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Occurrence of *Yersinia enterocolitica* and *Aeromonas hydrophila* in Clinical, Food and Environmental Samples in Gaza Strip

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By

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DEDICATION

I dedicate this thesis to my supervisor, Dr. Abdelraouf A. Elmanama. I would like to thank him so much for always helping me and being by my side.

Also, this thesis is dedicated to my family and to my friend Lila Basher who supported me all the way since the beginning of this study.

Finally, this thesis is dedicated to all those believe in the richness of learning

Declaration

"I herby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substational extent has been accepted for the award of any other degree of the university or other institute, except where due acknowledgment has been made in the text"

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Naima Ferwana

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Abstract

The interest on the occurrence of *Yersinia enterocolitica* and *Aeromonas hydrophila,* their pathogenicity and antimicrobial resistance is increasing worldwide because both were linked to acute and chronic gastroenteritis, septicemia and wound infections. Though reports on the occurrence of both pathogens among human that are available from certain areas, no published data are available from Gaza strip. Moreover, there are no routine methods for the detection of *Yersinia* and *Aeromonas* in clinical or environmental samples. Hence this study investigated the occurrence of both *Y. enterocolitica* and *A. hydrophila* in clinical and environmental samples. Of the 473 clinical and environmental samples, 28 (5.9%) were positive for *Y. enterocolitica* and 179 (38.1%) for *A. hydrophila.* With high incidence of *Y. enterocolitica* and *A. hydrophila* in sewage (19.1%) and water (46.9%) respectively. The overall incidence of *Y. enterocolitica* and *A. hydrophila* in clinical samples was 4.7% and 34.3% respectively, with high frequency of both pathogens in AL-Dorrah and AL-Nasser hospitals. Virulence of isolates was assessed and their antimicrobial resistance to 20 antimicrobial agents was evaluated. Both clinical and environmental isolates possessed virulence factors with higher frequency in clinical samples. Antibiotic susceptibility testing revealed that most of *Y. enterocolitica* isolates were sensitive to most antibiotics; on the other hand, most of *A. hydrophila* isolates showed multiple antibiotic resistances. The most effective antimicrobials on *A. hydrophila* were azetreonam, ciprofloxacin and ofloxacin.

KeyWords: *Yersinia enterocolitica*, *Aeromonas hydrophila*, clinical samples, Environmental samples, Gaza Strip, Antimicrobial resistance

تواجد اليرسينيا انتيروكولتيكا و الايروموناس هيدروفيلا في عينات سريرية و غذائية و بيئية في قطاع غزة

ملخص عربي

الإهتمام بوجود كل من اليرسينيا انتيروكولتيكا و الايروموناس هيدروفيلا و الامراض التي تسببها و مقاومتها للمضادات الحيوية في تزايد مستمر على المستوى العالمي نظراً لارتباط كلا الكائنين بمجموعة من الأمراض مثل الاتهابات المعوية الحادة و المزمنة, خمج الدم و التهابات الجروح. و بالرغم من وجود تقارير عن وجود هذين الكائنين في البشر في اماكن معينة من العالم لايوجد أي بيانات منشورة عن قطاع غزة. فضلا عن ذلك لا توجد طرق محددة للكشف عن اليرسينيا و الايروموناس في العينات السريرية والبيئية و لهذا السبب فقد بحثت هذه الدراسة تواجد كلٍ من اليرسينيا انتيروكولتيكا و الايروموناس هيدروفيلا في هذه العينات. البكتيريا المعزولة اجري لها اختبار الفوعة و مقاومتها لعشرين مضاد حيوي. و كانت نسبة البكتيريا المعزولة من العينات السريرية و البيئية كالتالي: 28 (5.9%) يرسينيا انتيروكولتيكا و 179 (38.1%) ايروموناس هيدروفيلا و كانت أعلى نسبة تواجد لليرسينيا انتيروكولتيكا و الايروموناس الهيدروفيلا في عينات المجاري (19.1%) وعينات الماء(46.9%) على التوالي. و كان المجموع الكلي لكلٍ من اليرسينيا انتيروكولتيكا و الايروموناس هيدروفيلا من العينات السريرية هو(3.7%) و(34.3%) على التوالي, و كانت أعلى نسبة لكلٍ من الكائنين في مستشفى الدرة ومستشفى النصر. احتوت كل من العزلات البيئية و السريرية على عوامل الفوعة مع زيادة واضحة لهذه العوامل في العزلات السريرية, وقد أظهرت نتائج حساسية المضادات بأن اليرسينيا انتيروكولتيكا حساسة لمعظم المضادات الحيوية المفحوصة بينما أظهرت عزلات الايروموناس هيدروفيلا نسبة عالية من المقاومة المتعددة. و كان الازتريونام و سبروفلوكساسين و افلوكساسين من بين المضادات الحيوية الاكثر فعالية ضد الايروموناس هيدروفيلا.

- ***الكلمات المفتاحية: يرسينيا انتيروكولتيكا, ايروموناس هيدروفيلا, عوامل الفوعة,*** قطاع غزة

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LIST OF ABBREVIATIONS

A	Ampicillin
AA	Autoagglutination
ADA	Ampicillin Dextrin Agar
AC	Amoxycillin/clavulanate
AFLP	Amplified Fragment Length Polymorphism
Ail	Attachment Invasion Locus
AK	Amikacin
Ao	Aztreonam
API	Analytical Profile Index
APHA	American Public Health Association
BHIA	Brain Heart Infusion Agar
BHIB	Brain Heart Infusion Broth
BIBG	Bile Salts Inositol Brilliant Green Agar
BOS	Bile-Oxalate-Sorbose Broth
C	Chloramphenicol
Ca	Ceftazidim
CDC	Center for Disease Control and Prevention
Cf	Ciprofloxacin
CFU	Colony Forming Unit
Ci	Ceftriaxone
CIN	Cefsulodin-Irgasan-Novobiocin agar plate
Co	Co-Trimoxazole
Cp	Cephalexin
Cu	Cefuroxime
CV	Crystal violet
Cz	Cefazolin
DNA	Deoxy Ribonucleic Acid
DC	Deoxycholate citrate agar
Do	Doxycycline
EPA	Environmental Protection Agency
EDTA	Ethylene Diamine Tetra Acetic Acid
FAME	Fatty Acid Methyl Ester Analysis
FDA	Food and Drug Administration
FISH	Fluorescent *In Situ* Hybridization
G	Gentamicin
GWWTP	Gaza Wastewater Treatment Plant
HCl	Hydrochloric Acid
HE	Hektoen Enteric Agar
HPI	High Pathogenic Islands
HUS	Hemolytic Uremic Syndrome
Inv	Invasin
ISO	International Organization for Standardization
ITC	Irgasan-Ticarcillin-Potassium Chlorate broth
KOH	Potassium hydroxide
LPS	Lipopolysaccharide
MCA	MacConkey agar

MEM	Meropenem
MF	Membrane Filtration method
MRB	Modified Rappaport Broth
Na	Nalidixic Acid
Nacl	Sodium chloride
NCCLS	National Committee for Clinical Laboratory Standard
Ofx	Ofloxacin
OMP	Outer Membrane Proteins
PAB	Precipitation After Boiling
PBS	Phosphate-Buffered Saline Broth
PBSSB	Phosphate-Buffered Saline Broth with Sorbitol and Bile Salts
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PMO	Pasteurized Milk Ordinance
PYZ	Pyrazinamidase
pYV	Plasmid for *Yersinia* Virulence
SEL	Selenite Broth
SAA	Starch Ampicillin Agar
SAP	Surface Array Protein
SGAP-10	Starch Glutamate Ampicillin Penicillin
SP	Self pelleting
SS	*Salmonella-Shigella* agar
SSDC	*Salmonella-Shigella*-Sodium Deoxycholate-Calcium Chloride agar
Te	Tetracycline
TSB	Trypticase Soya Broth
TSB-30	Tryptose Broth containing Ampicillin (ampicillin 30 mg/L)
Ure	Urease gene
USA	United State of America
USFDA	United States Food and Drug Administration
VYE	Virulent *Yersinia enterocolitica* agar
XLD	Xylose Lysine Deoxycholate agar
YadA	*Yersinia* Adhesin A
YER	Yeast Extract-Rose bengal Broth
Yop	*Yersinia* Outer Membrane Protein
Ybt	Yersiniabactin locus
Ysc	Yop Secretion
Yst	*Yersinia* Heat Stable Enterotoxin
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 Overview

Yersinia enterocolitica and *Aeromonas 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 **[1,2]**.

The genus *Yersinia* comprises an important group of bacterial pathogens, with *Yersinia enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* representing the species of interest. *Y. pestis* is the etiologic agent of plague, whereas *Y. enterocolitica* and *Y. pseudotuberculosis* are enteropathogens which cause a variety of intestinal and extraintestinal clinical symptoms of varying severity ranging from mild gastroenterititis to mesenteric lymphadenitis, which mimics appendicitis and septicemia **[3]**. *Y. enterocolitica* is the most common agent of this genus that are pathogenic for both humans and animals and have a nearly worldwide distribution. Human clinical infections with this species ensue after ingestion of the microorganisms in contaminated food or water or by direct inoculation through blood transfusion **[4]**.

Both pathogenic and nonpathogenic strains are frequently isolated from various animals (birds, mammals, and reptiles), foods (milk, meat, eggs, vegetables) contaminated with feces of infected animals or secondarily during the technologic process, as well as from the environment (water and soil). Rodents (mice and rats), hares, rabbits, and birds serve as reservoirs for *Y. pseudotuberculosis* **[5]**, with swine serving as a major reservoir for

human pathogenic strains of *Y. enterocolitica*, and the highest isolation rates have been reported during the cold season in temperate climates **[6]**.

Clinical illness is characterized by diarrhea and/or vomiting, fever and acute abdominal pain caused by mesenteric lymphadenitis, and it is often clinically indistinguishable from acute appendicitis. Sometimes post-infections, more specifically extra-intestinal sequelae, such as reactive arthritis, erythema nodosum, erythema multiforme, scarlatiniform exanthemata and septicemic types deserve particular clinical attention **[7]**.

The major mechanism of virulence of *Yersinia* species, is invasiveness **[8]**, which is mediated by genes *inv, ail,* and *yadA,* the first two of these are chromosomal and the last, plasmidial. These genes are responsible for the production of the proteins Inv (invasin), Ail (attachment invasion locus) and YadA (*Yersinia* adhesion) **[9]**. The virulence plasmid pYV (40-48 KDa) expresses different phenotypic characteristics such as autoagglutination at 37^{o}C, calcium dependence at 37^{o}C, and Congo red uptake. A set of three tests has been proposed to separate pathogenic from non-pathogenic *Yersinia* strains; pyrazinamidase activity, esculin hydrolysis and salicin fermentation **[10]**. Studies on the behavior of two species of *Yersinia* have demonstrated that the microorganisms are susceptible to large number of antibiotics and chemotherapeutic agent **[11]**.

The *Aeromonas* genus has been placed in its own family, the *Aeromonadaceae*. The aeromonads share many biochemical characteristics with members of the *Enterobacteriaceae*, from which they are primarily differentiated by being oxidase-positive. The genus includes at least 13 species, among which are the motile, mesophilic *A. hydrophila*, *A. caviae*, *A. sobria*, *A. veronii*, and *A. schubertii*, and the non-motile, psychrophilic *A. salmonicida* **[12]**. Seasonal variations in isolation of *Aeromonas* from stools have also been reported, with highest recovery during the warmer months. The mesophilic species have been associated with a wide range of infections

in humans, that have been isolated from freshwater, salt water, ground waters, drinking water (chlorinated and unchlorinated drinking water) **[13]**, and have been frequently isolated from various food products such as fish and shellfish, raw meat, vegetables and raw milk, and from patients with diarrhea **[14]**.

Among *Aeromonas* species, *Aeromonas hydrophila* is most commonly associated with human infections, leading to intestinal and non-intestinal diseases. Furthermore, increased resistance of this organism to antibiotics and chlorination in water presents a significant threat to public health. As a result, the Environmental Protection Agency (EPA) began the monitoring of United States water supplies for this organism in 2002 **[15]**. These pathogens have been associated with several categories of human infections, such as gastroenteritis ("summer diarrhea"), peritonitis, endocarditis, septicemia, septic arthritis, acute renal failure, and pneumonia **[16]**. Epidemiological studies implicated *Aeromonas* species in causing water and food-borne outbreaks and traveler's diarrhea **[17]**.

A. hydrophila produce an array of virulence factors, and the pathogenesis of *Aeromonas* infections is therefore complex and multifactorial. These factors include O antigens, capsules, the S layer, flagella, exotoxins such as hemolysins, and enterotoxin, and a repertoire of exoenzymes which digests cellular components. These virulence determinants are involved sequentially in enabling the bacteria to colonize, gain, entry, establish, replicate, and cause damage in host tissues and to evade the host defense system and spread, eventually killing the host **[18]**. Phenotypic characteristics of *Aeromonas* spp. have been used to differentiate between environmental strains and those strains causing gastroenteritis; including the lysine decarboxylase, Voges-Proskauer and autoagglutination positivity tests, congo red and crystal violet uptake and the production of a cell-free hemolysin and cytotoxin **[19]**.

Y. enterocolitica and *A. hydrophila* are important human pathogens that are increasingly recognized by researchers as a cause of various clinical syndromes **[20,21]**. The presence of *Y. enterocolitica* and *A. hydrophila* in food products is of a special concern since those organisms are capable of growth at refrigerator temperatures. The presence of these pathogens in clinical, food and environments represents possible hazard **[5,14]**. In several countries, *Y. enterocolitica* has eclipsed *Shigella* species and approaches *Salmonella* species and *Campylobacter* species as the cause of acute bacterial gastroenteritis **[5]**.

There is no published or unpublished data concerning these pathogens in Gaza strip and there are no routine methods for the detection of these pathogens in any of the concerned authorities (Ministry of health, Environmental Quality Authority).

1.2 Objectives

The objectives of this wok are to investigate the presence of *Y. enterocolitica* and *A. hydrophila* in clinical, food and environmental samples; to examine the distribution of these isolates in the different areas in Gaza strip; and to evaluate the methods for the recovery of *Y. enterocolitica* and *A. hydrophila* from clinical, food and environmental samples. The specific aims are as follows:

1- To determine the occurrence of *Y. enterocolitica* and *A. hydrophila* in clinical, food and environmental samples.
2- To evaluate the methods used to detect *Yersinia* and *Aeromonas* pathogens in clinical, food and environmental samples.
3- To compare the occurrence of both bacteria in different sample types and sources.
4- To examine the virulence factors of the isolates.
5- To examine antimicrobial resistance of the isolates

1.3 Significance

Y. enterocolitica and *A. hydrophila* are considered as emerging water and food-borne pathogens because it was shown that *Yersinia* and some *Aeromonas* food isolates can produce different virulence factors, not only at optimal growth temperature, but also at refrigeration temperatures, increasing concern about water and food-borne transmission.

These microorganisms are recognized as an important agent of diarrheal diseases associated with a wide spectrum of clinical and immunological manifestations. As a result of an episode of food poisoning in the United States caused by ingestion of *Yersinia* contaminated chocolate milk and the presence of *A. hydrophila* in fish and seafoods lead the U.S. Food and Drug Administration (FDA) to designate them as a "new" foodborne. At the beginning of the program it was known that the *Yersinia* and *Aeromonas* species associated with food poisoning were among the few enteric pathogens capable of growth at refrigeration temperature (4°C). Also resistance of *Aeromonas* spp. to water chlorination and to multiple antibiotics has resulted in listing the organism on the "Contaminant Candidate List" by the EPA.

This research attempted to detect these pathogens in clinical, food and environmental samples in different areas of Gaza strip and determine sources of *Y. enterocolitica* and *A. hydrophila*. Data generated from this work would be the first to highlight these important pathogens in Gaza strip and it is expected that the results would provide essential background for policy makers and health providers.

CHAPTER 2

LITERATURE REVIEW

This chapter is divided into two major parts; the first part is concerned with *Y. enterocolitica* and the second part is dedicated to *A. hydrophila.* Each part is discussing and reviewing details of the organism (Historical background and taxonomy, ecology and host range, pathogenesis, factors affecting growth, isolation, identification, conformation of pathogenicity, prevalence, infection and epidemiology of the organism).

2.1 *Yersinia enterocolitica*

2.1.1 Historical background and taxonomy

In 1944, **Van Loghem** proposed that a new genus, designated *Yersinia*, be separated from the genus *Pasteurella*. This proposition became effective in 1974. The first species identified in this genus by **Malassez** and **Vingal** in 1883 was *Yersinia pseudotuberculosis.* The second species, *Yersinia enterocolitica,* was identified in 1939 by **Schleifstein** and **Coleman**. This species was found to be heterogeneous and to contain several related species (*"Y. enterocolitica-like")* that were subsequently designated *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii, Y. aldovae, and Y. rhodei*. More recently, *Y. mollareii* and *Y. bercovieri* were also separated from *Y. enterocolitica.* Finally, the species *Y. ruckeri* was included in the genus *Yersinia* but its classification in this genus is controversial **[22]**. Wild-type *Y. pestis* and *Y. pseudotuberculosis* exhibit nearly identical chromosomal DNA relatedness **[23]**.

The genus *Yersinia* presently consists of 11 species, three of which can cause disease in humans and animals; *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* **[24]**. They are invasive pathogenic

bacteria, which have a common capacity to resist non-specific immune response and are lymphotrophic. These three pathogenic species differ considerably in invasiveness; while *Y. enterocolitica* and *Y. pseudotuberculosis* can cross the gastro-intestinal mucosa to infect underlying tissue, *Y. pestis* is injected into the body by an insect bite, and thus, does not have to penetrate any body surface on its own **[25]**.

Y. enterocolitica are included in the genus *Yersinia*, which are classified into the family *Enterobacteriaceae*, a group of gram-negative, oxidase-negative and facultatively anaerobic bacteria. All bacteria belonging to the genus *Yersinia* are catalase-positive, non-spore-forming rods or coccobacilli of 0.5-0.8 x 1-3 μm in size. Strains belonging to *Y. enterocolitica* are urease-positive and can be differentiated from *Y. pseudotuberculosis* with a positive result for fermentation of sucrose, and negative reactions for rhamnose and melibiose fermentation. *Y. enterocolitica Y. pseudotuberculosis* are more active biochemically at 25°C than at 37°C, giving, for example, a positive Voges-Proskauer test only at the lower temperature. Most of the strains are motile at 25°C but non-motile at 37°C **[25]**.

Y. enterocolitica and bacteria that resemble it are ubiquitous, being isolated frequently from soil, water, animals, and a variety of foods **[5]**. They comprise a biochemically heterogeneous group that can grow at refrigeration temperatures **[26]**. Based on their biochemical heterogeneity and DNA relatedness, members of this group were separated into four species: *Y. enterocolitica*, *Y. intermedia*, *Y. frederiksenii*, and *Y. kristensenii*. *Y. enterocolitica* strains and related species can be separated serologically into groups based on their heat-stable somatic antigens **[27]**.

Y. enterocolitica is divided into 18 serogroups. Presently, pathogenic strains belonging to serogroups O:1, 2a, 3; O:2a, 3; O:3; O:8; O:9; O:4,32; O:5,27; O:12,25; O:13a,13b; O:19; O:20; and O:21 have been identified. Therefore, pathogenic strains can belong to diverse serogroups. Serogroups that

predominate in human illness are O:3, O:8, O:9, and O:5,27 **[27]**. These bioserotypes have been shown to have different geographical distributions. Strains that are largely responsible for human yersiniosis in Europe, Japan, Canada and USA belong to bioserotype 4/O:3. Bioserotype 4/O:3 has been recovered in Japan and China, bioserotype 2/O:9 mostly in Europe, and bioserotype 2/O:5,27 is more widely distributed. Strains of bioserotype 1B/O:8 are mostly limited to the USA, but have sporadically appeared in France, Italy and Japan as well **[28]**. Biotype 1A is considered to be non-pathogenic; however, isolates of this biotype have constituted a sizeable fraction of isolates from patients with gastroenteritis **[29]**.

2.1.2 Ecology and host range

Y. enterocolitica is robust organisms capable of long-term survival in natural environments due, in part, to their minimal nutritional requirements and ability to remain metabolically active at extremes of temperature. This capacity to remain viable in nature for extended periods of time as a fecal contaminant is, of course, especially advantageous to these organisms, which are transmitted to hosts via the oral route **[30]**. *Y. enterocolitica* is widely distributed in nature in aquatic and animal reservoirs, with swine serving as a major reservoir for human pathogenic strains **[6,31]**.

2.1.3 Pathogenicity

Human clinical infections with *Y. enterocolitica* ensue after ingestion of the microorganisms in contaminated food **[32]**, or water **[33]** or by direct inoculation through blood transfusion **[34]**. In the gastrointestinal tract, *Y. enterocolitica* and *Y. pseudotuberculosis* can cause acute enteritis (especially in children), enterocolitis, mesenteric lymphadenitis, and terminal ileitis **[30,35]**.

2.1.3.1 Plasmid-encoded virulence factors

All fully virulent *Y. enterocolitica* strains carry an approximately 70-kb plasmid, termed pYV (plasmid for *Yersinia* virulence), which is required for full expression of virulence. Virulence plasmids of pathogenic *Yersinia* are closely related to each other, sharing functional similarities and a high degree of DNA homology. The presence of pYV enables *Yersinia* species to survive and multiply in lymphoid tissues of their host **[36]**. This pYV codes for an outer membrane protein YadA (*Yersinia* adhesin A), a set of secreted proteins called Yops (*Yersinia* outer membrane protein), and their secretion apparatus called Ysc (Yop secretion) **[37]**.

The YadA protein is multifunctional and promotes binding to epithelial cells. The gene *yadA* codes for the major outer membrane protein YadA, which forms a fibrillar matrix on the surface of *Y. enterocolitica* and is only expressed at 37°C. YadA plays a protective role in *Y. enterocolitica,* with several different functions such as, serum resistance, surface hydrophobicity autoagglutination, adhesion to epithelial cells, expression of fibrils on the surface, haemagglutination, binding to intestinal brush border membranes and resistance to killing by polymorphonuclear leukocytes **[38]**. One major role of YadA is to protect *Y. enterocolitica* against killing by polymorphonuclear leukocytes. Although the mechanism is unknown, YadA has been suggested to act by binding to eukaryotic cells, and in doing so, allow delivery of the Yops, thus preventing phagocytosis **[39]**.

The *yop* genes located on the pYV code for at least 14 Yops, which were originally described as *Yersinia* outer membrane proteins because they were detected in the outer membrane fraction of bacterial extracts. With the type III secretion system (Ysc), extracellularly located *Yersinia* that are in close contact with the eukaryotic cell deliver toxic bacterial proteins (Yops) into the cytosol of the target cell **[40]**. Some of the Yops form pores in the eukaryotic target cell membrane, while the other Yops are effector proteins that are

delivered through these pores into the cytosol of the target cell. At least six different Yop effectors are injected by the Ysc secretion translocation apparatus **[41]**.

Genes specifying the type III machinery (*ysc*) are also located on the pYV. The *yop* and *ysc* genes are temperature- and calcium-regulated, being expressed maximally at 37°C in response to the presence of a low calcium concentration **[42]**. All *Yersinia* strains carrying the virulence plasmid exhibit a phenotype known as low-calcium response because it manifests only when pYV-bearing strains are grown at 37°C in media containing a low concentration of Ca^{2+} **[43]**.

2.1.3.2 Chromosome-encoded virulence factors

Chromosome-encoded factors are also needed for pathogenicity. Virulence functions have demonstrated to be transferable with the virulence plasmid only to the plasmid-cured strains derived from virulent parenteral strains **[44]**. Adherence to and invasion of epithelial layers require at least two chromosomal genes, *inv* (invasion) and *ail* (attachment invasion locus) **[45]**.

A. The invasion (*inv*) codes for Inv, an outer membrane protein found on the surface of *Yersinia*, which appears to play a vital role in promoting entry into epithelial cells of the ileum during the initial stage of infection, that is responsible for binding to β_1-integrins on the apical surface of M cells and initiating uptake of the organism **[46]**. Migration through these cells leads to the accumulation of bacteria in the underlying lymphoid tissue (Peyer's patches) and spread to the mesenteric lymph nodes **[47]**. This gene is found in all *Yersinia* spp., however, non-pathogenic strains lack functional *inv* homologous sequences **[48]**. Expression of *inv* in *Y. enterocolitica* responds to both temperature and pH. *inv* expression is higher at 26°C than 37°C during *in vitro* growth, with maximal expression occurring during late logarithmic to early stationary phase; however, expression of *inv* at 37°C can

be restored to levels comparable to 26°C by adjusting the pH of the medium to 5.5 **[49]**.

B. The attachment invasion locus (*ail*) codes for the surface protein Ail, which is produced at 37°C. In contrast to the *inv*, the *ail* was shown to be restricted to strains of serotypes associated with disease **[50]**.

C. The heat-stable enterotoxin (Yst) of *Y. enterocolitica* is chromosomally mediated **[51]**. The role of enterotoxin in the pathogenesis of *Y. enterocolitica* infection is unclear. Non-pathogenic strains of *Y. enterocolitica* and strains of related species have been found to produce Yst using the infant mouse model, and the *yst* gene has been detected in strains of biotype 1A, *Y. kristensenii* and *Y. intermedia* **[52]**. Absence of enterotoxin production *in vitro* at temperatures exceeding 30°C suggests that this toxin is not produced in the intestinal lumen. However, it has been demonstrated with isogenic Yst+ and Yst- strains in young rabbits that, at least in this model, Yst was responsible for diarrhea **[53,54]**.

D. Lipopolysaccharide (LPS) is a major surface component of the outer membrane of gram-negative bacteria. In *Yersinia*, the genes directing the biosynthesis of LPS are chromosomally located. LPS is a complex molecule composed of three main parts: lipid A, oligosaccharide core and O-side chain (O-antigen). The lipid A part is believed to be responsible for endotoxin activity and to play a central role in sepsis and septic shock due to gram-negative bacteria **[55]**. LPS of *Y. enterocolitica* O:8 has a unique structure in which the outer core forms a branch. Serotypes of *Y. enterocolitica* are mainly determined by the variability of O-antigen. While the O-antigen is required for full virulence, its role has yet to be clarified, and absence of O-antigen affects the expression of other virulence factors. A total absence of O antigen in *Y. enterocolitica* has been shown to reduce virulence in the infected mouse model **[56]**.

E. Urease is produced by all clinical isolates of *Y. enterocolitica* and is encoded by the urease gene complex (*ure*) on the chromosome. Bacterial survival depends on the ability to tolerate changes in the environment such as temperature, pH, osmolarity, and nutrient availability **[57]**. Bacteria are able to coordinate appropriate physiological responses to non-life-threatening or gradual environmental changes. *Y. enterocolitica* is a good example of how survival depends upon the ability of the bacteria to adapt to environmental changes. While most environmental changes experienced by *Y. enterocolitica* going from the free-living environment to the host environment allow for a progressive coordinated bacterial response, some conditions, such as changes in pH, occur rapidly and are potentially lethal, requiring the bacteria to maintain presumptive mechanisms for survival **[30]**.

However, more recent studies have implicated urease as a factor that is necessary for survival and pathogenesis of some bacteria **[58]**. More recently, urease activity was shown to affect survival of *Y. enterocolitica* O:9 under acidic conditions both *in vitro* and *in vivo*. For *Y. enterocolitica* O:8 and *Morganella morganii*, the authers describe how this enzyme contributes to survival. In addition, the contribution of urease to acid tolerance was determined for other gram-negative bacteria that survive both free-living and in a susceptible host. The decrease in virulence after intragastric inoculation of *Y. enterocolitica* O:3 urease-negative mutant indicates that the main role of urease is during the initial stage of the bacterial infection, when the bacteria reach the stomach **[59]**.

F. Iron is an essential micronutrient for almost all bacteria, including *Y. enterocolitica.* A variety of alternative pathways have been elucidated for the uptake and utilization of iron by *Yersinia*. To capture iron, highly pathogenic strains of *Y. enterocolitica* biotype IB posses a genomic high-pathogenic islands (HPI) **[60]**. This 35 to 45-kb island carries a siderophore- mediated iron uptake system named the yersiniabactin (Ybt) locus, which is required for full virulence expression in *Yersinia*. The yersiniabactin biosynthesis and

transport genes are clustered within high-pathogenic islands. The less virulent strains of other bioserotypes of *Y. enterocolitica*, including bioserotype 4/O:3, are able to bind and internalize a number of exogenously produced siderophores such as ferrioxamine and ferrichrome **[61]**. Several studies reported *Yersinia* species in clinical states of iron overload, such as hemochromatosis, thalassemia and in children following accidental iron overdose **[62]**.

2.1.4 Factors affecting growth

Y. enterocolitica, as a psychrotrophic bacterium, has the ability to replicate at temperatures between 0 and 44°C **[26]**. The doubling time at the optimum growth temperature (approximately 28 to 30°C) is around 34 min. Although *Y. enterocolitica* can grow at temperatures as low as 0°C, the organism grows much more slowly as temperatures drop below 5°C **[63]**. **Goverde *et al.,*** demonstrated that pYV positive strains grow slower than pYV-negative ones at 30-35°C and 1-10°C. *Yersinia* withstands freezing and can survive in frozen foods for extended periods even after repeated freezing and thawing, but it is susceptible to heat and is destroyed by pasteurization at 71.8°C for 18 seconds **[64]**.

Y. enterocolitica is able to grow over a pH range from approximately 4 to 10, with an optimum pH of around 7.6. *Yersinia* can survive alkaline conditions better than other gram-negative bacteria **[65]**. However, since few foods have an alkaline pH, this high pH tolerance is relatively unimportant. The bacterium's tolerance of acidic conditions, on the other hand, is of great significance. Survival of the high acidity of some foods and the passage through the stomach suggests that *Y. enterocolitica* species are relatively acid-resistant. Although the mechanism of acid tolerance is unknown, it may be due to the activity of urease, which catabolizes urea to release ammonia, which in turn elevates the cytoplasmic pH **[57]**.

Tolerance of *Yersinia* to acid depends on the acidulent used, the environmental temperature, the composition of the medium, and the growth phase of the bacteria. Acetic acid has been shown to be a more effective inhibitor than either lactic or citric acid **[66]**.

Y. enterocolitica is a facultatively anaerobic bacterium that can grow in anaerobic conditions. This bacterium can also grow well in modified atmospheres at 8°C, but with higher levels of CO_2, the length of lag phase will increase and growth will be slower **[67]**. *Y. enterocolitica* has been shown to grow well on meat when packaged in vacuum or in modified atmosphere and stored at 5°C, even in the presence of high background flora **[68]**.

Several studies demonstrated that *Y. enterocolitica* can grow well on both decontaminated and untreated pork when packaged in vacuum and stored at 10°C. However, the growth of serotype O:3 in raw minced meat has been found to be inhibited by natural microflora of the meat in some studies **[69]**.

Y. enterocolitica can tolerate salt (NaCl) at concentrations of up to 5% **[70]**. The inhibition caused by NaCl is strongly dependent on storage temperature. Inactivation of *Y. enterocolitica* by chlorine (0.6 to 20 ppm) was investigated in distilled water and in Trypticase soy broth (TSB, 0.015%) at different temperatures (4, 20, and 40^{o}C). In distilled water, chlorine inactivation of *Y. enterocolitica* was enhanced by increasing the temperature from 4 to 20^{o}C. *Y. enterocolitica* can tolerate both sodium nitrate and nitrite of up to 20 mg/ml for 48 h *in vitro*. However, a nitrite concentration of only 80 mg/kg has been reported to inhibit the growth of *Y. enterocolitica* in fermented sausages **[71]**.

2.1.5 Isolation and identification of pathogenic *Yersinia enterocolitica* from clinical, foods and environmental samples

2.1.5.1 Isolation and enumeration

The source of *Y. enterocolitica* may markedly affect the methods of isolation. To find pathogenic isolates from food and environmental sources is generally more difficult than to find them from stools of infected individuals. During acute gastroenteritis or with organ abscesses, pathogenic *Y. enterocolitica* are often the dominant bacteria and can easily be isolated by direct plating on conventional enteric media **[72]**. Because of the high number of background flora and the low number of pathogenic strains of *Yersinia* in food and environmental samples, direct isolation even on selective media is seldom successful. To increase the number of *Yersinia* strains in these samples, enrichment in liquid media prior to isolation on solid media is required **[73]**.

Recovery of pathogenic *Y. enterocolitica* is contingent upon a number of factors including: the level of background flora on the product; the amount of background flora coming through enrichment and plating; the level of pathogenic *Y. enterocolitica* on the sample; the numbers of non-pathogenic *Y. enterocolitica* and non-pathogenic *Yersinia* spp. present on the product; and loss of virulence factors during enrichment and plating **[74]**. Furthermore, a recovery method which gives good recovery of one serotype of pathogenic *Y. enterocolitica* may not be suited to other serotypes. In order to recover any of the important pathogenic serotypes of *Y. enterocolitica* which might be present, multiple enrichment broths and plating media are usually recommended for the recovery of the organism from naturally-contaminated foods. Several different methods are available for isolation of *Y. enterocolitica* from clinical, food and environmental samples **[5]**.

2.1.5.1.1 Cold enrichment

The psychrotrophic nature of *Y. enterocolitica* is unusual among enteric bacteria, and consequently, enrichment in different solutions at 4°C for prolonged periods has been used for isolation of *Yersinia* spp. **[75]**. Cold enrichment in phosphate buffered solution (PBS) or in phosphate buffered saline with sorbitol and bile salts (PSB) has been widely used for clinical, food, and environmental samples **[76]**. In addition, nutritionally richer media, such as TSB, have been reported to yield better results, particularly when food and environmental samples are studied **[74]**. One major disadvantage encountered with cold enrichment is the long incubation period, typically 21 days, which is unacceptable for quality assurance of foods. **Doyle** and **Hugdahl** have shown that incubation in PBS for 1-3 days at 25°C is as efficient as enrichment at 4°C for some weeks **[77]**. Another problem with cold enrichment is the presence of other psychrotrophic bacteria in foods, which also multiply during the enrichment. By treating cold enrichments with potassium hydroxide (KOH), the background flora can be reduced, making selection of *Yersinia* colonies easier **[78]**. This alkali treatment was developed by **Aulisio *et al.,*** after they observed that *Yersinia* spp. are more tolerant of alkali solutions than many other gram-negative bacteria **[65]**.

2.5.1.1.2 Selective enrichment

Several selective media for isolation of *Y. enterocolitica* at higher temperatures have been developed **[79]**, with different antimicrobial agents being used as selective supplements in these media. **Wauters** formulated a modified Rappaport broth (MRB) containing magnesium chloride, malachite green and carbenicillin, in which the sample was incubated at 25°C for 2-4 days **[80]**. Later, **Wauters *et al.,*** developed an enrichment broth derived from the modified Rappaport base, supplemented with irgasan, ticarcillin and potassium chlorate (ITC) **[81]**.

Schiemann developed a bile oxalate sorbose (BOS) medium for the isolation of *Y. enterocolitica*, particularly for strains belonging to bioserotype 1B/O:8 **[82,83]**. Pre-enrichment in low-selectivity medium prior to selective enrichment in MRB or BOS has also been used for isolation of *Y. enterocolitica* from foods **[75]**.

2.5.1.1.3 Selective agar plates

Several different selective agar plating media have been used for isolation of *Y. enterocolitica.* Initially, plating media, such as MacConkey (MCA) agar, deoxycholate citrate (DC) agar and *Salmonella-Shigella* (SS) agar, developed for other enteropathogens were used. On these media, *Y. enterocolitica* and *Y. pseudotuberculosis* strains grow well but slowly and are easily overgrown by other enteric bacteria because of the low selectivity. Of the traditional enteric media, the most widely used is MCA agar **[82,83]**.

Both modifying existing enteric media and development of entirely new media have achieved improvements in selectivity. SS agar was made more selective for *Y. enterocolitica* by addition of sodium deoxycholate and $CaCl_2$. Used in combination with ITC enrichment, recovery of strains of bioserotype 4/O:3 is good. This agar is widely used because of its high selectivity and commercial availability (ISO1994). However, differentiation of *Yersinia* from competing organisms, such as *Morganella, Proteus, Serratia* and *Aeromonas,* can be difficult. Cefsulodin irgasan novobiocin (CIN) agar is one of the media developed for isolation of *Y. enterocolitica* and *Y. pseudotuberculosis* **[84]**.

In several comparative studies, CIN agar was found to be the most selective plating medium for *Yersinia* spp. **[85]**. Organisms capable of fermenting mannitol, like *Yersinia*, produce red “bull's eye” colonies on CIN agar. Only *Citrobacter freundii, Enterobacter agglomerans* and species of *Aeromonas* and *Klebsiella* produce similar colony morphology **[86]**. Other selective agar,

virulent *Yersinia enterocolitica* (VYE) **[5]**, hase been developed for isolation of *Y. enterocolitica* strains, but CIN agar is the most generally accepted because of its high selectivity and the high confirmation rate of presumptive isolattion. Moreover, the commercial availability of this medium makes it convenient to use **[84]**.

2.1.5.2 Identification

Johnson determined the minimum number of biochemical tests required for identifying *Yersinia* amongst bacteria growing and presenting similar colony morphology on CIN agar; two tests, Kligler iron and Christensen's urea tests, were sufficient. *Y. enterocolitica* can be identified with biochemical tests such as fermentation of sucrose, rhamnose and melibiose **[74]**.

Commercial rapid identification tests provide suitable alternatives to the conventional tube tests. The Analytical Profile Index (API 20E) system, widely used for identification of presumptive *Yersinia* isolates, has been shown to be accurate in identifying of *Y. enterocolitica.* This kit system has a positive identification rate of 93% for *Y. enterocolitica* incubated at 28°C instead of 37°C. In the study by **Sharma *et al*.,** identification of *Y. enterocolitica* biotypes 3, 4 and 5 was excellent, with a positive predictive value of 99% when the strips were incubated at 28°C for 18-24 h **[87]**.

2.1.6 Confirmation of pathogenicity

Y. enterocolitica is a ubiquitous microorganism and, although the majority of isolates recovered from non-human sources are non-pathogenic, thus having no clinical significance, it is important to assess the pathogenicity of isolates **[24]**.

2.1.6.1 Animal tests

The pathogenicity of *Y. enterocolitica* can be studied by animal tests such as the guinea pig conjunctivitis model (Sereny test) **[88]**, suckling mouse assay, mouse intraperitoneal challenge, and mouse diarrhea and splenic infection following oral challenge **[89,90]**. However, because animal testing tends to be costly and is subject to increasing public opposition, it has largely been replaced by *in vitro* tests.

2.6.1.2 Phenotypic tests

A number of phenotypic characteristics associated with the virulence plasmid have been described **[91]**. Calcium dependence, measured by growth restriction on magnesium oxalate agar **[92]**, autoagglutination at 35-37°C **[93]**, uptake of Congo red and crystal violet **[94]** are the most popular indirect markers for identifying pathogenic strains of *Y. enterocolitica*. The pyrazinamidase (PYZ) test and the tissue culture invasiveness assay are proven indicators of potentially pathogenic isolates **[10]**. However, both of these tests measures functions that are chromosomally mediated, and thus, cannot replace pathogenicity tests, since they are only correlated with the ability of the strain to harbor the plasmid, and not to the presence of the plasmid itself. No single phenotypic virulence-associated characteristic has been shown to be a reliable indicator of pathogenicity **[95]**.

2.1.6.3 Genotypic tests

Several colony hybridization, amplified fragment length polymorphism (AFLP) and polymerase chain reaction (PCR) assays have been designed to verify the pathogenicity of *Y. enterocolitica* isolates specifically and rapidly **[96-98]**. The methods are based on specific segments of the virulence plasmid or the chromosomal DNA that have known virulence functions.

2.1.7 Prevalence of *Yersinia enterocolitica*

2.1.7.1 In animals

Animals, especially domestic animals, have been suspected as transmitters of *Y. enterocolitica* to humans. *Y. enterocolitica* infection in animals is not notifiable, and reports on its prevalence are for the most part results from research projects obtained from institutions or authorities in the member countries. Various studies have investigated wild and farm animals for the presence of pathogenic strains of *Y. enterocolitica*, and only a few have been recovered **[99]**. **Shayegani *et al.,*** examined fecal specimens from 1,426 animals including mammals, birds, reptiles, fish and invertebrates throughout New York State. Eleven human pathogenic strains were isolated, including bioserotype 1B/O:8 , 4/O:3 and O:5,27 **[100]**. In various studies examining healthy domestic animals and animals with acute enteritis, including cattle, sheep, goats, deer, calves, broilers, hens, turkeys and ducks, only a few pathogenic isolates have been recovered, and they differed from those usually associated with human infections **[101,102]**.

Pet animals, such as cats and dogs, have been suspected of being reservoirs for human infections with *Y. enterocolitica*, because of their close contact with humans. Dogs and cats occasionally harbor *Y. enterocolitica* 4/O:3 **[103,104]**. **Fredriksson-Ahomaa, Korte** and **Korkeala** showed that raw pork was an important source of yersiniosis in dogs and cats. The infected dogs showed no clinical signs of infection. The duration of fecal shedding by the dogs varied between 7 and 23 days. These findings suggest that dogs can act as a potential source of the infection to humans **[6]**.

Pigs are healthy carriers of *Y. enterocolitica* 4/O:3 and are the only animal species from which the bacterium can be isolated frequently. Based on results from several studies, it can be concluded that *Y. enterocolitica* 4/O:3 is present in the pig population in many countries in the world. The

prevalence often varies herd-wise **[6]**. **Korte *et al.,*** found that the prevalence in fattening pigs in Finland, investigated in five slaughterhouses, increased from 33% to 64% between 1995 and 1999 **[105]**.

2.1.7.2 In foods

The primary route of human infection is most probably ingestion of food. Based on the information on domestic cases notified by clinicians for 2004 in Sweden, the Swedish Institute for Infectious Disease Control reported that 75% of the yersiniosis cases were suspected to be food- or waterborne infections. However, pathogenic strains of *Y. enterocolitica* are difficult to be isolated from food. In several studies, high frequencies of nonpathogenic strains have been isolated from food, whereas pathogenic strains are only occasionally recovered **[106]**. In studies from different countries, various ready-to-eat products have been investigated including, fresh salad, whole and sliced vegetables, sandwiches, milk, dairy products, desserts and soft cheese **[14]**. Only a few pathogenic strains were isolated from these foods.

Raw and pasteurized milk have been examined in several studies because outbreaks over a number of years in the United States were traced to milk. With the exception of a few isolates of O:5, 27, none of the strains isolated in these studies were identified as pathogenic **[107]**. Pork is likely to be an important vehicle of the infection to humans because pigs are the only animals consumed that frequently harbor the same bioserotype of *Y. enterocolitica* as is isolated from human yersiniosis cases. However, the problem is that only few pathogenic strains have been recovered from pork or pork products **[5]**.

From outbreaks and case-control studies, there are other indications that pork is involved in the transmission of the pathogen to humans. Two outbreaks have been traced to ingestion and/or handling of contaminated pork. In one outbreak, home prepared ‘pork cheese’ (a sausage variant) was identified as the source of the infection. Preparation of pork chitterlings (a

dish made from pig intestines) was the source of the infection in the other outbreak. Both outbreaks involved *Y. enterocolitica* 4/O:3 as the causative agent **[108]**.

2.1.7.3 In environment (including water)

Y. enterocolitica and other *Yersinia* species are ubiquitous in the natural environment and can be isolated from soil, foliage, surface water, sewage water and sludge, have been non-pathogenic **[109]**. However, the vast majority of the isolates lacks the classical markers of bacterial virulence and is considered nonpathogenic. The possibility for the microorganism to survive in this type of environment has been investigated **[109]**.

In a study carried out in Australia, 251 water samples tested by nested PCR. Eleven samples from 4 separate locations tested positive. One of the PCR-positive results was confirmed by culture. Some waterborne cases/outbreaks caused by the bacterium have been reported from North America. The isolated strains belonged to bioserotype 1B/O:8, a bioserotype not commonly isolated in the United States after the 1980. Bioserotype 4/O:3 was isolated from well water in a small family outbreak of gastroenteritis in Ontario, Canada **[110]**.

2.1.7.4 In human

Y. enterocolitica was first recognized as a human pathogen in the 1930 **[24]**. Bioserotype 4/O:3 is the most common type of *Y. enterocolitica* recovered from humans with diarrhea. The highest incidence of enteritis caused by this type has been found in young children. However, **Morris *et al*.,** have also isolated strains of bioserotype 4/O:3 at a high rate from asymptomatic children **[111]**.

The infection rate is probably much higher since only the most serious cases are registered. Only a few isolates of O:9 and O:5,27 are reported annually. During the 1980, several countries in Europe reported a dramatic increase in the number of recovered human cases. In the beginning of the 1990, the diagnosed yersiniosis cases in Sweden reached numbers exceeding 1000 and in 1996 it was classified a notifiable disease. In the United States, the Center for Disease Control and Prevention (CDC) estimates about 17,000 annual cases, *i.e*. an incidence of 6 per 100,000 inhabitants **[112]**.

2.1.8 *Yersinia enterocolitica* infections

2.1.8.1 In animals

Several reports have been presented on isolation of *Y. enterocolitica* strains from a variety of animals, but descriptions of observed clinical manifestations or patho-anatomical changes are sparse. Sporadic, small outbreaks of enteritis caused by *Y. enterocolitica* have been reported in chinchillas, hares, sheep and goats **[101]**. However, both biochemical and serological patterns deviated from those of human strains.

Pigs have been experimentally infected with bioserotype 4/O:3 in several studies **[113]**. Strains of *Y. enterocolitica* 4/O:3 have been shown to cause gastroenteritis in new-born, colostrum-deprived piglets, whereas full-term colostrum-fed piglets seem to be quite resistant to infection. In colostrum-fed piglets, colonization was typically restricted to the throat and intestinal tract without development of serious illness. Fattening pigs have been shown to excrete high numbers of *Y. enterocolitica* 4/O:3 in faeces for several weeks after infection mostly without any symptoms. However, **Thibodeau *et al,***. demonstrated that the fecal shedding stops soon after ingestion of bacteria and only tonsillar infection occurs **[114]**.

2.1.8.2 In humans

Y. enterocolitica can cause gastro-intestinal symptoms ranging from mild self-limited diarrhea to acute mesenteric lymphadenitis evoking appendicitis. Sometimes focal disease, such as pharyngitis, cellulitis, abscess, pneumonia and meningitis, may occur without gastro-intestinal illness **[30]**. The incubation period of *Y. enterocolitica* enterocolitis ranges from 1 to 11 days. The minimal infective dose for humans has not been determined. Symptoms of enterocolitis typically persist for 5 to 14 days, but they may occasionally last for several months. The duration of the excretion of the organism in stool has been reported to range from 14 to 97 days. The clinical manifestations of infection depend on factors such as the age and physiological state of the host and the pathogenic properties of the particular strain **[30]**.

Most commonly, *Y. enterocolitica* infections occur in young children **[25]**. In patients under 5 years of age, yersiniosis presents as diarrhea, often with low-grade fever and sometimes with abdominal pain. The symptoms can even be so faint and short-lived that yersiniosis is not diagnosed, despite fecal carriage. In older children and young adults, acute yersiniosis can be present as a pseudo-appendicular syndrome, which is frequently confused with appendicitis **[17]**.

Sepsis is a rare complication of *Y. enterocolitica* infection, except in patients who have a predisposing underlying disease or are in an iron-overloaded state **[115]**. Sepsis can also occur during blood transfusion. One source of *Y. enterocolitica* -contaminated red blood cell concentrate has been reported to be a blood donor with asymptomatic bacteremia **[116]**. Normally, yersiniosis is a self-limited disease, but sometimes long-term sequelae, including reactive arthritis, erythema nodosum, glomerulonephritis and myocarditis, will occur. Post-infection complications usually develop within one week to one month of initial infection, and these may be the only obvious clinical manifestation of *Yersinia* infection **[17]**.

2.1.9 Epidemiology and disease outbreaks

The distribution of *Y. enterocolitica* appears to be temperate, involving the United States, Northern Europe, Canada, and Australia. There is also some speculation that the seasonal incidence of disease peaks in cooler months. These observations have been linked to the fact that the organism is cold-adapted and can survive and grow at 4^{o}C. In Europe the serotypes O:3 and O:9 predominate, whereas in the United States serotype O:8 is the most frequently isolated. Serious disease has been linked, however, to all three of these serotypes, regardless of geographic locale **[28]**.

Epidemic outbreaks are usually fled to particular serotypes, but it is likely that other serotypes are involved in the sporadic occurrence of enterocolitis **[117]**. *Y. enterocolitica* is primarily a zoonotic disease. Humans are incidental hosts. Illness is usually sporadic and outbreaks are rare **[118]**.

2.1.9.1 Outbreak locations

In Denmark, to examine the general frequency of household outbreaks, the authors performed a retrospective search among cases of the five most frequent gastrointestinal bacterial pathogens in Denmark, a country of 5.3 million inhabitants. This was done for 57,667 cases registered from 1991 to 2001 by finding all cases that shared addresses and became infected within 3 weeks of one another. The percentage of cases that were part of household outbreaks was found to be 2.0% for *Y. enterocolitica* **[119]**.

In New York, USA, In September and October, 1976, an outbreak of illness due to chocolate milk contaminated with *Y. enterocolitica* resulted in hospitalization of 36 children, 16 of whom had appendectomies **[121]**. An epidemiologic investigation demonstrated that illness was associated with drinking of chocolate milk purchased in school cafeterias, and *Y. enterocolitica* O:8 was subsequently isolated from the milk. The investigation

suggested that the bacterium was introduced at the dairy during the mixing by hand of chocolate syrup with previously pasteurized milk **[121]**.

Gastrointestinal disorders of varying severity were observed in 239 (53%) of 455 campers and staff members at a coed summer camp in Sullivan County, New York, during July 1981. Five of seven hospitalized patients had appendectomies before the disease was recognized as yersiniosis. *Yersinia enterocolitica* serogroup O:8 (American strain) was isolated from 37 (54%) of 69 persons examined, including the head cook and 3 others of the 11-person kitchen staff. Of 48 food, water, and environmental samples collected from the camp area, *Y. enterocolitica* isolates belonging to the same serogroup and biogroup as the human isolates were recovered from dissolved powdered milk, a milk dispenser, and turkey chow mein **[120]**.

This laboratory finding supported the epidemiological data indicating a correlation between consumption of these foods and illness. *Y. enterocolitica* isolates of the same biogroup as the O:8 isolates but belonging to serogroup O:34 were also isolated from six campers and two samples of dissolved powdered milk **[120]**.

In June and July 1982, a large interstate outbreak of *Y. enterocolitica* infections caused by an unusual serotype occurred in Tennessee, Arkansas, and Mississippi. In three separate case-control studies, drinking milk pasteurized by plant A was statistically associated with illness. In a survey of randomly chosen households, 8.3% of persons who recalled having drunk milk from plant A during the suspect period experienced a yersiniosis-like illness. Inspection of the plant and cultures of the available raw and pasteurized milk did not reveal the source or mechanism of contamination or a breach in normal pasteurizing technique **[121]**.

Although outbreaks of enteric disease caused by pasteurized milk are rare in the United States, the ability of *Y. enterocolitica* to grow in milk at refrigeration temperatures makes pasteurized milk a possible vehicle for virulent *Y. enterocolitica*. The extent to which milk is responsible for sporadic cases of yersiniosis is unknown **[121]**.

Twelve cases of *Yersinia* infection in infants less than 1 year of age were identified in Tennessee with onset from November 15, 2001 to February 15, 2002. All cases were identified by stool culture. Six cases occurred in December, and 10 were medically evaluated in the same city. All case-patients were black. In comparison, 49% of the population of the urban county in which the outbreak was identified is black. In this case-control study of *Y. enterocolitica* infections among black infants, chitterling preparation was significantly associated with illness (p less than 0.001). Of 13 samples of chitterlings tested, 2 were positive for *Yersinia intermedia* **[122]**.

In Japan. On 3 August 2004, a local public health bureau in Nara Prefecture received a report of a food poisoning case at a nursery school. Of 182 nursery school children, 42 were infected; none of the 20 staff members were infected. From clinical symptoms and bacterial isolations, the patients were diagnosed as having been infected with *Y. enterocolitica* **[123]**.

Y. enterocolitica serotype O:8 was isolated from 16 of 32 patients, none of the 17 childcare workers, and none of the 3 cooking staff members from whom stool samples were taken. In addition, 5 strains were obtained from medical facilities. *Y. enterocolitica* serotype O:8 was also isolated from salads containing apples, cucumbers, ham, potatoes, carrots, and mayonnaise, which were served during lunch at the nursery school on 23 July **[123]**.

2.1.9.2 Possible transmission routes of sporadic *Yersinia enterocolitica* infections

Pigs are considered to be the main source of human *Y. enterocolitica* 4/O:3 infections, even though a definite connection between isolates from pigs and human infections has still to be established. Elevated serum antibody concentrations have been found among people involved in swine breeding or pork production, suggesting a direct transmission of this bacterium from pigs to humans. In Finland, slaughterhouse workers and pig farmers were observed to have elevated antibody levels to *Y. enterocolitica* O:3 twice as frequently as grain- or berry farmers. Similar differences have also been discovered between people involved in swine slaughtering practices and office personnel in Norway **[124]**.

Pet animals have also been suspected of being sources for human infections because of their close contact with humans. However, direct transmission from pets to humans has yet to be proven. The most common transmission route of pathogenic *Y. enterocolitica* is thought to be fecal-oral via contaminated food, although pathogenic isolates have seldom been recovered from food samples **[125]**.

Direct person-to-person contact has not been demonstrated, but **Lee *et al.,*** reported *Y. enterocolitica* O:3 infections in infants who were probably exposed to infection by their caretakers **[108]**. Indirect person-to-person transmission has apparently occurred in several instances by transfusion of blood products **[126]**. In these cases, the most likely source of *Yersinia* has been blood donors with subclinical bacteremia.

2.2 *Aeromonas hydrophila*

2.2.1 Historical background and taxonomy

Species of *Aeromonas* are Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacteria that occur ubiquitously and autochthonously in aquatic environments. Some species are pathogenic for animals and humans. Although historically the *Aeromonas* genus has been placed in the family *Vibrionaceae*, there have been proposals to place it in its own family, the *Aeromonadaceae*. The aeromonads share many biochemical characteristics with members of the Enterobacteriaceae, from which they are primarily differentiated by being oxidase-positive **[16]**. Earlier literature focused mainly on *A. hydrophila* but several later studies have shown that the majority of clinical isolates fall within three species **[127]**.

The genus *Aeromonas* includes at least 13 genospecies, among which are the mesophilic *A. hydrophila*, *A. caviae*, *A. sobria*, *A. veronii*, and *A. schubertii*, and the non-motile, psychrophilic *A. salmonicida*. *A. salmonicida* is a fish pathogen and has not been associated with human infection. By contrast, the mesophilic species have been associated with a wide range of infections in humans **[128]**. Although members of the genus have classically been divided into three biochemically differentiated groups (typified by *A. hydrophila*, *A. caviae*, and *A. sobria*), these contain a number of genospecies, to which new species have been added **[129]**.

The current taxonomy of the genus *Aeromonas* is based upon DNA-DNA hybridization and 16S ribosomal DNA relatedness studies. The genera of the family *Aeromonadaceae* now include *Aeromonas*, *Oceanimonas*, *Oceanisphaera*, and *Tolumonas* (*incertae sedis*). The current genomospecies and phenospecies within the genus *Aeromonas* are *A. hydrophila* ssp. *dhakensis* (subsp. nov.), *A. hydrophila* ssp. *ranae* (subsp. nov.), *A. culicicola* (sp. nov.), *A. simiae* (sp. nov.), and *A. molluscorum* (sp.

nov.) have been proposed as new species and subspecies of *Aeromonas*, and more will undoubtedly be described **[16]**.

The *A. hydrophila* causing human diseases are associated with a variety of infections including septicemia, wound infections, meningitis, peritonitis, and hepatobiliary infections. Some strains of *Aeromonas* produce enterotoxins responsible for causing gastroenteritis in humans, since these bacteria are widely distributed throughout the environment in water and foods, especially during summer months **[128]**.

The genus *Aeromonas* consists of straight, coccobacillary to bacillary gram-negative bacteria with rounded ends measuring 0.3-3.5 mm **[16]**. They occur singly, in pairs, and rarely as short chains. Motile strains produce a single polar flagellum, though peritrichous or lateral flagella may be formed on solid media by some species. *Aeromonas* spp. are facultatively anaerobic, catalase positive, oxidase positive, chemoorganotrophic bacteria that exhibit both oxidative and fermentative metabolism on carbohydrates **[129]**.

Serotyping is based upon somatic (O) antigen determinants as described by **Sakazaki** and **Shimada [130]**. Several typing schema have been proposed **[131]**, but only one comparison study of two of these schema has been published **[132]**. The schema of **Sakazaki** and **Shimada** recognizes 44 serogroups, with an additional 52 provisional serogroups proposed by **Albert [130]**. *Aeromonas* spp. are found to be serologically heterogeneous, with individual serogroups found in more than one species. Most type and reference strains were not serologically representative of a genomospecies. Three serotypes predominate in clinical samples, O:11 (24%), O:16 (14%), and O:34 (10%) **[128]**.

Korbsrisate characterized the distribution of *A. hydrophila* serogroups in clinical samples and developed polyclonal antibodies for rapid identification of clinical isolates by direct agglutination. Only 50% of strains fell into the

common serogroups O:11, O:16, O:18, O:34, or O:83. Rough strains (15.2%) and untypable strains (2.3%) reduced the effectiveness of serotyping for identification of clinical strains. A polyvalent antiserum was produced that resulted in positive agglutination of 102 or 105 strains, for a calculated sensitivity of 97.1% and specificity of 90.7%. This test could be useful in rapid identification of aeromonads to genus where they are isolated from samples that may also contain vibrios **[133]**.

2.2.2 Ecology and host range

A. hydrophila are found worldwide in aquatic environments, including ground water, surface waters, estuarine and marine waters, drinking water, and wastewater **[134]**. *A. hydrophila* are found in foods, including fresh grocery produce, seafood, raw meats, packaged ready-to-eat meats, cheese, and milk **[135]**. While *Aeromonas* spp. are not considered fecal bacteria, they are present in the feces of healthy animals and humans, presumably as the result of ingestion of food and water containing these organisms **[136]**.

They are present in high numbers in sewage before and after treatment, thus they have been proposed as an indicator of sewage-contaminated surface water. *A. hydrophila* may colonize drinking water distribution systems and produce biofilms that resist disinfection **[137]**.

2.2.3 Pathogenicity

Although most of the active research on *Aeromonas* species concerns the identification of virulence factors or mechanisms potentially operative in human or animal infections, only one factor, the S layer of *A. salmonicida*, has been linked to the overt pathogenicity of this species in causing serious infections in fish. Most of the other reputed virulence factors produced by *A. hydrophila* have been linked to pathogenicity in humans by inference; that is similar molecules have been shown to play important roles **[128]**.

Virulence factors are present in two forms, cell-associated structures, and extracellular products. Among the cell-associated structures are pili, flagella, outer membrane proteins, lipopolysaccharide, and capsules. The major extracellular products include cytotoxic, cytolytic, hemolytic, and enterotoxic proteins **[128,138]**.

2.2.3.1 Cell-associated structures

A. Pili. While early studies had indicated that a number of *Aeromonas* strains were piliated, a detailed analysis of such structures was not undertaken until recently. *A. hydrophila* produce an array of filamentous structures, including short rigid, and long wavy pili, and polar and lateral flagella. Removal of pili or neutralization of attachment sites by homologous antibody treatment limits or defeats adherence properties in cell culture systems. Polar flagella and lateral flagella were described by **Rabaan *et al*.,** and **Kirov *et al*., [139,140]**. Polar flagellins function as adhesions, while lateral flagellins are thought to serve as colonization factors **[141]**.

B. Capsule production has been reported for *A. hydrophila* serogroups, but the function of capsule material is vague. It is presumed to resist complement activity and perhaps enhance adherence **[142]**.

C. S-layers (originally termed A-layer in *A. salmonicida*) are paracrystalline structures made up of identical protein subunits that are translocated across the cell membrane and assembled on the cell wall surface via an interaction with O-polysaccharide side chains of lipopolysaccharide. *A. hydrophila* stains producing S-layers are more pathogenic for fish, but the role of S-layer in human infection is not clear. Studies suggest that strains containing S-layers autoagglutinate **[143]**.

D. Outer membrane proteins (OMP**)** of *A. hydrophila* are rather heterogeneous, although most strains produce a 36 k Da protein. In addition,

iron-starved *A. hydrophila* cells synthesize new OMP of 68 – 93 k Da. **Mittal *et al.,*** reported that a group of *A. hydrophila,* virulent fish pathogens, exhibited a number of unique phenotypic properties that were thought to be cell-surface associated, including autoaggregation during growth in static broth culture **[144]**.

E. Lipopolysaccharide (LPS) endotoxin. The O-antigen structure of virulent strains of *A. hydrophila* has been shown to have many points of similarity with that of the O-antigen of *A. salmonicida*. LPS endotoxin is an important component of the outer membrane of *A. hydrophila,* which has been shown to enhance red-leg disease in frogs. However, the importance of this toxin in human infections has not yet been elucidated **[145]**.

2.2.3.2 Extracellular products

Most aeromonads elaborate a large number of extracellular enzymes that actively degrade a variety of complex protein, polysaccharide, muco-polysaccharide, and lipid-containing molecules. Although these enzymes are, in many instances, useful in identification as in the case of DNase, their roles in the physiologic functions of the bacterium or in virulence are largely unknown. To date, with minor exceptions, most extracellular factors produced by *Aeromonas* species are thought to play a role in gastrointestinal disease; this association will remain unproved until suitable models are developed for their study. Another reason for the difficulty in understanding the role that various extracellular enzymes play in pathogenesis concerns their multifunctional nature. A prime example of this latter problem is the *Aeromonas* hemolysin(s), which appears to be not only cytolytic but also enterotoxigenic **[128]**.

A. Hemolysins. Probably the most striking cultural feature displayed by many *Aeromonas* strains is their ability to hemolyze erythrocytes when grown on a suitable agar-based medium. This characteristic is principally

associated with certain strains belonging to the phenospecies *A. hydrophila* and *A. sobria* and is linked to the elaboration of an extracellular hemolysin(s) **[146]**. Such a hemolysin, which typically belongs to a larger group of pore-forming bacterial cytolysins, causes leakage of the cytoplasmic contents from target cells via disruption of the normal integrity of the cell membrane **[147]**. The end result is death, either by osmotic lysis or by a nonosmotic process. At least two major classes of hemolysins expressed by *Aeromonas* strains have been reported. One class, originally termed "aerolysins" by **Bernheimer** and **Avigad [128]**, comprises typical beta-hemolysins that produce clear zones of hemolysis on blood agar.

The aerolysin is synthesized in a precursor form, from which the signal sequence is removed prior to export across the bacterial outer membrane. A second class of hemolysins, termed alpha-hemolysins, has been primarily studied by a number of Swedish investigators. The alpha-hemolysin is elaborated during the stationary phase and is not expressed when temperatures exceed 30°C. When observed on blood agar, this hemolysin produces an opaque, incomplete type of hemolysis that is often seen as the inner hemolytic zone of a strain producing "double-zone" hemolysis. Both alpha- and beta-hemolysins have observable but different effects on cell culture lines, although the effect of the beta-hemolysin appears irreversible **[128]**.

B. Proteases are enzymes that are capable of cleaving peptide bonds. A number of extracellular proteases produced by gram-negative bacteria are thought to play important roles in pathogenesis and virulence. *Aeromonas* isolates secrete at least four or five different proteases, as determined of pH optima and substrate specificities. Two major proteases produced by *A. hydrophila*. One enzyme is a heat-stable protease that is inactivated by EDTA and appears to belong to the general class of thermostable metalloproteases; the other protease is heat labile (56°C, 30 min) and belongs to the thermolabile serine protease family, the metallo- and serine

proteases are involved in toxin activation and have a protective role in inimical environments **[128]**.

C. Siderophores are low-molecular-weight compounds with high affinities (binding capacities) for various organic and inorganic forms of iron, particularly under iron-limiting conditions. These compounds are thought in many instances to play important roles in the establishment of infection, and the hydroxymate class of siderophores has been associated with gram-negative bacteria. Almost all strains of A*. salmonicida, A. hydrophila, A. sobria,* and *A. caviae* studied elaborate one or more types of siderophores **[148,149]**.

2.2.4 Factors affecting growth

A. hydrophila have their natural habitat in water and grow over a wide temperature range. Because *A. hydrophila* grow between 0ºC and 45ºC, with a temperature optimum of 22ºC to 32ºC, there are few environmental habitats where they are not found. Both high and low **[150]** survival rates have been reported. Nutrient availability, temperature, and water activity most affected growth rates. Growth was optimal at 30ºC at pH 7 and a water activity of 0.99. *A. hydrophila* have been shown to grow in foods held at refrigerator temperatures **[151]**.

Growth temperature is an important feature in differentiation of clinical and environmental strains. Approximately half of clinical isolates show some growth at 4-5 ºC, all food isolates grow at this temperature. While most clinical strains grow at 42 ºC, only a few isolates from vegetables stored at 5ºC grew at elevated temperature. The growth temperature range for *A. hydrophila* is from 4 to 44ºC, but individual strains typically have a restricted growth range according to their ecological niche, and growth of a strain at both extremes of the range are rare **[14]**. *A. hydrophila* are considered heat sensitive with respect to other foodborne pathogens.

A. hydrophila tolerate high pH well and this feature has been exploited by using alkaline peptone water at pH 8.6 for sample enrichment. *A. hydrophila* grow at pH 5.8 or higher, and may survive at pH 4.6 or higher according to computer modeling using Food Micromodel **[151]**. Species-specific acid tolerance is know to occur, since *Aeromonas* spp. grown on glucose or other simple sugars produces sufficient acetic acid to auto-sterilize a broth culture within 48 hr. in weakly buffered systems. This metabolic activity has been called the suicide phenomenon **[152]**.

Modified atmospheres are increasingly being used in food packaging. **Pin *et al.*,** studied the response of *A. hydrophila* to various combinations of pH, temperature, and CO_2 and O_2 concentrations. The results were used to develop and validate a predictive model for growth and death estimates under modified atmospheres at refrigerator temperature. Reduced oxygen levels do not exert a detrimental effect on survival and growth of aeromonads, and they may be isolated from vacuum packed foods **[153]**.

2.2.5 Isolation and identification of pathogenic *Aeromonas hydrophila* from clinical, foods and environmental samples

2.2.5.1 Isolation and enumeration

Isolation of *A. hydrophila* from food and environmental samples provides a challenge because of the presence of competing bacteria and the possibility of sample matrix interference with sample preparation and culture methods. The use of dilution schemes and enrichment media facilitate isolation of *A. hydrophila* from heavily contaminated samples such as sewage, sludge and sewage effluents. **Palumbo *et al.*,** compared several culture media for isolation and enumeration of aeromonads from water samples and concluded that ampicillin dextrin agar (ADA) produced the best overall results **[154]**.

Several culture enrichment and culture media have been evaluated for isolation of *A. hydrophila* from foods. Starch ampicillin agar (SAA) and bile salts inositol brilliant green agar (BIBG) with prior enrichment in tryptose broth containing ampicillin (TSB-30) (ampicillin 30 mg/L) **[155]** are recommended, together with commercially available media such as *Aeromonas* Medium (Ryan's Medium) **[156]**. Starch glutamate ampicillin penicillin (SGAP-10) medium was used to isolate aeromonads from sewage sludge. This medium is highly selective, and it has been used to isolate *A. hydrophila* from foods and other challenging matrices. Samples are prepared in dilution, inoculated into culture media with or without enrichment, and incubated aerobically at 35ºC for 24-48 hr. Colonies are screened by performing a spot oxidase test and identified using biochemical methods or commercially-available bacterial identification kits **[157]**.

Isolation of *A. hydrophila* from contaminated samples such as feces require the use of selective and differential plating media such as MacConkey agar, cefsulodin irgasan novobiocin (CIN) agar, or blood ampicillin agar (10 mg/L ampicillin) **[158]**. To facilitate recovery of *A. hydrophila* from heavily contaminated specimens such as feces, enrichment broths such as alkaline peptone water are incubated overnight and subcultured to blood ampicillin agar and CIN agar. Culture plates are incubated aerobically at 35ºC for 24-48 hr. *Aeromonas* spp. produce characteristic colonies, with or without hemolysis on blood agar, and colonies may be quickly screened using the spot oxidase test. Oxidase positive colonies are further screened using tube biochemicals or by inoculation of a cell suspension into one of the commercially available bacterial identification kits **[159]**.

2.2.5.2 Identification

Commercial systems for bacterial identification are notoriously inaccurate for identification of *Aeromonas* spp., since they do not incorporate the key substrates necessary for correct identification **[160]**. **Vivas *et al*.,** compared

MicroScan Walk/Away in conjunction with the MicroScan Combo Negative type 1S panels with conventional biochemical methods for identification of 85 environmental, clinical and reference strains of *Aeromonas* spp. Using the MicroScan Combo Negative type1S substrate panel, 67 of 88 (78.8%) of strains were correctly identified, 4 of 88 (4.7%) of strains were incorrectly identified, and 10 of 88 (11.8%) of strains represented rare biotypes that could not be identified **[161]**.

Carnahan and **Joseph** found that colistin resistance could be used as an additional phenotypic marker for identification of aeromonads. *A. hydrophila* group is 85.8% resistant, while *A. caviae* group is 2.1% resistant. When colistin was included in a 14 panel test format, 96.2% of strains could be identified to phenospecies and 93.6% of strains could be identified to genomospecies **[162]**.

Canonica *et al.,* used whole cell fatty acid analysis (FAME) by gas-liquid chromatography to correctly classify *A. hydrophila*, *A. sobria*, and *A. caviae*. While this method offers the advantages of an instrumental method with autosampling for unattended operation, the reliability of identifications does not compare to newer genomic methods **[163]**.

2.2.6 Confirmation of pathogenicity

One of the major drawbacks in studying virulence determinants related to *Aeromonas* pathogenicity has been the inability to establish appropriate organ or animal models that faithfully reproduce the specified disease observed *in vivo*. This situation is particularly acute in the case of *Aeromonas* associated gastroenteritis. Establishing such models is critical for identifying strains of high- and low-virulence potential and for comparing extracellular and cell-associated factors associated with these strains which lead to the recognition of determinants and gene products involved in microbial pathogenicity **[128]**. Once virulence determinants are identified, phenotypic

markers that allow accurate and rapid identification of pathogenic strains and the epidemiology associated with such infections can be sought **[128]**.

2.2.6.1 Animal tests

Most investigations determining the relative pathogenicities of individual *Aeromonas* strains have used either mouse or fish models **[149,164]** for assessing relative virulence, although limited studies have also been performed with chicks and turkey poults **[128]**. Values obtained from such investigations indicate that between 10- and 1,000-fold fewer bacteria are required to produce mortality in susceptible fish than in susceptible mice.

2.2.6.2 Phenotypic tests

While inherent pathogenic differences do exist among aeromonads, few virulence phenotypic markers reported in the literature go beyond the definition of pathogenicity at the phenospecies level. One group of major interest, however, is *Aeromonas* serogroup O:11 strains, which are primarily associated with severe invasive disease in both humans and animals **[165]**. These strains are characterized by their autoagglutination or aggregation in broth, the presence of an unusual LPS side chain architecture, and the possession of a SAP (surface array protein) in the form of an S layer **[166]**. Such strains, predominantly found in the *A. hydrophila* and *A. sobria* phenospecies, can be recognized in the clinical laboratory by phenotypic and serologic tests **[165]**.

2.2.6.3 Genotypic tests

Molecular systems such as PCR, DNA hybridization, microarrays of DNA probes and fluorescent *in situ* hybridization (FISH) have been developed for detection of aeromonads in variety samples **[18]**.

2.2.7 Prevalence of *Aeromonas hydrophila*

2.2.7.1 In animals

Aeromonas spp. have been recognized as animal pathogens since they were first isolated from diseased frogs and fish. They are now recognized to cause disease in birds and domestic animals **[167]**. *A. hydrophila* and *A. salmonicida* cause hemorrhagic disease, ulcerative disease, furunculosis, red sore disease and septicemia in fish. *A. hydrophila* has been isolated from diseased turtles, alligators, snakes, and frogs **[168]**. Populations in animals probably reflect the presence of *A. hydrophila* in their feed and water. In their study of *Aeromonas* spp. in the feces of domestic animals, **Figura** and **Marri** isolated *A. hydrophila* more frequently than *A. caviae*. **Stern *et al*.,** isolated aeromonads from 1 of 32 cows and 3 of 21 turkeys, but none were isolated from 22 pigs or 24 sheep. **Gray** and **Stickler** reported finding predominantly *A. hydrophila* in cow feces and *A. caviae* in pig feces. Diet and water sources influenced recovery of *A. hydrophila* from feces of domestic animals. *Aeromonas* spp. have been isolated from feces, bedding, and drinking water of health cows and pigs. They survive in soil for months. Both healthy and diseased animals shed *Aeromonas* spp. in feces **[169]**.

Nayduch *et al*., proposed that houseflies could serve as vectors for transmission of *Aeromonas* spp. since the bacteria multiplied in the gut and persisted for several days. Fly to fly transmission was demonstrated and transmission of *Aeromonas* spp. from fly to food was observed **[170]**. The use of medicinal leeches (*Hirudo medicinalis*) to treat vascular infiltration in surgical wounds has been recognized as a risk factor for *A. hydrophila* infections since 1983, and there are numerous reports of cellulitis and septicemia resulting from leech therapy **[171]**.

2.2.7.2 In foods

A. hydrophila have been isolated from fish, shellfish, meats, dairy products, and fresh vegetables, few foodborne outbreaks have been reported **[14]**. A growing body of epidemiological evidence supports the possibility of aeromonads causing foodborne gastroenteritis. While a plethora of putative virulence factors has been postulated and demonstrated in food isolates, the exact role and mechanism of aeromonads in causing diarrheal illness has not been elucidated. Evidence suggests that a high infective dose is necessary to produce gastrointestinal disease in a susceptible host, and the fact that aeromonads may survive and grow at refrigerator temperatures provides a reservoir of bacteria that may achieve an infective dose when foods are mishandled **[172]**.

United States Food and Drug Administrator (USFDA) reported *A. hydrophila* in fresh and fresh cut produce, and aeromonds have been isolated from lamb, oysters, cheese and raw milk, and fish and seafood **[173]**. **Szabo *et al.,*** isolated *Aeromonas* spp. from 70 of 120 samples of lettuce in Australia. Aeromonads are found in ready to eat foods, including seafoods **[174]**. Studies published before 1990 relied upon phenotypic identification, while several studies published after that time identified isolates to hybridization group. While hybridization groups containing virulence factors are found in environmental samples and foods, aeromonads only cause gastroenteritis when their presence exceeds an infective dose for a susceptible host **[14]**.

2.2.7.3 In environment (including water)

There are few studies of *A. hydrophila* in soil apart from the contribution of water. World Health Organization (WHO) reported the presence of aeromonads in pasture soil, probably as a contribution from manure **[150]**. *A. hydrophila* forms biofilms on surfaces and may pose a threat of contamination in food processing. Researchers found that heat and chlorine

were effective against biofilm on stainless steel surfaces, however older biofilm was more resistant to heat and less established biofilm. Eight-day old biofilm was destroyed by heating to 60ºC and by exposure to 75 mg/L chlorine for 1 min **[175]**.

2.2.7.4 In human

Humans carry *A. hydrophila* in their gastrointestinal tract both in the presence and absence of disease. The rates of fecal carriage in asymptomatic persons in developed countries range from 0% to 4.0%, while the isolation rate from persons with diarrheal illness ranges from 0.8 to 7.4% **[176]**. In Southeast Asia, asymptomatic carriage rates as high as 27.5% and recovery rates from patients with diarrhea as high as 34% have been reported. Among Western Peace Corp workers in Thailand, aeromonads were recovered from 8.5% of healthy persons and 30.8% of persons with diarrhea **[177]**. Recovery rates among children with diarrhea vary geographically: 0.62 to 4% in Malaysia, 2.3% in Taiwan, and 4.8% in Switzerland, **[178-180]**.

Sinha *et al.,* reported *Aeromonas* spp. in 6.5% of all patients in India **[181]**, and **Chan *et al.*** reported *Aeromonas* spp. in 6.9% of adult patients with acute diarrhea in Hong Kong **[182]**. Seventeen of 2,565 stool samples (0.66%) were positive for *Aeromonas* spp. **[183]**. **Agger *et al.,*** reported *A. hydrophila* in 1.1% of stools in Wisconsin **[184]**, and **Moyer** reported a fecal isolation rate of 7.1% in Iowa. **[185]**.

The clinical significance of isolates in these surveys is not clear, even when all patients in the surveyed population had diarrhea. Enteropathogenicity is influenced by growth temperature, where strains of O:34 grown at 20ºC exhibit enhanced virulence over strains grown at 37ºC. Strains isolated at 35-37ºC, the typical incubation temperature used in clinical laboratories may produce false negative tests for virulence factors, making the retrospective assessment of clinical significance impossible **[186]**.

2.2.8 *Aeromonas hydrophila* infections

2.2.8.1 In animals

A. hydrophila infection of aquatic animals has been recognized for over 100 years, but they are less commonly recognized in other vertebrates. While many animals shed aeromonads from their gastrointestinal tract, there is no evidence that they suffer from gastrointestinal disease. *Aeromonas* spp. cause outbreaks of disease and represent an economic threat to the aquaculture industry.

A. hydrophila has been reported to cause septicemia in snakes, turtles, and frogs **[187-189]**. **Forga-Martel *et al*.,** reported a case of infectious abortion caused by *A. hydrophila* in a mare. Contamination from an adjacent dairy farm was suspected as the source of infection for the mare and transplacental infection was thought to result in fetal sepsis and abortion **[169]**. Disease in aquatic animals is characterized by hemorrhagic lesions, ulcers, and septicemia in frogs and fish **[189,190]**. **Paniagua *et al.,*** determined that doses of 7 $\log^{10}$ CFU of *A. hydrophila* (72% of strains) infected intramuscularly produced disease in trout **[191]**.

2.2.8.2 In human

A. hydrophila has received particular attention because of its association with human diseases. It has been isolated form both polluted and unpolluted bodies of water through out the world **[192]**. A study showed that *Aeromonas* spp. was extremely common contaminants of human foodstuffs and that some of the strains appear to be virulent. In their study most of the toxigenic strains were isolated from seafood and they suggested that seafoods were potential sources of virulent aeromonads. Therefore, in cases of foodborne bacterial illnesses in which oysters are implicated, *Aeromonas* spp. should be included in the general screening for causative microorganisms **[190]**.

A common source of *A. hydrophila* in outbreaks of gastroenteritis is from water supplies such as mineral springs, seawater environments, chlorinated and unchlorinated domestic supplies and watersheds polluted by sewage effluents. Sources other than water from which this organism can be readily isolated include seafood, foods of terrestrial animal origin such as meats, dairy products and poultry and vegetables. Asymptomatic and immunocompromised human carriers involved with handling of foods are another potential source. The presence of *A. hydrophila* in the food chain should not be ignored. *Aeromonas* species are regarded as controversial primary pathogen but several worldwide investigations clearly now indicate that at least some strains are clearly enteropathogens and few cases are linked to gastroenteritis **[193]**.

Although the incidence of *Aeromonas* in foods is high, they vary between countries and also among the type of strains. Two factors appear to be affecting the significance of *A. hydrophila* as a re-emerging pathogen. The first is the consumer driven demand for less processed and more natural foods containing fewer additives, there in growing emphasis on, refrigeration as the primary means for controlling microbial growth in food. The second point is that during the warm season there is an increase in water associated *A. hydrophila* strains also in some cases associated with an increase in gastroenteritic patients **[193]**.

Gastroenteritis caused by *A. hydrophila* has been documented, and the incidence of gastroenteritis tends to be higher in summer than other seasons **[127]**. *A. hydrophila* may be present in the gastrointestinal tract of humans, and most epidemiological studies show higher numbers in stools of patients with gastroenteritis than in asymptomatic individuals. Acute self-limiting diarrhea occurs in children, and chronic gastroenteritis or enterocolitis may occur in children and the elderly. The presentation of gastroenteritis caused by aeromonads includes various combinations of fever, vomiting, and increased fecal leucocytes or erythrocytes **[128]**.

According to **Kirov**, the majority of aeromonads associated with gastroenteritis are *A. veronii* biovar sobria (HG-8/10), *A. hydrophila* (HG-1), and *A. caviae* (HG-4), though *A. veronii* biovar veronii (HG-8/10), *A. trota* (HG-13), and *A. jandaei* (HG-9) occur occasionally. Gastroenteritis attributed to *A. hydrophila* was characterized by acute watery diarrhea, vomiting abdominal pain, and fever. **[194]**.

Skin and soft tissue infection caused by *Aeromonas* spp. resulting in cellulitis and bacteremia. Skin and soft tissue infections may follow traumatic injury in environments where soil and water may contaminate the wound. A review of 32 foot injuries revealed that at least one-third of cases resulted from introduction of aeromonads in soil-contaminated glass, nails, or sticks. Infections from severe trauma associated with automobile accidents or other accidents resulting in crushing injury, compound fractures, or severe burns may lead to osteomylitis, myonecrosis, or gangrene **[195]**.

Necrotizing fasciitis is a rapidly advancing form of cellulitis characterized by muscle necrosis. **Tsai *et al*.,** reported necrotizing fasciitis caused by *A. hydrophila* in patients with suppressed immune systems, burns, and trauma in aquatic settings. The case of an 85-year old man with no history of trauma suggests that sepsis from an intestinal source resulted in soft tissue infection and subsequent necrotizing fasciitis **[196]**. **Furusu *et al.,*** reported a fatal case of necrotizing fasciitis accompanied by gas production caused by *A. hydrophila* in a 66 year old man who underwent valve replacement surgery **[197]**.

Burn infections caused by aeromonads are rare events – only 29 cases have been reported in English language literature. These authors reported 5 cases of *A. hydrophila* or *A. caviae* infections from burns associated with explosions (4 of 5) and a campfire accident (1 of 5). In 4 of 5 cases, water was used to quench the fire or as a first aid treatment. **Ko *et al.,*** reported *A. hydrophila* infection in 62-year old female suffered from a flame burn covered by 61% of her total body surface area **[198]**.

Trauma is most closely associated with *Aeromonas* wound infections. While the typical presentation in persons with normal immune status is cellulitis, more serious infections and sepsis can occur. Patients developing myonecrosis have a mortality rate exceeding 90%. The significance of *A. hydrophila* as a cause of skin and soft-tissue infections was made abundantly clear as a result of the tsunami that devastate Southeast Asia in December 2004. Among 777 patients hospitalized for injuries suffered as a result of the tsunami, 515 had skin and soft-tissue infections and 145 isolates for 305 patients were *A. hydrophila*. Many of these infections developed because traumatic wounds were not cleaned properly or because of delay in obtaining medical care **[199]**.

Pneumonia and lung abscess in adults has been reported. Pneumonia may also occur in children, and **Kao *et al*.,** reported a case of bacteremic pneumonia caused by *A. hydrophila* in a previously healthy 5-year old child. Predisposing conditions were present in 11 of 15 (73%) of cases. Reported predisposing conditions include alcohol abuse (20%), neurologic disease (20%), cardiovascular disease (27%), chronic renal failure (7%), chronic obstructive lung disease (20%), traffic accidents (7%), and malignancy (7%) **[200]**. **Murata *et al*.,** reported fulminant pneumonia caused by *A. hydrophila* in a patient undergoing hemodialysis with chronic renal failure and cirrhosis. The source of infection was not determined **[201]**.

Respiratory infections occur in the immunocompetent persons who involuntarily aspirate surface water while swimming or as the result of an accident **[202]**. **Miyake *et al*.,** reported *Aeromonas* pneumonia from near-drowning experiences. Respiratory infections may also occur in persons with underlying diseases placing them at risk for *Aeromonas* bacteremia originating from an intestinal source. Isolation of *A. hydrophila* from respiratory specimens must be interpreted together with clinical findings,

since the upper respiratory and nasopharyngeal tracts may be transitorily colonized **[203]**.

Meningitis is a rare complication of extraintestinal infections with *A. hydrophila* **[204]. Brouqui** and **Raoult** reviewed endocarditis and found only two cases caused by *A. hydrophila*, both in patients with cirrhosis **[205]**. Osteomyelitis has been reported following compound fractures or crushing trauma where wounds were contaminated by soil or water **[206].** Liver disease is a recognized predisposing factor leading to *Aeromonas* infection resulting from septicemia. Underlying hepatitis B infection and cirrhosis are predisposing factors for liver disease. Liver abscess, supperative cholangitis, and empyema may occur following septicemia in patients with underlying hepatobiliary disease. *A. hydrophila* was identified in all instances in which species identification was performed **[207]**.

Bacteremia resulting from *A. hydrophila* infection was reviewed by **Tsai *et al*.,** *A. hydrophila* sepsis is associated with gastrointestinal disease, liver cirrhosis, diabetes, malignancy, pancreatitis, trauma, cardiac anomalies, and respiratory disease. Sepsis is accompanied by fever, hypotension, jaundice, and chills, and complications of *Aeromonas* infection may include intravascular coagulation, purpura fulminans, and ecthyma gangrenosum. Disseminated infection progresses rapidly and has a high fatality rate **[208]**.

Peritonitis sometimes occurs as a secondary infection following colonization of the intestinal tract, and is also associated with peritoneal dialysis or intestinal perforation. Most infections occur in patients with chronic liver disease, where the case-fatality rate approaches 60% **[206]**. **Fang *et al.,*** reported a case of hemolytic uremic syndrome (HUS) caused by *A. hydrophila* in a 23-month old child that occurred six days following an episode of bloody diarrhea in a follow-up report **[209].**

The causal role of *A. hydrophila* in ocular disease must be evaluated in conjunction with clinical presentation, since *A. hydrophila* have been cultured from eye swabs of 73-year-old male with a history of myelodysplastic syndrome, suffered from periorbital swelling in the eye. Since *A. hydrophila* should be listed as an important pathogen in any soft tissue infection including eyelid infection **[210]**. Septic arthritis caused by *A. hydrophila* is relatively rare **[211]**.

2.2.9 Epidemiology and disease outbreaks

2.2.9.1 Outbreaks locations

A. hydrophila are frequently isolated from drinking water **[212]**, and temporal and seasonal associations between presence of aeromonads in drinking water and their presence in the stools of patients with gastroenteritis have been reported **[213]**. While some investigators claim that drinking water is responsible for outbreaks of *Aeromonas* gastroenteritis, epidemiological evidence linking water ingestion to gastrointestinal illness has been limited to untreated drinking water supplies**[183]**.

Molecular typing studies have shown that the strains most frequently found in feces belong to HG-1 and HG-4, while HG-2, HG-3 and HG-5A are more commonly found in drinking water and the environment, suggesting that environmental strains are fundamentally different from clinical strains. The high infectious dose, the differences in temperature optima, and the variation in expression of putative virulence factors between clinical and environmental strains suggest that outbreaks of gastrointestinal illness resulting from water ingestion are unlikely to occur **[214]**.

One report linked exposure to aquarium water to a case of gastroenteritis caused by *A. sobria* with fatal disseminated disease in a 6-month old child **[215]**. *A. hydrophila* have been reported as the cause of individual cases and point source outbreaks of foodborne disease. Seafood products are among the ideal substrates for proliferation of *Aeromonas* **[128]**.

In tropical countries like India, fishery products are contaminated by various food-borne pathogens. Pollution and cross contamination levels are very high in many developing tropical countries due to lack of infrastructure. It is important that fishery products should be maintained free from pathogens before consumption. Variations in the incidence level of *A. hydrophila*, the most common aeromonad in seafood can be attributed by secondary contaminations such as handling, usage of polluted/non-hygienic water, storage of seafood in inadequate facilities etc. Also, *A. hydrophila* may be introduced from water, animal faeces, or food-handlers. The ubiquity of this organism means it has the potential to be food-borne **[216]**.

In United States, food poisoning is responsible for a major loss of economic resources, being second to the common cold in causing time lost from work. About 35-40% of the reported food-borne outbreaks, which occur in the US each year, are of unknown etiology **[217]**. In the last few years, motile *A. hydrophila* have been included in the list of bacterial species that are considered enteric pathogens **[218]**. Here have also been several reports of *A. hydrophila* contaminated meat, poultry and raw milk but there have been few systematic studies on the incidence of *Aeromonas* spp. in foods although one report suggested that these organisms were very common contaminants of food **[14]**.

The much higher incidence of *Aeromonas* spp. in foods purchased at retail outlets suggests that the source of contamination may not be faeces and there is an increasing evidence that some strains of *Aeromonas* spp. cause diarrhea in human beings and it is likely that uncooked or cross contaminated foods act as a source of infection **[219]**. This organism has been isolated from all over the world, and its source is wild fish, and pond cultured edible and ornamental fish **[220]**. *A. hydrophila* was first reported as associated bacteria exclusively with diseased fish. Another known reservoir is shellfish, particularly, oysters **[221]**. **Vila *et al.,*** reported *Aeromonas* spp. as the cause of diarrhea in 2% of travelers to Africa, Latin America, and Asia **[222]**.

2.2.9.2 Possible transmission routes of sporadic *Aeromonas hydrophila*

A. hydrophila is ubiquitous in the environment and there are multiple opportunities for transmission to humans through food, water, animal contact, and direct human contact. Extra-intestinal infections are typically acquired following trauma in an aquatic environment, and intestinal infections are acquired by ingestion of contaminated food or water. Intestinal infections in immunocompromised patients may disseminate resulting in septicemia with multiple organ involvement. Inhalation of surface water in near drowning incidents has been reported to cause pneumonia.

A. hydrophila have been recognized as potential foodborne pathogens since 1984. **Kirov** reviewed the public health significance of *Aeromonas* spp. in foods, and **Merino *et al*.,** reviewed aeromonads as emerging pathogens present in foods, and are common on foods, especially green vegetables, and they are found in raw milk, ice cream, meats, and seafood **[150]**.

Diarrheal disease was associated with drinking untreated well water **[223]**. Many researchers used ribotyping to demonstrate that shrimp ingestion resulted in gastroenteritis in the first report of foodborne illness attributed to *Aeromonas* spp. Subsequently, others have shown the same ribotype in well water and stools of patients with gastroenteritis. Ribotyping was used to demonstrate that a patient with chronic diarrhea carried the same strain for years **[214]**, and ribotyping was used to demonstrate person-to-person transmission of *Aeromonas* between a foster child and a foster parent **[185]**.

Filler *et al*., reported a case of acute renal failure in a 6-month old infant caused by *Aeromonas* spp. acquired from aquarium water. Transmission among children in daycare centers, nursing homes, and patients in intensive care have been reported **[150]**. Animal-to-person transmission may occur through direct contact, or by ingestion of contaminated food products of animal origin. Extra-intestinal infections originate from environmental sources directly from soil or water contact, or indirectly by ingestion and bacteremic dissemination of *A. hydrophila* from the gastrointestinal tract **[224]**.

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

3.1.1 Equipment

a. Autoclave (Tuttnaur 3870 ELV)
b. Balance (sensitivity of ± 0.1 g) (Mettler tolado)
c. Binuclear microscope (Olympus ch20BIMF200)
d. Blinder (Memmolinix)
e. Freezer (-70°C) (Heraeus)
f. Fifteen ml plastic bottles
g. Incubators capable of holding temperatures at 4 ± 1°C, 25 ± 1°C, 28 ± 1°C, 30 ± 1°C, 32 ± 1°C, 35 ± 1°C and 37 ± 1°C (Memert BE400, Selecta 80067)
h. Inoculating needles and loops
i. Refrigerator (Kelvinator)
j. Sewage collection tool (Home made)
k. Sterile cellulose acetate membrane with a pore size of 0.45 (Millipore)
l. Sterile scissors, forceps, knives, pipettes, hockey sticks, and other supplies
m. Sterile tubes and cups
n. Vortex mixer (Snijers)
O. Water bath (Memert)

3.1.2 Reagents

a. Crystal violet (85 µg/ml aqueous solution)
b. Crystal violet (0.5 mg/ml aqueous solution)
c. Ferrous ammonium sulfate (1%)
d. Gram stain kit (Himedia, India)
e. HCl solution (1 N)

f. KOH (0.25%) in 0.5% NaCl aqueous solution

g. Kovacs' reagent

h. Oxidase reagent or reagent-impregnated disc/strip (Himedia, India)

i. Sterile mineral oil

j. Sterile Saline (0.85% NaCl)

k. Voges-Proskauer (VP) test reagents (KOH and Alpha naphthol)

l. Wayson stain (prepared from basic ingredients)

3.1.3 Media and biochemical tests

a. API 20E system (Biomeroux, France)

b. Brain heart infusion agar

c. Brain heart infusion broth

d. Blood agar

e. Cefsulodin-irgasan-novobiocin (CIN) agar

f. Christensen's urea agar slants

g. DNase test agar

h. Esculin agar

i. Hektoen Enteric agar

j. Kligler's Iron agar (KIA) slants

k. MacConkey agar

l. *Salmonella Shigella* (SS) agar

m. Phosphate Buffered Saline (0.01 M) (PBS, pH 7.6)

n. Simmon's Citrate agar slants

o. Thioglycollate broth

p. Trypticase Soy Broth (TSB)

q. Xylose Lysine Deoxycholate agar (XLD)

All media and antibiotics used were purchased from HiMedia (India).

3.2 Methods

3.2.1 Sample collection

A. **Appendiceal samples**: Twenty samples were collected from hospitalized patients after appendectomy from different hospitals in Gaza strip. Collection was performed by surgeons during appendectomy.

B. **Fecal samples:** Three hundreds diarrheal stools were collected from different hospitals (Kamal oudwan, Al-Shifa, Al-Nasser, Al-Dora, Al-Aqsa, Nasser, Gaza-European and Al-Najar) in sterile bottles.

C. **Sewage samples:** Twenty-six sewage samples were collected from six sources (a) Bietlahia WWTP, (b) El-shifa hospital, (c) Gaza European hospital, (d) Al-Aqsa hospital, (e) Al-Nasser hospital and (f) Shiek Ejleen in sterile 50 ml plastic bottles.

D. **Animal excreta samples:** Twenty-six samples were collected from slaughterhouses and houses using sterile bottles.

E. **Food samples:** Fifty samples from each the following materials; meat, turkey, chicken, sausage, ice-cream, cheese and milk samples were purchased from local supermarkets and houses.

F. **Water samples:** Two-liter samples of different water types (tap and well water) were collected in sterile bottles. Natural mineral water was purchased from a local supermarket. Tap water was obtained from municipal distribution system in various localities all over Gaza Strip. Seawater was taken at a depth of 1·5–2 m near the sewage discharge point of Gaza wastewater treatment Plant (GWWTP).

3.2.2 Sample transport

Clinical samples were collected and transported according to the recommended clinical laboratory practice **[5]**. Environmental samples were collected and handled according to the protocol outlined in Standard Methods for Examination of Water and Wastewater (APHA) **[224]**. Food samples were collected and handled in accordance with the procedures of the FDA Bacteriological Analytical Manual **[225]**. All samples were collected in sterile bottles and transported in an ice box until analyzed (No more than 3 hours were allowed between sample collection and processing).

3.2.3 Sample processing

3.2.3.1 Preparation of sample homogenate

Some of the clinical samples and environmental samples processing required preparation of serial dilutions, these were cultured directly, diluted, or by membrane filtration, followed by incubation of the filter membrane on culture media **[226]**.

A. Appendiceal samples

Appendix samples were grinded for 2 minutes in a sterile blinder and transferred into 10-fold volume of TSB and incubated for 48 h at 25 °C. In addition, 3 ml of culture was transferred to 15 ml of thioglycollate medium. Homogenate were allowed to stand undisturbed at room temperature for 10 min to allow settling of large appendix particles **[227]**.

B. Fecal samples

About one gram of fecal sample was placed into 10-fold volume of PBS **[5,157]**.

C. Animal excreta samples

Five-gram samples of animal excretion were homogenized with 20 ml of PBS **[5,157]**.

C. Sewage samples

Twenty five to thirty ml of the sample was inoculated into 10-fold volume of PBS **[33,157]**.

D. Water samples

Non-turbid water samples were processed using the membrane filtration (MF) method, 200 ml of the samples were filtered through sterile cellulose acetate membrane with a pore size of 0.45 (Millipore). One filter was aseptically placed onto the surface of selective media (CIN) agar in a 50-mm Petri plate which was incubated at 32°C for 48 h. The second membrane was aseptically placed into a bottle containing 10 ml of TSB enrichment broth and incubated at 25°C for 24 h. TSB enrichments were inoculated onto CIN agar which was incubated at 32°C for 24 h. Membrane filtration method (MF) has been validated for detection and enumeration of *Y. enterocolitica* and *A. hydrophila* from different type water **[33,150]**.

E. Food samples

Twenty five grams of each sample were added to 100 ml of 0.01 M PBS: pH 7.6 and homogenized for 2 minutes in a sterile blinder. Homogenates were allowed to stand undisturbed at room temperature for 10 min to allow settling of large meat particles **[77,225]**.

3.2.4 Isolation procedure

3.2.4.1 Enrichment and plating procedures

A. PBS: 0.1 ml of PBS homogenate was spread onto SS agar and incubated at 30°C for 24 h. 0.1 ml was spread onto CIN agar and incubated at 32°C for 18 h. 0.1 ml was spread on MCA agar and incubated at 25°C. 0.1 ml volumes were spread onto Hektoen Enteric (HE) agar, and Xylose Lysine Deoxycholate (XLD) agar, and incubated at 32°C for 18 h. In addition, 0.5 ml of the PBS enrichment was removed, treated with 4.5 ml KOH, and then streaked onto CIN agar only **[77,150]**.

B. TSB: 5 ml of PBS homogenate supernatant was transferred into 20 ml TSB. Incubated at 25°C for 24 h. 0.1 ml was spread onto SS agar and incubated at 30°C for 24 h. 0.1 ml was spread onto CIN agar, and incubated at 32°C for 18 h. 0.1 ml was spread on MacConkey agar and incubated the plates at 25°C. 0.1 ml volumes were spread onto HE, and XLD agar, and incubated at 32°C for 18 h. Also, 0.5 ml of the TSB enrichment was removed, treated with 4.5 ml KOH, and then streaked onto CIN. The TSB enrichment culture was re-incubated at 25°C for 2 additional days, and then was plated as previously described **[77,150]**.

C. Remainder of PBS: The remainder of the PBS homogenate was refrigerated at 4°C and subcultured after 1,4,7 and 14 days. 0.1 ml was spread onto CIN agar and the plates were incubated at 32°C for 18 h. Also, 0.5 ml of the PBS enrichment was removed, treated with KOH, and then streaked onto CIN. **[230,231]**.

D. KOH treatment: 0.5 ml of enrichment culture was added to 4.5 ml KOH/NaCl. Vortexed briefly (3-4 sec) and immediately a loop-full of the KOH-treated broth was streaked onto CIN agar **[81]**.

3.2.4.2 Selection of colonies from plating media

Due to the fact that SS, HE, XLD, MacConkey, and CIN agars are not completely inhibitory to non-*Yersinia* or *Aeromonas,* a variety of non-desired organisms may be recovered from these agars. Some of these organisms (e.g. strains of *Morganella*, *Citrobacter* and *Enterobacter*) have a colonial morphology similar to that of *Yersinia and Aeromonas species*. Care was exercised in the selection of suspect colonies from SS, HE, XLD, MacConkey and CIN agars in order to minimize picking non-*Yersinia* or *Aeromonas*.

A. SS: On SS, *Y. enterocolitica* and *A. hydrophila* colonies are typically round and opaque or colorless.
B. HE: On HE, *Y. enterocolitica* and *A. hydrophila* colonies have a salmon color.
C. XLD: On XLD, *Y. enterocolitica* and *A. hydrophila* colonies have a yellow color.
D. MacConkey: On MacConkey agar the colonies were invariably smaller than those on the CIN agar, non lactose fermenter, flat and without entire margins **[24,232]**.

E. CIN: On CIN, typical *Y. enterocolitica* and *A. hydrophila* colonies have a red bulls-eye which is usually very dark and sharply delineated. The bulls-eye is surrounded by a transparent zone with varying radii, with the edge of the colony either entire or irregular; colony diameter of *Y. enterocolitica* is 1-2 mm but the colony of *A. hydrophila* is larger than *Yersinia* species **[226,229]**.

3.2.4.3 Identification and confirmation procedures

3.2.4.3.1 Identification of *Yersinia and Aeromonas*

A colony on CIN, HE, XLD, or SS having morphology typical of *Y. enterocolitica or A. hydrophila* was selected and streaked on blood agar plates for pure culture, and a colony from blood agar plates was emulsified in about 1 ml of sterile saline (0.85%). This was used to first inoculate a slant of Simmon's citrate agar, then Kligler's iron agar, and a tube of urea agar. This procedure was repeated with 5 colonies having morphology typical of *Y. enterocolitica* and *A. hydrophila* selected from each plate of selective agar **[77,150]**.

a. **Simmon's Citrate:** The slant of a tube of Simmon's citrate agar was only streak-inoculated, and was incubated at 28°C for 24 h. Presumptive *Y. enterocolitica* and *A. hydrophila* are citrate negative.

b. Kligler's Iron Agar: The butt was stabbed and the slant streaked and was incubated at 28°C for 18-24 h. Presumptive *Y. enterocolitica* and *A. hydrophila* present an alkaline (red) slant and acid (yellow) butt (K/A), without gas or H_2S in KIA.

c. Christensen's urea agar: The slant of freshly prepared urea agar slant was heavily streaked with the test organism and was incubated at 28°C for 24-72 h. Presumptive *Y. enterocolitica* is (+) for urease and will turn the agar to an intense red-pink color while *A. hydrophila* is (-).

3.2.4.3.2 Confirmation of *Yersinia enterocolitica* and *Aeromonas hydrophila*

One well-isolated colony from each culture was used to inoculate 5 ml of 0.85% NaCl medium, pH 5.5 to 7.0. A humid atmosphere was provided, and, to identify the organisms, commercially available bacterial identification kit, API 20E test, a kit (Biomerieux, France) was used according to the instructions of the manufacturer. After 18 to 24 h, all reactions were analyzed according to the interpretation chart included in the package insert. Reagents were added to the TDA, Voges-Proskauer, and IND tubes, and the reactions were recorded. Inoculum for further testing was obtained from the KIA slant **[77,150]**.

For additional speciation of *Yersinia* and *Aeromonas*, the following tests were performed:

A. Oxidase test: A colony growing on KIA slant of any presumptive *Y. enterocolitica and A. hydrophila* isolates was tested for oxidase by the commercially available reagent-impregnated test discs. *Yersinia* is oxidase negative (-) while *A. hydrophila* is oxidase (+) **[77,150]**.

B. Deoxyribonuclease (DNase) test: *Yersinia* and *Aeromonas* strains were inoculated onto a plate of DNase test agar by streaking the medium in a band (about 1.9 cm length streak). Plates were incubated at 28°C for 18-24 h.

Following incubation, plates were examined as follows: Plate was flooded with 1 N HCl. A zone of clearing around a colony indicates a positive test. *Yersinia* and *Aeromonas* strains are DNase (+) **[77,150]**.

3.2.4.4 Testing for pathogenicity markers

A. Pyrazinamidase test: Isolates were inoculated over entire slant of pyrazinamide agar and incubated at 25°C for 48 h. Slant surface were flooded with 1 ml of freshly prepared 1% (w/v) aqueous solution of Ferric ammonium sulfate. Test results were read after 15 min; a pink to brown color indicates PYZ positive (+), (presence of pyrazinoic acid) while no color development is observed with PYR negative (-) strains. Pathogenic strains of *Yersinia* are PYZ negative and *A. hydrophila* are PYZ positive **[10,129,191]**.

B. Esculin hydrolysis: A plate of esculin agar was inoculated with the test strain. The plate was incubated at 25°C for 10 days, reading after 1,2,3,7 and 10 days. Blackening indicates esculin hydrolysis. Pathogenic *Y. enterocolitica* is negative for this test while A*. hydrophila* is esculin positive **[10,129,233]**.

C. Auto-agglutination in MR-VP and BHI broth: Individual isolates were evaluated for the ability to autoagglutinate in MR-VP broth for *Y. enterocolitica* and brain heart infusion broth (BHIB) for *A. hydrophila* as follows. For *Y. enterocolitica*; 2 tubes of MR-VP broth were inoculated; one was incubated at 25°C for 24 h, and the other at 35°C for 24 h. After 18 to 24 h incubation, the tubes were observed for agglutination, with care taken not to shake or disturb the sediment at the bottom and along the sides of the tube. The tube incubated at the lower temperature should exhibit turbidity from cell growth. The tube which had been incubated at 35°C should show agglutination (clumping) of bacteria along the walls and/or bottom of tube and clear supernatant fluid. Virulence plasmid agglutinates at 35°C but not 25°C. Isolates that lack the virulence plasmid do not agglutinate at either temperature **[10]**.

The autoagglutination test for selfpelleting (SP+) and precipitation after boiling (PAB+) phenotype characterization was carried out as described by **Janda *et al*.,**. Each *A. hydrophila isolate* was grown in 6 ml of BHIB for 18 h at 28°C. At the end of the incubation period, cultures were observed for evidence of self-pelleting, which was manifested as a large aggregate of cells at the bottom of the tube and the absence of turbidity in the medium. A 3-ml fraction was heated for 1 h at 100°C in a water bath and then cooled for 10 min and compared with the samples kept at room temperature for a control. Reduction in turbidity was considered positive for precipitation after boiling **[164,233]**. Both pathogenic *Y. enterocolitica* and *A. hydrophila* are positive for this test.

D. Crystal violet binding test: This rapid screening test differentiates potentially virulent *Y. enterocolitica* and *A. hydrophila* cultures. Suspected cultures were grown for 18 h at 22-26°C in BHI broth with shaking. Each culture was diluted in physiological saline. 0.1 ml of each culture was spread to each of two BHI agar plates. The plates were incubated at 25°C or 37°C for 30 h. Each plate was gently flooded with 8 ml of 85 µg/ml of crystal violet (CV) solution for *Y. enterocolitica* and 0.5 mg/ml for *A. hydrophila* for 2 min and the crystal violet uptake was qualitatively determined. Colonies were observed for their CV binding. The binding of CV to positive colonies was observed by their dark violet appearance, while negative colonies failed to bind the dye and remained white. Photographs of colonies were made for permanent records **[10,234]**.

E. Beta haemolysin production of *A. hydrophila*: Haemolytic activity of the organisms studied was detected on blood agar plates containing 5% human blood. All tests were incubated in air at 37°C for 18-24 h. Isolates exhibiting hemolytic zones in excess of 2 mm from the streak inoculum were considered positive **[137]**.

Yersinia and *Aeromonas* isolates were stored in TSB with 20% (v/v) glycerol at -80^{o}C until further testing.

3.2.4.5 Antimicrobial susceptibility testing

All *Y. enterocolitica* and *A. hydrophila* isolates were tested for antimicrobial susceptibility using disk diffusion method using Mueller Hinton agar and antibiotic disks procured from Hi-Media laboratories, India, according to National Committee for Clinical Laboratory Standard (NCCLS). 3-5 colonies of each isolate were introduced into a tube containing BHIB. These tubes were incubated for 4-6 h and the broth culture turbidity was adjusted to that of 0.5 McFarland standard. Muller Hinton plates were dried for a bout 30 min before inoculation and were used within one day of preparation. The standardized bacterial broth suspension was streaked on the surface of the medium with a cotton swab. After the inoculum had dried (3-5 min) the disks were placed on the agar with flamed forceps and gently pressed down to ensure contact **[235]**.

The plates were incubated for 24 h at 37^{o}C and the diameter of zone of inhibition of each antimicrobial agent was compared with the chart supplied by the manufacturer and interpreted as sensitive, intermediate or resistant. The following table includes the list of antimicrobials and their potencies used for the antimicrobial testing.

Table (3.1): Antimicrobial disks used in the susceptibility testing of *Y. enterocolitica* and *A. hydrophila*

Antimicrobial agents	Abbreviation	Disk potency
Amikacin	Ak	30 μg
Ampicillin	A	10 μg
Amoxycillin-Clavulanate	AC	30 μg
Aztreonam	Ao	30 μg
Cephalexin	Cp	30 μg
Cefazolin	Cz	30 μg
Cefotaxime	Ce	30 μg
Ceftazidim	Ca	30 μg
Ceftriaxone	Ci	30 μg
Cefuroxime	Cu	30 μg
Ciprofloxacin	Cf	5 μg
Chloramphenicol	C	30 μg

Doxocycline	Do	30 μg
Erythromycin	E	10 μg
Gentamicin	G	10 μg
Meropenem	MEM	10 μg
Nalidixic acid	Na	30 μg
Ofloxacin	Ofx	5 μg
Co-trimethoprim	Co	25 μg
Tetracycline	Te	30 μg

3.3 Permission and Ethics

Permission was taken from Helsinki Committee, Hospitals General Administration, patients or patients guardians and workers in GWWTP and Bit-lahia Wastewater Treatment Plant.

3.4 Questionnaire

The questionnaire used in this study included open and closed questions and data collected by interviewing patients or patients guardians to record address, age, sex, date of onset of illness and duration of illness. A checklist inquired about symptoms, including the presence of diarrhea, number of stools per day, consistency of stools, presence of blood or mucous, occurrence of abdominal pain or cramping, presence of vomiting, and fever. Epidemiological questions explored exposure to animals. Respondents were asked to specify the use of an untreated private or treated public drinking water supply. A cover letter accompanied each questionnaire explaining the purpose of the study. Questionnaires were tabulated to determine age and sex correlation with gastrointestinal disease, characteristic symptoms, and possible exposures and predisposing factors necessary to establish infection.

3.5 Data analysis

Data obtained from microbiological investigation and from the questionnaire survey were uploaded to (SPSS version 15) software and analyzed using cross tabulating and chi square test.

CHAPTER 4
RESULTS

This study was conducted on 300 patients to investigate the presence of *Y. enterocolitica* and *A. hydrophila* in clinical samples. 95 food samples and 84 environmental samples were also tested. All suspected *Y. enterocolitica* and *A. hydrophila* were identified using conventional microbiological techniques.

4.1 Identification of *Yersinia enterocolitica* and *Aeromonas hydrophila*

All suspected *Y. enterocolitica* and *A. hydrophila* were presumptively identified using colonial morphology and by the use of (urea agar, Simmon's citrate agar and Kligler's iron agar). All cultures of *Y. enterocolitica* were positive for urease while *A. hydrophila* were negative and all of *Y. enterocolitica* and *A. hydrophila* were negative for citrate utilization. On Kligler's iron agar after both pathogens produced K/A reaction without H_2S and gas. Figure (4.1).

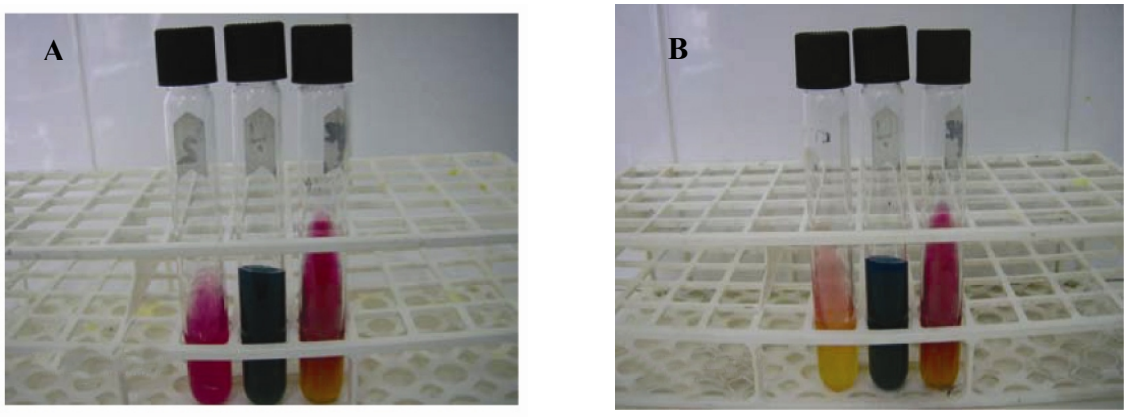

Figure (4.1): Presumptive identification of **(A)** *Yersinia* and **(B)** *Aeromonas*

Table 4.1 indicates the biochemical reaction of *Y. enterocolitica and A. hydrophila* isolated from clinical, food and environmental samples.

Table (4.1): Biochemical profiles and other properties of *Y. enterocolitica* and *A. hydrophila* isolated from different sources

Test	*Y. enterocolitica*	*A. hydrophila*
Ortho nitrophenyl-β-galactosidase (ONPG)	+	+
Arginine dihydrolase (ADH)	-	+
Lysine decarboxylase (LDC)	-	-
Ornithine decarboxylase (ODC)	+	-
Citrate utilization	-	-
H_2S production	-	-
Urease production	+	-
Tryptophane deaminase (TDA)	-	-
Indole production	+	+
Voges- Proskauer	+	+
Gelatinase production	-	+
D-Glucose	+	+
D-Mannitol	+	-
Inositol	+/-	-
D-Sorbitol	+/-	-
L-Rhamnose	-	-
D-Sucrose	+/-	+
D-Melibiose	-/+	-
amygdalin	+	+
L-Arabinose	+	+

Presumptive *Yersinia* and *Aeromonas* were identified biochemically by API 20E system (Figure 4.2).

Figure (4.2): API 20E reactions for **(A)** *Y. enterocolitica* and **(B)** *A. hydrophila*

All cultures of *Y. enterocolitica* and *A. hydrophila* grown on CIN agar plates were gram and wayson stained and showed negative reaction with rod to coccobacilli morphology with bipolar staining: All cultures of *Y. enterocolitica* were negative for oxidase, gelatin hydrolysis, lysine decarboxylase, arginine dihydrolase (ADH), and phenylalanine deaminase. On the other hand, all cultures of *A. hydrophila* were positive for oxidase and ADH. The results of the remaining tests are shown in Table (4.1).

4.2 Distribution of *Yersinia and Aeromonas* isolates

Yersinia species were isolated from all sampled sources. The highest incidence was from sewage (19.1%) followed by animal excreta (11.5%), while, clinical samples showed the lowest percentage (4.7%). With regard to *A. hydrophila*, meat and water showed the highest incidence (48.9% and 46.9% respectively). The overall frequency of *Yersinia* and *Aeromonas* isolates was 6.3% and 38.1% respectively (Table 4.2).

Table (4.2): Distribution of *Yersinia and Aeromonas* isolates according to sample type.

Sample type	No.	*Yersinia*		*Aeromonas*	
		No.	%	No.	%
Clinical samples	300	14	4.7	103	34.3
Animal excreta	26	3	11.5	10	38.5
Meat	45	3	6.7	22	48.9
Milk	50	3	6	18	36
Sewage	26	5	19.1	11	42.3
Water	32	2	6.25	15	46.9
Total	473	30	6.3	179	38.1

4.3 Recovery of *Yersinia enterocolitica* and *Aeromonas hydrophila* from clinical samples

Cultures of *Y. enterocolitica* and *A. hydrophila* were performed on 300 diarrheic stool samples and 20 appendiceal samples after appendectomy. The median age of the patient population was 3.6 years (range 40 days to 47 years), and 55% were males (Table 4.3 and Figure 4.3).

Table (4.3): Age distribution of the study sample

Age group	Frequency	Percent
Below 2 years	126	42
2 - less than 6 years	132	44
6 - less than 15 years	28	9.3
Over 15	14	4.7
Total	300	100

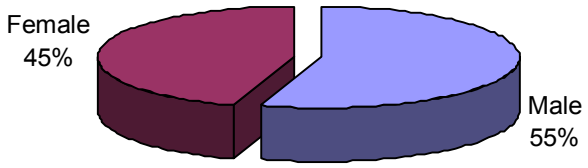

Figure (4.3): Sex distribution of the study population

One hundred fifty eight stool samples were collected from patients below the age of 6 and only 14 samples from patients older than 15 years. From table 4.4, it could be observed that both *Yersinia* and *Aeromonas* were isolated with a higher frequency from patients belonging to the age group 2 to less than 6 years (2.3% and 20.3% respectively). There was a decline in incidence of both pathogens with increasing age. A significant association was found among *Y. enterocolitica* and *A. hydrophila* isolation and age groups less than 6 years ($p < 0.05$).

Table (4.4): *Yersinia* and *Aeromonas* isolates distributed according to age (N=300)

Age range	*Yersinia*		*Aeromonas*	
	No.	%	No.	%
Below 2 years	5	1.7	31	10.3
2- less than 6 years	7	2.3	61	20.3
6- less than 15 years	1	0.3	8	2.7
Over 15	1	0.3	3	1.0
Total	14	4.7	103	34.3

P: 0.001

A total of 14 (4.7%) *Y. enterocolitica* and 103 (34.3 %) *A. hydrophila* isolates were recovered from 300 patients with acute diarrhea, whereas no isolates were recovered from appendiceal samples. Other bacterial enteropathogens were isolated from 300 patients with diarrhea; 3 isolates (1.0%) of *Salmonella* spp. and 3 isolates (1.0%) of *Shigella* spp. (Figure 4.4).

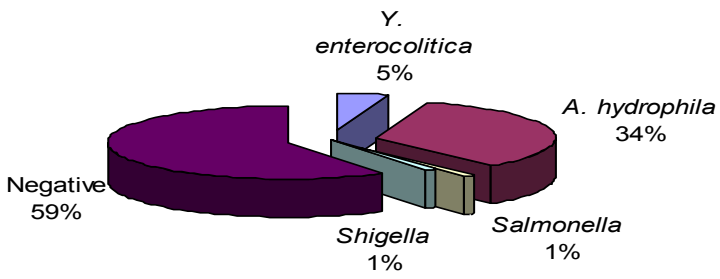

Figure (4.4): Frequency and distribution of *Y. enterocolitica* and *A. hydrophila* and other enteropathogens in clinical samples

4.3.1 Detection of *Yersinia enterocolitica* and *Aeromonas hydrophila* from different hospitals

Clinical samples were collected from eight hospitals located in various parts of Gaza strip. *Y. enterocolitica* was detected in 5 hospitals with high incidence at Al-Dorrah hospital. On the other hand, A. *hydrophila* was detected in all hospitals with the highest frequency from Al-Nasser hospital (Table 4.5). A significant association was found between the number of isolates and source of clinical samples (P<0.05).

Table (4.5): Distribution of *Yersinia* and *Aeromonas* according to hospital

Hospital	Location	No.	%	*Yersinia*		*Aeromonas*	
				No	%	No	%
AL-Aqsa	Deir-Albalah	101	33.7	4	4.0	41	40.6
Al-Nasser	Gaza	47	15.7	3	6.4	21	44.6
Al-Shifa	Gaza	32	10.7	0	0.0	4	12.5
Al-Najar	Rafah	30	10.0	2	6.7	8	26.6
Gaza-European	Rafah	23	7.7	0	0.0	5	21.7
Kamal-odwan	Bit-lahia	15	5.0	0	0.0	4	26.7
Nasser	Khan-Younes	36	12.0	2	5.6	13	36.1
Al-Dorrah	Gaza	16	5.3	3	21.4	7	43.8
Total		300	100.0	14	4.7	103	34.3

P = 0.001

4.3.2 Associated clinical features

All patients with *Y. enterocolitica* had watery diarrhea ranged from 3 to 7 days in duration. The frequency of defecations ranged from 3 to 7 daily. Most (92.2%) of the patients had vomiting, mucus and fever >39 ^{o}C. Other symptoms included

blood in stool (64.3%) and abdominal pain (28.6%). All patients with *A. hydrophila* had watery diarrhea of 3 to 7 days in duration. The maximum frequency of defecation was over 7 daily (95.1%). 66 % of the patients had vomiting and 77.7 % of the patients had fever > 39°C. Additional symptoms included, blood in the stool (43.7%), mucus (67%), and abdominal pain (26.2%), with significant association for both pathogens between the isolates and frequency of defecation ($p < 0.05$) (Table 4.6).

Table (4.6): Clinical features of 14 patients with *Y. enterocolitica* and 103 with *A. hydrophila* diarrhea

Clinical features		No. of positive isolates (%)	
		Y. enterocolitica	*A. hydrophila*
Frequency of defecation	3-7 daily	14 (100)	5 (4.9)
	>7 daily	0 (0.0)	98 (95.1)
Fever	<39°C	1 (7.1)	23 (22.5)
	>39°C	13 (92.2)	80(77.7)
Vomiting	Yes	13 (92.2)	68(66.0)
	No	1 (7.1)	35 (34.0)
Mucus	Yes	13 (92.2)	69 (67.0)
	No	1 (7.1)	34 (33.0)
Duration	3-7 days	14 (100)	103(100)
Bloody stool	Yes	9 (64.3)	45 (43.7)
	No	5 (35.7)	58(56.3)
Abdominal pain	Yes	4 (28.6)	27 (26.2)
	No	10 (71.4)	76(73.8)

4.4 Recovery of *Yersinia enterocolitica* and *Aeromonas hydrophila* from food samples

4.4.1 Milk and milk product samples

Fifty dairy product samples were collected from different places in Gaza strip. Of the 50 different milk samples analyzed, 3 displayed the presence of *Y. enterocolitica*, (6.0%) and 18 showed *A. hydrophila* growth (36%) (Figure 4.5).

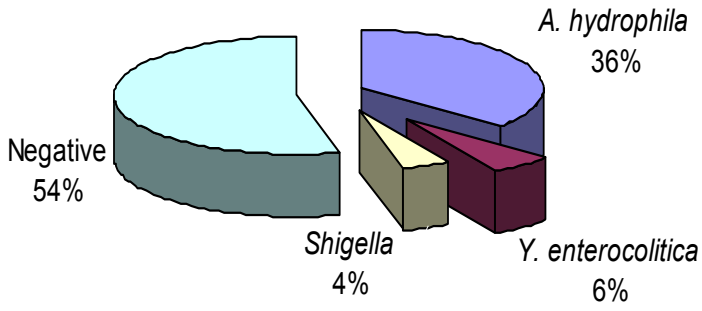

Figure (4.5): Frequency and distribution of *Y. enterocolitica* and *A. hydrophila* in milk and milk product samples

Y. enterocolitica was isolated only from cow milk (3 isolates, 16.7%), and the occurrence of *A. hydrophila* was slightly higher in cheese (75%) than in goat milk (66.7) and cow milk (22.2%). There was a significant relationship between number of isolates and type of dairy product ($P< 0.05$) (Table 4.7).

Table (4.7): Isolation of *Y. enterocolitica* and *A. hydrophila* from milk and milk product

Milk and milk products	Frequency			Isolates			
	No.	%		*Yersinia*	*Aeromonas*	*Shigella*	Negative
Cow milk	18	36.0	NO.	3	4	2	9
			%	16.7	22.2	11.1	49.9
Goat milk	6	12.0	NO.	0	4	0	2
			%	0.0	66.7	0.0	33.3
Pasteurized milk	5	10.0	NO.	0	0	0	5
			%	0.0	0.0	0.0	100
Powdered milk	2	4.0	NO.	0	0	0	2
			%	0.0	0.0	0.0	100
Ice cream	15	30.0	NO.	0	7	0	8
			%	0.0	46.7	0.0	53.3
Cheese	4	8.0	NO.	0	3	0	1
			%	0.0	75.0	0.0	25.0
Total	50	100	NO.	3	18	2	27
			%	6.0	36.0	4.0	54.0

P = 0.002

4.4.2 Meat samples

A total of 3 (6.7%) *Y. enterocolitica* isolates from different food samples were obtained from 45 food samples. One *Y. enterocolitica* was isolated from 20 cow samples (33.3%) and 2 isolates from 7 turkey samples, with no additional isolates from sausage, hamburger, chicken and packed meat samples. On the other hand, 22 *A. hydrophila* isolates were recovered from the same food samples. One *A. hydrophila* was isolated from 6 chicken samples, 7 from 20 cow samples, 5 from 5 hamburger samples, 2 from 2 packed samples, 2 from 7 turkey samples and 5 from 5 sausage samples. We were able to isolate two *Salmonella* species from these samples. These rates of isolation of pathogenic isolates were significantly different ($P < 0.05$) (Figure 4.6 and Table 4.8).

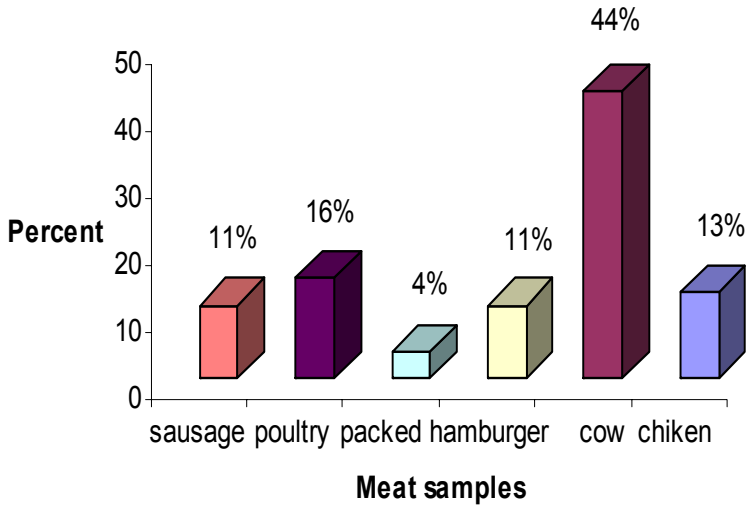

Figure (4.6): Frequency and distribution of tested food samples

Table (4.8): Number and percent of *Y. enterocolitica* and *A. hydrophila* recovered from various meat samples

Meat type	No.	%	Isolates				
				Yersinia	*Aeromonas*	*Salmonella*	Negative
Chicken	6	13.3	NO.	0	1	0	5
			%	0.0	16. 7	0.0	83.3
Cow	20	44.4	NO.	1	7	0	12
			%	5.0	35	0.0	60.0
Hamburger	5	11.1	NO.	0	5	0	0
			%	0.0	100	0.0	0.0
Packed	2	4.4	NO.	0	2	0	0
			%	0.0	100	0.0	0.0
Turkey	7	15.6	NO.	2	2	2	1
			%	28.6	28.6	28.6	14.3
Sausage	5	11.1	NO.	0	5	0	0
			%	0.0	100	0.0	0.0
Total	45	100	NO.	3	22	2	18
			%	6. 7	48. 9	4.4	40.0

P = 0.001

4.5 Recovery of *Yersinia enterocolitica* and *Aeromonas hydrophila* from environmental samples

4.5.1 Water samples

Figure 4.7 shows the distribution of *Y. enterocolitica* and *A. hydrophila* in different types of tested water. A total of 2 isolates of *Y. enterocolitica* were recovered from a total of 32 water samples, one from tap water and the other from well water with no isolate from sea water. On the other hand, 15 isolates of *A. hydrophila* were recovered from different types of water, 2 isolates from sea water, 6 isolates from tap water and 7 isolates from well water were obtained; with higher incidence of both organisms in tap and well water. There was a significant relationship between number of isolates and type of water $P < 0.05$ (Table 4.9).

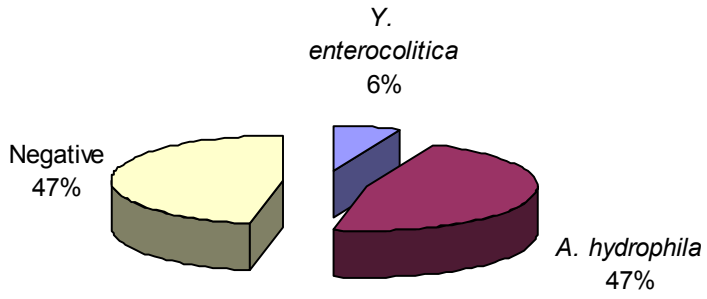

Figure (4.7): Frequency of *Y. enterocolitica* and *A. hydrophila* from water samples

Table (4.9): Number and percentage of *Y. enterocolitica* and *A. hydrophila* isolated from water

Water type	No.	%		Isolates *Yersinia*	Isolates *Aeromonas*	Isolates Negative
Sea water	5	15.6	NO.	0	2	3
			%	0.0	40	60.0
Tap water	13	40.6	NO.	1	6	6
			%	7.7	46.2	46.2
Wells water	14	43.8	NO.	1	7	6
			%	7.1	50.0	42.9
Total	32	100	NO.	2	15	15
			%	6.25	46.9	46.9

P = 0.004

4.5.2 Sewage samples

Five *Yersinia* spp. were recovered from 26 sewage samples. *Y. enterocolitica* was the most frequently isolated *Yersinia* spp. It was found in 3 (11.5%) of 26 samples. The other two isolates were identified as *Yersinia kristensenii* (7.7%). Eleven *A. hydrophila* (42.3%) were recovered from the same samples (Figure 4.8).

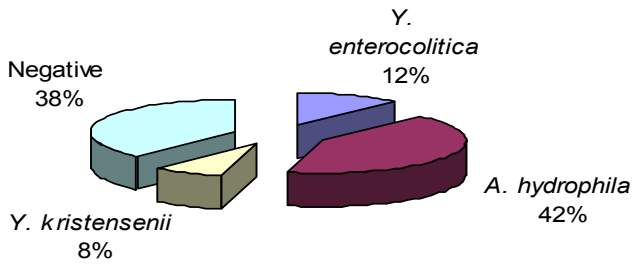

Figure (4.8): *Y. enterocolitica* and *A. hydrophila* isolated from sewage samples

4.5.2.1 Regional distribution of *Yersinia enterocolitica* and *Aeromonas hydrophila* isolated from sewage samples

A total of 26 sewage samples were collected from 6 different sampling points. From the 26 sewage samples, 11 (42.3%) *A. hydrophila*, 3 (11.5%) *Y. enterocolitica* and two environmental *Yersinia kristensenii* were isolated. The high incidence of *Y. enterocolitica* and *A. hydrophila* were from Al-Nasser hospital, 33.3% and 66.7% respectively, with no significant relationship between the number of isolates and source of sewage samples ($p > 0.05$) (Table 4.10).

Table (4.10): Number and percentage of *Yersinia* and *Aeromonas* isolated from each sampling points

Sewage			Isolates				
	No.	%		*Y. enterocolitica*	*A. hydrophila*	*Y. kristensenii*	Neg.
Bit-lahia WWTP	4	15.4	NO.	0	2	1	1
			%	0.0	50.0	25.0	25.0
European Hospital	4	15.4	NO.	0	2	0	0
			%	0.0	50.0	0.0	0.0
Al-Nasser Hospital	3	11.5	NO.	1	2	0	2
			%	33.3	66.7	0.0	66.7
El-shifa Hospital	3	11.5	NO.	0	1	0	2
			%	0.0	33.3	0.0	66.7
Al-aqsa Hospital	8	30.8	NO.	1	2	1	4
			%	12.5	25.0	12.5	50.0
Shiek Ejleen WWTP	4	15.4	NO.	1	2	0	1
			%	25.0	50.0	0.0	25.0
Total	26	100	NO.	3	11	2	10
			%	11.5	42.3	7.7	38.5

4.5.3. Animal excreta samples

Fecal specimens were collected from different animals of Gaza strip. A total of 3 (11.5%) *Y. enterocolitica* and 10 (38.5%) *A. hydrophila* were isolated from 26 animal excreta samples (Figure 4.9). Other enteropathogens; *Salmonella arizona* was recovered from 2 (7.7%) of the total samples.

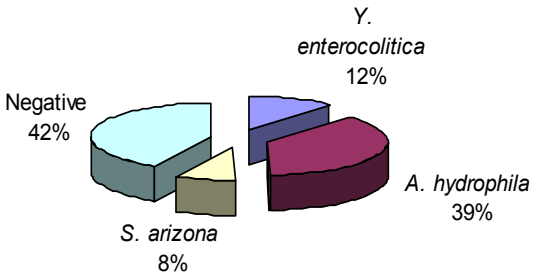

Figure (4.9): Frequency of *Y. enterocolitica and A. hydrophila* isolated from animal excreta samples

Twenty-six animal excreta samples were collected from different sources, 4 (15.4%) from chickens, 7 (26.9%) from cows, 8 (30.8%) from goats, and 7 (26.9%) from turkeys. The maximum number of *Y. enterocolitica* was recovered from cow's excreta (28.6%) and *A. hydrophila* from goat's excreta (62.5%) (Table 4.11). There is no statistically significant differences between occurrence of both pathogens and type of animal excreta ($P > 0.05$).

Table (4.11): The number and percentage of *Y. enterocolitica* and *A. hydrophila* isolated from animal excreta samples.

Animal excreta			Isolates			
	No.	%		*Y. enterocolitica*	*A. hydrophila*	*S. arizona*	Negative
Chicken	4	15.4	NO.	0	0	0	4
			%	0.0	0.0	0.0	100
Cow	7	26.9	NO.	2	3	1	1
			%	28.6	42.9	14.3	14.3
Goat	8	30.8	NO.	0	5	0	3
			%	0.0	62.5	0.0	37.5
Turkey	7	26.9	NO.	1	2	1	3
			%	14.3	28.6	14.3	42.9
Total	26	100	NO.	3	10	2	11
			%	11.5	38.5	7.7	42.3

4.6 Isolation of *Yersinia enterocolitica* and *Aeromonas hydrophila* using different enteric agar media

In this study, SS, HE, XLD, MCA and CIN agars were used to isolate *Y. enterocolitica* and *A. hydrophila.* The ability of enteric media to selectively support the growth of *Y. enterocolitica* and *A. hydrophila* was evaluated using clinical and food samples. Figures 4.10 (A,B,C,D) show the colony morphology of *Y. enterocolitica* and *A. hydrophila* on SS, HE, XLD and CIN agars respectively after incubation at 32°C for 24 h.

A: On SS agar: Both *Y. enterocolitica* and *A. hydrophila* appeared colorless.

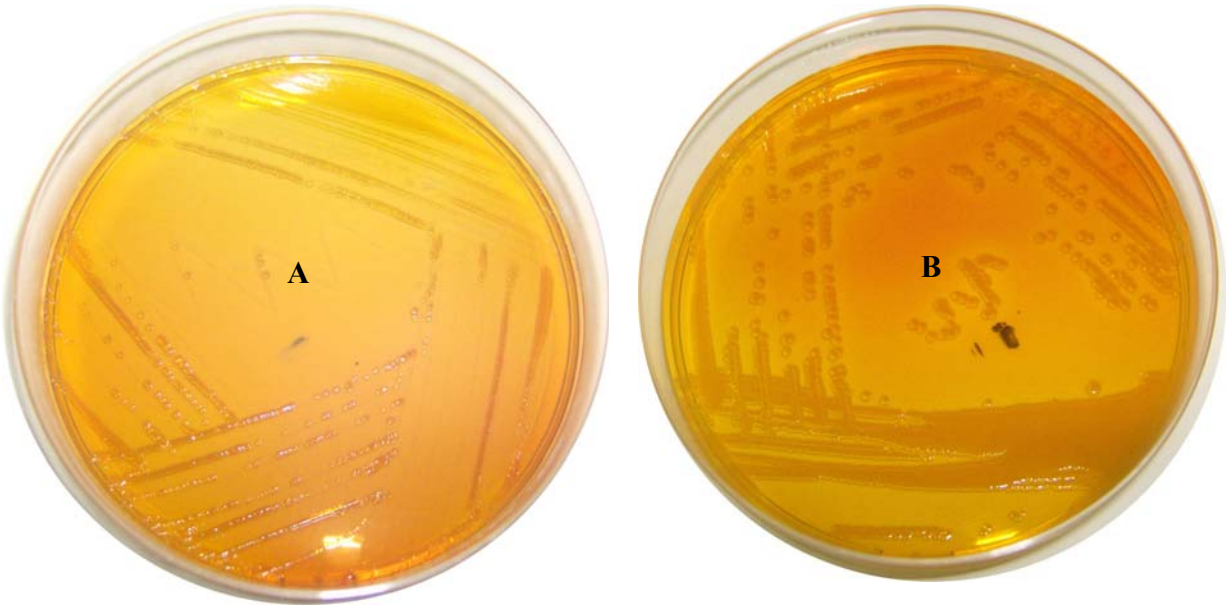

Figure (4.10.A): Appearance of *Y. enterocolitica* **(A)** and *A. hydrophila* **(B)** on SS agar after 24 h of incubation at 32°C

B: On HE agar: Both *Y. enterocolitica* and *A. hydrophila* showed salmon color on HE agar as normal coliform in the stool.

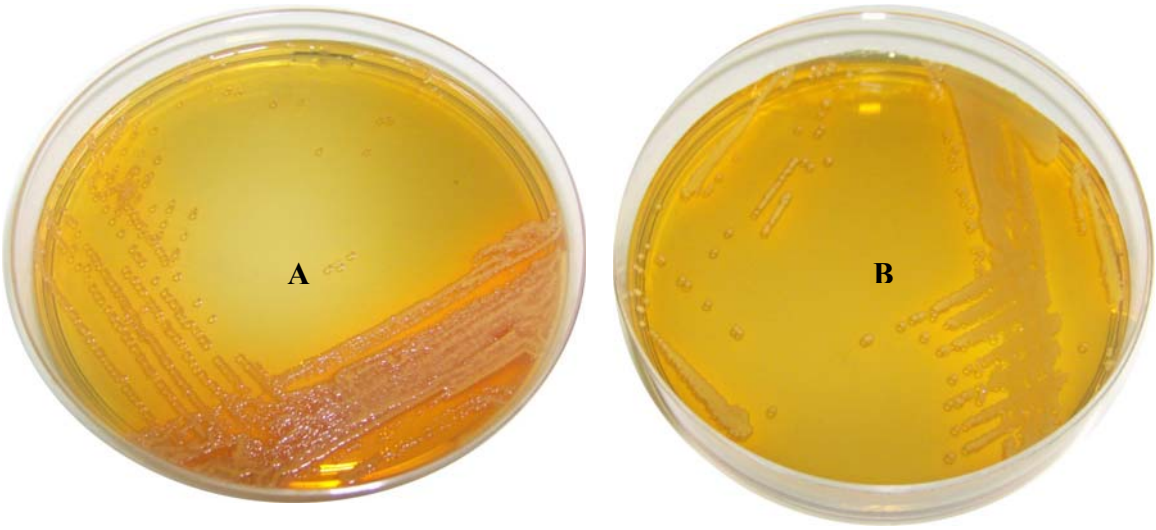

Figure (4.10.B): Appearance of *Y. enterocolitica* **(A)** and *A. hydrophila* **(B)** on HE agar after 24 h of incubation at 32°C.

C: On XLD agar: *Y. enterocolitica* and *A. hydrophila* appeared yellow color.

Figure (4.10.C): Appearance of *Y. enterocolitica* **(A)** and *A. hydrophila* **(B)** on XLD agar after 24 h of incubation at 32°C

D: On CIN agar: *Y. enterocolitica* and *A. hydrophila* appeared deep red center with a transparent margin, or "bull's-eye"

Figure (4.10.D): Appearance of *Y. enterocolitica* **(A)** and *A. hydrophila* **(B)** on CIN agars after 24 h of incubation at 32°C

4.6.1 Evaluation of the efficacy of selective media for the recovery of *Yersinia enterocolitica* and *Aeromonas hydrophila* from clinical and food samples

For the sake of comparison, CIN agar was considered as the golden standard for the isolation of both *Y. enterocolitica* and *A. hydrophila*. The results in table 4.12 clearly show different recovery efficacy for SS, HE, XLD and CIN agars in isolating *Y. enterocolitica* and *A. hydrophila*. However all three selective media showed inferior recovery when compared to CIN. They were even more inferior than CIN when dealing with food and environmental samples.

Table (4.12): Number and percentage of *Y. enterocolitica* and *A. hydrophila* using different culture method

Media			Clinical Isolates		Meat isolates	
			Yersinia N= (14)	*Aeromonas* N= (103)	*Yersinia* (3)	*Aeromonas* N= (22)
SS[a]	Growth	NO.	1	4	0	0
		%	7.1	3.9	0.0	0.0
	No growth	NO.	13	99	3	22
		%	92.9	96.1	100	100
HE[b]	Growth	NO.	1	3	0	0
		%	7.1	2.9	0.0	0.0
	No growth	NO.	13	100	3	22
		%	92.9	88.5	100	100
XLD[c]	Growth	NO.	1	3	0	0
		%	7.1	2.9	0.0	0.0
	No growth	NO.	13	100	3	22
		%	92.9	88.5	100	100
CIN[d]	Growth	NO.	14	103	3	22
		%	100	100	100	100
	No growth	NO.	0	0	0	0
		%	0.0	0.0	0.0	0.0

[a]SS, Salmonella Shigella agar, [b]HE, Hektoen enteric agar

[c]XLD, Xylose Lysine Dextrose agar, [d]CIN, Cefsulodin-Irgasan-Novobiocin agar

P value= 0.000

4.6.2 Efficacy of KOH treatment

Three methods were used to detect *Y. enterocolitica* and *A. hydrophila* from clinical, food and environmental samples. The three methods were; direct plating on CIN agar after KOH treatment, cold enrichment in PBS followed by plating on CIN agar and overnight enrichment in TSB followed by plating on CIN agar. *Yersinia* and *Aeromonas* organisms are more tolerant to alkali conditions than are most other bacteria, and enrichment cultures may be exposed to KOH to selectively reduce the level of competing microorganisms. All *Yersinia* and *Aeromonas* isolates in clinical and food samples were detected in large numbers on CIN agar on primary isolation, after treatment with KOH. No additional isolates were detected following cold enrichment in PBS and following overnight enrichment in TSB.

The percent recovery of *Yersinia* and *Aeromonas* using the KOH method was 100% as compared with 78.6%, 66.7% recovery for *Y. enterocolitica* and 76.7%, 77.3% for *A. hydrophila* after 14 days of incubation in PBS at 4^{o}C from clinical and food samples respectively. On the other hand, 100%, 66.7% recovery for *Y. enterocolitica* and 94.2%, 86.4% for *A. hydrophila* by TSB after 48 hr incubation from clinical and food samples respectively (Table 4.13). Direct plating on CIN agar after treatment with KOH was significantly ($p < 0.000$) more sensitive than cold enrichment in PBS and overnight enrichment in TSB.

Figure 4.11 illustrates a CIN agar plate after 24 h of incubation at 25^{o}C. One-half of the plate was streaked with alkali-treated inoculum, the other half with saline-treated inoculum. Many small, distinct, bulls eyes red colonies characteristic of *Yersinia* and *Aeromonas* were seen on the alkali treated inoculum; numerous large, pigmented, mucous colonies characteristic of non-*Yersinia* and *Aeromonas* colonies were seen on the saline-treated inoculum.

Table (4.13): Number and percentage of *Yersinia* and *Aeromonas* isolates from patient stools according to isolation technique

Enrichment techniques			Clinical Isolates		Meat isolates	
			Yersinia N= (14)	*Aeromonas* N= (103)	*Yersinia* N= (3)	*Aeromonas* N= (22)
KOH[a]	Growth	NO.	14	103	3	22
		%	100	100	100	100
	No growth	NO.	0	0	0	0
		%	0.0	0.0	0.0	0.0
Cold Enrichment[b]	Growth	NO.	11	79	2	17
		%	78.6	76.7	66.7	77.3
	No growth	NO.	3	24	1	5
		%	21.4	23.3	33.3	22.7
TSB[c]	Growth	NO.	14	97	2	19
		%	100	94.2	66.7	86.4
	No growth	NO.	0	6	1	3
		%	0.0	5.8	33.3	13.6

[a]*KOH, Direct plating on CIN agar with Potassium hydroxide,* [b]*PBS Cold enrichment in phosphate buffered saline for 2 weeks*

[c]*TSB, Overnight enrichment in TSB*

P value of KOH= 0.000

Figure (4.11): CIN agar plate after 24h of incubation at 26°C, streaked with *Y. enterocolitica* treated with saline (top section) and alkali (bottom section)

4.7 Virulence factors of *Yersinia enterocolitica* and *Aeromonas hydrophila*

Pyrazinamidase production, esculin hydrolysis, autoagglutination and crystal violet binding assay were tested in 28 *Y. enterocolitica* and 180 *A. hydrophila* isolates from various origins. Beta hemolysin production was tested on *A. hydrophila* isolates only. Pathogenic isolates of *Y. enterocolitica* are pyrazinamidase and esculin hydrolysis negative but positive for autoagglutination and crystal violet binding assay, while pathogenic *A. hydrophila* are positive for all virulence factors including beta hemolysin production. According to the results of this virulence factors, the highest percentage for the pathogenic strains of *Y. enterocolitica* (64.3%) and *A. hydrophila* (92.2%) was recovered from clinical samples, while environmental isolates showed virulence characteristics in some of these tests. Using the Chi square test, differences were significant for all tests in clinical samples ($P < 0.05$) whereas the difference was not significant ($P > 0.05$) with the environmental samples isolates.

In sewage samples, the two *Y. kristensenii* isolates were positive for Pyrazinamidase activity and esculin hydrolysis but negative for autoagglutination and crystal violet binding. Tables (4.14-4.17) present the virulence characteristics for *Y. enterocolitica* and *A. hydrophila.*

A. Pyrazinamidase production: Pyrazinamidase activity in *Y. enterocolitica* and *A. hydrophila* from clinical and environmental samples were reported in Table 4.14. Of the 14 clinical *Y. enterocolitica* isolates tested, 5 isolates (64.3%), 2 meat isolates (33.3%), 2 milk isolates (33.3%), 1 water isolates (50%), 2 sewage isolates (33.3%) and 2 animal excreta isolates (33.3%) were negative. On the other hand of *A. hydrophila*, 95 *Aeromonas* clinical isolates (92.2%), 4 meat isolates (18.8%), 6 milk isolates (33.3%), 5 water isolates (33.3%), 4 sewage isolates (36.4%) and 4 animal excreta isolates (40%) were positive.

Table (4.14): Pyrazinamidase production in relation to the source of *Y. enterocolitica* and *A. hydrophila*

Samples		*Y. enterocolitica*			*A. hydrophila*		
	N	N	Negative		N	Positive	
			N	%		N	%
Clinical samples	300	14	5	64.3	103	95	92.2
Meat samples	45	3	2	33.3	22	4	18.8
Milk samples	50	3	2	33.3	18	6	33.3
Water samples	32	2	1	50	15	5	33.3
Sewage samples	26	3	2	33.3	11	4	36.4
Animal excreta samples	26	3	2	33.3	10	4	40

B. Esculin hydrolysis: With esculin hydrolysis, 5 of 14 *Yersinia* clinical isolates (64.3%), 2 of 3 meat isolates (33.3%), 2 of 3 milk isolates (33.3%), 1 of 2 water isolates (50%), 2 of 3 sewage isolates (33.3%) and 2 of 3 animal excreta isolates (33.3%) were negative. On the other hand, 95 of 103 *Aeromonas* clinical isolates (92.2%), 14 of 22 meat isolates (66.7%), 10 of 18 milk isolates (55.6%), 8 of 15 water isolates (53.3%), 8 of 11 sewage isolates (72.7%) and 6 of 10 animal excreta isolates (60%) were positive (Table 4.15, Figure 4.12).

Table (4.15): Esculin hydrolysis in relation to the source of *Y. enterocolitica* and *A. hydrophila*

Samples		*Y. enterocolitica*			*A. hydrophila*		
	N	N	Negative		N	Positive	
			N	%		N	%
Clinical samples	300	14	5	64.3	103	95	92.2
Meat samples	45	3	2	33.3	22	14	66.7
Milk samples	50	3	2	33.3	18	10	55.6
Water samples	32	2	1	50	15	8	53.3
Sewage samples	26	3	2	33.3	11	8	72.7
Animal excreta samples	26	3	2	33.3	10	6	60

Positive reaction Negative reaction

Figure (4.12): Esculin hydrolysis by *Y. enterocolitica* and *A. hydrophila*

C. Autoagglutination: Table 4.16 and Figure 4.13 show the pattern of autoagglutination for clinical and environmental isolates. Of *Yersinia* isolates, 9 (64.3%) of clinical isolates, 1 (33.3%) of meat isolates, 1 (33.3%) of milk isolates, 1 (50%) of water isolates, 1 (33.3%) of sewage isolates and 1 (33.3%) of animal excreta isolates were positive for autoagglutination. On the other hand, 95 (92.2%) of *Aeromonas* clinical isolates, 9 (40.9%) of meat isolates, 6 (33.3%) of milk isolates, 5 (33.3%) of water isolates, 4 (36.4%) of sewage isolates and 4 (40%) of animal excreta isolates were positive.

Table (4.16): Autoagglutination in relation to the source of *Y. enterocolitica* and *A. hydrophila*

Samples		*Y. enterocolitica*			*A. hydrophila*		
	N	N	Positive		N	Positive	
			N	%		N	%
Clinical samples	300	14	9	64.3	103	95	92.2
Meat samples	45	3	1	33.3	22	9	40.9
Milk samples	50	3	1	33.3	18	6	33.3
Water samples	32	2	1	50	15	5	33.3
Sewage samples	26	5	1	33.3	11	4	36.4
Animal excreta samples	26	3	1	33.3	10	4	40

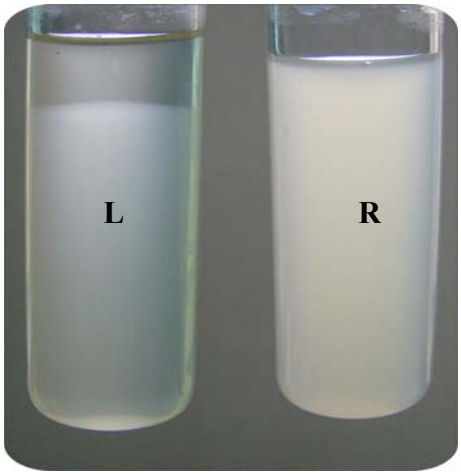

Figure (4.13): Appearance of autoagglutination phenomenon after 18 h of growth in MR-VR broth at 35°C. Positive test (left) and negative test (right).

D. Crystal violet binding: With crystal violet binding, of *Y. enterocolitica* isolates tested, 9 (64.3%) of clinical isolates, 1 (33.3%) of meat isolates, 1 (33.3%) of milk isolates, 1 (50%) of water isolates, 1 (33.3%) of sewage isolates and 1 (33.3%) of animal excreta isolates were positive for crystal violet binding. On the other hand, , 95 (92.2%) of *A. hydrophila* clinical isolates, 9 (40.9%) of meat isolates, 6 (33.3%) of milk isolates, 5 (33.3%) of water isolates, 4 (36.4%) of sewage isolates and 4 (40%) of animal excreta isolates were positive (Table 4.17, Figure 4.14).

Table (4.17): Crystal violet binding in relation to the source of *Y. enterocolitica* and *A. hydrophila*

Samples		*Y. enterocolitica*			*A. hydrophila*		
	N	N	Positive		N	Positive	
			N	%		N	%
Clinical samples	300	14	9	64.3	103	95	92.2
Meat samples	45	3	1	33.3	22	9	40.9
Milk samples	50	3	1	33.3	18	6	33.3
Water samples	32	2	1	50	15	5	33.3
Sewage samples	26	5	1	33.3	11	4	36.4
Animal excreta samples	26	3	1	33.3	10	4	40

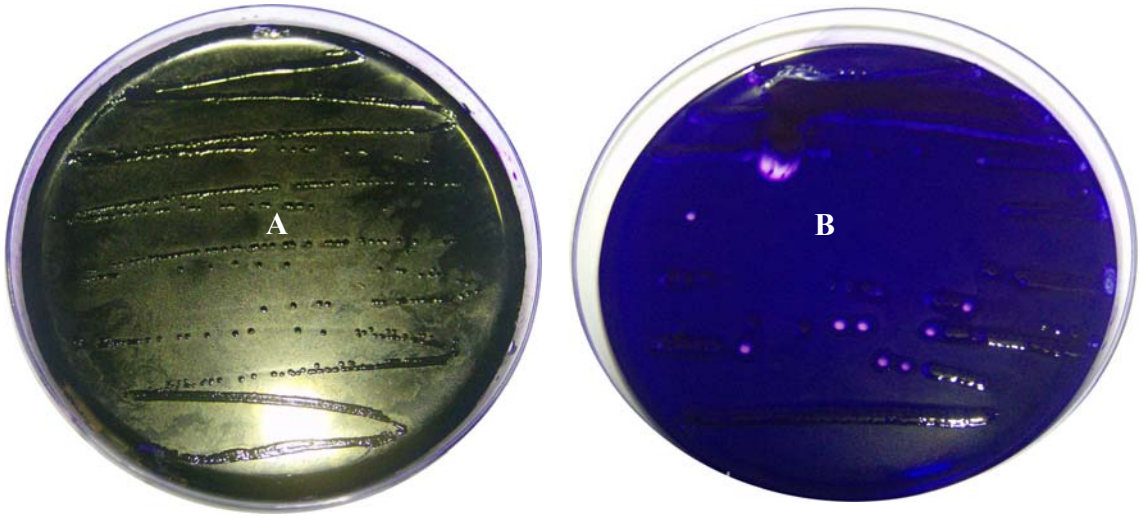

Figure (4.14): Positive **(A)** and negative **(B)** crystal violet binding of *Y. enterocolitica* and *A. hydrophila*

In this investigation, both environmental and clinical isolates were capable of producing virulence factors to various degrees. *Y. enterocolitica* and *A. hydrophila* isolates from the clinical samples proved the most virulent as judged by possession of all four virulence-associated factors.

E. Beta hemolytic activity of *Aeromonas hydrophila* as a virulence factor

Most of the isolates were beta-hemolytic when assayed on blood agar plates (Figure 4.15, 4.16)

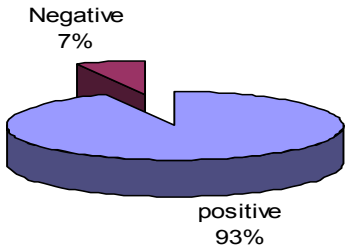

Figure (4.15): Frequency of β- hemolytic activity of *A. hydrophila*

Figure (4.16): β- hemolytic activity of *A. hydrophila* on blood agar plate

4.8 Susceptibility of *Yersinia enterocolitica* and *Aeromonas hydrophila* to various antimicrobial agents

In this test, all clinical *Yersinia* (14) isolates were resistant to amoxycillin/clavulanate, cefazolin and erythromycin. In contrast, all of the isolates were susceptible to co-trimoxazole, amikacin, gentamicin and cefotaxime. In addition, 90% or more of the isolates examined in this study were susceptible to aztreonam, ciprofloxacin, ofloxacin, ceftriaxone, and meropenem. Environmental *Y. enterocolitica* (14) isolates during the same periods displayed susceptibility patterns similar to those of the human isolates (Table 4.18). All of these isolates were not susceptible to cefazolin and erythromycin. With regard to the other antimicrobials agents (ampicillin cephalexin, ceftriaxone, ciprofloxacin, ofloxacin, and aztreonam), the susceptibility results were variable (Figure 4.17 and Table 4.18).

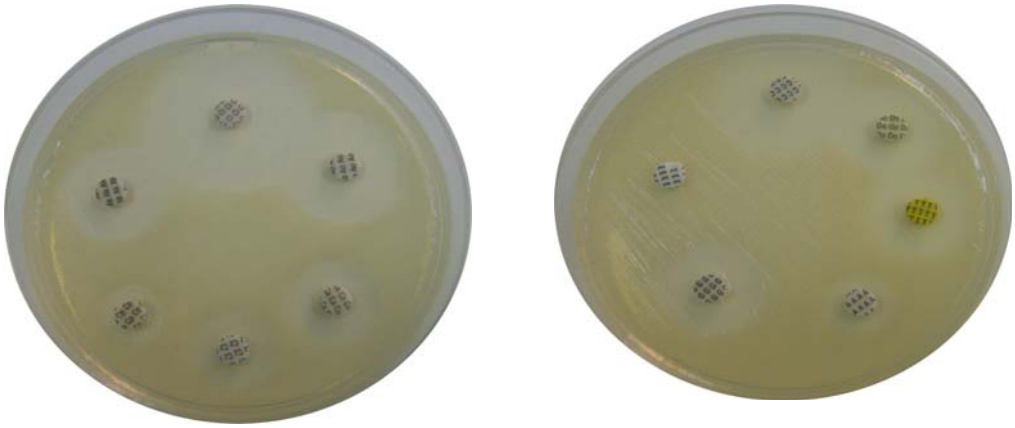

Figure (4.17): Antibiotic Susceptibility of *Y. enterocolitica*

All 103 clinical *A. hydrophila* isolates were resistant to ampicillin, amoxycillin/clavulanate, cefazolin, cephalexine, co-trimethoprim and erythromycin. In contrast, Most of the isolates were susceptible to the third generation of cephalosporins, Cefotaxime. Also, most of the isolates were susceptible to amikacin, aztreonam, ciprofloxacin and ofloxacin. The environmental isolates (84) included in this study showed the same or slightly or the same resistance compared to clinical isolates. All environmental isolates were resistant to ampicillin, amoxycillin/clavulanate, cephalexine, cefazolin, co-trimethoprim and erythromycin. Ciprofloxacin, Ofloxacin, azetronam, cefotaxime, meropenem, gentamycin and ceftriaxone were the most active antimicrobial agents tested (Figure 4.17 and table 4.18).

Figure (4.18): Antibiotic Susceptibility of *A. hydrophila*

Table (4.18): Susceptibility of *Y. enterocolitica* and *A. hydrophila* to various antimicrobial agents

Antimicrobial agents	Clinical Isolates						Environmental isolates					
	Yersinia N= (14)			Aeromonas N= (103)			Yersinia N= (14)			Aeromonas N= (76)		
	S	R	% R	S	R	% R	S	R	% R	S	R	% R
Amikacin	14	0	0.0	87	16	15.5	13	1	7.1	60	16	21.1
Ampicillin	5	9	64.3	0	103	100	10	4	28.5	0	76	100
Amox/clav	0	14	100	0	103	100	1	13	92.9	0	76	100
Aztreonam	13	1	7.1	95	8	7.8	13	1	7.1	64	12	15.8
Cefazolin	0	14	100	0	103	100	0	14	100	0	76	100
Cephalexine	4	10	71.4	0	103	100	4	10	71.4	0	76	100
Cefotaxime	14	0	0.0	84	19	18.5	14	0	0.0	64	12	15.8
Cefruxime	11	3	21.4	49	54	52.4	13	1	7.1	33	43	56.6
Ceftazidime	12	2	14.3	53	50	48.5	9	5	35.7	37	39	51.3
Ceftriaxone	13	1	7.1	65	38	36.9	13	1	7.1	54	22	28.9
Ciprofloxacin	13	1	7.1	93	10	9.7	13	1	7.1	66	10	13.2
Co-trimethoprim	14	0	0.0	0	103	100	8	6	42.8	0	76	100
Chloramphenicol	10	4	28.6	25	78	75.7	11	3	21.4	23	53	69.7
Doxycycline	8	6	42.9	22	81	78.6	8	6	42.8	30	46	60.5
Erythromycin	0	14	100	0	103	100	0	14	100	0	76	100
Gentamicin	14	0	0.0	86	17	16.5	13	1	7.1	56	20	26.3
Meropenem	13	1	7.1	75	28	27.2	13	1	7.1	57	19	25
Nalidixic acid	9	5	35.7	41	62	60.2	12	2	14.3	31	45	59.2
Ofloxacin	13	1	7.1	92	11	10.7	13	1	7.1	65	11	14.5
Tetracycline	8	6	42.9	22	81	78.6	7	7	50	29	47	61.8

CHAPTER 5

DISCUSSION

The primary goals of the present study are to investigate the occurrence of *Y. enterocolitica* and *A. hydrophila* in clinical, food and environmental samples in Gaza strip, to recommend a simple and reliable procedure for the detection of pathogenic *Y. enterocolitica* and *A. hydrophila* and to use the procedure as a diagnostic tool for the rapid identification of *Yersinia* and *Aeromonas* cultures. In the present study; *Y. enterocolitica* was isolated from all types of samples except seawater while *A. hydrophila* was isolated from all sampling sources.

5.1 Distribution of *Yersinia enterocolitica* and *Aeromonas hydrophila*

Y. enterocolitica and *A. hydrophila* are detected in a wide range of samples such as fresh waters **[13,33,109]**, vegetables **[5,14]**, meats and milk products **[32,121,138]**, fish **[14]**, shellfish, seawater **[5,168]** and clinical **[84,133]**. In this study, a high percentage of *Y. enterocolitica* was recovered from sewage (19.1%) followed by animal excreta (11.5%) and higher percentage (48.9 and 46.9%) of *A. hydrophila* isolates were identified in meat and water respectively.

5.2 Recovery of *Yersinia enterocolitica* and *Aeromonas hydrophila* from clinical samples

Of the 300 diarrheal stool samples tested, 14 (4.7%) were positive for *Y. enterocolitica* and 103 (34.3%) were positive for *A. hydrophila*. *Y. enterocolitica* and *A. hydrophila* were isolated either from stool samples from children or from older persons with diarrheal infections, with high incidence from patients below 6 years. *Y. enterocolitica* has been isolated from humans on all continents **[24]**. We found a frequency of 4.7% for this organism, which is lower than some parts of the world especially northern European countries

with a frequency up to 13% **[*5*]**. This might be partly due to the warmer climate in our country, wherein this study was carried out during summer. We would expect a higher frequency during autumn and winter, based on the fact that this organism increases greatly in comparison with other species in cold seasons **[*5*]**.

In a comparable study in Montreal, Canada, specimens from children with gastroenteritis were tested during a 15-month period for pathogenic enteric bacteria. *Y. enterocolitica* was isolated from 2.8% of the human fecal specimens and 2.1% from the Oneida County outbreak **[236]**. 1.04% were isolated from 7,290 black Atlanta children during the Thanksgiving-Christmas holidays in 1988 **[237]**.

The differences between the findings of various authors and those of this study might be due to several factors such as; isolation methods, number of analyzed samples, sources of samples, season, and geographical location. These factors may cause an increase or decrease in the incidence of the *Yersinia* spp. For instance, the present study was carried out in Gaza strip, where the weather is generally warm and humidity is high. It is known that the isolation ratio of *Y. enterocolitica* is higher in colder climates.

In case of *A. hydrophila,* our results were higher than the findings of 4.7 % incidence in Chennai, India, 2.8% in Los Angeles, California, hospital, 1.28 %, and 1.4 % of *A. hydrophila* from Mumbai, India. **Alavandi** and **Anandhan** reported *Aeromonas* associated diarrhea in 1 to 13 % of samples in Chennai, while **Kuijper *et al.,*** and **Ogunsanya *et al.,*** reported 3.7 % in Netherlands and 1.4 % in Lagos, Nigeria respectively. However, higher prevalence of 17.7 and 28.1 % were recorded during 2000 and 2001 in Kolkata, India. It is believed that gasteroenteritis caused by *A. hydrophila* occurs more commonly in children with acute diarrhea and adults with traveler's diarrhea **[238]**.

Among 1,821 patients with diarrhea visiting a clinic in La Crosse, Wisconsin, during an 18-month period, **Agger *et al.,*** identified 20 (1.1%) positive for *A. hydrophila*. **Moyer** examined 3,334 diarrheic stool specimens submitted by physicians over a 2-year period to an Iowa public health laboratory and found 238 (7.1%) positive for *A. caviae, A. hydrophila*, or *A. sobria*. Isolation in the latter study included an alkaline peptone water enrichment step, which may explain the higher prevalence rate. **Pazzaglia *et al.,*** reported that 23.1% of newborns in Peru demonstrated transitory gastrointestinal colonization with *Aeromonas* spp. during the first days of life **[177]**.

Worldwide, the isolation rate of *Aeromonas* from diarrheic stool has been reported as high as 10.8% and as low as 0%. A study conducted in Southeast Asia, 34% *A. hydrophila* have been recovered from patients with diarrhea **[177]**. Another study among Western Peace Corp workers in Thailand, showed that *Aeromonas* were recovered from 30.8% of persons with diarrhea which is similar to our results (34.3%) **[177]**.

This wide variation in the prevalence of *Aeromonas* among similar studies conducted on children with diarrhea may be attributed to the variation in fecal samples number and also to different methods used for isolation of *Aeromonas* as well as environmental condition, patient populations, food habits, and level of sanitation.

5.3 Recovery of *Yersinia enterocolitica* and *Aeromonas hydrophila* from food samples

In recent years, the number of studies on the prevalence of *Y. enterocolitica* and *A. hydrophila* in food products from various geographical regions has increased significantly **[14,121]**. In studies from different countries, *Y. enterocolitica* were isolated from various ready-to-eat products including, fresh salad, whole and sliced vegetables, sandwiches, milk, dairy products, desserts and soft cheese. In a study carried out in Finland, **Fredriksson-**

Ahomaa *et al.,* tested 200 samples of raw fish, 43 samples of raw chicken and 101 samples of lettuce by nested PCR targeting the *yad*A gene **[6]**. Three PCR-positive results were obtained, all recovered from the lettuce samples. **Logue *et al.,*** investigated Irish meat and meat products and isolated pathogenic serotypes of *Y. enterocolitica* from 10% of samples of cooked ham (n=20), corned beef (n=40) and pork sausage (n=20). **Wang, Cao** and **Cerniglia** examined seafood and found that none of the samples tested positive for pathogenic *Y. enterocolitica* by PCR **[125]**. In an Australian study, **Szabo, Scurrah** and **Burrows** examined 120 samples of minimally processed lettuce collected over an 8-month period and isolated 71 *Y. enterocolitica* strains, all belonging to nonpathogenic serotypes **[174]**.

Raw and pasteurised milk have been examined in several studies because outbreaks over a number of years in the United States were traced to milk. **Schiemann** and **Toma** examined 131 raw milk samples for the presence of *Y. enterocolitica*. Forty-two isolations were obtained from 19 pooled- (31.1% positive) and 10 individual-producer samples (14.3% positive) **[239]**. The other study by **Schiemann** reported a high incidence of *Y. enterocolitica* in raw milk (18.2%), 9.2% in cheese curd samples and 0.4% in pasteurized fluid dairy products from southern Ontario **[239]**.

Several studies have been conducted to isolate *Yersinia* spp. in ground beef and the isolation rate was reported to be 9-99.2%. Among these studies, some generated higher isolation rates than the results of this study. In the present study, *Y. enterocolitica* was isolated from 6.7% of meat samples. **Inoue** and **Kurose** and **Leistner *et al.,*** found that *Yersinia* spp. were recovered from 24 and 16% of samples, respectively. **Hanna *et al.,*** examined whole-sale cuts of vacuum-packaged fresh beef and reported that 10 out of 107 beef samples (9.3%) were positive for *Y. enterocolitica*. Similarly, **Ibrahim** and **MacRae** examined 50 beef samples for *Yersinia* spp. and the isolation rate was 20%. 9 (18%) were *Y. enterocolitica*. **Karib *et al.,*** also reported that 4 out of 30 beef meat (13.3%) and 3 out of 20 ground beef

samples (15%) were examined for *Yersinia* and *Y. enterocolitica* isolation rates were 13.3 and 15%, respectively. In another study by **Falcao**, 40 cultures of isolated ground beef, 9 (22.5%) were *Y. enterocolitica* **[106]**.

Although the results of this study showed higher isolation ratio, the examination of a limited number of samples might have caused misinterpretation. It might also have been related to the efficiency of the detection method.

In one study conducted in Norway, 47 samples of sausage meat and 99 samples of pork chop collected from five slaughterhouses and one retail outlet were analyzed **[125]**.The presence of pathogenic *Y. enterocolitica* was detected by PCR in 15% and 26% of these samples, respectively. In another study, **Nesbakken *et al.,*** examined 12 samples of pork cuts and 33 samples of sausage meat and obtained 5 and 23 positives, respectively **[88]**.

Ibrahim and **MacRae** reported that *Aeromonas* was present in 60, 58, 74 and 26% of investigated beef, lamb, pork and milk samples, respectively, whereas **Krovacek *et al.,*** found aeromonads in 42% of the food samples originating from a random selection of retail outlets in Sweden. *Aeromonas* were also found in fish and fresh salads, freshly dressed lamb carcasses, oysters, cheese and raw cow's milk **[14]**. In the present study, *Y. enterocolitica* and *A. hydrophila* were isolated from 6%, 36% of the milk samples and 6.7%, 48.9% of meat samples respectively. Because of the obvious differences in sampling period, geographical location, the origin of the samples and methodology for analysis, it is difficult to compare the level of *Y. enterocolitica* and *A. hydrophila* incidence published by different authors. However, the present data clearly confirm the widespread distribution of *Y. enterocolitica* and *A. hydrophila* in retail foods.

The recovery of pathogenic *Y. enterocolitica* and *A. hydrophila* is contingent upon a number of factors including the level of background flora on the sample, the amount of background flora coming from enrichment and plating,

the level of pathogenic *Y. enterocolitica* and *A. hydrophila* present in the sample, the numbers of non-pathogenic *Y. enterocolitica* and non-pathogenic *Yersinia* spp. present in the sample, and the loss of virulence factors during enrichment and plating. Furthermore, a difference in virulence factors may be to the different serotypes. Recovery method that gives good recovery of one particular serotype of pathogenic *Y. enterocolitica* may not suit another serotype.

5.3 Recovery of *Yersinia enterocolitica* and *Aeromonas hydrophila* from environmental samples

5.3.1 Water Samples

Y. enterocolitica and *A. hydrophila* isolates recovered from different water samples, including chlorinated and non chlorinated, fresh water, well water, seawater, wastewater and natural mineral water. Most of these microorganisms were found to be nonpathogenic **[5,13,33]**.

The possibility for these microorganisms to survive in this type of environment has been investigated. **Chao**, **Ding** and **Chen** showed that *Y. enterocolitica* could survive in soil and water systems, especially at low environmental temperatures **[125]**. **Karapinar** and **Gonul** found that a mixed culture of nonpathogenic and pathogenic strains held at 4ºC was recovered after 56 weeks incubation in sterile spring water. In our study, water isolates represented two (6.3%) of a total of 32 water samples which is lower than those reported in other parts of the world. **Shayegani *et al.,*** isolated 147 *Y. enterocolitica* and related species from 622 water samples; 23 (15.6%) were *Y. enterocolitica*. Some researchers reported that even lower isolation ratios were detected compared to the results of this study **[236]**. In a study carried out in Australia, **Sandery**, **Stinear** and **Kaucner** tested 251 water samples by nested PCR. Eleven samples (4.4%) from 4 separate locations tested positive **[125]**.

In this study, *A. hydrophila* was isolated (46.9%) from different water samples. *A. hydrophila* is also associated with the aquatic environment and has been isolated from tap water, well water, seawater and wastewater; sometimes at quite high levels **[134,151]**. **Knochel** and **Jeppesen** examined drinking-water in Denmark and found that only 28% of samples were positive; *A. hydrophila* made up 97% of isolates. In contrast, **Ghanem**, **Mussa** and **Eraki** reported that 90% of domestic water supplies in areas of Cairo contained *Aeromonas*, while from a survey of three distribution systems in Sweden, **Krovacek *et al.,*** reported that 85% of samples were positive for presumptive *Aeromonas*; *A. hydrophila* accounted for 67% of the strains isolated. **Stelzer *et al.,*** recorded a maximum count of 240 *Aeromonas*/100 ml in a drinking-water supply in Germany, with an isolation frequency for *A. hydrophila* of 37%. The highest counts were obtained from points furthest (>10km) from the treatment works. **Havelaar, Versteegh** and **During** reported regrowth of aeromonads in 16 of 20 distribution systems examined in the Netherlands **[150]**.

Legnani *et al.,* reported occurrence of *Aeromonas* spp. in drinking water supplies in a mountain area in northeast Italy (the Dolomites). Out of 7395 water samples analyzed over a 3 years period, 1623 (21.9%) were found to be positive for *Aeromonas*; 72.4% of the strains were identified as *A. hydrophila* **[240]**. **Ghenghesh *et al.,*** isolated *Aeromonas* in 48.7% of 1,000 water samples obtained from wells and other miscellaneous sources. *A. hydrophila* were detected in 59% of samples tested **[222]**. **Ormen *et al.,*** investigated the occurrence of *Aeromonas* spp. in Norwegian natural water sources. 42% of the total isolates were identified *A. hydrophila* **[241]**.

5.3.2 Sewage samples

In our study 5 *Yersinia* spp. were recovered from 26 sewage samples. *Y. enterocolitica* was found in 3(12%) whereas *Y. kristensenii* in 2 (8%). On the other hand; 11 *A. hydrophila* (42%) were recovered from the same samples.

Most of the *Y. enterocolitica* isolates recovered from environmental samples, including fodder, soil, foliage, surface water, sewage water and sludge, have been non-pathogenic. **Singh *et al.,*** reported isolation of *Y. enterocolitica* from sewage effluents collected from several sewage treatment plant in Delhi city (India), 9 (12.3%) from wastewater, 5 (2.8%) from groundwater and 4 (9%) from river Yamuna **[242]**, similar to the result of this study. In another study *Yersinia* spp. were detected in 90.6% out of 32 raw wastewater samples obtained within one year from two municipal sewage treatment plants. Moreover, *Yersinia* was isolated from 50% of 6 effluent samples. The remaining isolates were identified as *Y. frederiksenii* (24 isolates), *Y. intermedia* (22 isolates) and *Y. kristensenii* (3 isolates) **[243]**.

Aeromonas are widespread in wastewater treatment processes. **Burke *et al.,*** isolated 34% of *Aeromonas* spp. from the unchlorinated domestic water supply of a country center in Western Australia **[244]** yielding lower result than our study. The persistence and transmission of *Aeromonas* in a duckweed aquaculture-based hospital sewage water treatment plant in Bangladesh was studied. A total of 670 samples from different sites of the hospital sewage water treatment plant, from feces of hospitalized children suffering from diarrhea, from environmental control ponds, and from feces of healthy humans were collected over a period of three years. All samples (*n* = 86) from the sewage water treatment plant but only 27 out of the 68 (40%) samples from the control ponds were positive for *Aeromonas* similar to this study. The highest mean number of *Aeromonas* bacteria was found in untreated sewage samples **[245]**.

5.3.3 Animal excreta samples

Animals, especially domestic animals, have been suspected as transmitters of *Y. enterocolitica* and *A. hydrophila* to humans **[5,188,189]**. Published studies are contradictory in this regard. The Member States of the Europeans report annually to the EU-Commission on the Zoonoses situation in their country. However, *Y. enterocolitica* infection in animals is not notifiable, and

reports on its prevalence are for the most part results from research projects obtained from institutions or authorities in the member countries. Various studies have investigated wild and farm animals for the presence of pathogenic strains of *Y. enterocolitica*. **Shayegani *et al.,*** examined fecal specimens from 1,426 animals including mammals, birds, reptiles, fish and invertebrates throughout New York State. Strains of *Y. enterocolitica* and related species were isolated from 92 (11.3%) of 812 mammals, 36 (6.3%) of 573 birds and 5 (17.8%) of 28 reptiles and fish **[236]**.

In various studies examining healthy domestic animals and animals with acute enteritis, including cattle, sheep, goats, deer, calves, broilers, hens, turkeys and ducks, only a few pathogenic isolates have been recovered, and they differed from those usually associated with human infections. Virulent *Y. enterocolitica* was isolated from one or more sheep in 78 (17%) of 449 flocks **[101]**. These results are in agreement with our results, where we found that 11.5% of our studied animal excreta were positive to *Y. enterocolitica*.

In a German study, **Gurtler *et al.,*** found that the prevalence of *Y. enterocolitica* ranged between 0 and 65% in fattening pig herds and was present in 39% of pig tonsils at the abattoir. In Denmark and Norway, the frequency of *Y. enterocolitica* O:3 at herd level has been found to be 64% and 70% respectively. **Korte *et al.,*** found that the prevalence in fattening pigs in Finland, investigated in five slaughterhouses, increased from 33% to 64% between 1995 and 1999. In a study performed in Sweden between 1997 and 1998, the frequency at herd level was 67% **[125]**. These results are higher than our results and may be due to the fact that raw pork was main reservoir of *Y. enterocolitica* and transmitted to the other animals.

On the other hand, *Aeromonas* spp. have been recognized as animal pathogens since they were first isolated from diseased frogs and fish. They are now recognized to cause disease in birds and domestic animals **[189]**. **Gray** isolated *A. hydrophila* from feces of normal horses (7 of 110, 6.4%), pigs (11 of 115, 9.6%), sheep (10 of 111, 9.0%), and cows (26 of 123,

21.1%). The total fecal carriage rate in animals is slightly higher than the fecal carriage rate of normal humans, which is < 1 to 7% for most studies, although some studies report higher rates **[245]**. Populations in animals probably reflect the presence of aeromonads in their feed and water.

In the present study *Y. enterocolitica* was isolated from 11.5% of the total animal excreta samples. (14.3% of turkeys, and 28.6% of cows) and *A. hydrophila* was recovered from 38.5% of the total samples (28.6% of turkeys, 42.9% of cows and 62.5% of goats) but none were isolated from chicken in this study. Other studies reported that even lower isolation rates were detected compared to the results of this study. **Stern *et al.,*** isolated aeromonads from 1 (3.1%) of 32 cows and 3 (14.2%) of 21 turkeys, but none were isolated from 22 pigs or 24 sheep **[150]**.

In our study, isolates of *Y. enterocolitica* and *A. hydrophila* were found in human, animal and environmental sources. This suggests the possibility of transmission from environment or animals to humans.

5.6 Isolation of *Yersinia enterocolitica* and *Aeromonas hydrophila* using different enteric agar media

SS, HE, XLD, MCAand CIN were used to selectively isolate *Y. enterocolitica* and *A. hydrophila.*

5.6.1 Evaluation of the efficacy of selective media for the recovery of *Yersinia enterocolitica* and *Aeromonas hydrophila* from clinical and food samples

The number of proven bacterial gastroenteritis agents has increased over the past several years and now includes such diverse groups as certain serotypes of *Yersinia enterocolitica*, *Campylobacter* spp., invasive *Escherichia coli* (O:157, H:7), *Plesiomonas shigelloides*, new halophilic *Vibrio* spp., and *Aeromonas* spp. Because the development of enteric agars,

essentially designed for the primary isolation of *Salmonella* and *Shigella* spp., predated the discovery of these bacteria, either new selective agars are required to isolate these microorganisms (as in the case of *Campylobacter* spp.) or laboratory workers must make use of existing selective and differential agars **[232]**.

From this study, it is apparent that routine media used in our laboratory are unsatisfactory for the recovery of *Y. enterocolitica* and *A. hydrophila*, owing to the poor plating efficiency and recovery of these organism from stool specimens are difficult because not only are *Y. enterocolitica* and *A. hydrophila* indistinguishable from many other enteric organisms which do not ferment lactose on MCA or SS agar, but it may also be easily overgrown by most intestinal bacterial flora since the former grows relatively slowly. Additionally, *Y. enterocolitica* and *A. hydrophila* are capable of growth on XLD or H-E agar and ferment the sucrose and xylose in the former medium and salicin and sucrose in the latter, thereby rendering colonies indistinguishable from "coliforms".

The CIN agar used in this study provided the most effective medium for the recovery of *Y. enterocolitica* and *A. hydrophila.* The confirmation rate of identification of presumptive *Y. enterocolitica* and *A. hydrophila* from CIN was 100% but the isolation rate of *Y. enterocolitica* and *A. hydrophila* was 7.1% and 2.9% on HE and XLD agar and 8.1% and 3.9% on SS agar respectively. The greatest advantage of CIN agar is that *Y. enterocolitica* and *A. hydrophila* with red colonies on CIN agar are easily differentiated from most other gram-negative bacteria, which showed pink or dark-red colonies or transparent colonies with a peripheral dark zone as the result of mannitol fermentation and CIN agar dramatically inhibits normal flora organisms.

Head *et al.,* conducted comparative studies of several selective media including MacConkey agar for the recovery of *Y. enterocolitica*. They found that CIN agar was the most effective, yielding 100% recovery of *Y.*

enterocolitica in a test suspension containing 10 CFU/ml. Using a combination of CIN agar and cold enrichment during a 42-month period, these investigators isolated 80 *Y. enterocolitica* and 52 *Y. enterocolitica*-like strains (42 *Y. frederiksenii*, 8 *Y. intermedia*, and 2 *Y. kristensenii*) from 215 fecal specimens from 171 patients **[24]**.

Many investigators **[85,226,229]** reported that CIN agar was a more effective agar medium than SS and MCA agars for the isolation of *Y. enterocolitica* and *A. hydrophila* from various specimens. **Schiemann** reported that a color reaction resulting from the fermentation of mannitol presents a characteristic colony appearance which can serve to differentiate *Y. enterocolitica* and *A. hydrophila* from most other gram negative bacteria able to grow on CIN agar with added mannitol and differentiate *Y. enterocolitica* from *A. hydrophila* by oxidase test **[78]**. These findings suggest that CIN agar is a useful medium not only for isolation of *Y. enterocolitica* but also *A. hydrophila.*

5.6.2 Efficacy of KOH treatment

In our study the highest isolation rates of *Y. enterocolitica* and *A. hydrophila* were obtained after KOH treatment. All *Yersinia* and *Aeromonas* isolates were detected in large numbers on CIN agar on primary isolation, after treated with KOH. No additional isolates were detected following cold enrichment and following overnight enrichment in TSB. The percent recovery of *Yersinia* and *Aeromonas* by the KOH method was 100% as compared with 78.6% recovery for *Y. enterocolitica* and 76.7% for *A. hydrophila* after 14 days of incubation at 4^{o}C. We obtained a 100% recovery for *Y. enterocolitica* and a 94.2% for *A. hydrophila* by Trypticase soy broth after 48 h incubation.

Recovery of *Yersinia* and *Aeromonas* from environmental samples are complicated by a technical difficulty rather than by a nutritional requirement of *Yersinia* and *Aeromonas*. Because *Yersinia* and *Aeromonas* grows more slowly than non- *Yersinia* and *Aeromonas* organisms, their population is quickly overgrown and easily masked when streaked on a weakly selective

isolatory agar. By treating the inoculum with 0.5% KOH in 0.5% NaCl, the difficulty is largely overcome. The alkali treatment killed or inhibited a larger number of contaminating non- *Yersinia* and *Aeromonas* and thus facilitated the isolation of *Yersinia* and *Aeromonas* **[78,79]**.

The alkali method provides a simple, sensitive, and rapid technique for the recovery of *Y. enterocolitica* and *A. hydrophila* from mixed cultures, especially from food samples. A report has appeared describing the benefit of alkali treatment for recovery of *Y. enterocolitica* and *A. hydrophila* from feces **[78]**. These finding determined the usefulness of dilute alkali (KOH) treatment of meat samples for direct isolation of *Y. enterocolitica* and *A. hydrophila* without enrichment shortened the incubation period and appreciably decreased the growth of non-*Yersinia* and *Aeromonas* isolates from clinical and food samples.

5.7 Virulence factors of *Yersinia enterocolitica* and *Aeromonas hydrophila*

Virulence factors were compared for 14 *Y. enterocolitica* and 103 *A. hydrophila* isolated from clinical samples with 16 *Yersinia* spp. and 76 *A. hydrophila* isolated from environmental samples in the same area during the same period. *Yersinia* and *Aeromonas* spp. isolated from clinical samples showed differences in virulence characteristics when compared with strains isolated from environmental samples in the same environment **[95,127]**.

Isolates of *Y. enterocolitica* and *A. hydrophila* had several properties shown to be virulence-associated by other workers. Pathogenic *Y. enterocolitica* isolates autoagglutinated at 35°C, positive for crystal violet binding and negative for esculin hydrolysis and pyrazinamidase activity. On the other hand, pathogenic *A. hydrophila* isolates were positive for autoagglutination, esculin hydrolysis, pyrazinamidase activity and crystal violet binding.

A. Pyrazinamidase test

The pyrizinamidase test was easy to perform. The pyrazinamidase test correctly identified 94% *Yersinia* isolates **[10]**. Our results showed that both clinical and environmental isolates were positive for virulence test markers, with high percent in clinical isolates. 64.3% of clinical isolates were negative compared to only 33.3% of environmental isolates. This was in disagreement with the results of several authors.

Kandolo and **Wau-ters** investigated pyrazinamidase in 381 strains of *Y. enterocolitica* isolated from human, animal and environment sources from different geographical areas. They showed that pyrazinamidase test was negative (PYZ-) in all bioserogroups of *Y. enterocolitica*, in which is usually harbored the virulence plasmid, and was involved in human or animal diseases. The more ubiquitous bioserogroups of *Y. enterocolitica*, without naturally occurring virulence plasmid, and related species were all Pyz+. They found 100% accuracy in differentiating pathogenic and nonpathogenic serotypes. All *Y. kristensenii* isolates were positive for Pyrazinamidase activity similar to the results of the present study **[246]**. **Siriken** reported that none of the *Y. enterocolitica* isolates recovered from ground beef was positive for virulence assays **[106]**.

Our results were disagreement with other studies; 33.3% of *Y. enterocolitica* were virulent. **Vishnubhatla *et al.,*** reported that *Y. enterocolitica* was virulent in 30 (60%) of 50 ground beef samples **[106]**. **Riley** and **Toma** noted that 5 of their 21 strains of *Y. enterocolitica* serotype O:1,2,3 were pyrazinamidase positive, indicating a nonpathogenic serotype; lower than our results **[92]**.

Carnahan *et al.,* investigated pyrazinamidase activity of *Aeromonas* spp. Of the 37 *A. hydrophila* isolates tested, 35 (95%) were positive, which is similar to our result where we found that 92.2% of clinical isolates were positive for pyrazinamidase and 30.2% of environmental isolates were negative **[191]**.

B. Esculin hydrolysis

In our study a total of 35.7%, 92.2% of clinical *Y. enterocolitica* and *A. hydrophila* isolates were positive for bile esculin hydrolysis respectively. While, 64.3%, 60.5% of environmental *Y. enterocolitica* and *A. hydrophila* isolates were positive respectively. **Farmer *et al.,*** reported esculin hydrolysis of *Y. enterocolitica.* A negative reaction of esculin hydrolysis correctly identified all 63 strains of the pathogenic serotypes (100% sensitivity), and a positive results correctly identified 34 of 37 strains of nonpathogenic serotypes (92% specificity). Thus, esculin hydrolysis correctly identified 97% of the isolates **[10]**.

Carnahan *et al.,* investigated esculin hydrolysis of *Aeromonas* spp. Of the 167 clinical *Aeromonas* spp., isolates tested, 114 (68%) were positive. Most of *A. hydrophila* isolates were positive agreement with our result, (92.2%) *Aeromonas* clinical isolates were positive **[129]**. It can be concluded that esculin hydrolysis agar, available in most clinical laboratories for the identification of *Enterococcus* spp., can be used for differentiation of pathogenic from pathogenic aeromonads.

C. Autoagglutination

Farmer *et al.,* reported that only 8 (12.7%) of 63 *Yersinia* strains were positive for autoagglutination in MR-VP broth **[10]**. **Laird** and **Cavanaugh** pointed out that 25 (13.8%) of 180 *Y. enterocolitica* strains were found to be agglutinated **[247]**. These results disagree with our findings where 64.3% of clinical isolates were positive and only 35.7% of environmental isolates were positive. **Janda *et al.,*** described a group of *Aeromonas* strains; of 79 mesophilic aeromonads (13 environmental and 66 clinical isolates) evaluated for the ability to autoagglutinate in BHIB before (AA^+) or after boiling (PAB^+), 24 (30%) were positive (AA^+). Among *A. hydrophila,* 6 (55%) of 11 from invasive disease were AA^+, and 8 (28%) of 29 from noninvasive disease

were AA^+ **[164]**. These results are lower than our result, 92.2%, 36.8% were positive for clinical and environmental isolates respectively.

D. Crystal violet binding

CV binding was also tested by **Farmer *et al.,*** with mixed cultures of the Plasmid-bearing virulent strains of *Y. enterocolitica* (P+) and avirulent plasmidless derivatives (P-) strains. Average percent binding was 94% **[10]**. Our results showed that 64.3%, 92.2% of clinical *Yersinia* and *Aeromonas* isolates were able to bind crystal violet dye and 35.7%, 36.8% of environmental *Yersinia* and *Aeromonas* isolates were positive respectively. **Paniagua *et al.*** reported that 44 (49.5%) from 74 *A. hydrophila* isolated from rainbow trout fish were able to bind crystal violet dye **[234]**.

Differences in virulence marker activities, possibly due to differences observed between strains isolated from different geographic locations and between different species and the loss of virulence factors during enrichment and plating.

E. β- hemolysin production

The results of this study showed that 93% of *A. hydrophila* were hemolysin producer and this is in agreement with previous reports. **Subashkumar *et al.,*** reported that from 21 isolates of *A. hydrophila* tested 20 (95.2%) of them were hemolysin producers. The isolates varied in their ability to lyse the red blood cells of human origin. Overall 90.47, 4.76 and 4.76% isolates were beta, alpha and gamma hemolytic, respectively. Attention has been given on the hemolysin of motile *A. hydrophila* because the production of hemolytic toxin has been regarded as indication of pathogenic potential, though nonhemolytic aeromonads have also been implicated as human pathogens **[238]**.

As defined by **Wong *et al.,*** all *A. hydrophila* isolates with haemolysin positive genotype were virulent in the suckling mouse assay model. **Burke *et al.,*** reported 97% correlation between hemolysin and enterotoxin production determined by suckling mouse test. It was found that all enterotoxigenic *A. hydrophila* isolates produced hemolysins **[238]**.

Sixty-eight (91.89%) *A. hydrophila* strains were hemolytic, as shown on blood agar reported by **Paniagua *et al.,* [234]**. **Mateos *et al.,*** showed that *A. hydrophila* isolates from the environment were avirulent for mice, whereas human isolates caused lesions and death in these laboratory animals **[149]**.

5.8 Susceptibility of *Yersinia enterocolitica* and *Aeromonas hydrophila* to various antimicrobial agents

Yersinia enterocolitica and *Aeromonas hydrophila* have emerged as an enteropathogen associated with several types of human infections that often require antimicrobial therapy, but little is known about the antimicrobial susceptibilities of these pathogenes in Gaza strip.

In this study, total of 117 clinical isolates of *Y. enterocolitica* (14) and *A. hydrophila* (103) and a total of 90 environmental isolates (14 of *Y. enterocolitica* and 76 *A. hydrophila*) were tested for their susceptibility to antibiotics. All isolates were tested by a standard disk diffusion method for 20 antibiotics. The present study demonstrated a high susceptibility of clinical strains of *Y. enterocolitica* to most of the tested antibiotics. No major difference in susceptibility was observed between any of the isolates of human or environmental isolates included in this study.

These results are in agreement with those of previous investigations; **Rastawicki *et al.,*** demonstrated that almost all strains tested were resistant to ampicillin and cefazolin and susceptible to amoxycillin/clavulanate, cefaclor, cefamandole, cefuroxime, cefotaxime, ceftriaxone, aztreonam,

imipenem, gentamicin, amikacin, netilmicin, tetracycline, doxycycline, chloramphenicol, ciprofloxacin, sulphamethoxazole, trimethoprim, co-trimoxazole and furazolidone. The only disagreement was with amoxicillin/clavulanate **[248]**.

Preton *et al.*, showed that all strains tested were susceptible to ciprofloxacin and piperacillin, and 98% of the strains were susceptible to trimethoprim, sulfamethoxazole, cotrimoxazole, tetracycline, chloramphenicol, cefamandole, cefotaxime, aztreonam, and four aminoglycosides. In contrast, all strains were nonsusceptible to erythromycin, furazolidone, and clindamycin and 90% of the strains were non-susceptible to ampicillin, carbenicillin, ticarcillin, and cephalothin **[249]**. In agreement with our findings, **Kwaga** and **Iversen** reported that *Yersinia* isolated from slaughtered pigs and pork products in Canada in 1990 displayed little or no resistance to the aminoglycosides, cephalosporins (cefotaxime, ceftazidime, and ceftriaxone), imipenem, ticarcillin-clavulanic acid, aztreonam, ciprofloxacin, norfloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, tetracycline, and co-trimoxazole **[249]**.

Our data showed that pathogenic *Y. enterocolitica* isolates were susceptible to co-trimoxazole, amikacin, gentamycin, ciprofloxacin and meropenem, they also indicate that the agents used traditionally to treat human infections, including co-trimoxazole, tetracycline, chloramphenicol, and the third generation cephalosporins, retained their high levels of *in vitro* activities. However, our results do not rule out the potential for this species, like other members of the family *Enterobacteriaceae,* to acquire decreased susceptibility to multiple antimicrobial agents and emphasize the need for continued surveillance of the susceptibility patterns of *Y. enterocolitica* and *A. hydrophila* from both human and animal sources.

The spread of drug resistance among *Aeromonas* spp., is of concern because recent surveys indicate the emergence of these organisms as primary human pathogens. The presence of antibiotic resistant *A. hydrophila*

in natural habitats can pose a public health risk. Occurrence of multiple antibiotic resistant *A. hydrophila* in shoreline sediments, marine waters and shellfishes has been recognized as an important public health hazard. The prevalence of such multiple antibiotic resistant organisms in food and water is of considerable significance in relation to public health. Multiple antibiotic resistance of *A. hydrophila* strains from organs of infected catfish, *Clarius batrachus* has been reported **[127,218]**.

In this study, the frequency of resistance to some antimicrobial agents was much greater than previously reported. In other studies, frequency of resistance was exhibited against ampicillin followed by streptomycin, chloromphenicol and nalidixic acid. But all isolates exhibited susceptibility to tetracycline. Strains of *Aeromonas* spp. also showed high frequency of resistance to vancomycin, novobiocin, rifampicin and methicillin. **Son Radu *et al.,*** have reported resistance towards chloromphenicol, erythromycin, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole - trimethoprim and tetracycline among *A. hydrophila* isolates from cultured fish. **Pettibone *et al.,*** reported the susceptibility of *Aeromonas* spp. to chloromphenicol, erythromycin, kanamycin, polymyxin-B, streptomycin and trimethoprim. These antibiotics are generally active agents against strains of *Aeromonas* **[250]**.

Jones and **Wilcox** showed that aztreonam and the carbapenems, imipenem and meropenem remain highly active. Although resistance to the first and second generation cephalosporins is variable, more than 90% of *Aeromonas* spp. are susceptible to the third generation agents. While most strains are susceptible to chloramphenicol, ciprofloxacin, co-trimoxazole and the aminoglycosides, the activity of amoxycillin/clavulanate and the acylureidopenicillins is inconsistent **[218]**.

Imipenem and meropenem are also extremely active against all *Aeromonas* spp. Meropenem is generally more active than imipenem. More than 90% *of Aeromonas* spp. are susceptible to the third generation cephalosporins, such as cefotaxime, cefoperazone, cefpirome and ceftazidime. The activity of the first and second generation cephalosporins differs more widely between the three clinically important species *of Aeromonas* **[218]**.

Subashkumar *et al.,* reported that *A. hydrophila* isolates from children with acute diarrhea exhibited resistance to bacitracin (95.2%), novobiocin (95.2%), vancomycin (90.5%), methicillin (85.7%), cefazoline (85.7%), kanamycin (81%), rifampicin (76.2%), erythromycin and tetracycline (71.4% each) and nalidixic acid (62%). All the isolates were resistant to ampicillin as has been reported earlier **[251]**. The isolates exhibited susceptibility to polymyxin B (95.3%), chloramphenicol (90.5%) and gentamicin (76.2%). Earlier studies revealed the incidence of chloramphenicol resistance strains **[238]**.

Most of the isolates were from the high risk source contamination like fecal-oral contamination. Due to indiscriminate use of antibiotics; the microorganisms might have developed resistance towards several antibiotics. Differences in resistance patterns were observed between strains isolated from different geographic locations and between different species.

Antibiotic resistance in *Aeromonas* spp. poses a potential problem in the antimicrobial therapy of infections caused by these organisms. From our results, it is wise to avoid the use of broad-spectrum ampicillins, amoxycillin/clavulanate, co-trimethoprim, first and second generation of cephalosporins as first choice agents, particularly for invasive infections. Fluoroquinolones such as ciprofloxacin and Intravenous cefotaxime and meropenem are recommended for the treatment of serious *Aeromonas* infections. Ciprofloxacin and ofloxacin are good choices for oral therapy.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

To our knowledge, this is the first study that investigated the occurrence of *Y. enterocolitica* and *A. hydrophila* in clinical, food and environmental samples in Palestine.

1. The results of this study confirmed the presence of pathogenic *Y. enterocolitica* and *A. hydrophila* in clinical, food and environmental samples.

2. Conventional microbiological techniques used in the present study were shown to be an efficient tool for isolating and identifying both *Y. enterocolitica* and *A. hydrophila* isolates.

3. The percentages of *Y. enterocolitica* and *A. hydrophila* in clinical samples (diarrheal stool) were 4.7% and 34.3% respectively with no isolates from appendiceal samples collected from 8 hospitals located in various parts of Gaza strip and examined with enrichment and selective culture procedures. Other enteropathogenes, *Salmonella* (1%) and *Shigella* (1%) were recovered from the same samples.

4. The age group 2-6 years was shown to have the highest incidence rates of *Y. enterocolitica* and *A. hydrophila* infection.

5. Al-Dorrah hospital showed the highest incidence rate of *Y. enterocolitica* (21.4%) while Al-Nasser hospital showed the highest incidence of *A. hydrophila* (44.6%).

6. The results showed the presence of *Y. enterocolitica* and *A. hydrophila* in milk samples with high level of *Y. enterocolitica* (16.7%) in cow's milk, and 66.7% of *A. hydrophila* in goat's milk.

7. From meat samples we obtained an average of 6.7% of *Y. enterocolitica* and 48.9% of *A. hydrophila* with high incidence of *Yersinia* in turkey's and cow's meat samples and high incidence of *Aeromonas* in most meat samples.

8. *Y. enterocolitica* and *A. hydrophila* were isolated from 32 various water samples. The isolation rate was 6.25% for *Y. enterocolitica* and 46.9% for *A. hydrophila* with the highest incidence in tap and well water.

9. *Y. enterocolitica* and *A. hydrophila* were also found in sewage samples with isolation rate 12% of *Y. enterocolitica* and 42% of *A. hydrophila.* The high isolation rate was in Al-Nasser hospital. Other two environmental *Yersinia* species, *Yersinia kristensenii (*7.7%) were recovered from the same samples.

10. *Y. enterocolitica* (11.5%) and *A. hydrophila* (38.5%) were also detected in animal excreta samples from different animals in Gaza strip. The highest occurrence of *Y. enterocolitica* was in cow's excreta, while goat's excreta showed the highest incidence of *A. hydrophila*.

11. The CIN agar used in this study provided the most effective medium for the recovery of *Y. enterocolitica* and *A. hydrophila* from clinical and food samples. The confirmation rate of identification of presumptive *Y. enterocolitica* and *A. hydrophila* from CIN was 100%.

12. This study showed that direct plating on CIN agar after treatment with KOH treatment was more efficient than the widely accepted method of cold enrichment in PBS and overnight enrichment in TSB for the detection of *Y. enterocolitica* and *A. hydrophila* from clinical and food samples.

13. KOH method has the following advantages: (i) it uses a single enrichment medium, (ii) it eliminates 1 day of enrichment and another for the presumptive isolation, and (iii) it uses a single medium (CIN) for direct detection and isolation. This procedure is a practical alternative to many other recovery methods (PBS, TSB) which require significantly more time for completion, reducing the time required for detection up to 72 h by TSB and up to three weeks by PBS.

14. Virulence factors were detected among clinical isolates as well as among isolates from other sources with higher frequency in clinical isolates.

15. *Y. enterocolitica* retained its susceptibility to antimicrobials traditionally used treat human infections, including cotrimoxazole, tetracycline, chloramphenicol, and the aminoglycosides (amikacin, gentamicin).

16. High incidence of multiple drug resistant and β- hemolysin producing *A. hydrophila* was noticed.

6.2 Recommendations

In light of the result of this study and the above listed conclusions, the following actions are recommended.

• Further epidemiological studies are necessary to elucidate the public health significance of infections caused by *Y. enterocolitica* and *A. hydrophila.*

• The results of the present investigation suggest that further epidemiological studies are necessary to elucidate the public health significance of *Yersinia* and *Aeromonas* in food and water samples.

• Further studies are also needed to determine contamination routes and transmission pathways of *Yersinia* and *Aeromonas*.

• Further studies are needed to assess the clinical significance of the virulence factors in both food and water isolates.

• More studies should be performed to indicate the relative importance of *Y. enterocolitica* and *A. hydrophila* in acute diarrhea in Gaza strip in both humans and animals.

• Further careful epidemiologic studies are needed to determine the impact of restriction of antimicrobial use in limiting the spread of multi-drug resistance *Aeromonas*.

• *Y. enterocolitica* and *A. hydrophila* isolation and identification services should be offered by the Ministry of Health Laboratories (Both medical and food microbiology laboratories).

• We thus recommend the direct plating on CIN agar after KOH treatment method for routine *Yersinia* and *Aeromonas* screening and propose that this method could be used to detect the occurrence of *Yersinia* and *Aeromonas* in clinical and environmental samples.

• This study indicates that the rate of *Y. enterocolitica* and *A. hydrophila* is high in cow's meat and cow's excreta. This may suggests that the Monitoring Authorities to take serious procedures in order to protect consumers from the presence of *Y. enterocolitica* and *A. hydrophila*.

The author would like to recommend the establishment of a local culture collection for the preservation of local isolates.

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