and placed in McCartney bottle containing 1ml sterile distilled water. Contents of the bottle were mixing for 20-30 seconds before 4-5 ml of sterile distilled water were added with continuous mixing. Then the suspension were transfered carefully to another clear sterile McCartney bottle. Opacity of the bacterial culture was then adjusted by the addition of DW to obtain a concentration of 1mg/ml of tubercle bacilli by matching with McFarland stand0ard .(These steps were performed with the assistant of TB institute staff). Rifampicin (RP) 40µg/ml, as a specific antibiotic for *M.tuberculosis,* was added to the medium by sterile Millipore filter (0.45micron). The cultures were incubated at 37 $^{\circ}$C for 30 days.

Chapter Three

Results and Discussion

3-Results and Discussion

3-1 Histidine of mother colostrum and milk sample

3-1-1 Free Histidine

Table 3.1 shows the concentrations of free His in some randomly selected samples from colostrum and ripe milk. The average of free His concentration in the colostrum was 222 mg / l, which is more than four times than that of ripe milk 51 mg/l. These results confirm the healthy importance of free His in colostrum on for the infants in the first days of birth as an antimicrobial agent and also for the safety of colostrum. It seems that the average concentration of His in the colostrum samples for Iraqi lactating women was much higher than mentioned in the scientific references .The available references found that the concentrations of free His in colostrum did not exceed the range of 3.2–4.1 mg/l and which was not change substantially during the lactation period from women in different areas of Japan (Yamawaki *et al*. 2005).

Table(3.1): **Free histidine concentration in mother colostrum and ripe milk samples.**

Sample No.	Concentration of free Histidine (mg/l)	
	Colostrum	**Ripe milk**
1	47.2	78.63
2	90.2	71.47
3	114.7	34.02
4	710.9	42.2
5	150.2	33.11
Average	**222.64**	**51.88**

3-1-2 Combined histidine

Table (3.2) shows the approximate concentrations of combined His in colostrum and milk samples. It was observed that the average concentration of combined His in the colostrum was 94 mg/l which higher than that in milk (88mg/l) . Baldeón *et al* (2014) recorded lower than this average when is decreased to 29 mg/l.

Table (3.2): Combined histidine concentrations of mother colostrum and ripe milk samples.

Sample No.	**Combined Histidine (mg/l)**	
	Colostrum	**Milk**
1	75.4	42.8
2	110.6	259.4
3	47.8	45.3
4	62.8	32.4
5	177.3	64.5
Average	**94.78**	**88.88**

The results of high concentrations of free His in the Iraqi women breastfeeding may be belong to either normal hereditary or to relationship of genetics with the surrounding environment, and the need for immunity, and this requires a study.

3-2 Physical treatment of Histidine

3-2-1 Ultraviolet Radiation

Figure (3.1, a, b, c, d, e, f) shows the results of FTIR analysis for the pure purchased His after exposing to the UV radiation at 256_{nm} for (0, 5, 10, 30 min ,1 and 1.5 hr). It is clear from the figure that no change occurred on

chemical structure and active groups of His after each exposure time compared with control treatment. The free His used in an aqueous solution demonstrates the stability and resistance to UV radiation. These results insure the importance of His in maintaining and protecting the biomaterials from the effect of UV- radiation of the far wavelength. Moreover, no possibility for the condensation or polymerization of His exposed to UV radiation .

Synthesis of polyhistidine or condensation requires special chemical and physical treatments that cannot be performed in our laboratories, in addition to the fact that polyhistidine substance is highly expensive.

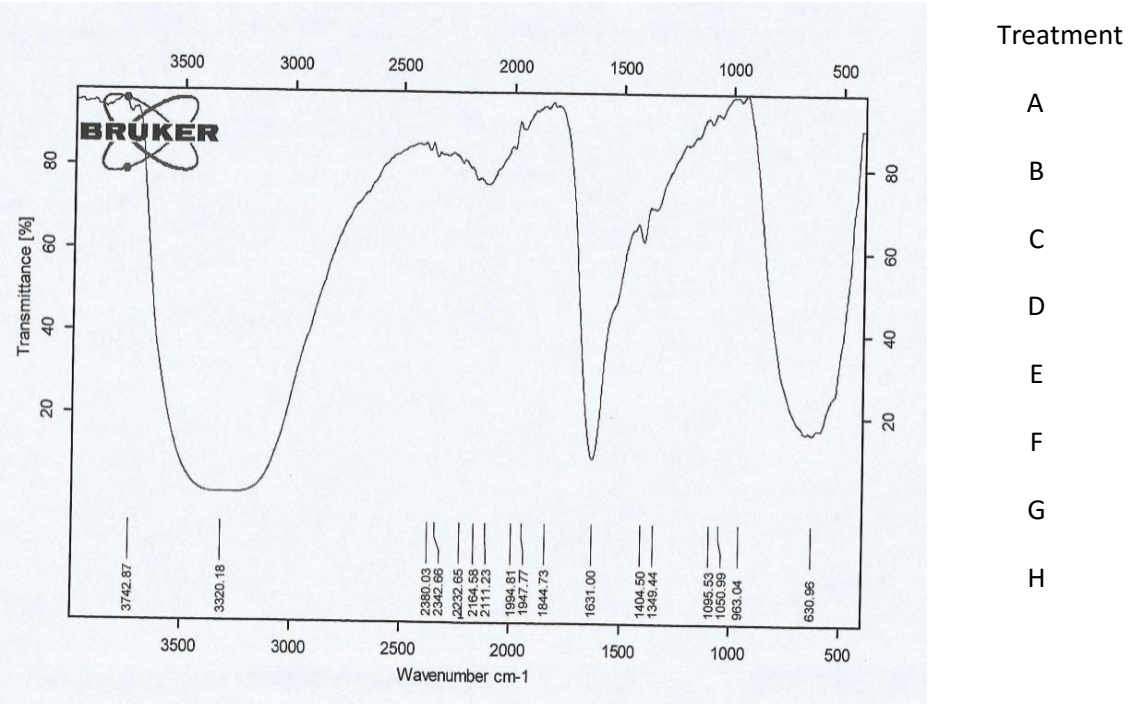


Figure (3.1): FTIR graphic for Histidine solution after exposed to UV radiation and microwave radiation (A: 0 time for UV and microwave , B: 5 min UV, C: 10 min UV, D: 30 min UV, E: 1 hr UV, F: 1.5hr UV, G: 1min microwave and H: 2 min microwave).

3-2-2 Microwave radiation

Figure (3.1 a, g, h) shows the results of FTIR for His solution after analysis of the samples that exposed to the microwave radiation for (0, 1, 2) min. Result show that His also did not suffer any chemical change in the structure and in active groups. The results of exposing the His to UV and microwave are conserved a new observation in the supporting the protection systems for the milk and human by the ability of this amino acid to resist these radiations .

3-3 Susceptibility of pathogenic bacteria against antibiotic

Table (3.3) contains the results of susceptibility of *E.coli* and *S. aureus* against sensitive and resistant antibiotics included in the study. There are matching with those of the standards mentioned in (Clsi, 2014) for both bacteria. Each isolates was highly sensitive against CIP and LEV but highly resistant to MZ , CX and VA. *E. coli* was moderate resistant against RP While *S. aureus* was complete resistant.

3-4 Antibacterial capability of Histidine

3-4-1 Histidine

When Huayong *et al* (2014) tested some strains of staphylococcal groups and *E. coli*, histidine kinases domain was found to poses bactericidal activity.

Result show that no effects was recorded on the use of His in concentrations 0-1 mg / ml and 1-10 mg / ml of Mueller Hintone agar by using wells and mixing techniques on the growth of *E.coli* and *S. aureus*. This may be belong to resistance of the local isolates to His.

Table(3.3): Susceptibility of pathogenic bacterial isolate toward antibiotics .

Antibiotics	Inhibition zone (mm)					
	E.coli			*Staph. aureus*		
	Local isolate	Standard*		Local isolate	Standard	
		S	R		S	R
Streptomycin(S10)	**14 (R)**	≥15	≤ 11			
Rifampicin (RP5)	**8 (R)**	≥ 12		**0 (R)**	≥ 17	≤ 19
Metronidazole(MZ5)	**0 (R)**			**0 (R)**		
Cloxacilline (CX5)	**0 (R)**			**0 (R)**		
Levofloxacin (LEV5)	**44 (S)**	≥ 37	≤ 29	**40 (S)**	≥ 16	≤ 18
Ciprofloxacin (CIP5)	**35 (S)**	≥ 21	≤ 15	**22 (S)**	≥ 21	≤ 15
Tetracycline (T30)	**24 (S)**	≥ 15	≤ 11	**10 (R)**	≥ 19	≤ 14
Clarithromycin(CLA15)				**0 (R)**	≥ 18	≤ 13
Nalidixic acid (NA30)	**28 (S)**	≥ 19	≤ 18			
Penicillin (P10)	**16 (R)**	≥ 17	≤ 24			
Vancomycin (VA30)	**14 (R)**	≥ 17	≤	**10 (R)**	≥2	≤16
Gentamicin (GM120)				**30 (S)**	≥15	≤12
Chloramphenicol(C30)				**22 (S)**	≥18	≤12

* **(Clsi, 2014)**

3-4-2 Histidine combined with antibiotics

3-4-2-1 Against *E.coli*

Table (3.4) shows results of susceptibility the sensitivity test of *E.coli* toward the antibiotic in presence of His at concentrations 1 to 10 mg/ml in comparison with the control treatment. The control treatment showed that sensitivity of *E. coli* use sensitive toward LEV, CIP, T, VA, S and NA and resistance to MZ, CX and P as mentioned in table (3.3).

There was no effect of His at different concentrations on the response of *E. coli* toward (VA, S, MZ, CX, NA and P).These antibiotic are not belonged to only one group. Instead they belong to different antibiotics.

Different hypothesises of antagonistic and synergistic behavior of His to interact with antibiotics or with physiological and genetic components of the bacterial cells, could be used to interpret the results.

Nicolas *et al* (2013) found that the sensitivity of isolates against RP5 and T30 was increased but reduced toward LEV and CIP in presence of 1 mg/ml of His and more**.** RP and T antibiotics are belong to different groups, while LEV and CIP return to the same group fluoroquinolones.

The quinolone class of antibiotics interferes with the maintenance of chromosomal topology by targeting DNA gyrase (topoisomerase II) and topoisomerase IV (topoIV). These enzymes at the DNA cleavage stage and preventing strand rejoining Despite the general functional similarities between topoIV and gyrase, the susceptibility of these targets to quinolone antibiotics varies across bacterial species. gyrase is the primary target and topo the secondary target of these drugs in Gram-negative bacteria (Kohanski *et al*.,2007).

Rifampicin inhibits bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerase (The American Society of Health-System Pharmacists, 2015) .

It binds to RNA polymerase at a site adjacent to the RNA polymerase active center and blocks RNA synthesis by physically blocking the formation of the phosphordiester bond in the RNA backbone, preventing extension of RNA products beyond a length of 2-3 nucleotides ("steric-occlusion" mechanism) (Campbell *et al*., 2001;

RP are considered bactericidal against Gram-positive bacteria and bacteriostatic against Gram- negative bacteria, a difference that has been attributed to drug uptake and not β subunit affinity (Feklistov *et al*., 2008).

Three types of antibiotics were selected for the subsequent studies for *E. coli* according to their changing in response in presence of Histidine . These are :

Increases in sensitivity: RP

Decreases in sensitivity: LEV

No change in sensitivity: CX (Resistant)

Changes in the sensitivity in table (3.4) has been showed for selected antibiotics where the 5 mg/ml for RP and 7 mg/ml for LEV and no change for CX sensitivity at all concentrations of **His.**

3-4-2-2 Against *S. aureus*

Table (3.5) shows result susceptibility test of *Staph. aureus* against antimicrobial agents in presence of His concentrations of 1 to 10 mg/ml in comparison with the control treatment (without His). In the control treatments *S. aureus* was sensitive toward G, LEV, VA, CIP, T, C and resistant to MZ, CX, CLA, RP according to standard values of antibiotics (Clsi, 2014).

The sensitivity varied in presence of His. It increased for RP (> 3mg/ml), T (>1 mg/ml) and decrease for LEV (>1mg/ml) and GM (>1 mg/ml).Antibiotics belong to different groups. So, same interpretations were applying as mentioned above for *E. coli* on the behavior of His with antibiotics.

Interpretation change of susceptibility toward the antibiotics may be due to influence of synergistic or antagonistic of His with antibiotics.

Table (3.4):Effect of the different concentrations of histidine on susceptibility of *E. coli* .

Antimicrobial Agent	Concentration of Histidine (mg/ml) / Diameter of Inhibition zone (mm)										
	Control (0)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Streptomycin(S10)	14 (R)	**22 S**	20	20	19	20	20	18	17	14	12
Rifampicin (RP5)	8(R)	**11**	12	11	12	**13 S**	13	13	13	14	18
Metronidazole(MZ5)	0 (R)	0	0	0	0	0	0	0	0	0	0
Cloxacilline (CX5)	**0 (R)**	0	0	0	0	0	0	0	0	0	0
Levofloxacin (LEV5)	44 (S)	**35 (S)**	40	36	40	37	31	26 **R**	22	23	24
Ciprofloxacin (CIP5)	35 (S)	**25 (S)**	25	23	26	20	22	25	24	24	24
Tetracycline (T30)	24 (S)	**35 (S)**	30	28	25	32	30	30	27	30	32
Nalidixic acid (NA30)	28 (S)	24	28	28	30	20	20	22	25	26	25
Penicillin (P10)	16 (R)	14	14	13	14	13	12	13	13	13	13
Vancomycin (VA30)	14 (R)	14	14	13	14	12	13	14	12	13	14

LEV, which belong to the quinolone group of antibiotics is affected by interfering with the maintenance of chromosomal topology by targeting DNA gyrase (topoisomerase II) and topoisomerase IV (topoIV). These enzymes at the DNA cleavage stage and preventing strand rejoining Despite the general functional similarities between topoIV and gyrase. The susceptibility of these targets to quinolone antibiotics varies across bacterial species declare that topoIV is the primary target of quinolones in Gram-positive bacteria(Kohanski *et al*., 2007).

Rifampicin inhibits bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerase.

Rifampicin binds to RNA polymerase at a site adjacent to the RNA polymerase active center and blocks RNA synthesis by physically blocking the formation of the phosphordiester bond in the RNA backbone, preventing extension of RNA products beyond a length of 2-3 nucleotides ("steric-occlusion" mechanism) (Campbell *et al*., 2001).

Rifampicin is considered as a bactericide against Gram-positive bacteria and bacteriostatic against Gram- negative bacteria, a difference that has been attributed to drug uptake and not β subunit affinity (Kohanski *et al*.,2010)..

Three types of antibiotics were selected for subsequence studies according to the changing in response Of *Staph. aureus* in presence of **His** :

a- increasing in sensitivity : Rp
b- Decrease in sensitivity: LEV
c- No change in sensitivity: CX

3-5 Genomic profile

The aim of this experiment was to lay a foundation for comparison the experiences of use the His under different conditions of curing and mutation and then monitoring the sensitivity tests against the selected antibiotics as indicators. Figure(3.2) shows the genomic profile of *E. coli* and *Staph. aureus* isolates. There are 5 bands for each isolate in addition to the ladder lane (100- 1000bp).

For *E. coli* isolate, the first lane represents the control treatment without any treatment. This lane and another contain two bands of plasmids (200 and 500bp).

The presence of His alone or with selected antibiotics had no effect on bands intensity and distant or may be lost .

S. aureus genome did not show any plasmid bands. This result is differed from that obtained by Kennedy *et al* (2010) on the 300 clinical isolates obtained from different geographic locations in the United States. All isolates tested contained small (2- to 3-kb) and/or large (27- to 30-kb) plasmids. The large plasmids encoded heavy metal and/or antimicrobial resistance elements.

Table (3.5): **Effect of the different concentrations of histidine on susceptibility of *S. aureus.***

Antimicrobial agent	**Concentration of Histidine (mg/ml)** / **Diameter of Inhibition zone (mm)**										
	Control (0)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Metronidazole (MZ5)	0 (R)	0	0	0	0	0	0	0	0	0	0
Vancomycin (VA30)	10(R)	10	10	10	12	10	10	10	10	10	10
Cloxacillin (CX5)	**0 (R)**	0	0	0	0	0	0	0	0	0	0
Clarithromycin (CLA 15)	0 (R)	0	0	0	0	0	0	0	0	0	0
Rifampicin (RP5)	0 (R)	0	0	**8**	12	15	**16S**	15	16	15	16
Gentamicin (GM120)	30(S)	30	27	30	27	**20 S**	20	23	20	23	20
Levofloxacin(LEV5)	40(S)	35	40	35	**30**	**25 R**	25	27	30	25	30
Ciprofloxacin (CIP5)	22(S)	22	24	26	28	26	25	26	25	24	26
Tetracycline (T30)	10 (R)	13	14	14	14	13	**15S**	15	15	15	15
Chloramphenicol(C30)	22(S)	21	21	21	21	21	21	21	21	21	21

3-6 Curing treatments

The Curing treatments were aimed to study the role of presence and absence of **His** in isolated cultures under the effect of physical and chemical curing agents on plasmids stability . It also for studying the antimicrobial response of isolates against the selected antibiotics after curing treatment .

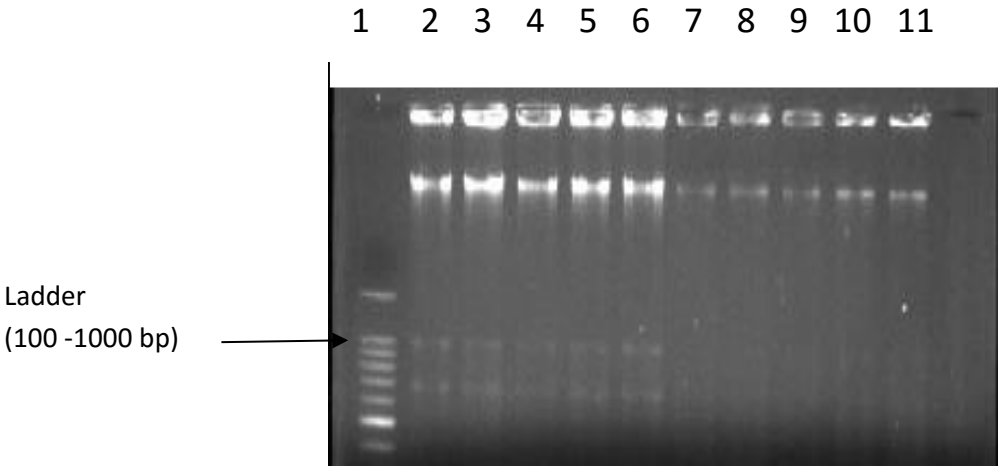


Figure (3.2): **Genomic profile of *E .coli* and *Staph. aureus* isolates. (From the left Lanes: 1, ladder(100-1000 bp); Lane2, *E. coli* (-His) ; lane3, *E. coli* (+His) ;lane4 *E. coli* + RP ; lane5 *E .coli* +LEV; lane 6, *E .coli* + CX ; lane7, *Staph. aureus* (-His); lane 8, *Staph. aureus* (+His) ; lane 9, *Staph. aureus* + RP; lane10, *Staph. aureus* + LEV;Lane11, *Staph. aureus* +CX , (Agarose, 2%,100 V and 70 minutes).**

3-6-1 Physical curing by heating

3-6-1-1 *E. coli*

Figure (3.3) illustrates the results of physical curing by heating (2-2-9). It is clear from this figure that curing was achieved by heating and no plasmid bands were appeared under the presence or absence of His.

The sensitivity test in table (3.6) shows no change in the inhibition zones before and after heat treatment. So, this result declares the resistant is carried on the chromosome not on the plasmid.

Table (3.6) : Effect of histidine on antibiotic susceptibility of *E. coli* exposed to heat curing .

Antibiotic *E.coli*	Inhibition zone (mm)				
	Standard	(-) His		(+) His (10mg/ml)	
		Control	Heat	Control	Heat
LEV	29- 37	44 (S)	42 (S)	24 (R)	30 (I)
RP	12	8 (R)	12 (I)	**18 (S)**	**12 (S)**
CX	0	0 (R)	0 (R)	0 (R)	0 (R)

3-6-1-2 *S. aureus*

Figure (3.4) illustrates that the effect of heating was not as curing but may be mutagenic due to the absence of plasmid.

Table (3.7) shows that the heat had effect on the sensitivity of the bacteria against the RP in the absence of His . It appeared that the effect of His was contradictory, since the absence of His and exposing the bacteria to thermal treatment resulted in increasing the sensitivity against RP and turned from resistant into sensitive . But in the presence of His and exposing to the heat , it became resistant to the RP.

These results indicate that presence of His under heat treatment lead to change the bacteria to the natural phenotype of resistance to the RP (control without any treatment). This may be related to the reverse mutation by heating with the interaction of His or to the His in prevention the penetration of RP inside the cells**.**

Table (3.7): Effect of histidine on antibiotic susceptibility of *S. aureus* exposed to heat curing.

Antibiotic	**Inhibition zone (mm)**				
	Standard	**(-) His**		**(+) His (10mg/ml)**	
		Control	**Heat**	**Control**	**Heat**
LEV	16-18	**40 (S)**	**45 (S)**	**30 (S)**	**35 (S)**
RP	17-19	**0 (R)**	**15 (R)**	**16 (S)**	**0 (R)**
CX	0	0 (R)	0 (R)	0 (R)	0 (R)

3-6-2 Chemical curing by SDS

3-6-2-1 *E. coli*

Figure (3.4) illustrate the results of physical curing by SDS. It is clear from this figure that curing of plasmids has succeeded with SDS and no plasmid bands found under the presence or absence of the His.

Sensitivity test in table (3.8) shows no change in the inhibition zone before and after treatment with SDS in the absence of His. So, this result proved that the resistance was carried on the chromosome and not on the plasmid. But in presence of His , change in the sensitivity of the bacterium obtained upon exposure to SDS where the sensitivity decrease to LEV and increased to RP compared with the control treatment with the presence of His.

These results may explain the role of His in the bacterial culture which has restored sensitivity to LEV by the presence of SDS. It may be also interpreted to reveres mutation to sensitive (control - His) . No change was found in resistance against to the CX for all treatments.

Table (3.8): Effect of histidine on antibiotic susceptibility of *E. coli* exposed to chemical curing agent SDS.

Antibiotic	**Inhibition zone (mm)**				
	Standard	**(-) His**		**(+) His (10mg/ml)**	
		Control	**SDS**	**Control**	**SDS**
LEV	29- 37	**44 (S)**	37 (S)	**24 (R**)	**38 (S)**
RP	12	**8 (R)**	10 (R)	**18 (S)**	**20 (S)**
CX	0	0 (R)	0	0	0

3-6-2-2 *S. aureus*

Figures (3.4) show the results of treatment *S. aureus* with SDS in presence and absence of His. It was found that SDS not just play a curing agent, but may be mutagenic agent because the absence of plasmid in bacteria. So, the effect may be at the genetic level or on the physiological level.

Table (3.9) shows that the effect of SDS was clear on the sensitivity of the bacteria against the RP in the absence of His. It was appeared that His have a contradictory effects, since the absence of His and exposing the bacteria to SDS treatment resulted in decreasing the resistance against RP and turned into high resistance in presence of His and treatment with SDS. This contradiction in the behavior of His under chemical curing conditions for bacteria and contrasting in sensitivity to RP need in-depth studies to determine the mechanics of the work and the interaction of His and identify the genetic variations and physiological sources. No change in responses of bacteria to the CX under curing conditions.

Table (3.9): Effect of histidine on antibiotic susceptibility of *S. aureus* exposed to chemical curing agent SDS.

Antibiotic	Inhibition zone (mm)				
	Standard	(-) His		(+) His (10mg/ml)	
		Control	SDS	Control	SDS
LEV	16-18	40(S)	35(S)	30	28(S)
RP	17-19	**0 (R)**	**11(R)**	**16(R)**	**0(R)**
CX	0 (R)	0	0	0	0

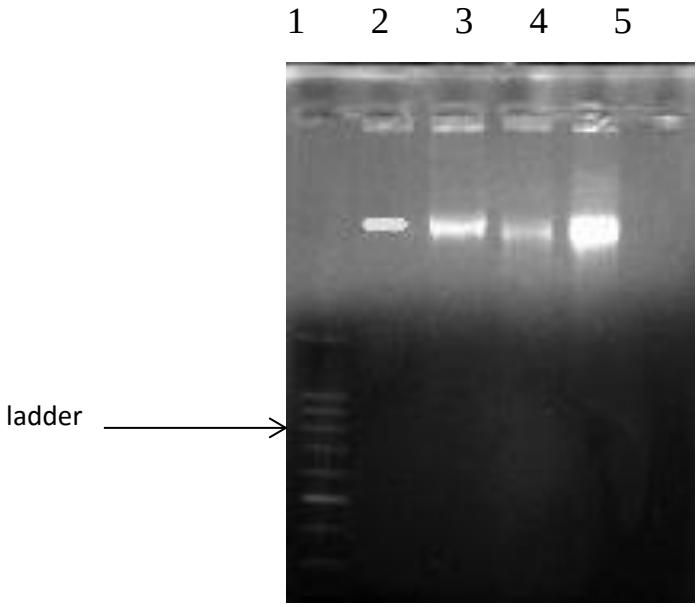


Figure (3.3): Genomic profile of *E. coli* and *Staph. aureus* isolates after physical curing by heating. Lanes: 1, Ladder ; 2 and 3 *E. coli*; 4 and 5 *Staph. aureus* (Agarose, 2%, 100 V and 70 minutes)

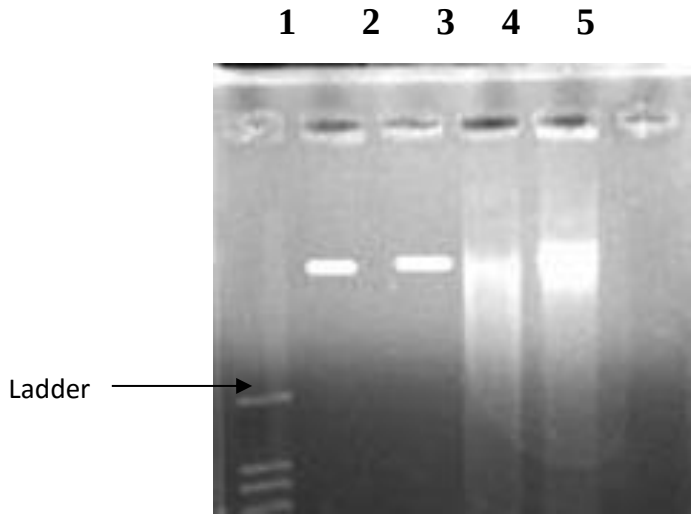


Figure (3.4): Genomic profile of *E. coli* and *Staph. aureus* isolates after chemical curing by SDS. Lanes : 1,Ladder; 2and 3 *E. coli*,4and5 *Staph. aureus*(Agarose, 2%, 100 V and 70 minutes).

3-7 Mutagenesis of bacterial isolate

Mutagenesis was aimed to investigate the behavior of His on the cells suspension of bacterial isolates after exposing to the UV radiation and evaluating their susceptibility toward the selected antibiotics. Natural and curing of *E.coli* and *S. aureus* were used in these experiments .

3-7-1 Mutagenesis with physical mutagens

3-7-1-1 Against *E. coli*

Table (3.10) shows the effect of exposure the *E. coli* suspension to the UV-radiation at 254nm for different periods (30 and 60sec) in presence and absence of His (10mg/ml). Based on previous results on the His resistance to ultraviolet radiation after exposure for a period of 1.5 hr and without the occurrence of any change in the structure or in the active groups, it has been used in exposing the bacterial suspension to UV- radiation.

It seems that the isolates became more resistant when exposed to radiation for 60 seconds in the presence and the absence of His compared to 30 seconds exposure to LEV, RP. The sensitivity was increased against CX in presence of His when no much different from exposure treatment for 30

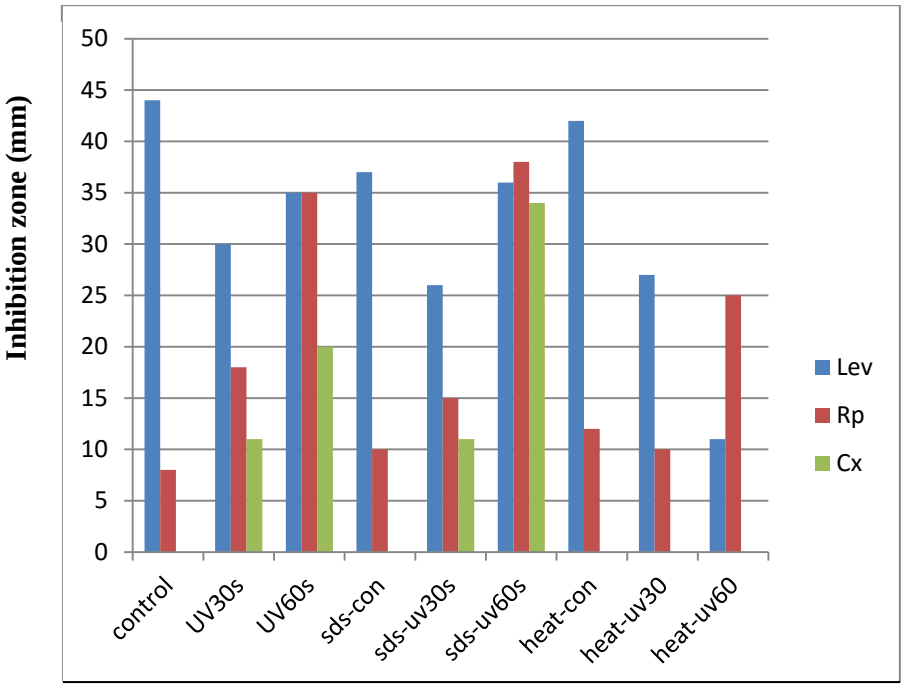
seconds was recorded. The explanation for this result may be related to the clear difference in the behavior of isolates toward antibiotics in presence and the absence of His after exposed to ultraviolet mutagenesis. However, this behavior did not seem that much difference from that of exposure to curing agents(Arshad, 2010).

From results illustrated in figures(3.5 and 3.6) it can be concluded that isolates of *E.coli* cured by physically and chemically agents showed same behavior of sensitivity and resistance to antibiotics when exposed for a period of 30 seconds to UV irradiation, but with little change when exposed for a period of 60 seconds. This demonstrates that changes have occurred in the chromosomes or internal pathways or transport systems of the material across cell membrane.

Table (3.10): Effect UV- radiation on antibiotic susceptibility of *E. coli* in presence and absence of Histidine.

Antibiotic	Inhibition zone (mm)				
	Control	UV 254 $_{nm}$ for 30 Sec		UV 254$_{nm}$ for 60 Sec	
		(-)His	+ (His)	(-)His	+ (His)
LEV	44 (S) (St= (29-37)*	40 (S)	30 (I)	**44 (S)**	35 (I)
RP	8 (R) (St=12)	**15 (S)**	**18 (S)**	**20 (S)**	**35 (S)**
CX	0 (R)	0 (R)	11	0 (R)	20

*** St: Standard inhibition zone(Clsi, 2014)**



Figure(3.5): comparesion susceptibility response *E.coli* to the selected antibiotics in absence of Histidine at the different treatments .

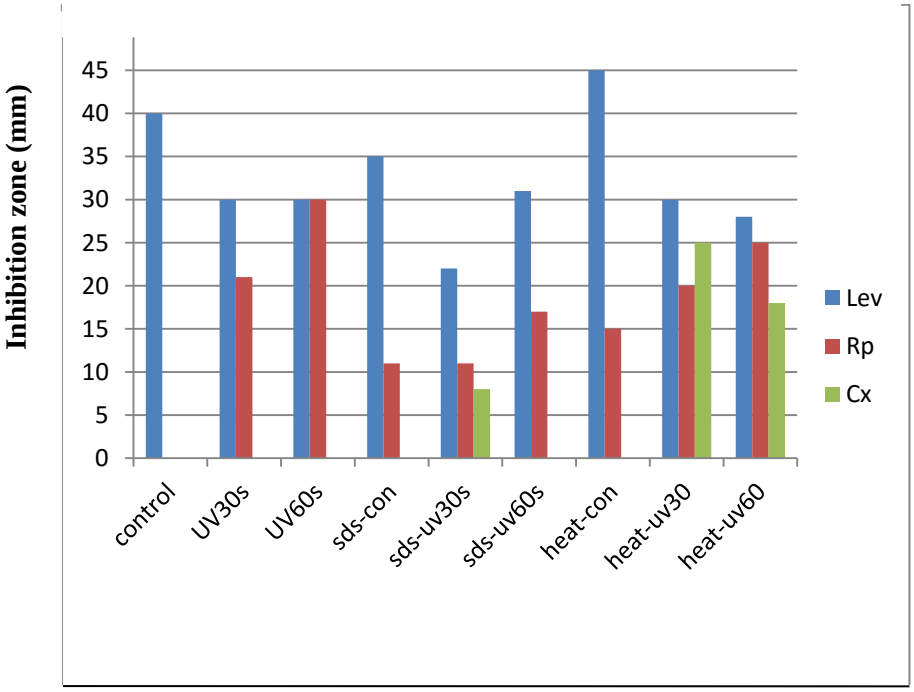


Figure (3.6) : Comparesion of susceptibility response *E.coli* to the selected antibiotics in presence of Histidine at the different treatments (+HIS)

Table (3.11) illustrates the behavior of *S. aureus* when exposed to UV-radiation for 30 and 60 Second. It seems that isolate more resistant when exposed to radiation for 60 seconds in the presence and the absence of His compared to exposure for 30 seconds against LEV, RP and increasing the sensitivity against CX in presence the His where the results did not much different from exposure treatment for 30 seconds. The explanation for this result may be indicated to the clear difference in the behavior of isolate toward antibiotics in presence and the absence of His when exposed to ultraviolet mutagenesis and this behavior does not seem that much different from the exposure to curing agents.

As it noted from the figures(3.7 and 3.8) that the cured isolates of *Staph. aureus* by physically and chemically curing agent showed the same cases of sensitivity and resistance to antibiotics when exposed for a period of 30 seconds to UV irradiation with little change when exposed for a period of 60 seconds. This demonstrates that changes have occurred in the chromosomes or internal pathways or transport systems of the material across cell membrane.

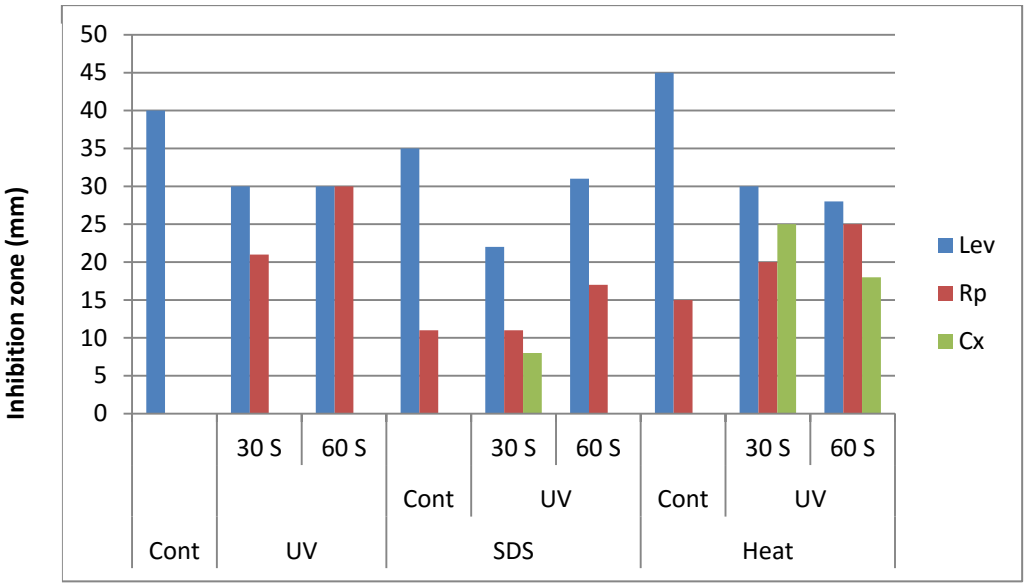
3-8 Histidine biosynthesis

The result of mutation experiment showed that the mutagenesis by physical (UV- Radiation at 254 nm at different times) and chemical Acridine orange. The mutagenic agent had no influence on His biosynthesis in each isolates. The growth was similar in M9 media in presence and absence of His. So the genetic or physical level of His biosynthesis were not appeared to be influenced by these mutagenic agent**s.**

Table (3.11): Effect UV- radiation on antibiotic susceptibility of *S. aureus* in presence and absence of Histidine.

Antibiotic	Inhibition zone (mm)				
	Control	UV 254 nm for 30 Sec		UV 254nm for 60 Sec	
		(-)His	+ (His)	(-)His	+ (His)
LEV	40 (S) St(16-18)*	30 (S)	32 (S)	30 (S)	**35 (S)**
RP	16 (R) S(17-19)	**21 (S)**	**35 (S)**	**30 (S)**	**30 (S)**
CX	0 (R) St (0)	0 (R)	**28 (S)**	0 (R)	30

St : Standard inhibition zone (Clsi, 2014)



Figure(3.7):Comparesion of susceptibility response *Staph. aureus* to the selected antibiotics in presence of Histidine at the different treatments (+HIS)

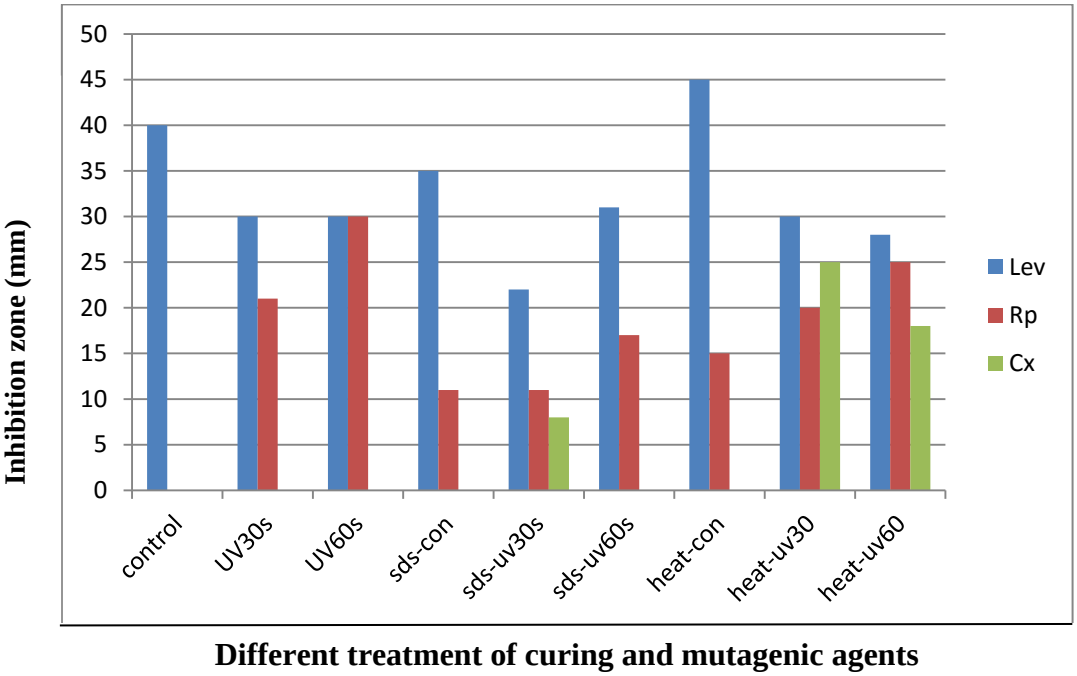


Figure (3.8): Comparesion of susceptibility response *Staph. aureus* to the selected antibiotics in absence of Histidine at the different treatments .

3-9 Effect of Histidine on response of TB

Results of tables (3.4 and 3.5), declared that sensitivity of *E.coli* and *S. aureus* toward RP antibiotic. In presence of low concentrations of His (>1 and >3 mg/ml, respectively) was increased. RP is a broad-spectrum antibiotic that is used in the therapy of many infectious diseases, in particular, tuberculosis. In this research two concentration of His (5 and 10 mg/ml) were mixed with RP. While results shows that the sample which was sensitive to RP still sensitive, while samples resistant to RP were converted to sensitive.

The mode of action of rifampicin in *M.tuberculosis* is by binding to the β-subunit of the RNA polymerase, inhibiting the elongation of messenger RNA The majority of rifampicin-resistant clinical isolates of *M.tuberculosis* harbor mutations in the rpoB gene that codes for the β-subunit of the RNA polymerase. As a result of this, conformational changes occur that decrease the affinity for the drug and results in the development of resistance

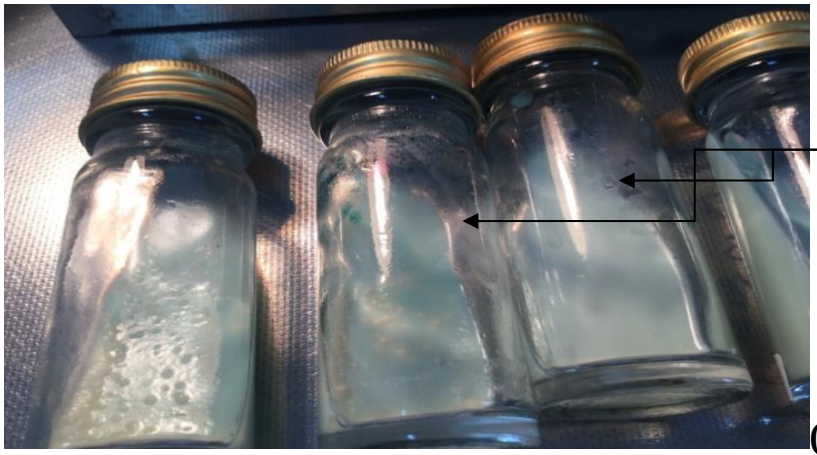
In about 96% of *M.tuberculosis* isolates resistant to rifampicin, there were mutations in the so-called―hot-spot region‖ of 81-bp spanning codons 507–533 of the rpoB gene. This region is also known as the rifampicin resistance-determining region. (Palomino and Martin, 2014)

The monoresistance to rifampicin is quite rare and almost all rifampicin-resistant strains are also resistant to other drugs, especially to isoniazid(an anti-TB agent). This is the reason why rifampicin resistance is considered as a surrogate marker for MDR-TB (Traore *et al*., 2000).

Table (3.12) :Susceptibility of local isolates *Mycobacterium. tuberculosis* to RP in presence of His.

Sample +RP(40µg/ml)	**RP(40µg/ml) + His (5mg/ml)**	**RP(40µg/ml) + His (10mg/ml)**
S	S	S
R	S	S

(a)



(b)

Control **RP** **RP+5mg/ml His** **RP +10mg/ml His**

Figure (3.9 a,b): Growth of *Mycobacterium tuberculosis* in LJ-medium in presence of Histidine. a: Rifampicin sensitive isolate ; b : Rifampicin resistant isolate.

Chapter Four

Conclusions and Recommendations

4-1 Conclusions

1- Concentrations rate of free and combined histidine in the samples of Iraqi mother's milk were increased in comparison with references.

2- Concentrations rate of the free histidine in colostrum increased four times the concentration of combined histidine and the concentrations in the natural milk of the mother in the breast-feeding.

3- Resistance of histidine to the short wavelength of UV and high frequencies of microwave.

4- Histidine was found to be not effective as antibacterial agents against *E.coli* and *S. aureus*.

5- Variation of histidine behaviors in increase the sensitivity or resistance of *Staph. aureus* and *E.coli* bacteria pathological life to antibiotics under natural and artificial conditions of chemical and physical curing and mutagenic agents.

6- Increase the sensitivity of bacterial isolates against the Rifampicin and resistance to the Livofloxacine and is not affected the resistance against to Cloxacilline in presences of **His**.

7- Rp- resistant isolates of *M. tuberculosis* were turned to sensitive in presence of His.

4-2 Recommendations

1- Using modified methods of His measurement in milk and other liquid.

2- Advanced molecular and biochemical studies to determine the target effect of histidine inside and outside the cell.

3- Molecular studies of His effect on the resistance of TB.

4- Conducting an instrumental analysis studies to find the interactions of the histidine with the antibiotics and, in particular, rifampicin and its relationship to change the sensitivity of the tuberculosis bacteria.

5- determine the mechanics of the work and the interaction of His and identify the genetic variations and physiological sources.

6- *In vivo* studies to complete the production of an effectively histidine-rifampicin against resistant tuberculosis.

7- Study the effect of the histidine on anti-mutations and cancers therapies.

8- Using the histidine and their polymers in the synthesis of protective material against radiation.

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الخلاصة

انجزت الدراسة الحالية لمعرفة سلوكيات الهستدين تجاه نوعين من البكتريا المرضية عند تعرضها لظروف مختلفة وتأثير ذلك على حساسيتها لبعض المضادات الحياتية ذات العلاقة. تم تحوير طريقة تقدير الهستدين الحر والمرتبط في عينات اللبأ و الحليب الطبيعي للام العراقية المرضعة وذلك بنقل عمود الفصل من جهاز Amino Acid Analyser (AAA) الى جهاز High Performance Liquid Chromatography (HPLC) الذي يحتوي على متحسس فلورسنت. اظهرت نتائج التحليلات ارتفاع معدلات تراكيز الهستدين الحر الى الضعف في اللبأ ليصل الى 222 مقارنة بالمرتبط الذي بلغ 94 ملغم/لتر. ولم تظهر اية فروقات ملحوظة بين الهستدين الحر والمتربط في الحليب حيث كان معدلي تركيزيها 51 و 88 ملغم /لتر على التوالي ، كما وظهر ان الهستدين الحر في اللبأ قد بلغ اربع اضعاف الهستدين الحر في الحليب العادي. وبينت نتائج الدراسة مدى تأثير التعرض للأشعة فوق البنفسجية (254نانومتر) وترددات الموجات الدقيقة العالية على تركيب الهستدين بأستعمال تحليل FTIR عدم تاثر تركيب الهستدين ومجاميعه الفعالة اذ لم تتحقق البلمرة او تكثيف الحامض بهذه الطريقة المتيسرة. وبينت النتائج عدم تحسس البكتريا المرضية *Staph. aureus* و*E. coli* للهستدين كمضاد حياتي بتراكيز وصلت الى 10ملغم /مل. لوحظ ان وجود الهستدين في الوسط الزرعي ادى الى حصول تغيير كبير في تحسس العزلات للمضادات الحياتية. فقد ادى الهستدين الى حصول توافق في زيادة حساسية العزلات للمضاد الحياتي الريفامبسين بينما انخفضت ضد الـ ليفوفلوكساسين والجنتامايسين و السبروفلوكساسين ولم تتغير ضد الستربتومايسين والميترانيدازول والكلوكساسلين والتتراسايكلين و النالدكسك اسيد والبنسلين والفانكومايسين والكلاريثرومايسين والكلولرمفينيكول. بينت نتائج النسق الوراثي وجود حزم لبلازميدات صغيرة الحجم الجزيئي في *E. coli* وعدم احتواء *Staph. aureus* على بلازميدات. بينت تجارب التحييد الكيميائي والفيزيائي بوجود وعدم وجود الهستدين حصول تغيير في حساسية الـ *Staph. aureus* للمضادات الحياتية المنتخبة بالرغم من عدم احتواءها على بلازميدات وكذلك بالنسبة للـ *E.coli* التي اظهرت تغييرات بوجود وعدم وجود الهستدين وهذا يدلل على ان التغييرات كانت على مستوى الكروموسوم ان كان التأثير في الجانب الوراثي. انحصر دور هذه التفاعلات بين الحامض الاميني مع الكائنات الحية الدقيقة. وأظهرت النتائج أن *E.coli* تحولت إلى حساسية ضد الريفامبسين والكلوكساسلين ومقاومة ضد ليفوفلوكساسين. عكس الحامض الاميني سلوكيات غير متجانسة كبيرة، بعد خلطه مع عالق العزلات والتعريض للمطفر الفيزيائي (UV على طول موجي 254نانومتر)، في العلاقة مع المضادات الحياتية المختارة (Rp ، Lev و Cx). انحصر دور هذه التفاعلات بين الحامض الاميني مع الكائنات الحية الدقيقة. أظهرت النتائج أن *E. coli* قد تحولت إلى

حساسة ضد الريفامبسين والكلوكساسلين ومقاومة لليفوفلوكساسين. تحولت المكورات العنقودية *Staph. Aureus* الى اكثر حساسية ضد الريفامبسين والكلوكساسلين في حين لم تتغير مع اليفوفلوكساسين. وقد استثمر دور الهستدين في فحص العزلات الحساسة والمقاومة لبكتريا السل (TB) ضد الريفامبسين في مركز الصحة الصدرية والتنفسية في بغداد. وأظهرت النتائج أن وجود الهتسدين بتراكيز5 و 10 ملغم / مل حولت عزلات بكتريا السل من المقاوم للريفامبسين (40 مايكروغرام /مل) إلى حساسة. هذه النتائج لها تأثير كبير في تجنيب المرضى من المستويات اللاحقة من المضادات الحياتية عالية الخطورة.

الإهداء

إلى من كنت اتمنى وجودهم بقربي أبي .... جدي... جدتي رحمهم الله

إلى البساطة والطيب...إلى جنتي في الأرض... امي حفظها الله

الى الهبة التي وهبها الله لي الى من شجعني في كل خطوة اخطوها...

زوجي الغالي طه

الى احبائي ..و قرة عيني .. أخواتي .. دانية، شيماء، مها ، ندى و فرح

الى فلذة كبدي ... ونوري عيني ابنتي نوال

الى أحبتي ... خوالي وخالاتي و عماتي

الى اللاتي كن عونا في كل المراحل صديقاتي واخواتي زينب ، صابرين ، تمارا ، ريم ، فرح ، بيادر، ميس ، رشا، مياسة ، نور، ريام ، نهى ، شهد وغفران .

اهدي ثمرة جهدي المتواضع

فاطمة

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

تأثيرات الهستدين المركب المضاد للبكتريا والطفرات

رسالة
مقدمة الى كليه العلوم/ جامعه النهرين
كجزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل
فاطمه أحمد عبد الجبار
بكالوريوس في علوم الحياة والاحياء المجهرية/كلية العلوم/جامعة ديالى
2006

إشراف
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