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Genetic study on virulence factors and cytotoxicity for *Aeromonas hydrophila* isolates

A thesis

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by

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Hind

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List of Abbreviation

Abbreviations	Full name
ATCC	American Type Culture Collection
Api	Analytical Profile Index
EUS	epizootic ulcerative syndrome
AML	acute myelogenous leukemia
ELISA	Enzyme-linked immonosorbent assay
BSA	Bovine Serum Albumin
bp	Base pair
CHO	*Chinese hamster ovary*
DNA	Deoxyribonucleic acid
ECPs	Extracellular products
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
HepG2 cell line	human liver carcinoma cell line
Kb	Kelo base
LPS	lipopolysaccharide
nm	Nanometer
MMC	Mitomycin C
MDR	multiple-drug resistant
MAS	motile aeromonad septicaemia
Mabs	Monoclonal antibodies
OMPs	outer-membrane proteins
pH	Power of Hydrogen

PBS	Phosphate buffered saline
PCR	*Polymerase Chain Reaction*
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
RPMI-medium	Roswell Park Memorial Institute-
R-plasmid	Resistant plasmid
Spp.	Species
TCBS	Thiosulphate citrate bile sucrose
T3SS	type III secretion system
TNF	Tumor necrosis factor
TBE	Tris-Borate-EDTA
TCA	Trichloroacetic acid
Tris-base	Tris (hydroxymethyl)aminomethane base
WTA	wall teichoic acid

Summary

For the isolation of *Aeromonas hydrophila,* one hundred and twenty samples were collected from different locations in Baghdad governorate. These samples include 80 samples of fish gills and intestine, 20 samples of water, and 20 samples of chicken and meat. From the overall samples a total of 136 isolates were obtained. These isolates were further identified according to their morphological, microscopical characteristic and biochemical tests. Results of identification showed that twelve of these isolates were belonged to *A.hydrophila*. These results were confirmed by identification using Api20E system.

Several virulence factors in local isolates of *A.hydrophila* were investigated, which were includes haemolytic activity, slime production and protease production. Results showed that these isolates of *A. hydrophila* were able to produce haemolysin type β, and have the ability of congo red uptake and produce viscous slime around their colonies in addition to protease production. Among these isolate, *A. hydrophila* H4 was the most virulent because of its high ability in production of these virulence factors and was selected for farther study.

Antibiotic susceptibility of *A. hydrophila* H4 against different antibiotics was examined. Results showed that this isolate was resisting to penicillin, ampicillin, amoxicillin, cefepime, ciphradine, cephalothin and cefotaxime, while it was sensitive to other antibiotics.

Plasmid profile of *A.hydrophila* H4 was studied by extraction of plasmid DNA according to salting out procedure. Results showed that this isolate harboring two plasmids conferring to cefotaxime and cephradine resistance, according to the curing results by using SDS.

Cytotoxic effect of *A. hydrophila* H4 crude extracts and partially purified extracts against HepG2 tumor cell line were investigated. Results

showed that both crude extract and partially purified extract have an inhibitory effect against HepG2 cell line after incubation for 72 hours. Inhibitory effect was increased with the increase of crude filtrates concentration, and the maximum inhibitory effect was reached 80% and 70% at wave length of 450 and 492nm respectively for crude extract of *A.hydrophila* at a concentration of 57.5 mg/ml then decreased gradually with the increase of crude extract concentration, while the maximum inhibitory effect for the partially purified extract of *A. htdrophila* was 55% and 60% at wave length of 450 and 492 nm respectively at a concentration of 20 mg/ml, then inhibitory effect was also decreased with the increase of the partially purified extract concentration.

1-1 Introduction:

Genus *Aeromonas* has undergone a number of taxonomic and nomenclature revisions over the past 15 years. Although originally placed in the family *Vibrionaceae*. The subsequent phylogenetic investigations indicated that the genus *Aeromonas* is not closely related to vibrios and necessitated the removal of *Aeromonas* from the family *Vibrionaceae* and transfer to a new family, the *Aeromonadaceae* (Abulhamd, 2009).

A. hydrophila are Gram-negative, facultative anaerobic bacteria that can be isolated from many sources, such as food, drinking water, sewage, environmental water and human clinical samples with a world-wide distribution. These bacteria can develop in refrigeration temperatures and are responsible for food and water-borne diseases that can cause a range of human diseases that vary in severity from a self-limiting gastroenteritis to potentially fatal septicemia (Tsai *et al*., 2006).

The family *Aeromonadaceae* shares many biochemical characteristics with members of the *Enterobacteriaceae* family, from which they are primarily differentiated by being oxidase-positive (Janda and Duffey, 1988).

Among all species of *Aeromonas*, *A. hydrophila* is the most studied due to its presence in food (Radu *et al.,* 2003), water (Asmat and Gires, 2002), estuary (Odeyemi *et al.*, 2012), antibiotic resistance and it ability to cause infections in human and animals (Evangelista-Barreto *et al.*, 2010). *A. hydrophila* has been identified as causative agent of human diseases such as septicemia, meningitis, wound infections as a result of exposure to contaminated marine environment and diarrhea (*Odeyemi et al. ,2012*).

A. hydrophila inhabit a wide variety of sources and has been implicated in a variety of infections in humans such as gastroenteritis, wound infections, septicemia and occasionally others including urinary tract infection, meningitis, and peritonitis. *A. hydrophila* is capable of expressing a number of virulence factors such as haemolysin, aerolysin, cytotoxin, enterotoxin, cytotonic enterotoxin, endotoxin lipopolysaccharide, outer membrane proteins and enzymes such as proteases, lipases, DNases, elastase and gelatinase (Seethalakshmi *et al*., 2010).

In addition, exoenzymes such as proteases have been implicated as virulence factors, as species deficient in proteases show reduced virulence. It has been shown that proteases are also required for the activation of other virulence factors such as aerolysin which form pores in the cell membrane leading to cell lysis (Gillespie and Hawkey, 2006).

The pathogenicity of aeromonads is attributed to a series of factors, including cell structural lipopolysaccharides (LPS), outer-membrane proteins (OMPs), pili and flagella, a type III secretion system (T3SS) acting as adhesion structures, and extracellular factors such as enzymes and toxins (Ottaviani *et al*., 2011). Aerolysin is a representative virulence factor of *Aeromonas* and was reported to function as hemolysins and cytolytic enterotoxins (Aberoum and jooyandeh, 2010).

Although plasmids are reported to be present in several fish pathogenic bacteria, their role in the expression of virulence factors and also in the pathogenicity of *Aeromonas* is poorly understood. *Aeromonas* strains are known to harbor stable plasmids which play an important role in antimicrobial multiple-drug resistant (MDR) (Majumdar *et al*., 2007). *Aeromonas* spp. can possess a conjugative plasmid that confers multiple antibiotic resistances (Chang and Bolton, 1987).

According to these mentioned above, this study was aimed to:

1- Isolation and identification of *Aeromonas hydrophila* from different aquatic and meat samples.
2- Detection of the virulence factors produced by the bacterial isolates and selection the most virulent isolate.
3- Study the plasmid profile of the most virulent isolate.
4- Curing of plasmid DNA of the selected isolate.
5- Investigation the responsibility of plasmid DNA for the production of virulence factors.
6- Study the cytotoxicity of cell free culture supernatant and partially purified supernatant against HepG2 cell line.

1-2 Literatures review

1-2-1 Genus *Aeromonas*

The genus *Aeromonas* was first described by Zimmermann (1890), who isolated the bacterium from the drinking water supply of Chemnitz in Germany using gelatine agar. He named the bacterium "*Bacillus punctatus*". Sanarelli (1891) isolated a similar bacterium from the blood and lymph of frogs, which he called "*Bacillus hydrophilus fuscus"*, but in 1901 Chester proposed a name change to "*Bacterium hydrophilium"* (Caselitz, 1966). In the first edition of the Bergey´s manual, this species was erroneously designated as "*Proteus hydrophilus*". However, in the Sixth Edition the genus *Proteus* was reclassified as *Pseudomonas* (Speck and Stark, 1942; Rustigan and Stuart, 1943). The genus *Aeromonas* was finally adopted in the Seventh Edition of *Bergey´s* manual (Stainer, 1943), According to molecular genetic studies, Messner and Sleytr (1992) proposed that the genus *Aeromonas* might be placed in a new family, the *Aeromonadaceae*. This genus was previously placed in the family, *Vibrionaceae* (Farmer, 1992) based on its phenotypic expression. The family is sub-divided into psychrophilic and mesophilic species. The psychrophilic group is non-motile, does not grow at 37°C and is therefore unimportant to clinical microbiology. Members of the mesophilic group grow at 37°C and are motile using polar flagella. This group is divided into three principal groups, *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* (Poobalane, 2007).

1-2-2 *Aeromonas hydrophila*

Aeromonas hydrophila is a gram-negative, facultative anaerobic rod approximately 0.8-1.0 × 1.0-3.5 μm in size that is motile via single

polar flagella that morphologically resembles members of the family Enterobacteriaceae. Certain strains are able to produce two distinct types of flagella; polar flagella for swimming in liquids and lateral flagella for swarming over surfaces (Altarriba *et al*., 2003). *A. hydrophilia* is a primary, secondary and opportunistic pathogen of a variety of aquatic and terrestrial animals, including humans (Joice *et al*., 2002), show figure (1-1). It is a ubiquitous, free living, mainly found in water and water-related environments and causes a wide variety of symptoms. It has also been found in a variety of food products producing a range of toxins such as haemolysin, enterotoxin and cytotoxin (Yucel *et al*., 2005; Daskalov, 2006).

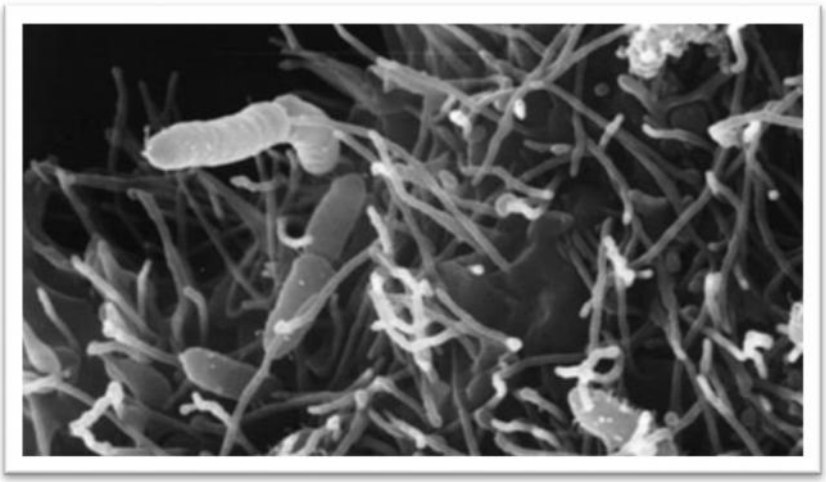


Figure (1-1) Scanning electron micrographs of *A. hydrophila* adhering to human epithelial cells (Yucel *et al*., 2005)

This bacterium can be found in fresh, brackish, estuarine, marine, chlorinated and unchlorinated water supplies worldwide, with highest numbers obtained in the warmer climates (Mathewson and Dupont, 1992). The bacterium has optimal growth at 28°C but can also grow at the limits from 4°C to 37°C (Rekha *et al*,. 2006). Some strains of *Aeromonas hydrophila* are capable of causing disease in fish and

amphibians as well as in humans who may acquire infections through open wounds or by ingestion of an adequate number of the organisms in water or food. This bacterium can digest materials such as gelatin, hemoglobin, and elastin. *A.hydrophila* was isolated from diseased cold- and warm- blooded animals for over 100 years and from humans since the early 1950s (Vilches *et al,.* 2007). It is also hard to kill because it is a resistant bacterium. *A.hydrophila* is resistant to chlorine and refrigeration or cold temperatures (Johanna *et al.,* 2007).

1-2-3 Diseases caused by *A. hydropila*

Members of the genus Aeromonas have received increasing attention as opportunistic as well as primary pathogens in humans and aquatic and terrestrial animals. Human infections include gastrointestinal tract syndromes, wound and soft tissue infections, blood-borne dyscrasias and various other infections (Janda and Abbott, 2010). However, gastroenteritis is the most common of these diseases, affecting mainly young, elderly and immunocompromised individuals (Cascon, *et al.*, 2000).

The disease caused by *A. hydrophila* is called motile aeromonad septicaemia (MAS) and this pathogen is associated with number of other diseases in fish, for example, epizootic ulcerative syndrome (EUS) as a secondary pathogen. The clinical signs in fish vary from tissue swelling, necrosis, ulceration and haemorrhagic septicemia (Azad *et al.*, 2001).

A. hydrophila is associated with disease in humans and domestic animals including sheep, dogs and cats, especially when exposed to periods of stress (Ilhan *et al.*, 2006). Two major diseases associated with *A. hydrophilia* are gastroenteritis and wound infections, with or without bacteremia. Gastroenteritis typically occurs after the ingestion of contaminated water or food, whereas wound infections result from

exposure to contaminated water. One of the diseases it can cause in humans is gastroenteritis. This disease can affect anyone, but it occurs most in young children and people who have compromised immune systems or growth problems. This bacterium is linked to two types of gastroenteritis. The first type is a disease similar to cholera, which causes rice-water diarrhea. The other type of disease is dysenteric gastroenteritis, which causes loose stools filled with blood and mucus. Dysenteric gastroenteritis is the most severe out of the two types, and can last for multiple weeks. *A. hydrophila* is also associated with cellulitis, an infection that causes inflammation in the skin tissue. It also causes diseases such as myonecrosis and eczema in people with compromised or suppressed (by medication) immune systems (Guimaraes *et al*., 2002).

1-2-4 Isolation and identification of *A. hydrophila*

A number of methods have been reported for the detection and identification of this pathogen, including traditional (phenotypic and biochemical characteristics), immunological and molecular techniques. Traditional methods to detect and identify *A. hydrophila* include examination of the shape and color of colonies on nutrient agar, Gram staining, morphology and motility of the bacterium and various biochemical analyses (Yambot, 1998). A rapid method based on biochemical analysis using an API strip containing premixed chemicals has been routinely used for the identification of *A. hydrophila* (Hettiarachchi and Cheong, 1994). Immunological detection methods such as enzyme linked immunosorbent assay (ELISA) were developed for the detection of *A. hydrophila* by Merino *et al*. (1993) and Sendra *et al*. (1997). Korbsrisate *et al*. (2002) produced polyclonal antibodies against *A. hydrophila* for use in a specific direct agglutination test to identify *A. hydrophila*. Monoclonal antibodies (Mabs) have also played a

vital role for the identification of fish pathogens (Adams and Thompson, 2006), Molecular methods have been recommended for the identification *A. hydrophila* to overcome possible problems encountered with traditional or immunological methods. Sugita *et al*. (1994) suggested a deoxyribonucleic acid (DNA) based hybridisation method for the identification of *A. hydrophila*, while amplification of specific genes (e.g. haemolysin) of *A. hydrophila* by polymerase chain reaction (PCR) has been recommended for detection of the bacterium (Xia *et al*., 2004). In addition, a rapid identification method was developed by sequencing 16s ribosomal DNA (rDNA) regions of *A. hydrophila* (Dorsch *et al*., 1994). More recently, Chu and Lu (2005) developed a multiplex PCR method to amplify the 16s rDNA gene and the aerolysin gene of *A. hydrophila* to detect pathogenic strains of *A. hydrophila*. A method combining immunological and molecular techniques (immuno-capture assay with PCR) has also been developed to provide a quick, sensitive and reproducible way of detecting *A. hydrophila* (Peng *et al*., 2002).

1-2-5 Environment of *A. hydrophila*

The distribution of *A. hydrophila* in many aquatic systems globally indicates the successful adaptation of the bacterium to such environment. It is a common contaminant of fresh foods, including fish and other seafood (Poobalane, 2007). Fish disease is a major risk factor in commercial aquaculture with millions of dollars lost annually (Fang *et al.,* 2004). *A. hydrophila* infection is the scourge of fresh and warm water fish farming worldwide and is considered as a significant economic problem (Rahman *et al.,*2001; Hu *et al*., 2005), They are commonly isolated from normal healthy fish, with only certain strains possessing the virulence factors necessary to induce disease (Vivekanandhan *et al*., 2005).

1-2-6 Virulence factors and pathogeneses of *A. hydrophila*

The pathogenicity of aeromonads has been linked to exotoxins such as cytolytic enterotoxin, hemolysin/aerolysin, lipases and proteases. The detection method of aerA was recently proposed as a reliable approach by which to identify a potential pathogenic *Aeromonas* strain by using methods involving PCR and restriction fragment length polymorphism analysis, the virulence genes of *Aeromonas* spp. were grouped as aerolysins-hemolysins, cytolytic enterotoxins, or cytotonic enterotoxins (Yogananth *et al*,.2009).

Show figure (1-2). These virulence factors are used as survival means, self defense mechanism and establishment of pathogenicity. In a research in 1995, some researchers stated that virulence factors are determinant of bacterial pathogenicity (Vadivelu *et al.*, 1995). These are mostly found in bacteria including *Aeromonas* spp. (Singh *et al.*, 2010). In 2004, Subashkumar and colleagues, stated protease, aerolysin, hemolysin, enterotoxins, lipases, gelatinase and biofilm formation as virulence factors in *Aeromonas* spp (Odeyemi *et al*., 2012).

The main pathogenic factors associated with *A. hydrophilia* are:

õ **The capsule**

The capsule is a structure composed of polysaccharides that usually covers the outer membrane of the bacterial cell. It has been described as a major virulence factor of many pathogens, as they prevent phagocytosis, favor interactions to other bacteria and host tissue, and act as a barrier against hydrophobic toxins (Merino and Tom, 2010). *A. hydrophila* are also able to produce a capsule when grown in glucose rich media (Tom´as, 2012).

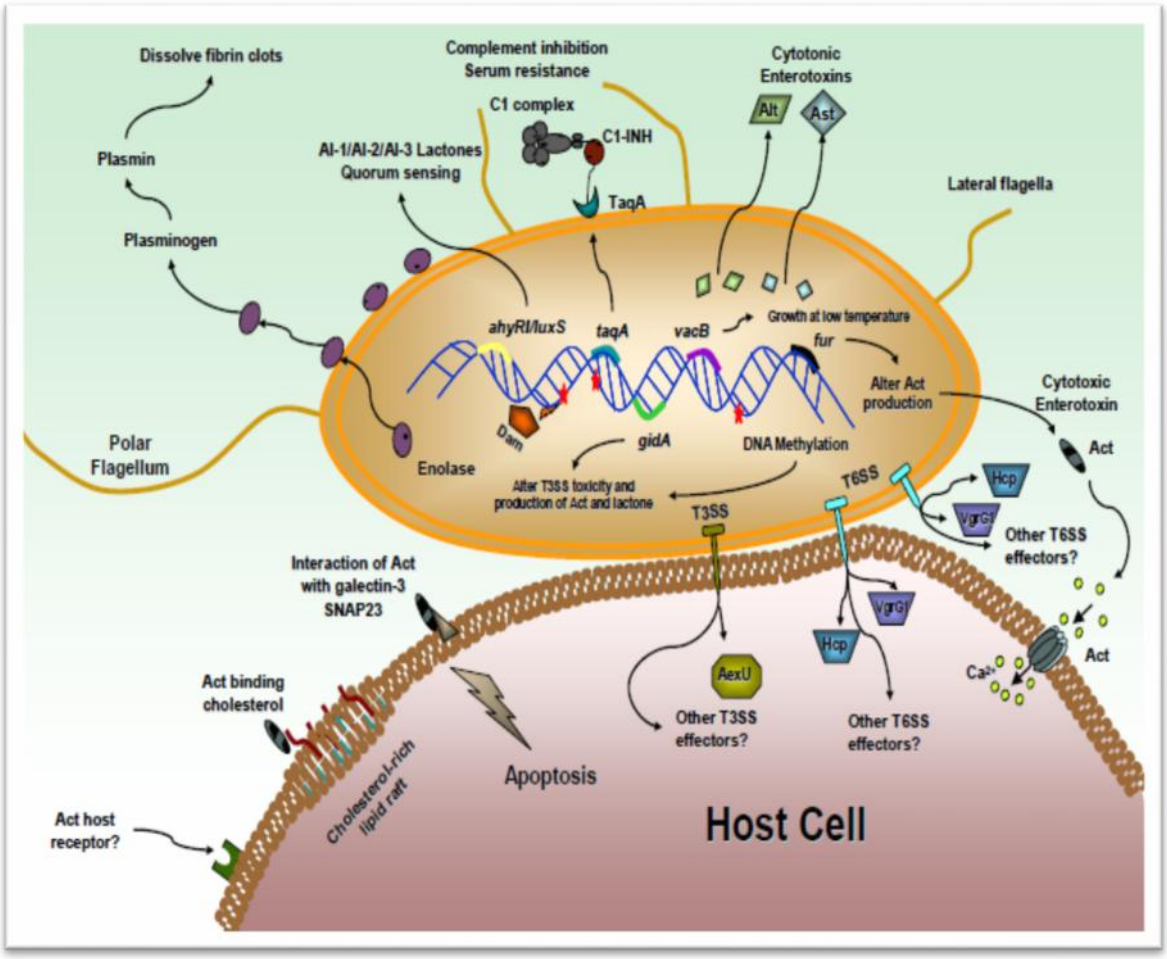


Figure (1-2): Virulence factors associated with *Aeromonas hydrophila* (Giovanni, 2010)

õ Adherence factors

The bacterial capacity to adhere and colonize the hosts' mucosa is a critical step in the infection process. Two classes of adhesins which allow bacteria to bind to specific receptors on the eukaryotic cell surface have been described in *A. hydrophilia*: those associated with filamentous structures and those associated with proteins of the outer membrane or other structures (Tom´as, 2012).

õ **Outer membrane and other surface components**

The outer membrane is the outer most structure on the surface of Gram-negative bacteria. Proteins present in the outer membrane are composed of two classes: lipoprotein, which are anchored into the outer membrane via N-terminal lipid tail, and integral proteins that contain membrane-spanning regions. The LPS is an important component of the outer membrane it plays an important role in the pathogenesis of the bacterium including having a role in adhesion and its ability to cause gastroenteritis (Bos and Tommassen, 2004). Also Knirel *et al*., (2002) mentioned that *A. hydrophila* express a unique O-antigen, and were able to differentiate between virulent and less virulent strains on the basis of serogrouping and cell surface characteristics. Many of the properties which facilitate the colonization of the bacterium on its host are associated with the cell surface of *A. hydrophila*, and are very important in host-pathogen infection (Bos and Tommassen, 2004). The S-layer proteins are considered to play a major role in infection for a number of bacteria; it is believed to influence the interaction between the bacterial cell and its environment (Austin and Austin, 1999). The localization of the S-layer on the surface of the cell suggests it has an important role in the growth and survival of bacteria, and is the site of interaction between the bacteria and the external environment. It possesses anti-phagocytic activity which may aid in the systemic dissemination of bacteria once invasion through the gastrointestinal mucosa has occurred (Janda *et al*., 1994).

õ **Biofilm**

Biofilm is an irreversible growth of aggregated bacterial micro-colonies on surfaces embedded in extracellular polysaccharide matrix.

Biofilm formation results into resistance of bacteria to conventional antibiotics and persistent infections (Rodney 2001). Biofilm helps in recycling of minerals (Brown *et al.*, 1999).

õ **Extracellular toxins**

A. hydrophila is very well known for producing a wide range of extracellular toxins such as enterotoxin, aerolysin, cytotoxin, and haemolysin (Sha *et al*., 2003). Aerolysin involved in the virulence of *A. hydrophila* by binding to specific glycoprotein receptors on the surface of eukaryotic cells, and inserts itself into the lipid bilayer forming holes approximately 3 nm in diameter (Tom´as, 2012). This in turn leads to destruction of the permeability membrane barrier and ultimately cell death. Thus, aerolysin kills cells by forming discrete channels in their plasma membranes (Buckley and Howard, 1999). Studies mentioned that there are three types of exotoxin produce by *A. hydrophila*, first type like cholera toxin (CT) which it is lose its activity at 56°C for 10 min. (Mehdi, 1998). Second type is like Hemolysin which is active against red blood cell and its molecular weight 3000-5000 Dalton, active at 56°C for 10 min but its activity was unstable on 60C for 20 min. Third type it's called cellulertoxin which is induce secretion and fluid collection inside the intestine without tissue damage, this type of toxin lose its activity at 56°C for 10 min. (Trower *et al*. 2000).

õ **Extracellular Enzymes**

A.hydrophila is very well known for producing a wide range of extracellular enzymes such as haemolysin, protease, amylase, acetylcholine esterase, lipase/acyltransferase, leucocidins, enolase (Sha *et al*., 2003), nucleases, chitinases (Khalil and Mansour, 1997). Strains of *A.hydrophila* also produce gelatinase, caseinase, elastase, lecithinase and

deoxyribonuclease (Favre *et al*., 1993). The role of these enzymes is to provide nutrients to the bacterium by breaking down host proteins into smaller molecules which are then capable of entering into the bacterial cell. Proteases are considered to be the main virulence factors in Extra cellular Products ECPs implicated in the pathogenicity (Viji, 2011).

1-2-6-1 Haemolysin

Haemolysin is an important virulence factor, especially for bacteria that invade the mucous membranes to reach the blood (Ketyi, 1984). *A. hydrophila* produces two type of haemolysin, the first type is beta haemolysin which named Aerolysin (Chakraborty *et al*.1987). This kind shows cytotoxic effect to different types of cell lines, haemolysin was sensitive to the temperature of 56°C for 10 minutes. (Khalil and Mansour, 1997). The second type is alpha haemolysin, which plays a minor role in the pathogenesis of the bacteria and also be sensitive to the temperature of 56°C for 10 minutes. (Mateos *et al*., 1993; Palumbo *et al*., 1996)

A. hydrophila produces haemolysin on brain heart infusion agar medium. As it was observed that most strains of the bacteria *A.hydrophila* produced a wide range of blood haemolysis type beta on the blood agar medium. (Graevenitz and Bucker, 1983; Pollard *et al*., 1990).

1-2-6-2 Slime production

Slime is another type of virulence factors, which is a viscous glycoconjugate material, produced by most of the Gram negative bacteria. It is also helpful in the formation of biofilm. The slime is highly significant to the pathogenesis, it appears to inhibit the neutrophil,

chemotaxis, phagocytosis and antimicrobial drugs (Seethalakshmi *et al*,. 2010).

Uptake of Congo red dye has been shown to be a virulence marker for several pathogenic bacteria, the ability to take up dye is associated with the presence of a virulence plasmid. The utility of Congo red uptake as a virulence marker and the lack of clearly defined virulence markers or factors in motile aeromonads prompt to determine whether dye uptake might be a useful marker for virulence of *Aeromonas* spp (Statner and George 1987).

1-2-6-3 Protease

Proteases are highly complex group of enzymes which occupy a central position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes, which are produce intracellularly and extracellularly, play an important role in the metabolic and regulatory processes of animal and Plant cells, as well as in those of prokaryotic and eukaryotic microorganisms. Extracellular proteases are involved mainly in the hydrolysis of large polypeptide substrates, such as proteins into smaller entities which can subsequently be absorbed by the cell. There are different types of protease include serine, cysteine, aspartic and metallo proteases (Barret, 1999). Serine and cysteine protease use these amino acid side chains as nucleophile to attack a peptide bond carbonyl, while aspartic and metallo proteases use water molecules as the nucleophile. The protease enzymes constituted two thirds of the total enzyme used in various industries (Gupta *et al*., 2002).

This dominance in the industrial market has probably increased during the last three years. They have several applications, mainly in detergent and food industries. In view of the recent trend of developing

environmentally friendly technologies; proteases have extensive application in leather treatment and in several bioremediation processes. Proteases are used extensively in the pharmaceutical industry for preparation of medicines. Sharmin and Rahman , in 2007 mentioned that proteases are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations; whereas those are used in medicine are produce in small amounts but require extensive purification before they can be used.

1-2-7 Antimicrobial Resistance of *Aeromonas* spp

In the last two decades, high rates of resistance to commonly used, cheap oral antibiotics among enteric pathogens has been reported from several developing countries (Urio *et al.*, 2001; Rahman *et al.*, 2001). High resistance rates to antimicrobial agents appear to be common among aeromonads isolated from fish in developing countries. Antimicrobial agents are used extensively in fish farms to treat and prevent fish diseases and also as feed additives. Such practice has been shown to increase drug resistant bacteria as well as R plasmids (Hayashi *et al.*, 1982)

A. hydrophila exhibits resistance to multiple drugs (Son *et al.*, 1997; Kaskhedikar and Chhabra, 2010). It has been well documented that multiresistance in most Gram-negative bacteria is mainly contributed to expression of multidrug efflux systems (Poole, 2005). These systems simultaneously extrude the structurally-unrelated substrates including drugs in different classes (Poole, 2004).

Such differences in the frequency of resistance may well be related to the source of the *Aeromonas* isolates and the frequency and type of antimicrobial agents prescribed for treating *Aeromonas* infections in different geographical areas (Son *et al.*, 1997). Resistance of most

aeromonads to ampicillin is generally considered to be intrinsic or chromosomal mediated (Aoki *et al.*, 1971).

The antibiotic sensitivity of an isolate is usually required for effective clinical control, especially when it is from a clinical specimen. Tetracycline and oxytetracycline are usually used to treat the infections (De Paola *et al.*, 1988). Antibiotic-susceptibility pattern is also important for selective isolation of microorganisms. The aeromonads have been regarded as universally resistant to penicillins (penicillin, ampicillin, carbenecillin, and ticarcillin) for quite a long time. For this reason, ampicillin has been generally incorporated in the culture media for selective isolation of the aeromonads from contaminated. Resistance of the aeromonads to penicillins and other antibiotics has been explained to be due to presence of plasmids samples (Awan *et al.*, 2009).

DePaola *et al.* (1988) stated that *A. hydrophila* acquires resistance to commonly used antibiotics through transfer of R-factors. The occurrence of plasmids resistant to ß-lactam antibiotics and other drugs in the aeromonads has also been described (Aoki *et al.*, 1971). The resistance markers harbored on such elements include those for tetracycline, ampicillin, tobramycin and kanamicin (Janda, 1991).

The resistance of the aeromonads to ampicillin and related drugs is due to the presence of at least four β-lactamases (von Graevenitz and Altwegg 1991; Sykes and Mathew 1976).

In addition, most of *Aeromonas* species are susceptible to aminoglycosides, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, and quinolones (Motyl *et al.*, 1985; Renhardt and George 1985). They are also susceptible to azlocillin, piperacillin and the second and third generation of cephalosporins (von Graevenitz and Altwegg, 1991).

The patient was given oral ciprofloxacin, 500 mg every 12 h, for 2 weeks. A repeat urine culture after completion of the antibiotic therapy did not grow any bacteria. The patient remained well during the 3-month follow up. Recently, Rodriguez *et al*.(2005), in Venezuela, reported the isolation of *A. hydrophila* from human blood that is resistant to amikacin, gentamicin, oxacillin, piperacillin, ampicillin-sulbactam, cefotaxime, levofloxacin, and ciprofloxacin, but susceptible to imipenem and cefoperazone sulbactam (Khalifa, 2008).

1-2-8 Genome structure of *A.hydrophila*

The complete genome of *A.hydrophila* strain ATCC 7966T was sequenced (Rekha *et al.,* 2006). The genome is comprised of a single circular 4,744,448 bp chromosome with 61.5% GC content (Albert *et al*., 2000). Its entire genome consists of 4,122 protein coding genes and 159 RNA genes: 128 tRNA genes and 30 rRNA genes. The complete genome sequence of *A.hydrophila* ATCC 7966T reveals mechanisms contributing to virulence and metabolic condition that allow the organism to grow in a variety of environments and explains how *A.hydrophila* is able to survive in polluted or oxygen- poor environments and to colonize and cause illness in humans and other hosts. However, two recognized virulence markers, a type III secretion system and a lateral flagellum, that are reported in other *A.hydrophila* strains are not identified in the sequenced isolate, ATCC 7966T Given the ubiquity and free- living lifestyle of this organism, there is relatively little evidence of fluidity in terms of mobile elements in the genome of this particular strain. Thus, the *A. hydrophila* genome sequence provides valuable insights into its ability to flourish in both aquatic and host environments (Rekha *et al.,* 2006).

1-2-9 Plasmid profile and its role in pathogenicity

Plasmids are extra chromosomal circular DNA molecules, (some bacteria have linear plasmids), found in most bacterial species and in some species of eukaryotes. They have their origin of replication, autonomously replicate with respect to chromosomal DNA and stably inherited. Normally, plasmids are dispensable to their host cell, though many plasmids contain genes that may be essential in certain environment. Plasmid often carry virulence gene required for bacterial pathogenicity, which is include enzymes for the utilization of unusual carbon sources such as toluene, resistance to substances such as heavy metals and antibiotics, synthesis of antibiotics, and synthesis of toxins and proteins that allow the successful infection of higher organisms. For example, to be pathogenic, strains of the genus *Shigella* must carry a large plasmid longer than 200 Kb that contain gene for cell invasion and cell adhesion, the genes for regulating the virulence gene on the plasmids are in the chromosome rather than in plasmid (Snyder and Champness, 1997).

Several bacterial phenotypic properties, such as antimicrobial resistance or virulence factors, have been demonstrated to be plasmid encoded. The presence of plasmids in these potentially pathogenic micro-organisms like *A.hydrophila* may present a potential public health hazard, because they may be transferred from animals to man either directly or indirectly (Aoki, 1977; Toranzo, 1984). Several reports have shown that the presence of plasmids in clinically important bacteria increases their virulence (Crosa, 1999). Various virulence factors of *Vibrio* and *Aeromonas* spp. have been characterized, including extracellular products and protein molecules of the cell envelope (Santos, 1988). One of the most important factors involved in the virulence of the pathogenic strains

is the possession of an efficient iron-sequestering system mediated by siderophores, which allows the bacteria to grow in the iron-limiting conditions imposed by the high-affinity iron-binding proteins present in organic fluids (Borrego *et al.,* 1991).

In *A. hydrophila* investigators have detected only a very large plasmid, while other investigators found small plasmids ranging from 2 to 8 MDa (Marshall *et al.*, 1996).

1-2-10 Role of bacteria in the treatment of cancer

Bacteria or their extracts have been used in the treatment of cancer for a hundred years because of its ability to shrink tumors (Chakrabarty, 2003). Gram-positive and gram-negative bacteria produce many chemical components include, carbohydrate and lipids, all of them used as inhibition agents against tumor cells, by stimulate immune response that help in tumors regression. In earlier studies, Bogdanov (1978) observed that *Lactobacillus bulgaricus* possessed potent antitumor activity. Three glycopeptides which showed biological activity against sarcoma-180 and solid Ehrlich ascites tumor were isolated. Some strains of *Salmonella* spp. and *Listeria monocytogenses* used as vaccine vectors to delivery drugs in treatment cancer (Yamada *et al.*, 2002). Anaerobic bacteria, such as *Clostridium novyi* and *Bifidobactrium* were used to consume the interior of oxygen-poor tumors, these bacteria can target core tumor, and replicated in it and produce anti-vascular growth factors to prevent the metastasis (Dang *et al.*, 2001). Lipopolysaccharide (LPS) that extracted from *P.aeroginosa* used in treatment of acute myelogenous leukemia (AML) (Clarkson *et al*., 1975; Chakrabarty, 2003). Also using LPS of *Pseudomonas aeruginosa* to stimulate blood cells to release Tumor

necrosis factor (TNF), which promotes an inflammatory response to fight the infection and kill tumor cells by enhancing the apoptotic signals carried by TNF (Goodsell, 2006).

Toxins play role in cancer treatment as exotoxin of *P. aeruginosa* has antitumor effect in vivo specially breast cancer by inducing cell apoptosis (Jain and Forbes, 2001). Purified proteins such as azurine have been shown to allow cancer regression in nud mice harboring human melanoma.

Azurin enters into the cytosol of human melanoma cell line, and transport to the nucleus and form a complex with the tumor suppressor protein P53, therapy stabilizing it that allow the significant generation or reactive oxygen species, which is a potent inducer of apoptosis(Chapes and stillman. 2007). Microcin is bacteriocins produce from *Klebsiella pneumonia* have antitumor activity against some type of cell lines, it can stimulate the apoptosis (Hetz *et al*., 2002). Pyocyanin produced by *P. aeroginosa* exposed high efficiency against proliferation of several types of cancer cells and induced apoptosis (AL-Azawi. 2006, Hassani *et al.*, 2012). Crude and partial purification of wall teichoic acid (WTA) from *Enterococcus faecalis*, were exhibited high inhibitory activity on some cell lines than normal cell line at exposure time 24h (AL-Hassini, 2008).

1-2-11 *Aeromonas* spp mediated cell contact cytotoxicity

Some *Aeromonas* species are opportunistic pathogens that have been implicated as etiological agents of human diseases. The most common infection is gastroenteritis, mainly in young, elderly or immunocompromised patients. The clinical symptoms of the infection varied from watery, self-limited diarrhea to chronic intestinal or cholera-like and dysentery-like disease. The most serious complications potentially resulting from gastroenteritis is ileal ulceration, inflammatory

bowel disease, cholangitis, cirrhosis and peritonitis, which may result in septicemia and mortality (Janda and Abbott 2010; Parker and Shaw 2011). The most prevailing species isolated from patients with gastroenteritis is *Aeromonas caviae,* followed by *A.hydrophila* (von Gravaenitz, 2007). Epidemiological studies suggested that there were differences in the predominating species depending on geographical area (Szczuka and Kaznowski, 2004).

The pathogenicity of *Aeromonas* spp is complex and multifactorial, with the involvement of multiple potential virulence factors. These bacteria produce a variety of biologically active extracellular products similar to the virulence factors of enteropathogenic bacteria (von Gravaenitz, 2007; Janda and Abbott, 2010). They include lipopolysaccharide, flagellae, proteases nucleases, and siderophores. Their role in pathogenesis has not been elucidated. Some strains possess type III secretion systems (TTSS) that can deliver virulence factors directly into the host cell. The contribution of TTSS to bacterial virulence has been proven for fish pathogens *A. hydrophila* (Yu *et al*. 2004). There exists a broad clinical spectrum of diseases caused by TTSS containing pathogens for example infections with enteropathogenic *Escherichia coli, Shigella, Salmonella, and Yersinia* species result in serious intestinal diseases (Coburn *et al*., 2007). Some bacteria cause diarrhea by production of enterotoxins or by invasion of the gastrointestinal epithelium (Janda and Abbott 2010). Two categories of enterotoxins, cytotoxic and cytotonic, have been discovered in culture filtrates of *Aeromonas* spp isolates (Krzymin´ska *et al*., 2003; von Gravaenitz 2007; Janda and Abbott 2010). The cytotoxic enterotoxins cause extensive damage to epithelia. The toxins include heat-labile and stable enterotoxins with hemolytic and cytotoxic activities, the pore-forming toxin aerolysin and different α- and β-hemolysins (von Gravaenitz, 2007).

Aeromonas spp strains produce also cytotonic enterotoxins that, like cholera toxin, cause increase in the level of cAMP in intestinal epithelial cells (Galindo *et al*., 2006).

1-2-12 Human liver carcinoma cell line HepG2

Hep G2 is a perpetual cell line which was derived from the liver tissue of a 15 years old Caucasian American male with a well differentiated hepatocellular carcinoma. These cells are epithelial in morphology and are not tumorigenic in nude mice (Udeanu *et al.*, 2011). The cells secrete a variety of major plasma proteins; e.g., albumin, transferrin and the acute phase proteins fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferrin and plasminogen. HepG2 cells are a suitable *in vitro* model system for the study of polarized human hepatocytes. (Ihrke *et al*., 1993). With the proper culture conditions, HepG2 cells display robust morphological and functional differentiation with a controllable formation of apical and basolateral cell surface domains (van IJzendoorn and Mostov, 2000) that resemble the bile canalicular and sinusoidal domains, respectively, *in vivo*.

Because of their high degree of morphological and functional differentiation *in vitro*, HepG2 cells are a suitable model to study the intracellular trafficking and dynamics of bile canalicular and sinusoidal membrane proteins and lipids in human hepatocytes *in vitro*. HepG2 cells and its derivatives are also used as a model system for studies of liver metabolism and toxicity of xenobiotics, the detection of cytoprotective, anti (environmental and dietary) genotoxic and cogenotoxic agents, understanding hepatocarcinogenesis, and for drug targeting studies. HepG2 cells are also employed in trials with bio-artificial liver devices (Mersch-Sundermann *et al.*, 2004).

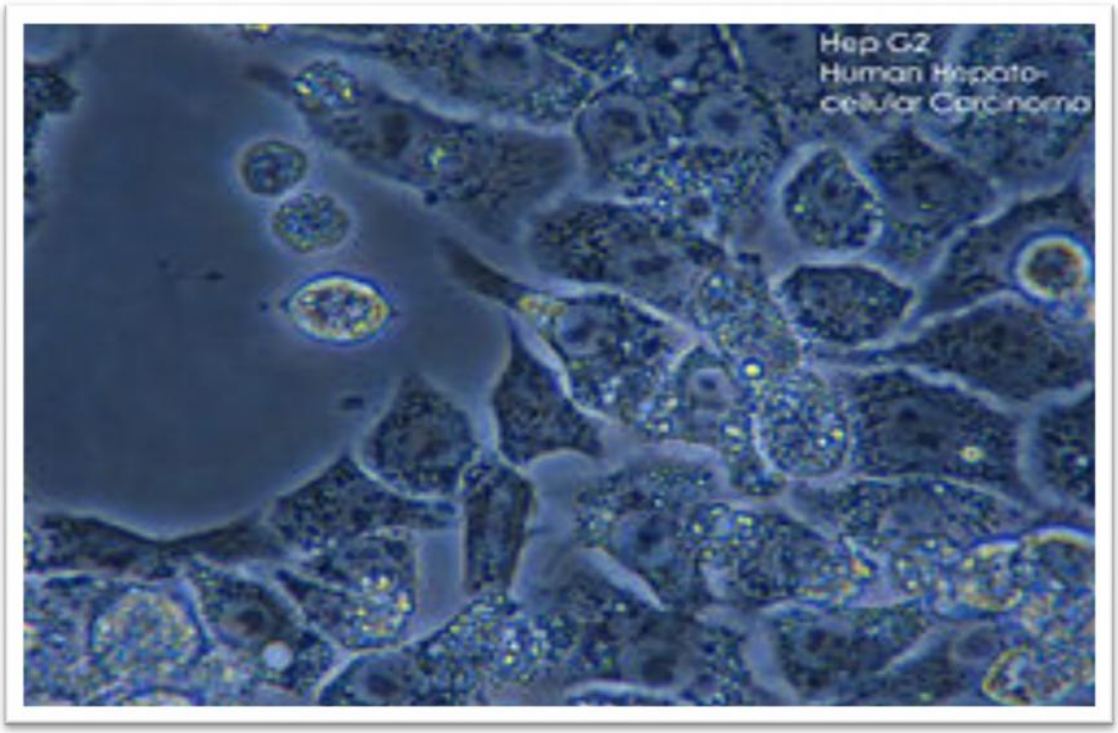


Figure (1-3) Morphology of Hep G2 Cell line (Nguyen, 2012).

1-2-13 Molecular Basis of Cancer

Cancer is a polygenic disease where tumor progression carries a seemingly endless combination of genetic and epigenetic alterations, these alterations affect the six hallmarks of cancer which are defined by Hanahan and Weinberg: (A,B) disregard of signals to stop proliferation and differentiation; (C) capacity sustained proliferation; (D) evasion of apoptosis; (E) invasion; and (F) angiogenesis (Hanahan and Weinberg, 2000).

1-2-14 Genetic Basis of Cancer

Cancer is a genetic disease caused by genomic instability. In many cancers, this instability is manifested by chromosomal reconfigurations and karyotypic complexity (Roschke *et al*., 2005).

Many types of genes play a role in cancer induction particularly those responsible for growth stimulation known as (Proto-oncogenes), whose normal alleles may be activated and mutated into oncogens. Other genes are tumor suppressor genes, sometimes called "Anti-oncogenes". When these genes are lost or inactivated by mutation, the absence of their products allows malignant growth to occur (Miller and Therman, 2001).

2- Materials and Methods

2-1 Materials

2-1-1 Apparatus and equipments

The following apparatus and equipments were used in this study:

Equipments	Company/ Country
Autoclave	Express/Germany
Balance	Satorius/ Germany
Centrifuge	Hermile Z200A /Germany
CO_2 Incubator	Sanyo / Japan
Compound light microscope	Olympus/ Japan
Cooling centrifuge	Hermile/ Germany
Distillator	GFL/ Germany
Electrophoresis Unit	Bio Rad/ Italy
ELISA Reader	Asys / Austria
Hot plate with magnetic stirrer	GallenKamp / England
Incubator	Sanyo / Germany
Inverted Microscope	Meiji/ Japan
Laminar air flow hood	Sanyo / Germany
Micropipettes	Ependrof / Germany
Microwive	Kenwood / China
Millipore filter unit	Millipore corp / USA
Oven	Gallenkamp / England
pH-meter	Martini/ Germany
Power supply	LKB/ Sweden
Refrigerator	Ariston / Japan
Sensitive balance	Denver/ Germany

Shaker incubator	GFL / Germany
Spectrophotometer	Aurora instruments Ltd / England
U V Transilaminator	Vilber Lourmat/ France
Vacuum pump	Scuco Inc. / England
Vortex	Stuart/ England
Water bath	Grant/ England

2-1-2 Chemicals and Biological materials

The following chemicals and biological materials were used in this study

Material	Company/ Country
Agar, Trypton, TCA,	Biolife/ Italy
Agarose, Ethidium bromide, Lysozyme, tetramethyl-p- phenylenediamine dihydrochloride, hydrogen peroxide, casein, Phosphate Buffer Saline,	Sigma/ USA
Boric acid, Bromo phenol blue	Riedel-Dettaen/ Germany
Ethanol, Sodium chloride, Versene, Trypsin	Sigma /USA
Ethylene diamine tetra-acetic acid, Sodium dodecyl sulphate(SDS), Tris-hydrochloride,	Fluka/ Switzerland
Fetal calf serum, trypsin, trypan blue	Gibico/ Canada
Glucose, Congo red, brillent blue	Analar/ U.K.
HCl, Isopropanol, Chloroform, KCl, NaCl, Glycerol, Sucrose, K_2HPO_4	BDH/ U.K.
Peptone, Yeast extract, Urea,	Himedia/ India

2-1-3 Antibiotics

2-1-3-1 Antibiotic discs

The following antibiotic discs were used in this study:

Antibiotics	**Abb.**	**Concentration (µg/ Disc)**	**Company (origin)**
Azithromycin	AZM	15	Oxoid (England)
Amikacin	AK	30	Oxoid
Amoxicillin	AX	25	Oxoid
Ampicillin	AM	25	Oxoid
Chloramphenicol	C	10	Oxoid
Cefepime	FEP	30	AL - Razzi (Iraq)
Cephradine	CE	30	AL - Razzi
Cephalothin	KF	30	AL - Razzi
Cefotaxime	CTX	5	AL - Razzi
Ciprofloxacin	CIP	5	Oxoid
Clindamycin	DA	2	AL - Razzi
Doxycycline	DO	30	Oxoid
Imipenem	IPM	10	AL – Razzi
Gentamicin	GM	30	AL - Razzi
Nalidixic acid	NA	30	AL – Razzi
Nitroflurantion	F	100	Oxoid
Norfloxacillin	Nor	10	AL – Razzi
Novobiocin	NV	30	Oxoid

Penicillin	P	10	AL – Razzi
Rifampin	RA	30	Oxoid
Tetracycline	TE	30	Oxoid
Trimethoprim	TMP	5	AL – Razzi
Vancomycin	VA	30	AL – Razzi

2-1-3-2 Antibiotics Powders:

Antibiotic	Cod	Company (origin)
Amoxicillin	AX	Oxoid (England)
Cefotaxime	CTX	Oxoid
Cephradine	CE	Oxoid

2-1-4 Bacterial strain:

bacterial strain	type	source
A. hydrophila	ATCC 35654 standard strain	the central public health laboratory

2-1-5 Media

2-1-5-1 Ready to use culture media

Medium	Company/Origin
Blood agar base	Difco/ USA
Brain Heart Infusion agar	Oxoid/England

Brain heart infusion broth	Oxoid
Kligler Iron Agar	Difco
MacConky agar	BDH/England
Muller Hinton Agar	Oxoid
Nutrient agar	Difco
Nutrient broth	Oxoid
RPMI1640 medium	PAA/ Austria
Simmon citrate agar	Difco
TCBS Kobayashi agar	Biolife/ Italy
Urea agar base	Difco

2-1-5-2 Laboratory prepared media

- **Pepton water (Collee *et al*., 1996)**

This medium was prepared to be consisted of the following components:

Component	Weight (g)
Peptone	20
Sodium chloride	5

Ingredients were dissolved in 900 ml of distilled water, pH was adjusted to 9.8, mixed thoroughly, and then volume was completed to 1000 ml and distributed into test tubes and sterilized by autoclaving.

- **Urea agar medium (Collee *et al*., 1996)**

It was prepared by adding 24 g of urea agar base to 950 ml of distilled water, pH was adjusted to 6.8-7.0 and sterilized by autoclaving.

After cooling to 50 °C, 50 ml of 20% urea solution sterilized by filtration was added, mixed gently then medium was distributed into sterile test tubes and left to solidify in slant position.

õ **Semi- solid agar medium (KoBy and Ronald, 1974)**

This medium was prepared to be consisting of the following components:

Component	Weight (g)
Glucose	20
Yeast extract	2
Peptone	3
Agar	5

All components were dissolved in 900 ml of distilled water, pH was adjusted to 7, then volume was completed to 1000 ml, sterilized by autoclaving, and left to solidify in vertical position.

õ **Protease production medium** (**Weihua and Chengping, 2003**)

This medium was prepared to be consisting of the following components:

Component	Weight (g)
Trypton	1.5
K_2HPO_4	0.006
KCL	0.0002

All components were dissolved in 900ml of distilled water, pH was adjusted to 8, and then volume was completed to 1000ml with distilled water, and sterilized by autoclaving. This medium was used for protease production by the selected isolate.

- **Skim milk agar medium (Sneath *et al*., 1986)**

This medium was prepared by dissolving 5g of skim milk in 50 ml distilled water (D.W.) and sterilized by autoclaving (5min), then 2 g of agar were dissolve in 50 ml D.W. and sterilized by autoclaving, After cooling to 45°C, medium was mixed gently, then distributed into sterilized petridish plates and stored at 4°C until use.

- **Blood Agar Medium (Atlas *et al.*, 1995)**

It was prepared by autoclaving blood base agar after adjusting pH to 7.0, cooled to 45 °C, then 5% blood was added and mixed well.

2-1-6 Reagents

The following reagents used in this study were prepared according to Atlas *et. al*. (1995) as follows:

2-1-6-1 Oxidase reagent

This solution was freshly prepared in a dark bottle by dissolving 1g of tetramethyl-p- phenylenediamine dihydrochloride in 100 ml D.W

2-1-6-2 Catalase reagent

Catalase reagent was prepared to be consisting of 3% hydrogen peroxide.

2-1-6-3 Kovacs reagent

This reagent was prepared to be consisting of the following components**:**

Component	Quantity
Isoamyl alcohol	150 ml
ρ –Dimethyl-aminobenzaldehde	10 g
Hydrochloric acid	50 ml

ρ –Dimethyl-aminobenzaldehyde was dissolved in isoamyl alcohol with heating in water bath at 50°C, and then acid was added slowly, mixed gently and stored at 4°C until use.

2-1-7 stains and dyes

The following stains and dyes used in this study were prepared according to Freshney (2000) as follows:

2-1-7-1 Gram stain

Its compose of 4 reagents;

- A primary stain- crystal violet
- A mordant- Gram's iodine solution
- A decolorizing agent- an organic solvent (alcohol)
- A secondary stain or counter stain- safranin

2-1-7-2 Neutral red

This stain was freshly prepared by dissolving 10mg of neutral red in 100 ml of phosphate buffer saline and filtered through Whatman filter paper No.1.

2-1-7-3 Trypan blue stain

Stock solution of trypan blue was prepared by dissolving 1 g of the stain in 100ml of phosphate buffer saline and filtered through Whatman filter paper No.1, and stored at 4°C, and then it was diluted (1:10) in PBS to prepare working solution.

2-1-8 Buffers and solutions

2-1-8-1 Protease assay solution (Atlas *et al*., 1995)

- **Tris-HCl buffer** (0.1M), pH 8

- **Casein solution (1%):** It was prepared by dissolving 1g of casein in 100 ml of 0.1 M Tris-HCl buffer solution.

- **5% (Trichloroacetic acid):** It was prepared by dissolving 5 g of TCA in 90 ml of D.W., and then the volume was completed to 100 ml with D.W., and stored at 4°C.

2-1-8-2 Protein concentration solution (Atlas *et al*., 1995)

- **Coomassie Brilliant Blue G-250**

It was prepared by dissolving 0.1 g of brilliant blue in 50 ml of 95% ethanol, then 100 ml of 85% phosphoric acid was added, and the volume was completed to 1litter with D.W.

- **0.1M Tris-HCl buffer (0.1M) and pH 8.0**

- **Bovine Serum Albumin**

Attended gradual concentrations of bovine serum albumin (20, 40, 60, 80, and 100µg/ml) of Tris-HCl buffer pH 8.

2-1-8-3 Buffers and Solution for extraction of plasmid DNA

Buffers and solution for extraction of plasmid DNA were prepared according to Sambrook and Russell, (2001) as follows:

- **SET buffer**

This solution was prepared to be consist of 75 mM NaCl, 25 mM EDTA and 20mM Tris-HCl, pH was adjusted to 8 and sterilized by autoclaving.

- **Lysozyme solution (10mg/ml)**

It was freshly prepared by dissolving 10 mg of lysozyme in 1ml of sterilized distilled water.

- **Sodium dodecyl sulphate solution (10%)**

It was freshly prepared by dissolving 10g of SDS in 100 ml of distilled water.

- **Sodium chloride solution (5M)**

It was prepared by dissolving 29.2 g of NaCl in 80 ml of distilled water, and then volume was completed to 100 ml with distilled water and sterilized by autoclaving.

- **TE buffer**

It was prepared to be consist of 10mM Tris-HCl and 1mM EDTA, pH was adjusted to 8 and sterilized by autoclaving.

2-1-8-4 Gel electrophoresis buffers and solutions

Gel electrophoresis buffers and solutions were prepared according to Sambrook and Russell, (2001) as follows:

- **Tris-Borate-EDTA (TBE) buffer (5X)**

Tris-Borate-EDTA (TBE) Buffer (5X) was prepared by dissolving 54g Tris-HCl and 27.5 g boric acid in 900 ml distilled water, then 20 ml of 0.5 M EDTA was added, volume was completed to 1000 ml with distilled water, pH was adjusted to 8 and sterilized by autoclaving.

- **Loading buffer (6X)**

This solution was prepared by dissolving 0.25 gm of bromophenol blue and 40 g of sucrose in 80 ml of distilled water, then volume was completed to 100 ml with distilled water.

- **Ethidium bromide solution (10mg/ml)**

It was prepared by dissolving 1 g of ethidium bromide in 100 ml of distilled water and stirred on magnetic stirrer until complete dissolving, and then it was filtered through a wattman filter paper No.1 and store in dark bottle at 4°C until use.

2-1-8-5 Antibiotic solutions (Sambrook and Russell, 2001)

Stock solution (100mg/ml) of Amoxicillin, Cefotaxime and Cephradine were prepared by dissolving 1 g of each antibiotic in 10 ml distilled water, sterilized by filtration and stored at –20 °C until use.

2-1-8-6 Phosphate Buffer Saline

One tablet of phosphate buffer saline dissolved in 200 ml distal water sterilized by autoclave then stored until using.

2-1-8-7 Trypsin solution

It was prepared by dissolving 2.5 grams of trypsin powder in 100 ml of distilled water. Then, it was sterilized by (Millipore filter; 0.22 µm) and kept at 4°C (Freshney, 2000).

2-1-8-8 Trypsin – Versene Solution

It was freshly prepared by mixing 20 ml of trypsin solution, 10 ml of versene solution and 370 ml phosphate buffer saline and kept at 4°C until use.

2-1-8-9 Versene Solution

Versene solution was prepared by dissolving 1g of ethylene-diamine-tetraacetic acid (EDTA) in 100ml of phosphate buffer saline, then sterilized by autoclaving and stored at 4°C.

2-1-8-10 Elution Buffer

It was freshly prepared by mixing phosphate buffer saline to absolute ethanol (v/v) then used directly.

2-1-9 Api 20 E kit (Api Bio merieux, lyon, France):

Api 20 E kit consists of:-

A. Galleries: The gallery is a plastic strip with 20 microtubes containing dehydrated reactive ingredients.

B. Api 20 E Reagent :

- Kovac's reagent (p-dimethyl amino benzaldehyde at 4% in HCl isoamyl alcohol).

- Voges – proskauer reagent :
  - VP1 (40% potassium hydroxide).
  - VP2 (5% alpha - naphthol).

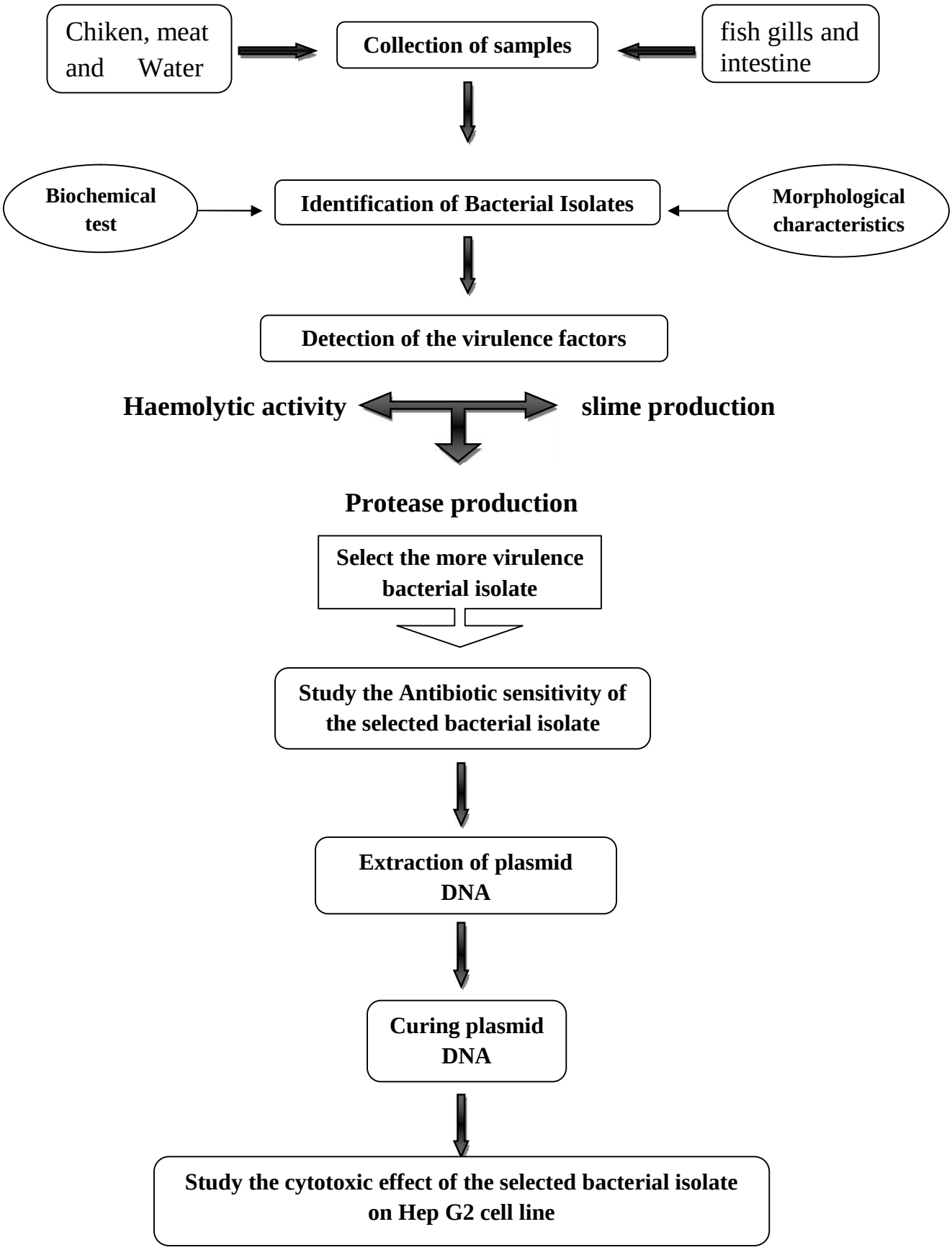
õ Ferric chloride 3.4 %.

2-1-10 Cell Line

The Hep G2 cell line (human liver carcinoma cell line) was kindly provided by Animal cell culture laboratory, Biotechnology Research Center / AL- Nahrain University.

2-2 Methods

The main steps of the research plan were summarized in scheme (2-1), beginning with the collection of samples, isolation and identification of bacterial isolates, Screening of bacterial isolates according to their: Haemolytic activity, slime production and Protease production, selection of the more virulence bacterial isolate, antibiotic sensitivity, plasmid profile and curing of the plasmid DNA and finally the cytotoxic effect of the selected bacterial isolate on Hep G2 cell line.



Scheme (2-1): Main steps of the research plan

2-2-1 Sterilization methods (Colline and Lyne, 1987)

- **Moist heat sterilization (Autoclaving)**

Media, buffers and solutions were sterilized by autoclaving at 121°C and (15 Ib/in^2) for 15 minutes, except some heat sensitive solutions.

- **Dry heat sterilization**

Electric oven was used to sterilize glassware and some other tools at 180°C for 2 hours.

- **Membrane sterilization (Filtration)**

Millipore filter unit was used to sterilize heat sensitive solutions by using millipore filters (0.45 µm).

2-2-2 Collection of samples

A total of 120 samples of fresh fish samples (80 sample), and water samples (20 sample) from pools of living fish were collected from lake of fish farm in Baghdad governorate during the period from 10-2011 to 1-2012, and 20 samples of chicken and meat during the same period. These samples were transferred quickly in sterilized tube containers and nylon bags to the lab for further analysis.

2-2-3 Isolation of *A. hydrophila*

For the isolation of *A. hydrophilia*, one gram of each fish sample (pieces of gills), and one milliliter of each water sample were transferred aseptically to test tubes containing 9 ml of alkaline peptone water (pH 8.9) and incubated at 35ºC for 4-6 h. under aerobic conditions, then 1 ml aliquots of each sample was added to test tubes containing 9 ml of

distilled water and mixed vigorously. Each sample was serially diluted, then 0.1 ml aliquots from the appropriate dilution were taken and spread on blood and MacConky agar medium as differential medium, and on TCBS agar medium as a selective medium for *Aeromanas* ssp. and incubated at 35ºC for 24h., then the suspected colonies were selected and subjected to identification according to their morphological and culture characteristics and biochemical tests.

2-2-4 Identification of the isolates

In order to identify the bacterial isolates, the following examinations were performed:

2-2-4-1 Microscopical and morphological characteristics (Atlas *et al.*, 1995)

Morphological characteristics (size, shape, edge, color, and margin) of the bacterial isolates were studied. Microscopicl characteristics were also examimed by transferring a loop full of bacterial suspension and bacterial suspension was fixed on a clean slide to study the Gram stain to examine Gram reaction and spore forming under light microscope.

2-2-4-2 Biochemical tests

- **Catalase Test (Atlas *et al.*, 1995)**

A single colony of different isolates was smeared onto a clean glass microscopical slide with a sterile toothpick, and then drop of hydrogen peroxide (3%) was placed onto the colony. Production of gaseous bubbles indicates a positive result.

õ **Oxidase Test (Atlas *et al*., 1995)**

This test was achieved by using moistened filter paper with few drops of freshly prepared solution of tetramethyl-ρ-phenylene diamine dihydrochloride. Aseptically, clump of cells was picked up from slant of each bacterial isolate with a sterile wooden stick applicator and smeared on the moistened paper. Development of a violet or purple color within 10 seconds indicates a positive result.

õ **Indole Test (Collee *et al*., 1996)**

Peptone water was inoculated with a single colony of each bacterial isolates and incubated at 35°C for 24hr., and then 50µl of Kovacs reagent was added and mixed gently. Appearance of a red ring on the surface of liquid medium indicates a positive result.

õ **Citrate Utilization Test (Atlas *et al*., 1995)**

Simmon s citrate agar slants were inoculated with a single colony of each bacterial isolates, and incubated for 24 hrs at 35°C. changing of color to royal blue indicates a positive result.

• **Urease Test (Atlas *et al*., 1995)**

Urease activity was detected by streaking Christensen urea agar slants with each bacterial isolate, and incubated at 35°C for 24 hrs. Appearance of a red-violet color indicates a positive result.

õ **Motility test (Collee *et al*., 1996)**

Semi-solid agar medium was inoculated with each bacterial isolates using a straight wire to make a single stab down the center of the tube to about half the depth of the medium. Motile bacteria typically give diffuse,

hazy growth that spreads throughout the medium rendering it slightly opaque.

õ **Haemolysine test (Atlas *et al.*, 1995)**

Each bacterial isolate was cultured on blood agar medium and incubated at 35°C for 24 h under aerobic condition. The appearance of complete blood lyses around the colonies indicates a positive result.

2-2-4-3 Identification by using Api 20E (Overman, *et al.*, 1985**)**

1- Preparation of galleries :-

Five ml of tap water dispensed into incubation tray to provide a humid atmosphere during incubation.

2- Preparation of bacterial suspension :-

By using sterilized loop, a well – isolated colony from plating medium was picked. The inoculums was emulsified in 5 ml suspending medium (sterile distilled water) by rubbing against the side of the tube and mixed thoroughly with the water.

3- Inoculation of the galleries :-

With sterile pasture pipette, a twenty microtubes were inoculated. According to the manufactures instructions both the tube and the cupule section of CIT, VP and GEL microtubes were filled.

After inoculation cupule section of ADH, LDC, ODC, H_2S and URE microtube were completely filled with sterile mineral oil, then the plastic lid was placed on the tray and the galleries were incubated overnight at 35 °C.

4- Reading of the galleries :-

All the reactions not requiring reagent were recorded first, then the following reagents were added to the corresponding microtubes:-

a- One drop of 3.4% ferric chloride to the TDA microtube.

b- One drop of Kovac's reagent to the IND microtube.

c- One drop of Vogas – Proskauer reagent to VP microtube.

The biochemical reaction performed by the API 20E and their interpretation are listed in appendix I.

2-2-5 Maintenance of bacterial isolates

Maintenance of bacterial isolates was performed according to Maniatis *et al*., (1982) as follows:

2-2-5-1 Short-Term storage

Bacterial isolates were maintained for few weeks on Nutrient agar slant. They were tightly wrapped with parafilm, and then stored at 4°C.

2-2-5-2 Medium- Term Storage

Bacterial isolates were maintained as stab cultures for few months. Such cultures were prepared in small screw-capped bottles containing 2-3 ml of Nutrient agar medium and stored at 4°C.

2-2-5-3 Long-Term Storage

Single colonies were cultured in nutrient broth and incubated for 24h, and then 8.5 ml of bacterial culture mixed with 1.5 ml of glycerol, then stored for a long time.

2-2-6 Detection of virulence factors

Virulence factors produced by bacterial isolates of *A. hydrophila* were detected as follows:

2-2-6-1 Haemolytic activity

The haemolytic activity of the *A. hydrophila* isolates was detected by blood agar plate assay (Seethalakshmi *et al.*,2010). Pattern of haemolysis around the colonies on blood agar plates containing 5% (v/v) human blood was recorded after 24 hr incubation at 35 °C.

2-2-6-2 Slime production

A. hydrophila isolates were plated on the surface of Brain heart infusion agar (HiMedia) plates prepared with 0.8 g/L Congo red and incubated at 35 °C for 48 hr., and then colonies were examined for Congo red uptake (seethalakshmi *et al*.,2010). under obliquely reflected light on a black background.

2-2-6-3 production of protease

- **Semi quantitative screening (Sneath *et al*., 1986)**

Local isolates were streaked on nutrient agar medium and incubated at 35 °C for 24h. A single colony was then taken and placed on the center of skim milk agar medium. Plates were then incubated at 35°C for 24h. Ability of bacterial isolate of *A. hydrophilia* to produce protease was estimated.

õ **Quantitative screening (Manachini *et al*., 1989)**

After the appropriate incubation period, bacterial cultured was centrifuged at 15000 rpm for 20 min, at 4°C. The clear supernatant was assay for proteolytic activity by casein digestion method. Activity of protease was assayed in triplicate by measuring the release of TCA soluble peptides from 1% (w/v) casein solution. The assay mixture consisted of 0.8 ml casein solution and 0.2 ml of enzyme solution, incubated at 35°C for 30 min. The reaction was terminated by addition of 1ml of TCA reagent, incubated in ice bath for 10-15 min, and centrifuged at maximum speed for 20 min. The control was prepared using the same steps except the addition of 1ml of TCA reagent into casein solution before the addition of 0.2ml of enzyme solution. The absorbance was measured at 280 nm since one unit (U) of enzyme activity was defined as amount of enzyme required to produce an increase in absorbance at 280 nm equal to 0.01 in one minute under experimental conditions according to the following equations (Whitaker and Bernard, 1972):

$$Enzyme\ activity(U/ml) = \frac{Absorbance\ at\ 280\ nm}{0.01 \times 30 \times 0.2}$$

0.01: Constant

30: Reaction time (min)

0.2: enzyme volume (ml)

$$Specific\ activity(U/mg) = \frac{Activity(U/ml)}{Protein\ concentration(mg/ml)}$$

õ **Determination of protein concentration (Bradford, M., 1976)**

After the preparation of standard curve by bovine serum albumin BSA to estimate protein concentration in the samples, which occurred by adding 1ml of the tested sample to the 0.4ml of Tris-HCl and 2.5 ml of Coomasi blue Dye and vortex the tubes for 2 min. The absorbance for all the test tubes was measured at 595 nm with spectrophotometer.

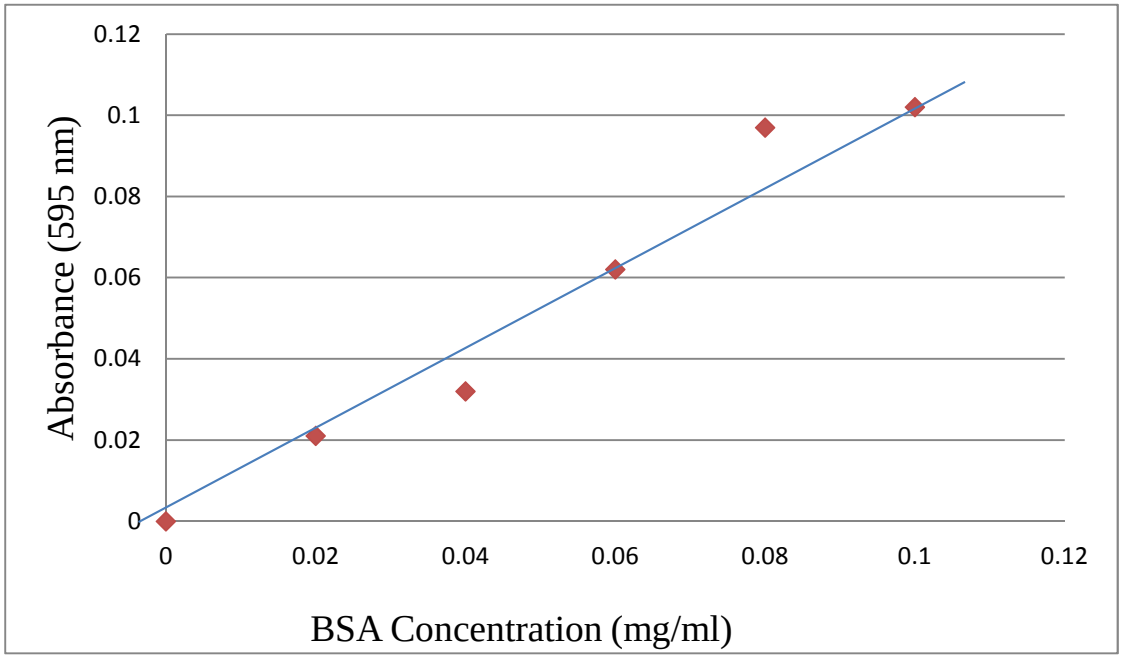


Figure (2-1): Bovine Serum Albumin Standard Curve

2-2-7 Antibiotic susceptibility test (Atlas *et al.*, 1995)

A sterile cotton swab was dipped into inoculums of fresh culture of each bacterial isolate, and the entire surface of the Mullar hinton agar plates was swabbed three times by rotating the plate approximately 60º between streaking to ensure even distribution, then the discs of antibiotics were applied and incubated at 37°C for 24 hr. The zone of inhibition

(clear area around disks) indicates the sensitivity of bacteria to that antibiotic.

2-2-8 Growth curve (Rodrigues and Tait, 1983)

To determine growth phases of the selected isolate of *A. hydrophila*, the selected isolate was grown by inoculating 100 ml of brain heart infusion broth (dispensed into 250 ml Erlenmeyer flask), with 1 ml of bacterial culture (18 hrs old) and incubated in shaker incubator (180 rpm) at 37°C for 24 hrs. A aliquots of 0.1ml of bacterial culture were taken every two hours to measure the absorbency using spectrophotometer at wave length 600 nm.

2-2-9 Extraction of plasmid DNA

Plasmid DNA extraction was done by salting out method from *A.hydrophila* manually by using Kiesser method (Kiesser, 1995), as follow:-

1- Culture of the selected isolate was grown in brain heart infusion broth for 24hrs. at 37 °C then culture was pelleted by centrifugation at 6000 rpm for 15 min.
2- The pellet was washed with 3 ml of SET buffer, centrifuged and then cells were resuspended in 1.6 ml of SET buffer, then freshly prepared lysozyme (final concentration 1 mg /ml) was added, and incubated at 37°C ºC for 30 min.
3- One ml of 10% SDS was added, mixed by inversion, and then incubated at room temperature for 15 min.
4- An equal volume of chloroform was added, mixed by inversion for 15 min. Then centrifuged (6000 rpm. at 4°C) for 20 min

5- The aqueous phase (upper) was transferred to another sterile epprndorff tube, and 0.6 volumes of isopropanol was added, mixed by inversion, and kept at room temperature for 5 minutes.
6- Eppendorff tubes were centrifuged at 13000 rpm for 15 min at 4˚C.
7- The isopropanol layer was discarded gently and the precipitated DNA was dissolved in 100 µl of TE buffer and stored at -20 ˚C.

2-2-10 Extraction of plasmid DNA from *A.hydrophila* by using Qiagen kit

This protocol is designed for purification of total DNA from Gram-negative bacteria:

1- Cells were harvested in a microcenterifuge tube by centrifuge for 10 min at 5000 × g (7500 rpm), the supernatant was discarded.
2- Pellet was resuspednded in 180µl buffer ATL.
3- 20µl proteinase K was added, mixed thoroughly by vortex, and incubate at 56 °C for 1hr, vortex occasionally during incubation to disperse the sample.
4- The sample was Vortex for 15s, 200µl AL buffer was added to the sample, and mixed thoroughly by vortex, and then 200µl ethanol (96-100%) was added, and mixed again thoroughly by vortex.
5- Pipet the mixture from step 4 (including any precipitate) in to the DNeasy mini spin column placed in 2ml collection tube. Centrifuge at ≥ 6000 × g(8000 rpm) for 1min. discard flow-through and collection tube.
6- The DNeasy mini spin column was placed in a new 2ml collection tube, 500µl AW1 buffer was added, and then centrifuge for 1min. at ≥ 6000 × g (8000 rpm) discard flow-through and collection tube.

7- The DNeasy mini spin column was placed in a new 2mll collection tube, add 500µl AW2 buffer, and centrifuge for 2min. at 20,000 × g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.
8- The DNeasy mini spin column was placed in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 200 µl buffer AE directly to the DNeasy membrane. Incubate at room temperature for 1min, and centrifuge for 1min at ≥6000 × g (8000 rpm) to elute.
9- Recommended: for maximum DNA yield, repeat elution ones as described in step 8.

2-2-11 Agarose gel electrophoresis (Maniatis *et al.*, 1982)

Plasmid content for the selected isolate was detected on agarose gel (0.8 %). Gel was run horizontally in 1 X TBE buffer. Electrophoretic buffer was added to cover the gel. Samples of DNA were mixed with loading buffer (1:10 v/v) and loaded into the wells and run for 2 hours at 5 V/cm, then agarose gel was stained with ethidium bromide by immersing in distilled water containing the dye at a final concentration of 0.5 µg/ml for 30-45 minutes. DNA bands were visualized by UV transilluminator. Gel was de-stained using distilled water for 30- 60 min. to get rid of background before photographing of DNA bands.

2-2-12 Curing of plasmid DNA

In order to determine the relationship between plasmid content and virulence factors of *A.hydrophila*, curing experiment was performed on the selected isolate and standard strain ATCC by using SDS as a curing agent (Trevors, 1986).

Fresh culture of these isolates were grown in 10 ml of brain heart infusion broth to mid log phase, then 0.05 ml inoculum of the culture was

inoculated in a series of 10 ml fresh BHI broth tubes containing various concentration of SDS (1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%). All tubes were incubated at 35ºC for 24 – 48 hrs.

The growth density of different tubes were observed by naked eye and compared with the control (without SDS) to determine the effect of SDS on bacterial growth. The lowest concentration of SDS that inhibited the growth of the bacterial strain considered as the minimum inhibitory concentration (MIC).

Samples were taken from tubes containing the highest concentration of SDS that still allow bacterial growth, and diluted appropriately then 0.1 ml samples from proper dilutions were spread on brain heart infusion agar plates and incubated overnight at 37 ºC to score the survived colonies.

õ **Selection of Cured Cells:**

After treatment of bacterial isolates with SDS and the isolation of survivors on BHI agar, survivors were analyzed for the presence or absence of antibiotics resistance as a result of eliminating the plasmid by selecting 100 colonies from bacterial isolates. Those colonies were replica plated (using toothpick) on brain heart infusion agar (master plates), and on brain heart infusion agar containing antibiotic to which the original isolate was resistant. If colonies were able to grow on the master plate but not on the selective agar containing the appropriate antibiotic, it was regard as cured cells that lost the resistance to this antibiotic.

The suspected cured colonies were picked up and their sensitivity to several other antibiotics were tested and compared to that of the original isolate in order to determine which antibiotic marker had been lost in addition to the original one as a result of treatment with the curing agent

(SDS), then plasmid profile for these colonies was examined as in item (2-2-8).

2-2-13 Method of Cytotoxicity

2-2-13-1 Maintenance of the cell lines

Cancer cell lines were monitored to form a confluent monolayer. Sub-culture was established by discarding the old medium. This is followed by washing the cells with sterile PBS under aseptic conditions, then 3 ml of trypsine–versine solution was discarded by washing, using growth medium followed by the addition of new growth medium, redistributed in special falcons and incubated at 37°C (Freshney, 2000).

2-2-13-2 Cell Culture and Culture Conditions

HepG2 cell line was used in this study, the cells were grown as a monolayer, spindle like cells. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 mg/ml streptomycin and 1000U/L penicillin. Cell line was grown as a monolayer in humidified atmosphere at 37°C with 5% CO_2. The experiments were performed when cells were healthy and at logarithmic phase of growth. Hep G2 cell line at passage (40) used in this study they were supplied by Animal cell culture laboratory, Biotechnology Research Center / AL- Nahrain University.

2-2-13-3 Cytotoxicity Assay

This method was carried out according to Freshney (2000). The cells suspension was prepared by treating 25 cm^3 cell culture flask with 2 ml of trypsin solution when a single cell suspension appeared 20 ml of growth medium supplemented with 10% fetal calf serum added to the flask to inactivate the trypsin effect then the viability of the cells counted by using trypan blue dye the viability should be more than 95%. Cell

suspension was well mixed followed by transferring 200 µl/well into each well of the 45 well flat bottom micro titer plate using automatic micropipette containing (1×10^5 cell/well). Plates were incubated at 37°C until 60 -70% confluence of the internal surface area of the well for Hep G2 cell line. Two types of bacterial extract were used for this purpose, the first is the crud extract obtained directly after centrifugation of fresh bacterial culture in a concentration of (230, 115, 75.5, 28.75, 14.37 µg/ml). The second was the partially purified bacterial extract obtained after filtration throughout Millipore membrane tube (30 000MW cutoff) in a concentration of (70, 35, 17, 8.75, 4.37 µg/ml). the cells were then expose to different concentrations of these two extracts prepared by serial two fold dilutions using maintenance media from stock solution of test sample in triplicate form of each concentration. The negative control wells which contained only the cells with culture media, then the plates were incubated at 37°C in an incubator supplemented with (5%) CO_2 for 72 hrs, after elapsing the incubation period, 50 µl/well of neutral red dye were added and incubated again for 2 hrs. The contents of the plate were removed by washing the cells 3 times with PBS then 100 µl of elution buffer added to each well (PBS and absolute ethanol 1:1) to remove the excess dye from viable cells. Optical density of each well was read by using ELISA reader at a transmitting wave length on 492 and 450 nm, then inhibition rate were determined for each concentration according to the formula (Gao *et al*., 2003):

$$Ihibition\ Rate\% = \frac{\text{Abs.at 492 nm of control} - \text{Abs.at 492 nm of test}}{\text{Abs.at 492 nm of control}} \times 100$$

Abs = Absorance

2-2-14 Experimental design and statistical analysis

The experiments were designed as factorial experiments with a completely randomized design. Analyses were done using the SPSS var. 12 software. Differences between means were determined and least significant differences were compared at probability level of 0.05 (Steel and Torrie, 1982).

3- Results and Discussion

3-1 Isolation of *Aeromonas hydrophila*

In order to isolate *Aeromonas hydrophila*, one hundred and twenty samples were locally collected from different locations in Baghdad governorate during the period from 10-2011 to 1-2012. As indicated in table (3-1).

Table (3-1): Bacterial isolates from different sources.

Source	No. of sample	No. of isolate	No. of *Aeromonas. spp*
Fish gills and intestine	80	55	20
water	20	36	15
Chicken and meat	20	45	___
total	120	136	35

From all of these samples, one hundred thirty six isolates were obtained, these isolates were maintained and grown on blood and MacConky agar medium as differential medium, and on TCBS agar medium as a selective medium for *Aeromonas* spp. Among the resulted isolates, thirty five isolates were suspected to be belonging to *Aeromonas* spp. and were subjected to identification according to their morphological and cultural characteristics and biochemical tests.

3-2 Identification of the bacterial isolates

The selected isolates were firstly identified according to their morphological, cultural, and biochemical characteristics.

Results showed that these isolates had rod shape, non-spore forming and occurs singly when examined under light microscope, and appears as small, straight, gram-negative rod, (figure 3-1).

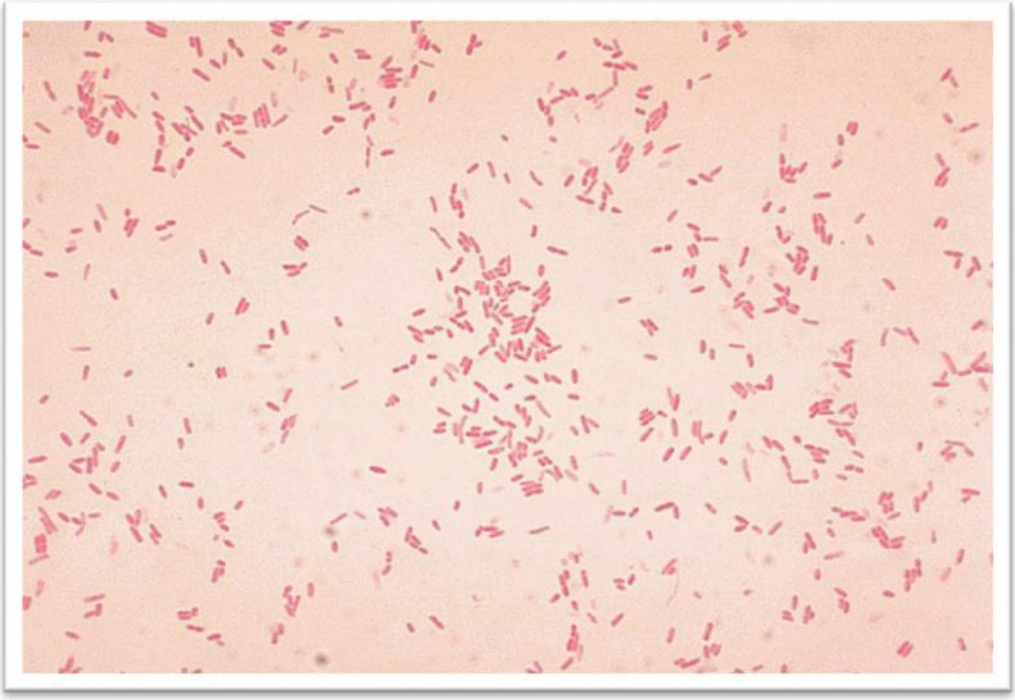
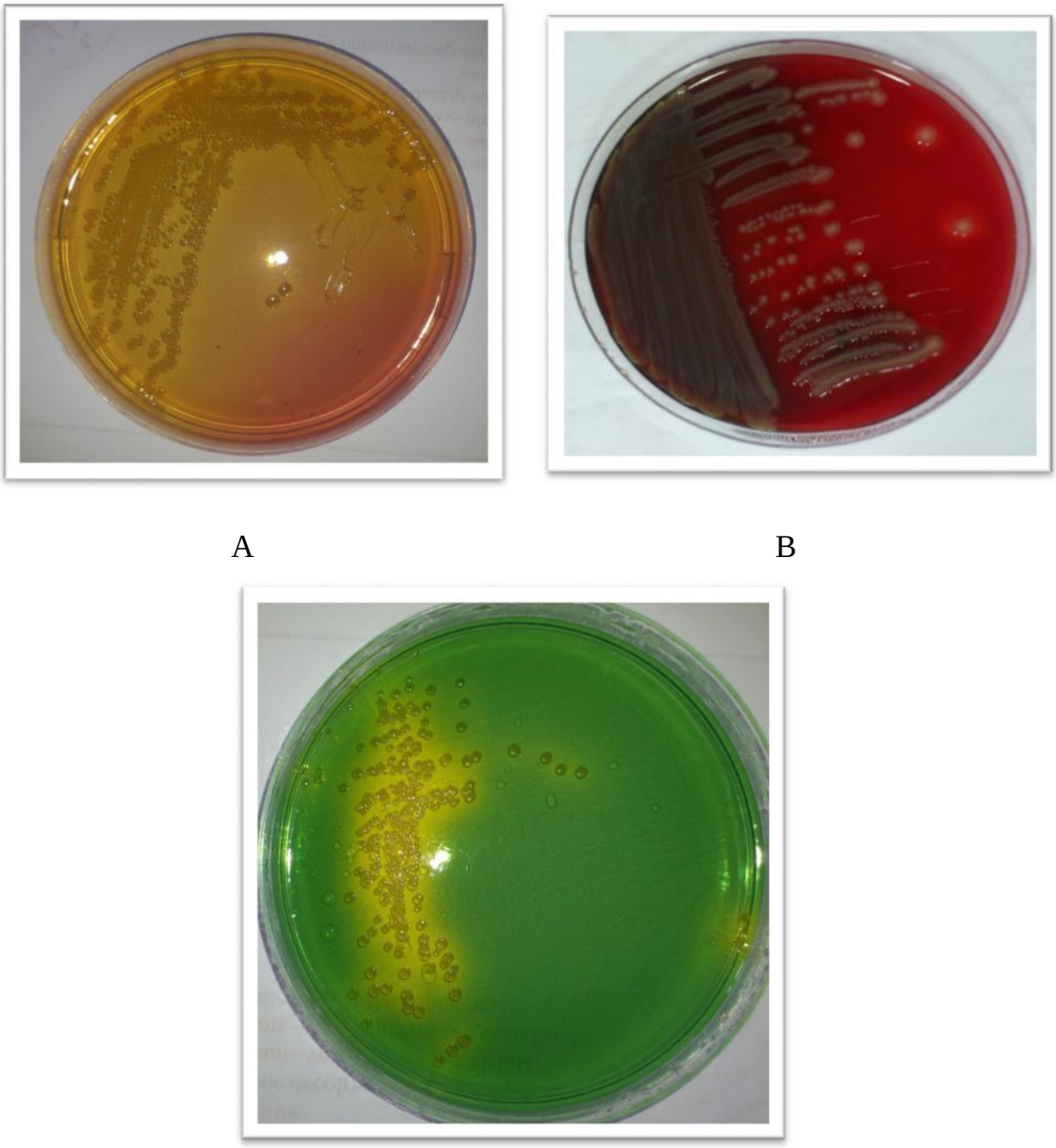


Figure (3-1): Gram stain of *Aeromonas* spp appear under light microscope (1000X).

Bacterial isolates reveals different colonial appearance depending on the selective or differential media, as shown in figure (3-2). Colonies of these isolates on MacConky agar are colorless and lactose non-fermenting, while on blood agar medium colonies of *Aeromonas* isolates are approximately 4mm in diameter, round, raised, opaque, and β-hemolytic, while on TCBS medium the colonies of *Aeromonas* isolates appeared as yellow colonies because of their ability to ferment sucrose.



A B

C

Figure (3-2): Growth of *Aeromonas* spp after incubation for 24 hours at 37îC on:

(A): MacConky agar medium (lactose non-fermenting).

(B): Blood agar medium (round, raised, opaque and beta-hemolytic).

(C): TCBS agar medium (sucrose fomenters with golden to yellowish colonies).

These isolates were subjected to biochemical tests for full-identification. Results mentioned in table (3-2) showed that twelve of these isolates were positive for catalase, oxidase and indole tests, while all the twelve isolates were negative for urease test, as shown in figure (3-3), and they gave a variable results for citrate utilization in which ten of them were negative and two isolates were positive for this test, this may be attributed to the different ecotypes of *Aeromonas* spp in different environments as mentioned by Mange *et al.*,(1998).

According to these results of biochemical tests, which were agreed with Al-taee, (2005) and Urriza *et al*. (2001) , it could be concluded that these twelve isolates were belong to *A.hydrophila*.

The positivity for catalase test is due to the ability of these bacterial isolates to produce catalase responsible for the reduction of hydrogen peroxide to water and hydrogen gas, while the positivity for oxidase is due to the production of cytochrome c oxidase, an enzyme of the bacterial electron transport chain that oxidizes the test reagent. The positive result for citrate utilization is due to the ability of bacterial isolates to utilize citrate as a sole source for carbon and energy. On the other hand the positivity for indole test was due to the ability of the bacterial isolates to produce tryptophanse enzyme that reduces tryptophan to indole. Results indicated in table (3-2) also showed that all the bacterial isolates were able to ferment glucose, sucrose, and manitol, but they are non-lactose fermenters.

Table (3-2): Morphological and biochemical properties of *A. hydrophila* isolates

Test \ Isolate Symbol		standard strain	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
shape		**Rod**	**Rod**	**Rod**	**Rod**	**Rod**	**Rod**	**Rod**	**Rod**	**Rod**	**Rod**	**Rod**	**Rod**	**Rod**
motility		+	+	+	+	+	+	+	+	+	+	+	+	+
Gram stain		-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase		+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase		+	+	+	+	+	+	+	+	+	+	+	+	+
Indole		+	+	+	+	+	+	+	+	+	+	+	+	+
urease		-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization		-	-	-	-	+	-	-	-	-	-	+	-	-
Glucose		+	+	+	+	+	+	+	+	+	+	+	+	+
manitol		+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose		+	+	+	+	+	+	+	+	+	+	+	+	+
lactose		-	-	-	-	-	-	-	-	-	-	-	-	-
Kligler iron agar	**Slant/ Butt**	**K/A**	**K/A**	**K/A**	**K/A**	**K/A**	**K/A**	**K/A**	**K/A**	**K/A**	**K/A**	**K/A**	**K/A**	**K/A**
	H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-
	CO_2	-	-	-	-	-	-	-	-	-	-	-	-	-
Hemolysis		**β**	**β**	**β**	**β**	**β**	**β**	**β**	**β**	**β**	**β**	**β**	**β**	**β**

(-): negative result, (+): Positive result, β**:** beta (Complete haemolysis),

K/A: alkaline/acid

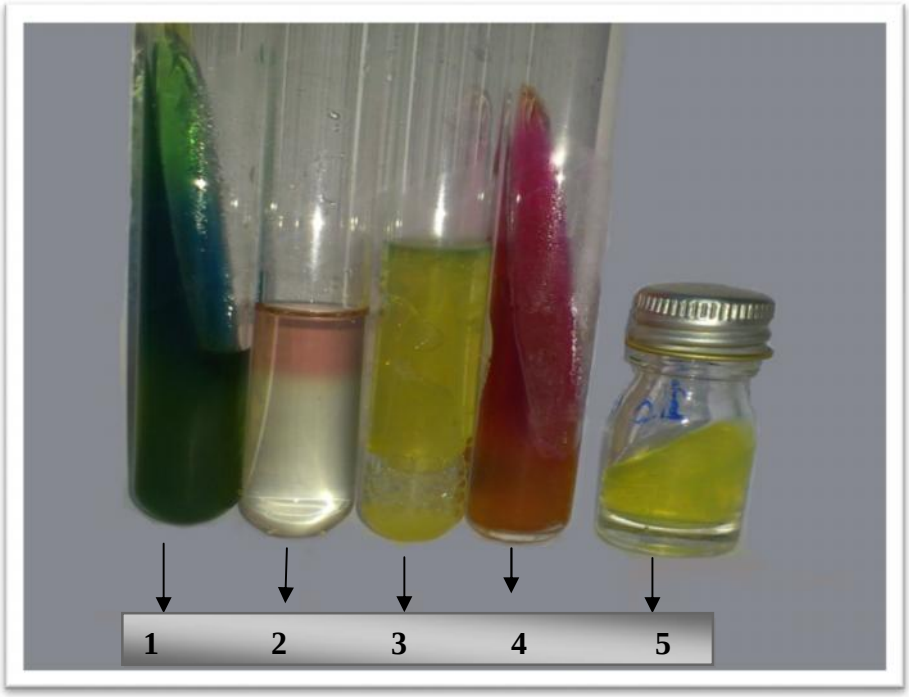


Figure (3-3) Biochemical characteristics of *A.hydrophila* isolates:

(1): Negative result for Citrate utilization. (2): positive result for indol test.

(3): Positive result for manitol test. (4): Kligler Iron agar (alkaline/acid).

(5): Negative result for Urease test.

To confirm the identification of the twelve isolates of *A.hydrophila*, these isolates were re-identified by using Api 20E system. Results indicated in figure (3-4) and table (3-3) showed that these isolates gave the same results of identification given by the standard isolate of *A. hydrophila*(ATCC35654).

Figure (3-4): Identification of *A.hydrophila* using API 20E system.

Table(3-3): Biochemical properties of *A.hydrophila* using API 20E system.

Test	Isolates												
	Standard Strain	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
ONPG	+	+	+	+	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+	+	+	+
LDC	+	-	-	-	+	-	-	-	-	-	+	+	+
ODC	-	-	-	-	-	-	-	-	-	-	-	-	-
CIT	-	-	-	-	+	-	-	-	-	-	+	-	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-
URE	-	-	-	-	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-	-	-	-
IND	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	+	+	+	+	+	+	+	+	+	+	+	+	+
GEL	+	+	+	+	+	+	+	+	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+	+	+	+
INO	-	-	-	-	-	-	-	-	-	-	-	-	-
SOR	-	+	+	+	-	+	+	+	+	+	-	-	-
RHA	-	-	-	-	-	-	-	-	-	-	-	-	-
SAC	+	+	+	+	+	+	+	+	+	+	+	+	+
MEL	-	-	-	-	-	-	-	-	-	-	+	-	-
AMY	+	-	+	+	+	+	+	+	+	+	+	-	-
ARA	-	+	+	+	-	+	+	+	+	+	-	+	+

3-4 Detection of the virulence factors produced by *A. hydrophila* isolate

Virulence factors produced by local isolates of *A.hydrophile* were investigated. These virulence factors include haemolytic activity, slime production and protease production and as follows:

3-4-1 Haemolytic activity

Ability of local isolates of *A. hydrophila* in haemolysin production as a major virulence factor was studied. Haemolytic activity was examined by measuring the diameter of hydrolysis zone around each bacterial isolate on blood agar medium containing 5% human blood after 24 hours of incubation at 35°C. Results indicated in figure (3-5) and table (3-4) showed that the local isolates were able to produce haemolysin type β to hydrolyze the blood constituent of the medium and forming a halo of hydrolysis zone around each colony ranged between 5mm to 10 mm.

Figure(3-5): β haemolytic type zone of *A. hydrophila* incubated on blood agar medium for 24hrs at 35 îC.

Table(3-4): Diameter of hydrolysis zones around each bacterial isolate of *A.hydrophila* on blood agar medium after incubation for 24hrs at 35îC

Isolate Symbol	Diameter (mm)
H1	8
H2	8
H3	5
H4	10
H5	9
H6	8
H7	10
H8	9
H9	6
H10	10
H11	5
H12	5
Std. Strain	12

Among these isolates, the bacterial isolate symbolized by H4 was the most virulent because of its high ability in haemolysin production that gives the highest zone of hydrolysis (10mm) around its colony, in comparison with the other isolates and with the standard strain ATCC35654.

3-4-2 Slime Production

Slime production in terms of the uptake of Congo red dye has been shown to be a marker for virulence in several enteropathogenic and nonenteropathogenic bacteria (Maurelli *et al.*, 1984).

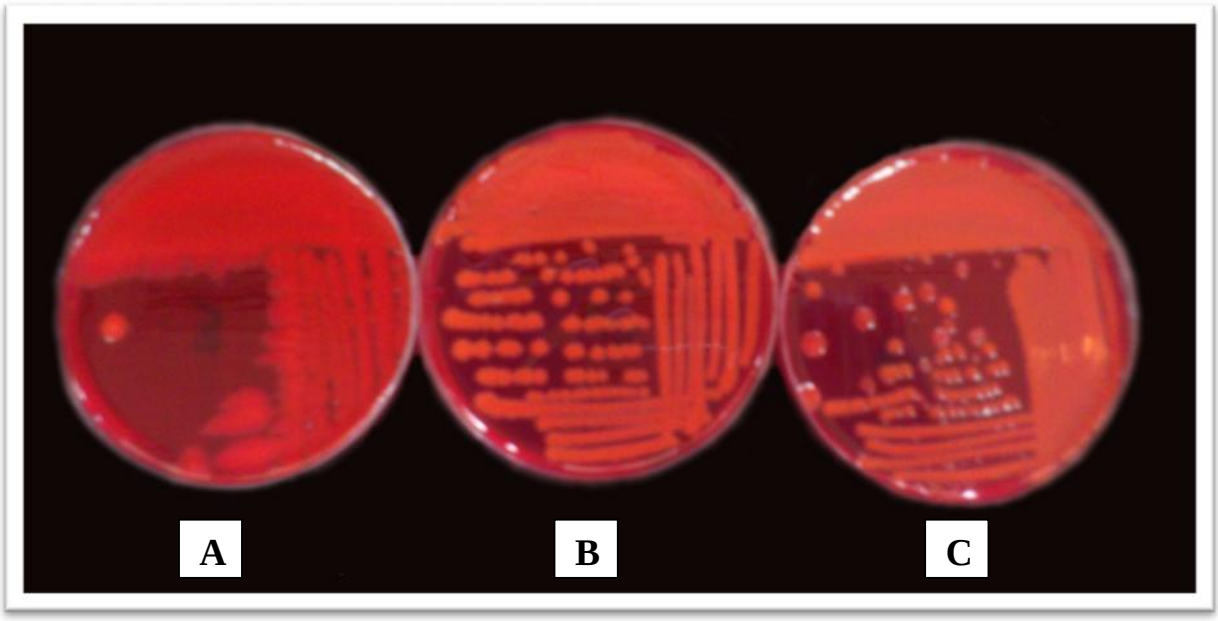


Figure (3-6): Slime Production by *A. hydrophila* isolates

A- High. B-Moderate. C- Low.

Results indicated in figure (3-6) and table (3-5) showed that these local isolates of *A.hydrophila* were able to took up congo red and produce viscous slimes around their colonies with variable degrees. This means that these isolates differ in pathogenesis, and this may be associated with the presence of virulence plasmid. Barry and George, (1987) found that motile *Aeromonas* strains of diverse clinical origin universally took up this dye, this finding suggests that the all isolates were potential enteric pathogens.

Table (3-5): Slime Production by *A. hydrophila* isolates after 48hrs of incubation on Brain heart infusion agar medium with 0.8g/l congo red at 37îC

Isolate Symbol	Slime Production
H1	++
H2	++
H3	+++
H4	++
H5	++
H6	+
H7	+
H8	+++
H9	+++
H10	+
H11	+++
H12	+++
Std. Strain	++

+ = Low viscous growth,
++ = Moderate viscous growth,
+++ = High viscous growth

3-4-3 Screening the ability of *A. hydrophila* isolates in protease production

Two methods for screening the ability of the local isolates of *A.hydrophila* for protease production as indicator for the virulence and pathogenesity of these isolates. The first was semi-quntitative screening which depends on the formation of halo of hydrolysis around colonies on skim milk agar medium, and the second was the quantitative screening which depends on the determination of specific activity of protease produced by these local isolates of *A. hydrophila*.

3-4-3-1 Semi- quantitative screening

Semi-quantitative screenings for protease production by the local isolates of *A. hydrophila* was achieved by detecting the ability of these isolates to produce protease enzyme and the formation of halo of hydrolysis around each colony when grown on skim milk agar medium. Results mentioned in figure (3-7) and table (3-6) showed that these isolates were able to hydrolyze skim milk agar medium around each colony and forming halo of hydrolysis with variable degrees.

Results mentioned in table (3-6) also showed that the diameter of zone of hydrolysis ranged between 6 and 24mm for different isolates, among them the isolates H4 (isolated from fish gills) was the most efficient in protease production because it gives the highest diameter of hydrolysis (24 mm) on skim milk agar.

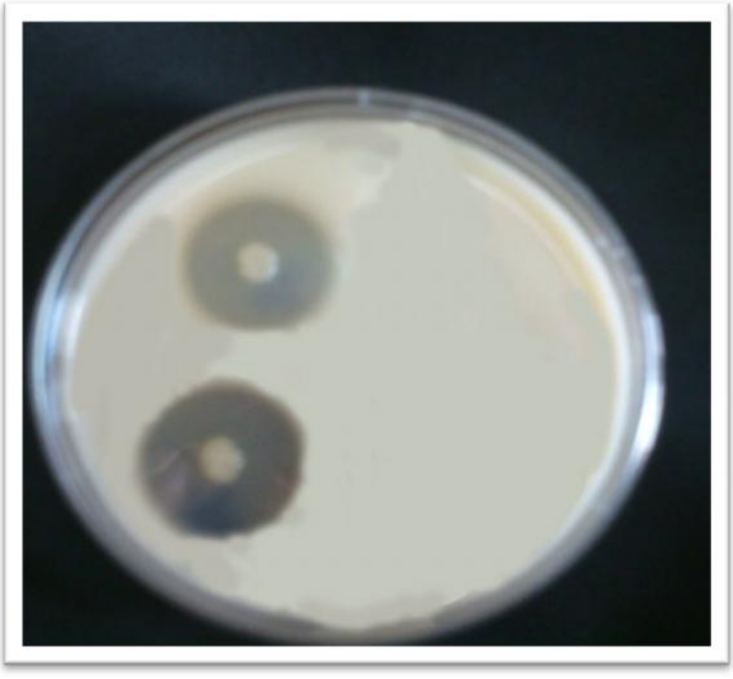


Figure (3-7): Proteolytic activity of protease produced by *A. hydrophila* on 10% skim milk agar after incubation at 35°C for 24h.

Table (3-6): Diameter of clear zone around colonies of *A. hydrophila* grown on skim milk agar medium for 24 hrs at 35°C.

Isolate Symbol	Diameter of clear zoon (mm)
H1	6
H2	16
H3	14
H4	24
H5	19
H6	10
H7	10
H8	18
H9	17
H10	7
H11	14
H12	12
Std. Strain	20

On other hand results mentioned in table (3-6) showed that the other isolates of *A. hydrophila* were less efficient in protease production due to the lower formation of zones of hydrolysis around their colonies.

It was well known that protease is a proteolytic enzyme that responsible for hydrolyzing proteins by attacking peptide bounds in the basic structure of proteins, so protease produced by bacterial isolates was attack casein (the constituent of skim milk protein) in the culture medium and forming a halo of hydrolysis around each colony (Viji *et al.*, 2011).

3-4-3-2 Quantitative screening for protease production by local isolates of *A. hydrophila*

Local isolates of *A. hydrophila* were screened quantitatively to examine their ability in protease production. This was achieved by growing each of the twelve isolates in protease production broth medium for 24 hours at 35°C, then they were centrifuged and the specific activity of protease in crude filtrates was determined. Results indicated in table (3-7) showed that all the isolates were protease producers with variable degrees. Specific activity of protease in culture filtrates was ranged between 2.11 and 30.01 U/mg protein. Among them, *A. hydrophila* H4 was the most efficient in protease production because the specific activity of protease in crude filtrate of this isolate was 31.01 U/mg protein, while the specific activity of protease in culture filtrates of the other isolates were ranged between 0.1 and 12 U/mg protein. According to these results, the isolate H4 was regarded as the most virulent because of its high ability in protease production.

The differences in the ability of the isolates to produce protease are due to genetic variations of the genes responsible for the production of protease (Whealer *et al*., 1991). In another study carried out by Hynes and Tagg, (1986) about isolates of *Streptococcus pyogenes* they found that 81% of these isolates were able to produce protease. Whealer *et al*., (1991) stated that 73% of the *S. pyogenes* isolates producing enzyme that causing cases of bacteremia in children.

Table (3-7): Specific activity of protease produced by local isolates of *A.hydrophila* after 24h.of incubation at 35°C in protease production medium (pH 8.0) in shaker incubator at 150 rpm.

Isolate	Specific activity (U/mg protein)
H1	0.121
H2	12.03
H3	3.86
H4	31.01
H5	0.06
H6	5.03
H7	7.89
H8	3.07
H9	1.69
H10	4.55
H11	6.04
H12	5.78
Std. Strain	12.01

According to the results of the detection of virulence factors for the bacterial isolates of *A. hydrophila*, the isolate H4 was selected for further studies because it was the most virulent isolate among the other local isolates of *A. hydrophila*.

3-5 Antibiotic susceptibility of *A. hydrophila*

The standard disk diffusion method was used to determine the susceptibility of *A.hydrophila* H4 and the standard strain to several antibiotics. The susceptibility of *A.hydrophila* H4 and the standard strain against twenty three antibiotics were studied. Results indicated in table (3-8) showed that these bacteria were resistant to seven antibiotics (penicillin, ampicillin, Amoxicillin, Cefepime, Cephradine, Cephalothin, and Cefotaxime), while it was sensitive to the other fourteen antibiotics (Azithromycin, Amikacin, Chloramphenicol, Ciprofloxacin, Clindamycin, Doxycycline, Imipenem, Gentamicin, Nitroflurantion, Norfloxacillin, Rifampin, Tetracycline, Trimethoprim, and Vancomycin). On the other hand the susceptibility of local isolate H4 and the standard strain to Naldixic acid and Novobiocin was varied. Resistance to these antibiotics may be encoded by chromosomal and/ or plasmid genes. This resistance could be attributed to degradation of these antibiotics by β-lactamase enzyme or to lack of penicillin binding protein or the microorganisms change outer membran permeability to the drug (Scott *et al*.,1999;Avesion *et al*., 2000). *Aeromonas* spp. can possess a conjugative plasmid that confers multiple antibiotic resistances (Chang and Bolton, 1987).

Ferer (1995) found that the major mechanism for the resistance to ampicillin and penicillin in gram negative bacteria causing clinically significant infection is the expression of beta lactamases, of which there are several classes including plasmid and chromosomally encoded enzymes.

Table (3-8): Antibiotic susceptibly of the*A.hydrophila* H4

Antibiotic	Abb.	susceptibility	
		Standard strain	H4
Azithromycin	AZM	S	S
Amikacin	AK	S	S
Amoxicillin	AX	R	R
Ampcillin	AM	R	R
Chloramphenicol	C	S	S
Cefepime	FEP	R	R
Cephradine	CE	R	R
Cephalothin	KF	R	R
Cefotaxime	CTX	R	R
Ciprofloxacin	CIP	S	S
Clindamycin	DA	S	S
Doxycycline	DO	S	S
Imipenem	IPM	S	S
Gentamicin	GM	S	S
Nalidixic acid	NA	S	R
Nitroflurantion	F	S	S
Norfloxacillin	NOR	S	S
Novobiocin	NV	S	R
Penicillin	P	R	R
Rifampin	RA	S	S
Tetracycline	TE	S	S
Trimethoprim	TMP	S	S
Vancomycin	VA	S	S

R: Resistance, S: Sensitive

3-6 Growth curve of *A.hydrophila* H4

Growth curve of the selected isolate (*A.hydrophila* H4) was studied to determine the growth phase (lag phase, log phase, stationary phase and death phase) of this bacterium by propagation in brain heart infusion broth at 35°C for 24 hours. Results shown in figure (3-8) indicates that lag phase takes about four hours from the beginning of incubation which shows slight increase in cells number according to the slight increase in optical density of the growth culture, then logarithmic phase was started and continued for six hours. In this phase optical density (which represents cells number) was increased in constant rate. After that, growth was entered the stationary phase for eight hours characterized with constant number of bacterial cells because the cell division was slowed down, then growth was entered the decline phase and the total count was decreased according to the decrease of the optical density for the growth culture.

This experiment was achieved to determine the mid log phase for the selected isolate for determining the mid log phase to achieve curing experiment.

Most of microorganisms produce secondary metabolites (eg. Antibiotics, enzymes, virulence factors ….etc) in the late exponential phase in culture medium before entering the stationary phase (Mateos *et al.*1993).

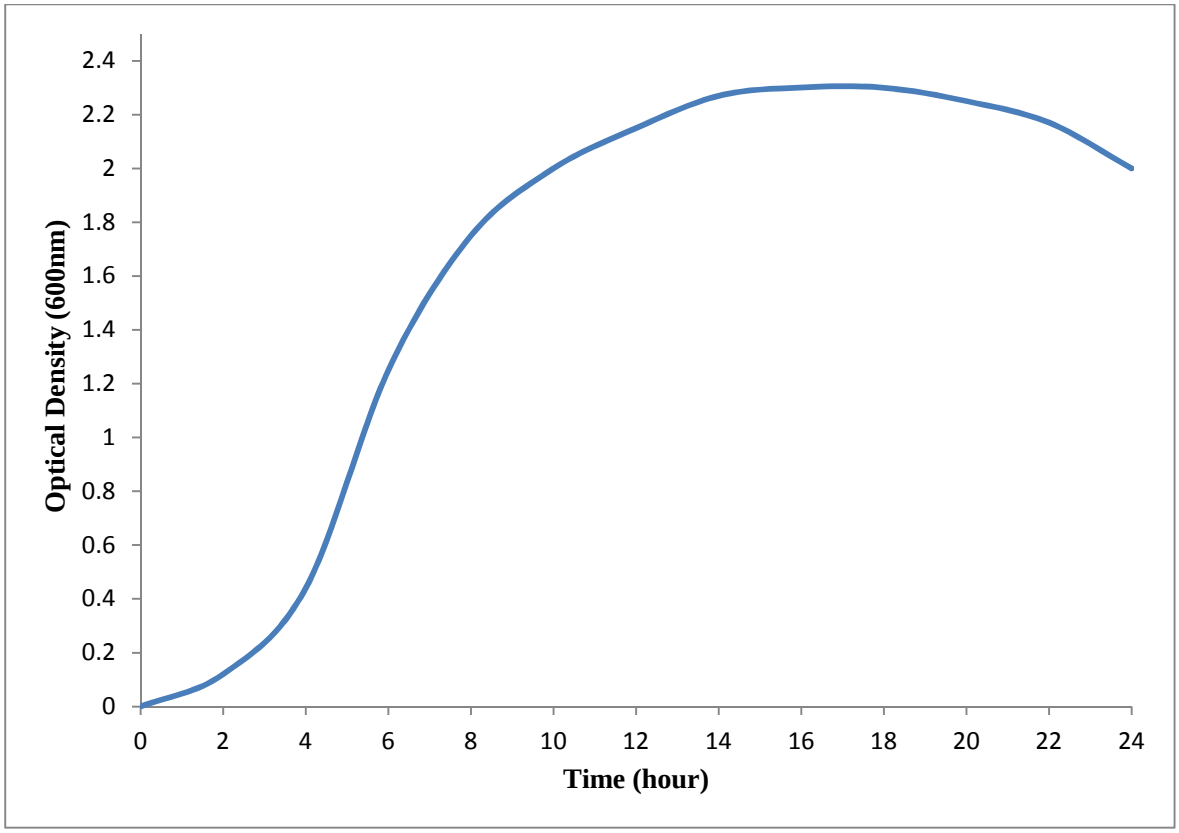


Figure (3-8): Growth curve for *A.hydrophila* H4 grown in brain heart infusion broth for 24 hours with shaking (180 rpm) at 35 °C.

3-7 Plasmid profile of A. *hydrophila*

Plasmid profile of locally isolated *A. hydrophila* H4 and standard strain (ATCC35654) were studied by extraction genomic DNA according to salting-out procedure described by (kieser, 1995). Results mentioned in figure (3-9) showed that these bacteria have two plasmids after electrophoresis on agarose gel, and indicated that both of the bacterial isolate H4 and the standard strain containing small plasmids. These plasmids were approximately in the same size compared with each other. The molecular sizes of *A.hydrophila* plasmids ranged from approximately 2 to 110 megadaltons (Chang and Botton, 1987).

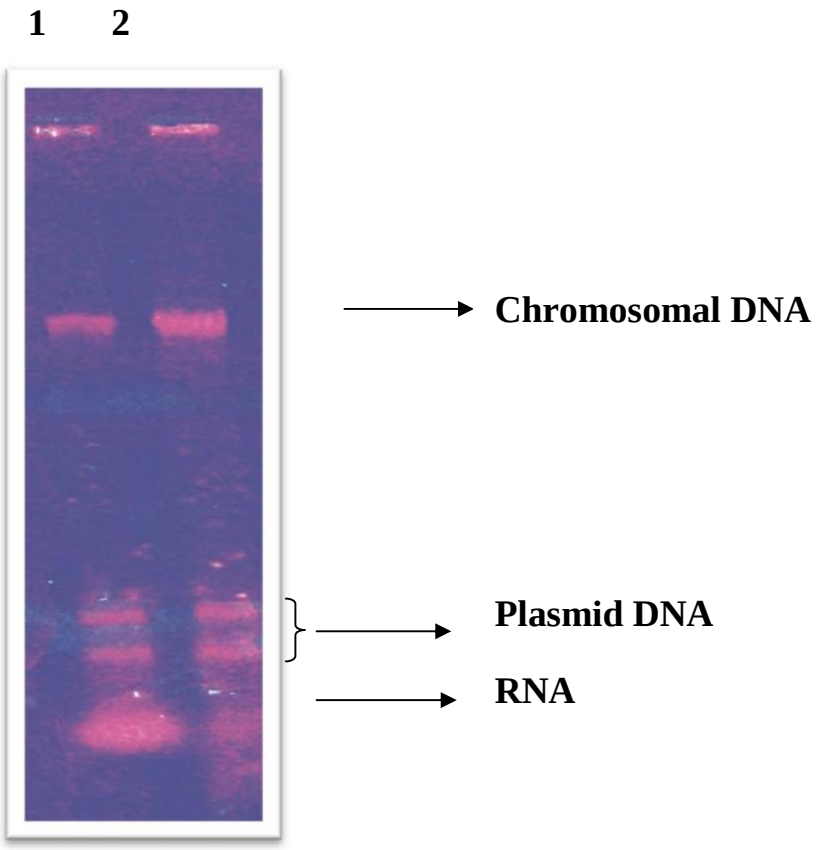


Figure (3-9): Gel electrophoresis of isolated plasmid from the bacterial isolates migrated on agarose gel (0.8%) in TBE buffer at 5V/cm.)

Lane (1) *A.hydrophila* H4.

Lane (2) *A.hydrophila* standard strain ATCC.

3-8 Curing of plasmid DNA

To establish the role of plasmids on the antibiotic resistance of A. *hydrophila*, curing experiment with SDS was performed. So plasmid curing of the locally isolated *A. hydrophila* H4 and the standard strain ATCC was achieved to determine whether the genes responsible for virulence factors and antibiotic resistance are chromosomally located or encoded by plasmid. For that, many attempts were done in order to cure *A.hydrophila* H4 and standard strain plasmids by using SDS. Isolate H4 was selected to perform curing experiment for their multi-resistant to antibiotics and the more virulence characteristics from the other isolates. Results indicated in table (3-9) showed that the highest concentration of SDS that allows the growth of *A.hydrophila* H4 was 8%, and the highest concentration of SDS that allows the growth of the standard strain was 6%. From this treatment, appropriate dilutions were done and spread on brain heart infusion agar medium in order to investigate cured colonies.

After treatment with SDS, a total of 100 colonies were selected and tested on a selective medium containing the appropriate antibiotic to which the wild type was resist (Amoxicillin, Cefotaxime, Cephradine) in order to detect the cured colonies which lost their ability to conferring the resistance phenotypes to those antibiotics.

Out of 100 colonies, 18 colonies were unable for growth in the presence of Cefotaxime, Cephradine and became sensitive to these antibiotics. One of these colonies was selected randomly and examined for the presence of its own plasmid by extraction of genomic DNA and electrophoresis on agarose gel.

Table (3-9): Effect of SDS on the growth of *A. hydrophila* H4 and the standard strain

SDS Con.(B)	Bacterial growth	
	***A. hydrophila* H4**	**Std. strain**
1	+++	+++
2	+++	+++
3	+++	+++
4	++	++
5	++	+
6	+	±
7	+	–
8	±	–
9	–	–
10	–	–

(+++): very good growth, (++): Good growth, (+): Moderate growth, (±): Slightly growth, (-): No growth

Table (3-10): Curing strains of *A. hydrophila* that lost resistance to antibiotics after treatment with SDS

Antibiotic and virulence factors	***A. hydrophila* H4**		**standard strain**	
	Wild-type	**Cured**	**Wild-type**	**Cured**
Haemolysin	β	β	β	β
protease	+	+	+	+
Amoxicillin	R	R	R	R
Cefotaxime	R	S	R	S
Cephradine	R	S	R	S

β: beta (Complete haemolysis), R : Resistance , S : Sensitive, (+):Existance

Result mentioned in figure (3-10) showed that this cured colony of *A. hydrophila* was lost their own plasmid, and this referred that the plasmid is responsible for resistance Cefotaxime, Cephradine in *A. hydrophila* show table (3-10). In other word, gene(s) responsible for resistance of these antibiotics are located on plasmid in this bacterium.

The results showed that environmental and pathogenic *A.hydrophila* strains were naturally resistant to ampicillin, cephalothin, Amoxicillin and prystanamycin, and susceptible to amikacin. These results are in agreement with those of other authors (Seidler *et al., 1980)* for ampicillin and cephalothin.

The correlation between the virulence of *A. hydrophila* for fish, or its enterotoxigenicity, with the production of haemolysins, enterotoxins and cytotoxins is variable and contradictory (Santos *et al., 1988).* In the present study, the results are in agreement with observations of Borrego *et al*., (1991) who stated that haemolytic activity was more prevalent in strains of *A. hydrophila*. This property seems to be chromosome-encoded because plasmid-free strains maintained the haemolytic activity. The role of haemolysins in the pathogenicity of *A. hydrophila* is difficult to establish. As a suggestion, the haemolysins could increase the availability of iron for the microorganisms by erythrocyte lysis.

Ibraheem, (2006) showed that SDS is a powerful agent in eliminating different plasmids, in the curing experiment for *P. shigelloides* at a concentration of 7% this result was agreement with Trevors (1998) who found that SDS was used successfully as a curing agent.

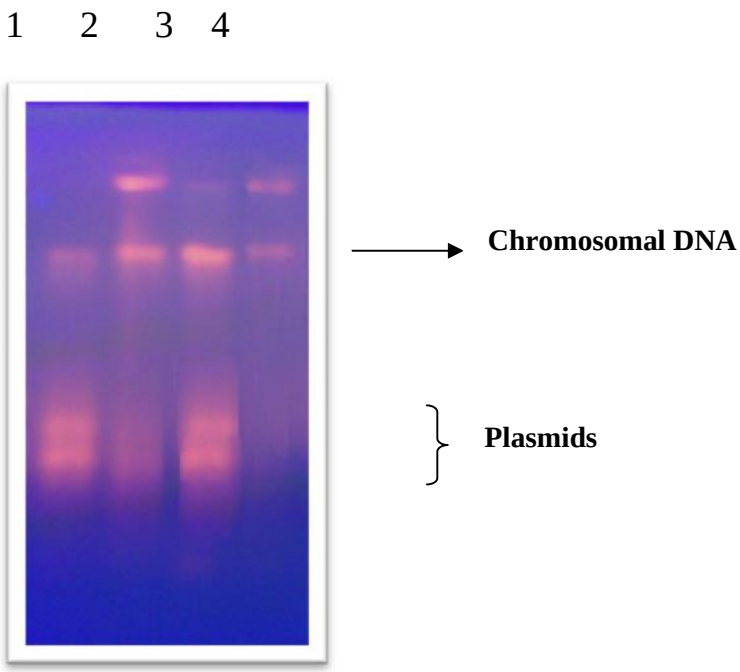


Figure (3-10): Plasmid profile of wild type and cured *A. hydrophila* on agarose gel (0.8%) at (5 v/cm) for 2 hrs.

Lane (1): *A. hydrophila* H4 (wild-type).

Lane (2): *A. hydrophila* H4 (cured).

Lane (3): Standard strain of *A .hydrophila* (wild-type).

Lane (4): Standard strain of *A. hydrophila*(cured).

3-9 Cytotoxic effect of *A. hydrophila* against tumor cell line

A variety of exotoxins such as cytolytic enterotoxin, hemolysin/aerolysin, lipase and protease produced by *Aeromonas* spp. have been implicated in the pathogenesis of gastroenteritis. It is important to determine the activity of toxins and virulence factors to establish their role in pathogenesis. Therefore, two types of bacterial extract were used to study the cytotoxic effect on tumor cell line, the first is the culture extract obtained directly after centrifugation of fresh bacterial culture, and the second was the partially purified bacterial extract obtained after filtration throughout Millipore membrane tube (30 000MW cutoff).

The cytotoxic activity of the cell-free culture supernatant and partially purified supernatant was determined by evaluating its effect on growth of HepG2 cell line after incubation for 72 hours with diferrent concentrations of crude extract (230, 115, 57.5, 28.75, 14.37 µg/ml) and partially purified extract (70, 35, 17, 8.75, 4.37 µg/ml). Optical density of tumor cell line culture was measured at transmitting wave length of 450nm and 492nm.

The cytotoxic effect expressed by the percentage of inhibition growth rate (I.R) which represents the cytotoxicity of bacterial extracts.

Results indicated in that cytotoxicity of culture supernatant was increased with the increase of bacterial extract concentration. Growth inhibition was decreased significantly ($P \leq 0.05$) with the increase of extract concentration after 72 hours of incubation, see appendix II, III and IV (figure 3-11), (table 3-10).

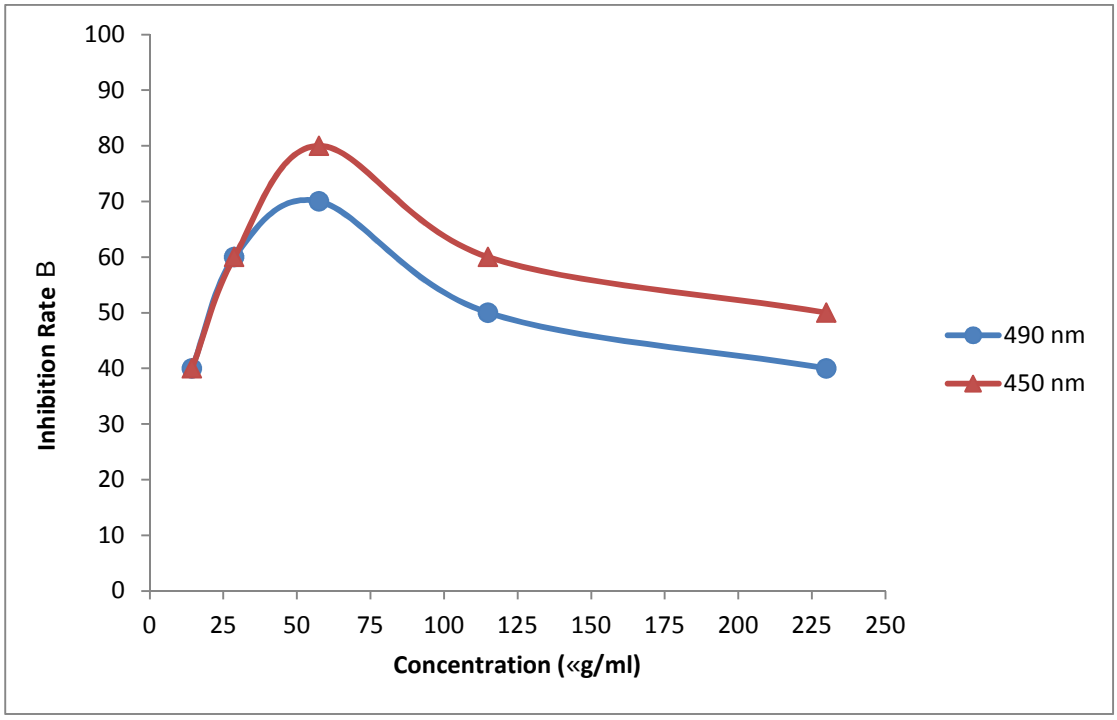


Figure (3-11) Cytotoxic effect of *A. hydrophila* H4 culture filtrates on growth of HepG2.

Effect of bacterial crude extract against HepG2 cell line increased with the increase of crude extract concentration until it reaches the maximum effect at the concentration of 57.5 µg/ml with an inhibition rate of 80% and 70% at wave length of 450 and 492nm respectively, then the cytotoxic effect was decreased and still constant at higher concentrations ranged between 100 and 225 µg/ml of crude filtrate as indicated in figure (3-11), which means that there is no an inhibitory effect against tumor cell line at these concentrations. On the other hand the effect of partially purified bacterial culture filtrate was also increased with the increase of culture filtrate concentration until reaches the maximum effect at the concentration of 20 µg/ml with an inhibition rate of 55% and 60% at wave length of 450 and 492 nm respectively as indicated in figure (3-12). These results showed that partially purified extract was more effective than the crude extract, and this may be due to the higher concentrations of

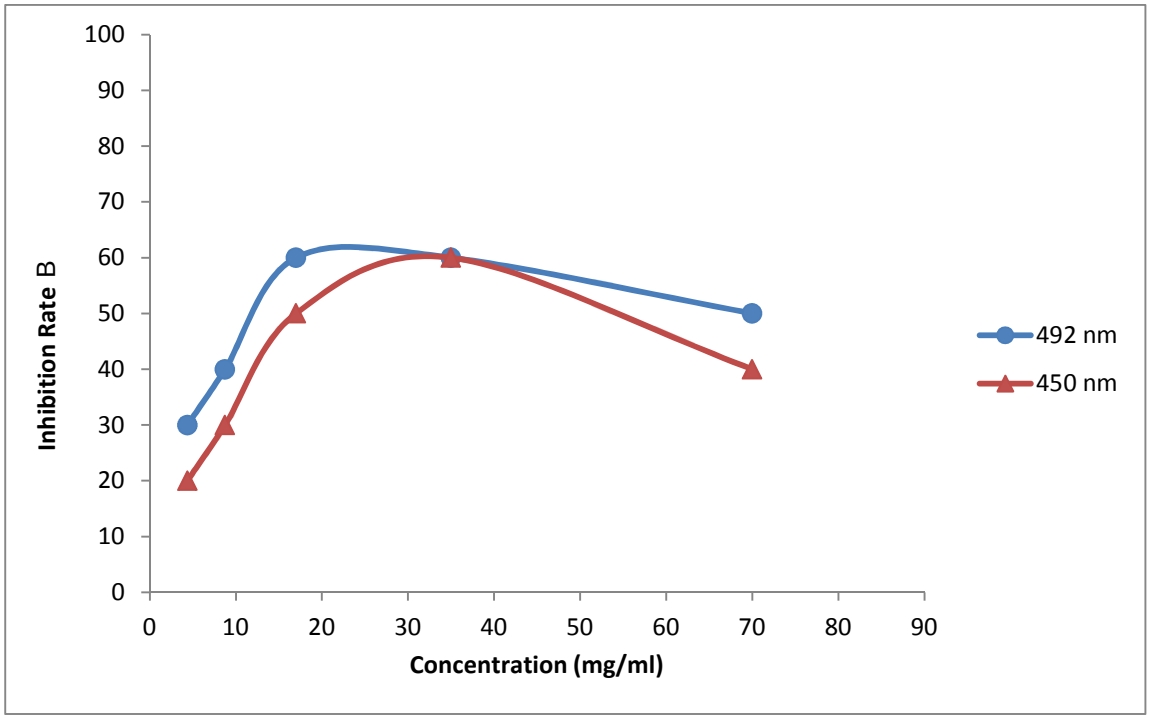


Figure (3-12) Cytotoxic effect of partially purified *A. hydrophila* culture filtrates on HepG2 tumor cell line

proteins (enterotoxines, haemolysin, lipase and protease) in the partially purified crude extract in comparison with the crude filtrate that contains culture medium components and some other low molecular weight secondary metabolites.

Zychlinsky, (1999) indicated that tumor cells differ in morphology than normal cells and one most important difference is that tumor cells highly express receptors on their membranes than normal cells which may allow the attachment of different components. In addition to that, DNA of tumor cell found in relaxant shape, and the DNA molecule was found in unstable figure because of the far away between the H-bond which connect the both strands of DNA. This makes it easy for compounds to interfere or to be associated with both strands of DNA. Whereas DNA of normal cell has a strong H-bond connect the both

strands to each other and make it more stable, so the compounds cannot interfere or associated with DNA strand (Belijanski, 2002).

Krzymin´ska in 2012 observed low cytotoxic activity of *Aeromonas* spp. cell-free culture supernatant at 4 hrs after infection that suggested production of extracellular toxins. The highest activity of the toxins was observed at 24 hrs of incubation. The strains, as with many enteropathogens, displayed a variety of virulence factors involved in the infection process, showing the ability to damage host tissues as well as to evade the host defense system. One of the most potent cytotoxic factors produced by Aeromonas spp. strains is a cytotoxic enterotoxin

Rose *et al*. (1989) observed that cytolytic toxin from *A. hydrophila* strain revealed cytotoxic and enterotoxic activity as well as mice lethality, their results showed a decrease of specific cytotoxic activity to Vero and CHO cells during purification which could have been due to removal of other cytotoxic enterotoxins from culture supernatants of examined strains. Evidence for the existence of more than one cytolytic toxin has been reported previously (Asao *et al*., 1984; Chopra and Houston 1999). Interestingly, partially purified cytolytic toxins in thier study demonstrated also low cytotonic activity revealed to CHO cells only after preheating (56°C for 20 min) of culture supernatant and fractions of purified toxins. Preheating of these samples caused inactivation of heat-labile toxins which destroyed CHO monolayer. Cytotonic activity of these toxins could be associated with an increase of cAMP concentration. Fujii *et al*. (2003) demonstrated that hemolysin produced by sobria strains increased intracellular cyclic AMP concentration in cultured colonic epithelial cells. Also their results showed that cytolytic toxins produced by isolates of *A. hydrophila* and *A. veronii* biotype sobria revealed

hemolytic, cytotoxic and cytotonic activities. These observations suggested that cytolytic toxins play an essential role in *Aeromonas sp.* associated gastroenteritis.

4- Conclusions and recommendations

4-1 Conclusions

1. Gills of fresh fish and water of pool of living fish is a major source for isolation of *A. hydrophila*.
2. Local isolates of *A. hydrophila* are able to produce different virulence factors such as haemolysin, slime production and protease production with variable degrees.
3. Local isolate of *A. hydrophila* harboring two plasmids responsible for antibiotic resistance.
4. Virulence factors of *A. hydrophila* are chromosomally encoded.
5. Crude extracts of locally isolated *A. hydrophila* has an inhibitory effect against HepG2 cancer cell line.
6. Partially purified extract of locally isolated *A. hydrophila*H4 had inhibitory effect against HepG2 than unpurified crude.

4-2 Recommendations

1. Study the genetic polymorphisim of local isolates of *A.hydrophila* using polymerase chain reaction.
2. Cloning of *A. hydrophila* H4 protease gene into *E.coli* or other protease producing bacteria for large scale production of this enzyme.
3. Detection of the extracellular protein produced by locall isolate *A. hydrophila* H4.
4. Study the antitumor activity of purified extracellular protein from *A. hydrophila* against different cell lines and in vivo.

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Appendix I: The biochemical reaction performed by the API 20E and their interpretation

Test	Substrate	Enzyme reaction	Result	
			Positive	Negative
ONPG	Ortho - nitro Phenyl-galactosidase	Beta-galactosidase	Yellow	Colorless
ADH	Arginine	Arginine dehydrolase	Red-Orange	Yellow
LDC	Lysine	Lysine decarboxylase	Orange	Yellow
ODC	Ornithine	Ornithine decarboxylase	Red-Orange	Yellow
CIT	Sodium citrate	Citrate utilization	Blue-Green / Green	Pale Green / Yellow
H_2S	Sod-Thio-ulphate	H_2S production	Black Deposite	Colorless / Grayish
URE	Urea	Ureas	Red-Orange	Yellow
TDA	Tryptophane	Tryptophane deaminase	Dark Brown	Yellow
IND	Tryptophane	Indol production	Red Ring	Yellow Ring
VP	Sodium pyruvate	Aceton production	Pink / Red	Colorless
GEL	Gelatin	Gelatinase	Diffusion of Black Pigment	No diffusion
GLU	Glucose	Fermentation oxidation	Yellow	Blue/Blue-Green
MAN	Mannitol	Fermentation oxidation	Yellow	Blue/Blue-Green
INO	Inositol	Fermentation oxidation	Yellow	Blue/Blue-Green
SOR	Sorbitol	Fermentation oxidation	Yellow	Blue/Blue-Green
RHA	Rhamnose	Fermentation oxidation Yellow	Yellow	Blue/Blue-Green
SAC	Sucrose	Fermentation oxidation	Yellow	Blue/Blue-Green
MEL	Melibiose	Fermentation oxidation	Yellow	Blue/Blue-Green
AMY	Amygdalin	Fermentation oxidation	Yellow	Blue/Blue-Green
ARA	Arabinose	Fermentation oxidation	Yellow	Blue/Blue-Green

Appendix II: Growth inhibition of HepG2 tumor cell line incubated with *A. hydrophila* culture filtrates (FA1 - FE1) and partially purified *A. hydrophila* culture filtrates (FA2 – FE2) at 492nn and 72hr.

group	**concentration (µg/ml)**	Mean	t cal.	t tab.	P	Std. Deviation	Std. Error mean
C	Control	1.21350		3.182	0.00	0.16203	4.68E-02
FA1	230	0.41733	8.258	3.182	0.00	2.45E-02	1.42E-02
FB1	115	0.51267	7.192	3.182	0.00	6.10E-02	3.52E-02
FC1	57.5	0.39733	8.245	3.182	0.00	9.20E-02	5.31E-02
FD1	28.75	0.42700	8.019	3.182	0.00	7.52E-02	4.34E-02
FE1	14.37	0.70500	5.038	3.182	0.00	0.12053	6.96E-02
FA2	70	0.51767	7.136	3.182	0.00	6.25E-02	3.61E-02
FB2	35	0.40833	8.269	3.182	0.00	5.91E-02	3.41E-02
FC2	17	0.49033	7.018	3.182	0.00	0.14571	8.41E-02
FD2	8.75	0.73333	4.356	3.182	0.01	0.21258	0.12273
FE2	4.37	0.80267	4.050	3.182	0.01	0.12712	7.34E-02

Significant differences (p≤0.05).

Appendix III: Growth inhibition of HepG2 tumor cell line incubated with *A. hydrophila* culture filtrates (FA1 - FE1) and partially purified *A. hydrophila* culture filtrates (FA2 – FE2) at 450nn and 72hr.

group	**concentration (µg/ml)**	Mean	t cal.	t tab.	P	Std. Deviation	Std. Error mean
C	control	1.621533		3.182	0.00	0.2058037	5.94E-02
FA1	230	0.614333	8.229	3.182	0.00	2.76E-02	1.59E-02
FB1	115	0.751333	7.000	3.182	0.00	9.02E-02	5.21E-02
FC1	57.5	0.584000	8.220	3.182	0.00	0.125000	7.22E-02
FD1	28.75	0.603667	8.167	3.182	0.00	9.69E-02	5.59E-02
FE1	14.37	0.996000	4.858	3.182	0.00	0.1603621	9.26E-02
FA2	70	0.743333	7.048	3.182	0.00	0.2058037	5.57E-02
FB2	35	0.618000	7.965	3.182	0.00	0.1213549	7.01E-02
FC2	17	0.738666	6.515	3.182	0.00	0.2313878	0.1335918
FD2	8.75	1.024333	4.135	3.182	0.01	0.304291	0.1756410
FE2	4.37	1.150000	3.607	3.182	0.003	0.1836627	0.106377

Significant differences (p≤0.05).

Appendix IV: The LSD values of the 72hr incubation of HepG2 cell line with:

(1) *A. hydrophila* culture filtrates (Factor A1) at 492nm

Multiple comparisons

(I) METHOD	(J) METHOD	Mean Differences (I-J)	Std.Error	Sig.	95% Confidence Interval	
					Lower bound	Upper bound
LSD 1.00	2.00	-9.5E-02	0.066	0.181	-0.24809	5.24E-02
	3.00	2.00E-02	0.066	0.769	-0.12775	0.16775
	4.00	-9.7E-03	0.066	0.887	-0.15742	0.13809
	5.00	-0.28767*	0.066	0.001	-0.43542	-0.13991
2.00	1.00	9.533E-02	0.066	0.181	-5.2E-02	0.24309
	3.00	0.11533	0.066	0.113	-3.2E-02	0.26309
	4.00	8.567E-02	0.066	0.225	-6.2E-02	0.26309
	5.00	-0.19233	0.066	0.016	-0.34009	0.23342
3.00	1.00	-0.2E-02	0.066	0.796	-0.16775	0.12775
	2.00	-0.11533	0.066	0.113	-0.26309	3.24E-02
	4.00	-3.0E-02	0.066	0.664	-0.17742	0.11809
	5.00	-0.30767*	0.066	0.001	-0.45542	-0.15991
4 .00	1.00	9.667E-03	0.066	0.887	-0.13809	0.15742
	2.00	-8.6E-02	0.066	0.225	-0.23342	6.21E-02
	3.00	2.967E-02	0.066	0.664	-0.11809	0.17742
	5.00	-0.27800	0.066	0.002	-0.42575	-0.13025
5.00	1.00	0.28767*	0.066	0.001	0.13991	0.43542
	2.00	0.19233*	0.066	0.016	4.46E-02	0.34009
	3.00	0.30767*	0.066	0.001	0.15991	0.45542
	4.00	0.27800*	0.066	0.002	0.13025	0.42575
Dunnett 1.00	5.00	-0.28767*	0.066	0.005	-0.47934	-9.6E-02
(2-sided)[a] 2.00	5.00	-0.19233*	0.066	0.049	-0.38401	-6.6E-04
3.00	5.00	-0.30767*	0.066	0.003	-0.49934	-0.11599
4.00	5.00	-0.27800*	0.066	0.006	-0.46968	-8.6E-02

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-test one group as control, and compare all other groups against it.

(2) partially purified *A. hydrophila* culture filtrates(factor A2) at 492 nm

Multiple comparisons

(I) METHOD	(J) METHOD	Mean Differences (I-J)	Std.Error	Sig.	95% Confidence Interval	
					Lower bound	Upper bound
LSD 1.00	2.00	0.10933	0.110	0.342	-0.13472	0.35338
	3.00	2.733E-02	0.110	0.808	-0.21672	0.27138
	4.00	-0.21567	0.110	0.077	-0.45972	2.84E-02
	5.00	-0.28500*	0.110	0.026	-0.52905	-4.1E-02
2.00	1.00	-0.10933	0.110	0.342	-0.35338	0.13472
	3.00	-8.2E-02	0.110	0.471	-0.32605	0.16205
	4.00	-0.32500*	0.110	0.014	-0.56905	-8.1E-02
	5.00	-0.39433*	0.110	0.005	-0.63838	-0.15028
3.00	1.00	-2.7E-02	0.110	0.808	-0.27138	0.21672
	2.00	8.200E-02	0.110	0.471	-0.16205	0.32605
	4.00	-0.4300	0.110	0.051	-0.48805	1.05E-03
	5.00	00.31233*	0.110	0.017	-0.55638	-6.8E-02
4 .00	1.00	0.21567	0.110	0.077	-2.8E-02	0.45972
	2.00	0.32500*	0.110	0.014	8.09E-02	0.56905
	3.00	0.24300	0.110	0.051	-1.1E-03	0.48705
	5.00	-6.9E-02	0.110	0.541	-0.31338	0.17472
5.00	1.00	0.28500*	0.110	0.026	4.09E-02	0.52905
	2.00	0.39433*	0.110	0.005	0.15028	0.63838
	3.00	0.31233*	0.110	0.017	6.83E-02	0.55638
	4.00	6.9333E-02	0.110	0.541	-0.17472	0.31338
Dunnett 1.00	5.00	-0.28500	0.110	0.080	-0.60160	3.16E-02
(2-sided)[a] 2.00	5.00	-0.39433*	0.110	0.016	-0.71094	-7.8E-02
3.00	5.00	-0.31233	0.110	0.053	-0.62894	4.27E-03
4.00	5.00	-6.9E-02	0.110	0.917	-0.38594	0.24727

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-test one group as control, and compare all other groups against it.

(3) *A. hydrophila* culture filtrates (Factor A1) at 450nm

Multiple comparisons

(I) METHOD	(J) METHOD	Mean Differences (I-J)	Std.Error	Sig.	95% Confidence Interval	
					Lower bound	Upper bound
LSD	1	2	-0.13700	0.089	0.155	-0.33566	6.17E-02
		3	3.033E-02	0.089	0.741	-0.16832	0.22899
		4	1.067E-02	0.089	0.907	-0.18799	0.20932
		5	-0.38167*	0.089	0.002	-0.58032	-0.18301
	2	1	0.13700	0.089	0.155	-6.2E-02	0.33566
		3	0.16733	0.089	0.090	-3.1E-02	0.36599
		4	0.14767	0.089	0.129	-5.1E-02	0.34632
		5	-0.24467	0.089	0.021	-0.4432	-4.6E-02
	3	1	-3.0E-02	0.089	0.741	-0.22899	0.16832
		2	-0.16733	0.089	0.090	-0.36599	3.13E-02
		4	-2.0E-02	0.089	0.830	-0.21832	0.17899
		5	-0.41200	0.089	0.001	-0.61066	-0.21334
	4	1	-1.1E-02	0.089	0.907	-0.20932	0.18799
		2	-0.14767	0.089	0.129	-0.34632	5.10E-02
		3	-2.0E-02	0.089	0.830	-0.17899	0.21832
		5	-0.41200*	0.089	0.001	-0.59099	-0.19368
	5	1	0.38167*	0.089	0.002	0.18301	0.58032
		2	0.2447*	0.089	0.021	4.60E-02	0.44822
		3	0.41200*	0.089	0.001	0.21334	0.61066
		4	0.39233*	0.089	0.001	0.19368	0.59099
Dunnett (2-sided)[a]	1	5	-0.38167*	0.089	0.005	-0.63938	-0.12395
	2	5	-0.24467	0.089	0.063	-0.50238	1.30E-02
	3	5	-0.41200*	0.089	0.003	-0.66971	-0.15429
	4	5	-0.39233*	0.089	0.004	-0.65005	-0.13462

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-test one group as control, and compare all other groups against it.

(4) partially purified *A. hydrophila* culture filtrates(factor A2) at 450 nm

Multiple comparisons

(I) METHOD	(J) METHOD	Mean Differences (I-J)	Std.Error	Sig.	95% Confidence Interval	
					Lower bound	Upper bound
LSD 1	2	0.12533	0.165	0.465	-0.24201	0.49268
	3	4.667E-03	0.165	0.978	-0.36268	0.37201
	4	-0.28100	0.165	0.119	-0.64835	8.63E-02
	5	-0.40667*	0.165	0.033	-0.77401	-3.9E-02
2	1	-0.12533	0.165	0.465	-0.49268	0.24201
	3	-0.12067	0.165	0.481	-0.48801	0.24668
	4	-0.40633*	0.165	0.003	-0.77368	-3.9E-02
	5	-0.53200*	0.165	0.009	-0.89935	-0.16465
3	1	-4.7E-03	0.165	0.978	-0.37201	0.36268
	2	0.12067	0.165	0.481	-0.24668	0.48801
	4	-0.28567	0.165	0.114	-0.65301	8.177E-02
	5	-0.41133*	0.165	0.032	-0.77868	-4.4E-02
4	1	0.28100	0.165	0.119	-8.6E-02	0.64835
	2	0.40633*	0.165	0.033	3.90E-02	0.77368
	3	0.28567	0.165	0.114	-8.2E-02	0.65301
	5	-0.12567	0.165	0.464	-0.49301	0.24168
5	1	0.40667*	0.165	0.033	3.93E-02	0.77401
	2	0.53200*	0.165	0.009	0.16465	0.89935
	3	0.41133*	0.165	0.032	4.40E-02	0.77868
	4	0.12567	0.165	0.464	-0.24198	0.49301
Dunnett (2-sided)[a] 1	5	-0.40667	0.165	0.099	-0.88322	6.99E-02
2	5	-0.53200*	0.165	0.029	-1.00855	-5.5E-02
3	5	-0.41133	0.165	0.095	-0.88788	6.52E-02
4	5	-0.12567	0.165	0.858	-0.60222	0.35088

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-test one group as control, and compare all other groups against it.

الخلاصه

لعزل بكتريا *Aeromonas hydrophila* جمعت 120 عينه من مناطق مختلفه من محافظه بغداد شملت 80 عينه من خياشم وامعاء الاسماك، و20 عينه ماء من احواض تربيه الاسماك و20 عينه من اللحوم الحمراء والبيضاء (الدجاج). ومن المجموع الكلي لتلك العينات تم الحصول على 136 عزله بكتيريه أخضعت جميعا للتشخيص وفقا لخصائصها المظهريه والمزرعيه وصفاتها الكيموحيويه وقد شخصت 12 عزله بكتيريه منها على انها *A.hydrophila* . وقد تم تأكيد نتائج التشخيص بأستخدام العده التشخيصيه Api20E.

أختبرت قابليه عزلات بكتريا *A.hydrophila* على انتاج عدد من عوامل الضراوه كالهيمولايسين واختبار اللزوجه وانزيم البروتييز. وقد اظهرت النتائج قابليه تلك العزلات على انتاج عوامل الضراوه ولكن بنسب متفاوته. وقد تميزت من بين تلك العزلات البكتيريه العزله *A.hydrophila* H4 بضراوتها العاليه على اساس كفائتها العاليه في انتاج الهيمولايسين وانزيم البروتييز.

أختبرت حساسيه العزله *A.hydrophila* H4 ل23 نوع من مضادات الحياه، وقد اظهرت النتائج ان هذه العزله كانت مقاومه للبنسلين والامبسلين والاومكسسلين والسيفبيم و السيفرادين والسيفالوثين والسيفوتاكسيم، بينما كانت حساسه لبقيه المضادات المدروسه.

درس المحتوى البلازميدي للعزله *A .hydrophila* H4 بأستخلاص الدنا البلازميدي بطريقه الاخراج الملحي (salting out). وقد اظهرت النتائج أمتلاك هذه العزله بلازميدان يشفران لصفه المقاومه السيفوتاكسيم و السيفرادين، بينما لم يكن لهذين البلازميدين دور في صفه المقاومه لبقيه المضادات او في انتاج عوامل الضراوه وفقا لنتائج التحييد للدنا البلازميدي بأستخدام ماده SDS.

درس التأثير السمي الخلوي للمستخلصات الخام والمنقاه جزئيا للعزله البكتيريه *A.hydrophila*H4 تجاه خط الخلايا السرطانيه HepG2. وقد اظهرت النتائج ان لكلا المستخلصين تأثير مثبط النمو ضد خلايا الخط السرطاني بعد حضنها مع تلك المستخلصات لمده 72ساعه. اذ لوحظ زياده التأثير المثبط للمستخلص الخام والمستخلص المنقى جزئيا من العزله البكتيريه *A.hydrophila* H4 بزياده تركيز المستخلص. وقد تم الحصول على اعلى نسبه تثبيط (80%) بعد الحضن مع المستخلص الخام بتركيز57.5مايكروغرام/مل ثم انخفظت نسبه التثبيط

بزياده تركيز المستخلص، بينما كانت اعلى نسبه تثبيط (60%) لخط الخلايا السرطانيه HepG2 بعد الحضن مع المستخلص المنقى جزئيا بتركيز 20مايكروغرام/مل للعزله البكتيريه *A.hydrophila* H4 لمده 72 ساعه ثم انخفظت بنسبه التثبيط ايضا بزياده تركيز المستخلص المنقى جزئيا.

الأهــداء

اهدي ثمره جهدي المتواضع هـذا

الى سيد الخلق ونورالهدى ورسول المحبه

محمد(صلى الله عليه وسلم)

إلى من كلّت أناملة ليقدم لي لحظة سعادة

الى الذي علمني ان ارتقي سلم الحياه بالحكمه والصبر

إلى القلب الكبير

والدي العزيز

إلى رمز الحب وبلسم الشفاء إلى القلب الناصع بالبياض الى من كان دعائها سر نجاحي

والدتي الحبيبة

إلى سندي وقوتي وملاذي بعد الله إلى القلوب الطاهرة الرقيقة والنفوس البريئة

إلى رياحين حياتي

اخي واخواتي

إلى الروح التي سكنت روحي إلى من آنسني في دراستي وشاركني همومي تذكاراً وتقديراً

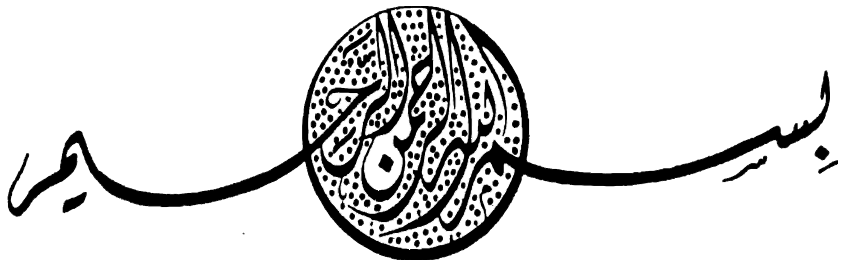
زوجي

إلى الأخوات اللواتي لم تلدهن أمي .. إلى من تحلو بالإخاء وتميزوا بالوفاء

الى من عرفت كيف أجدهم وعلموني أن لا أضيعهم

صديقاتي

هند



وأَنْزَلَ اللّٰهُ عَلَيكَ الكِتابَ و الْحِكْمَةَ و عَلَمكَ ما لَمْ تَكُنْ

تَعلَمْ وَ كانَ فَضْلُ اللّٰه عليكَ عَظيماً

صدق الله العظيم

سورة النساء (113)

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

قسم التقانة الأحيائية

دراسه وراثيه على عوامل الضراوه والسميه الخلويه لعزلات بكتريا *Aeromonas hydrophila*

رسالة

مقدمة الى كلية العلوم - جامعة النهرين وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الأحيائية

من قبل

هند قتيبه محمد

بكالوريوس تقانة احيائية (2010)

بإشراف

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