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Formulation of Selective Media for the Isolation of Fastidious Gram Negative Bacteria

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Formulation of Selective Media for the Isolation of Fastidious Gram Negative Bacteria (FGNB)

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Dedication:

I dedicate my work to my affectionate parents who supported me all the way long, to my wife and my family, and to my brothers.

Also I dedicate this to all my teachers and supervisors who lightened the way with their wisdom, patience and knowledge.

With my Respect and Love

Abdallah Rashad Saad Mohammad

Declaration

I certify that this thesis submitted for the degree of master in medical laboratory science, is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Name: Abdallah Rashad Saad Mohammad

Signed: 

Date: 21 /12/2016

Acknowledgments

After an intensive period of more than one year, today is the day: Writing this note of thanks is the finishing touch on my thesis. It has been a period of intense learning for me, not only in the scientific area, but also on personal level. Writing this thesis has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period.

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Abstract

Three fastidious Gram negative bacteria (FGN) were utilized in this study to test the ability of new formulated media to selectively support their growth while controlling the growth of most Gram positive and fungi. Several media were formulated by modifying MacConkey agar to be a chocolate agar with the addition of 8-10 % sheep blood, yeast extract, glycerol, and vitamins. Dent's, Line's, and Skirrow's antibiotics were used during the study.

The results showed that media formulated with reduced bile salt and crystal violet and supplemented with vitamins, yeast extract, and glycerol were able to support the growth of *Campylobacter jejuni, Helicobacter pylori,* and *Haemophilus influenza* while reducing contamination associated with blood agar or chocolate agar media.

It was revealed in this study that these FGN bacteria are sensitive to bile salt and crystal violet since their growth was enhanced when they were absent from the media or reduced from their original concentrations used in dehydrated Difco media.

In conclusion media containing 70% MacConkey with vitamins, and two new formulated media number 1 and number 2 (NM2 and NM4) are useful media for growing FGN bacteria especially they alleviate the need to incorporate several of the antibiotics which used to inhibit growth of Gram positive bacteria and fungi. This work should stimulate the formulation of new selective media for selected pathogens.

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List of abbreviation

Sympol	Abbreviation representation
ATCC	American type culture collection
BHI	Brain-heart infusion
C. jejuni	*Campylobacter jejuni*
°C	degrees Celsius
CA	Chocolate Agar.
CAB	Columbia agarbase
CAL	Chocolate agar with Line's antibiotics
CAN	Colistin and Nalidixic acid
CAP	Chocolate agar plate
CCCYG	Chocolate Colombia Charcoal Yeast Glycerol
CGF	comparative genomic fingerprinting
ChMYG	Chocolate MacConkey Yeast Glycerol
ChMYGV	Chocolate MacConkey Yeast Glycerol Vitamin
CLED	Cystine Lactose-Electrolyte-Deficient
CMG	Chocolate MacConkey Glycerol
CO_2	Carbon dioxide.
CV	Crystal Violet
DNA	Deoxyribonucleic acid
EMB	Eosin methylene blue
GBS	Guillain- barre syndrome
GN	Gram-Negative
GNR	Gram Negative rods
H. Pylori	*Helicobacter pylori*
H_2O_2	Hydrogen peroxide
HACEK	Haemophilus species., Aggregatibacter species, Cardiobacterium species, Eikenella corrodens, and Kingellakingae)
HE	Hektoen Enteric
IE	Infective endocarditis
IU	International unit
JB	Gel DNA Binding Buffer (Buffer GB)

L	Liter
µL	Micro liter
mCCDA	modified Charcoal Cefoperazone Deoxycholate Agar
ME	Mercaptoethanol
Mg	Milli gram
MH	Ministry of Health
Ml	milli liter
Mm	mill meter
MRSA	Methicillin resistant *staphylococcus aureus*
MSA	Mannitol salt agar
MTM	Modified Thayer-Martinagar
NA	Nalidixic Acid
NAD	Nicotin amide adenine dinucleotide
NaOH	Sodium hydroxide
NH	*Neisseria-Haemophilus*
NM	New media
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEA	Phenylethyl Alcohol Agar.
PFGE	Pulsed field gel electrophoresis
RO	reverses osmoses
RT-PCR	Real time polymerase chain reaction
S.aureus	*Staphylococcus aureus*
SBA	sheep blood agar
SDS	sodium dodecyl sulfate
SOP	standard operation procedure
TAE	tris acetate EDTA
TAE Buffer	Tris acetate EDTA
V/V	Volume/ Volume
VBNC	viable but non – culturable
XLD	Xylose-Lysine-Desoxycholate Agar

Chapter One

1. Introduction

Historically, bacterial growth media has evolved with time since the discovery of bacteria[1].Complex broth media were prepared from regular foods; potato soups, meat extract, beef extract, egg yolk, and others. As the need to isolate a single type of bacterium was recognized, researchers sought a solid medium to grow and isolate bacteria[1, 2]

Robert Koch realized the importance of solid media; he used slices of potato to grow bacteria [1]. Agar is currently used as a solidifying agent in the formulation of solid and semi-solid media. Agar has replaced less useful agents such as gelatin and starch which are susceptible to bacterial hydrolyzing enzymes among other unsatisfactory properties, such as clarity (starch being opaque). Gelatin becomes liquid at high temperatures used normally as incubation temperatures (35-37ºC)[1].Agar is a mixture of a long polymer which is made of agrobiose (D-galactose and 3,6-anhydro-L-galactopyranose), and small polysaccharides known collectively as agropectins; agropectins are polymers of a disaccharides made of D- and L-galactose (Figure1:1 of agrobiose below)[3].Agar is melted at 95^{o}C-100^{o}C (liquid) and solidifies at 42^{o}C-45^{o}C (gel), Only few microorganisms can liquefy agar[1, 4]

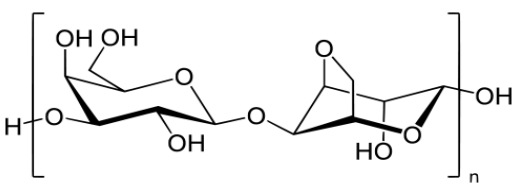

Figure 1.1: The structure of agarose polymer.

However, it may be a source of calcium and organic ions. Most commonly, it is used at concentration of 1-3% to make a solid agar medium[1]

The invention of selective media represented a powerful concept, if fully attained; a selective medium should allow direct and selective isolation of each pathogen or group of pathogens. Unfortunately, selective media are limited in number and quality and their selectivity is limited as well. MacConkey agar is one of the best selective media that allows the growth of Gram negative but not Gram positive bacteria with few exception including *Enterococcus faecalis* which is gram positive cocci but able to grow on MacConkey agar[4, 5]

MacConkey agar has several formulations [6]available from suppliers such as : MacConkey Agar which is used for isolating and differentiating lactose-fermenting from lactose-non fermenting gram-negative enteric bacteria, MacConkey Agar Base which is used with added carbohydrate in differentiating coliforms based on fermentation reactions, MacConkey Agar without Crystal Violet which is used for isolating and differentiating enteric microorganisms while permitting growth of staphylococci and enterococci. The medium can be used also to separate *Mycobacterium fortuitum* and *Mycobacterium chelonae* from other rapidly growing mycobacteria.Another formulation which is MacConkey Agar without bile salt which is used for isolating and differentiating Gram-negative bacilli while suppressing the swarming of most *Proteus* species[6]

Typically MacConkey agar contains a fermentable sugar (lactose) as a source of both carbon and energy; when fermented producing acidic pH that can be detected by the neutral red pH indicator (Red at pH below 7 and yellow above pH 7). The medium is rendered selective against Gram positive bacteria and fungi due to the incorporation of crystal violet and bile salts (detergent like in action). Sodium chloride: supplies essential electrolytes for transport and osmotic balance. When lactose is fermented, the pH of the medium decreases, changing the color of neutral red to pink [8-10].Another modification was made to allow the differential isolation of *Escherichia coli* O157:H7, by adding sorbitol instead of lactose which is fermented by *E. coli* strains but not by O157:H7 [7, 8].

A limited effort was recently reported by (Barghouthi and Younis 2014) where a combined MacConkey-Blood medium was used successfully to isolate *Pseudomonas argenteniensis*(aerobic Gram negative non-fastidious bacterium), the authors reported the selectivity of the medium, Qualitatively, it inhibited the growth of several Gram positive while several Gram negative species were able to grow aerobically or under microaerophilic conditions[9].

In this research the modification of MacConkey agar medium to selectively isolate fastidious Gram negative bacteria of clinical importance was attempted.

1.1 Review of the literature

Fastidious Gram negative bacteria encompass several important pathogenic bacteria (table 1:1), each bacterium has special requirements for its growth.

Table 1.1: Fastedious Gram Negative bacteria and other important Gram negative

Bacterium (Fastedious Gram Negative)	**Species**	**Shape**	**Growth requirement**	**Reference**
Bordetella	*Pertusis,parapertusis, bronchiseptica*	Coccobacillus	Aerobic	[10]
Brucella	*abortus,canis, melitenesis, suis*			[11]
Francisella	*Tularensis*			[12, 13]
Actinobacillus	*Actinomycetemcomitans*	sphericalor rod-shaped	facultative anaerobic	[14]
Aggregatibacter (Haemophilus)	*aphrophilus,influenzae,paraphrophilus, segnis*		Factor Vand or factor X	[14]
Legionella	pneumophila.	Coccobacillus	Aerobic need cysteine and iron salt	[15, 16]
Capnocytophaga	*Canimorsus*			[17]
Helicobacter	*Pylori, spp.*	Helical,Spiral	Microaero-philic	[18]
Campylobacter	*Jejuni , spp.*	Helical, spiral	Microaero-philic	[10, 18]
Neiserria	*meningitides, spp.*	Coccoid		[10]

1.2 Selective media used for isolation of fastidious Gram negative bacteria

Media used for isolation of Fastidious Gram negative bacteria depend largely on MacConkey with added antibiotics to which the target bacterium is resistant.

These media are highly selective and are designed for each individual bacterium. Therefore cannot be used with others. This fact limits their application to the intended use only. Searching the literature showed that there is no General Purpose Media for Fastidious Gram Negative bacteria such as those listed in table 1:1

1.2.1 *Bordetella* growth requirements and selective media

There are several selective media used to isolate *Bordetella*, these contain Cefalexinas antibioticas selective agent for the selective isolation of *Bordetella pertussis* and *B. parapertussis,* and suppression of other bacteria species. Among *Bordetella* selective media are : Regan-Lowe agar which contains blood charcoal agar and cephalexin [19] and Bordet Gengou Agar with 15% sheep blood[19] and BD Bordetella agar with charcoal and 7% horse blood.[20]

1.2.2 *Brucella* growth requirements and selective media

5% inactivated horse serum and several antibiotic were added to this medium ,including natamycin, vancomycin, nystatin, nalidixic acid, cycloheximide, polymyxin b, bacitracin for selective isolation of *Brucella*[21].

1.2.3 *Legionella*growth requirements and selective medium

Buffered Charcoal Yeast Extract (BCYE)with antibioticscontaining: cephalothin, colistin, vancomycin, cycloheximide(CCVC) is used as a selective medium for *Legionella*. *Legionella* requires L-cysteine as a growth factor [22].

1.2.4 *Helicobacter* and *Campylobacter* growth requirements and selective media

Media used for the growth of *Helicobacter pylori*[23]are suitable for the growth of *Campylobacter* as well. A patent was made by Lines for a *Campylobacter* selective

medium which contained rifampin in addition to other antibiotics used by Dent's and Skirrow's selective antibiotic mixes. Dent's selective mix contains Trimethoprim (inhibiting synthesis of tetrahydrofolic acid; thymine biosynthetic pathway), vancomycin (mostly active against Gram positive bacteria), cefsulodin (a cephalosporin active against *Pseudomonas aeroginosa*, and the antifungal amphotericin B[24, 25].

Skirrow's selective antibiotic mix for both C*ampylobacter* and *Helicobacter* contains vancomycin, amphotericin B, trimethoprim, and polymyxin B (antifungal).

1.2.5 *Haemophilus*growth requirements and selective media

As Chocolate agar contain haemin (heme) and nicotinamide adenine dinucleotide (NAD) which are the two important factors for growth of *Haemophilus*[26],chocolate agar is considered the selective media for *Haemophilus.*Antibiotic such as vancomycin, bacitracin and or clindamycine are added to the media to suppress growth of other bacterial species[27]

In general, pathogenic fastidious GNR are slow-growing organisms, which generally need supplemented media and carbon dioxide ($CO_{2)}$ enriched atmosphere, reduced oxygen, and are unable to grow on MacConkey agar. Most studies have focused only on one organism or genus of the FGN bacteria.[28]

Fastidious Gram-negative bacteria involve a number of unrelated genera and species that may cause different types of diseases including systemic infections. Their fastidious nature often makes them difficult to grow and identify in the routine microbiology laboratory.

*Haemophilus*specie, *Aggregatibacterspp, Cardiobacterium species, Eikenellacorrodens,*and *Kingellakingae* (the HACEK group of bacteria) is an example of FGN[28, 29] which includes members of the normal flora of the human oral cavity which can cause diseases; infective endocarditis (IE), periodontal infections, abscesses, and non-endocarditis bacteremia secondary to focal infections[30].

Most if not all FGN bacteria such as *Haemophilus influenzae, Campylobacter jejuni, Helicobacter pylori,* and *Neisseria gonorrhoeae* are grown under microaerophilic environment containing 5% to 10% CO_2 which can be supplied by using candle jar that can generate carbon dioxide in concentration of approximately3%[31].

The three pathogens selected as tester strains in the development of FGN medium were *Campylobacter jejuni*,*Haemophilus influenzae*, and *Helicobacter pylori* (see methods section 3.2)

1.3 Pathogenicity and characteristics of FGN bacteria used in this study

1.3.1 *Campylobacter jejuni (C.jejuni)*

*C. jejuni*is a Gram negative motile helical bacterium optimally grows at 37°C or 42°C, usually no growth at 25°C.It grows slowly, about 72- 96 hours is required for primary isolation, and its isolation from the blood takes longer [32-37]. It is oxidase positive but catalase negative (or weak catalase positive), and it causes about166 million food borne cases annually around the world. *C.jejuni* can be isolated from contaminated or undercooked poultry, meat, unpasteurized milk, and water. It persists in domestic animals, wildlife, and environmental sources and lives commensally in the intestine of food production animals such as livestock and poultry.[32, 34, 36, 38-44]. *C.jejuni*and*C.coli*iscommonlyresponsibleforhumaninfection.*Campylobacter*is the main risk factor of Guillain–Barré syndrome (GBS), a neurological disorder, as a post-infection complication[34-36, 39, 42, 45-47].

1.3.2 *Helicobacter pylori*

In 1875 the German Scientists found a spiral bacterium in human stomach, then in 1982 the two Australian researchers (Warren and Marshall, 1984) were able to isolate and cultivate the micro organism from human stomach (Kidd and Molding, 1998)[37].

Helicobacter pylori are fastidious microaerophilic human pathogen. It is a major cause of chronic gastritis, peptic ulcer disease, gastric neoplasia, and hepatic and colon cancer in humans. The bacterium has been categorized by the World Health Organization and International Agency for Research on Cancer harmony group as class I carcinogen[18].It's worldwide prevalence ranges between 20%-80% [48, 49].

*H. pylori*is motile, oxidase and catalase positive, that grows slowly (3-5days) to form translucent to grey small colonies. Young cells show spiral S shape and curved rods under microscope, yet they become coccoid as the culture age exceeds 5 days and turn into the viable but non culturable coccoid form (VBNC) as *campylobacter* does as well. Coccoid

and VBNC forms may be critical stage that permits undetectable transfer and survival of the bacterium, this vision is supported by all over distribution of such bacteria. Other examiner views these forms as dead bacteria , it causes a chronic low level of inflammation in the stomach lining [18].

1.3.3 *Haemophilus influenzae(H.influenzae)*

H. influenzae is fastidious microorganism, small, pleomorphic, gram-negative bacilli or coccobacilli with random arrangements. The growth requirements determined for *H. influenza* are heme (X factor) and NAD (V factor). Vitamins supplements are added (e.g.IsoVitaleX, Table 3.1).These requirements are added to chocolate agar to enhance the growth of *Haemophilus spp*. Then chocolate agar plates are incubated at 35-37°C under approximately5% CO_2.

Crystal violet, lincomycin,spectinomycin and bacitracin are incorporated as selective agents in media for separation and isolation of *Haemophilus* species. These compounds represent the best selectivity potential of the media[50].

Among the species of the normal flora of the upper respiratory tract in human that belongs to the genus *Haemophilus*, certain *Haemophilus influenzae* serotypes *b, f*, and less frequently *c* serotype are known etiologies of meningitis, sepsis, epiglottitis. Until the implementation of vaccination in many countries, *H. influenzae* was one of the three leading causes of airborne bacterial meningitis [51-55].

1.4 Problem Statements: and aim of the study

The effectiveness and impact of selective media that allow the growth of fastidious gram negative pathogenic bacteria and the speed of their isolation has been given minimal attention in the past decades. Selective media used for growing fastidious pathogenic gram negative bacteria (FGN) requires revision, evaluation, and improvement. In this work, FGN bacteriaare operationally defined as: bacteria that will not grow on plain MacConkey medium and generally require blood or serum (or their substitutes) for their growth.

1.4.1 Problem statements

No progress has been made during the past few decades in the area of developing a general purpose selective medium for the FGN pathogenic group of bacteria.

1.4.2 Aim: This study was focused on the development of antibiotic free selective medium for the growth and maintenance of fastidious pathogenic Gram negative bacteria that share several biological aspects.

1.5 Justifications

Fastidious Gram negative bacteria do not keep well in storage. Some studies found 100% culture of *H. pylori*-positive patients from gastric biopsies stored in a cysteine and 2% glycerol transport medium stored at -20°C for 4 weeks versus only 57% after 12 weeks storage[56].

They usually grow slowly, poorly, and they do not compete well with other bacteria and fungi which make their isolation and maintenance a major hindrance in research and clinical applications, and related studies.

1.6 Rational

Bacterial isolation is the primary step in perusing bacterial identification through morphological, biochemical, and molecular characterization. Anti-biograms of pathogenic bacteria cannot be fully determined without having pure bacterial cultures. In addition, biotechnological applications including vaccine and byproduct development are dependent on pure cultures. Complete or partial genomic DNA sequencing requires pure DNA is obtained from pure cultures as well. Also, storage and maintenance of bacteria is usually done for pure cultures as well.

The basic composition of Gram negative selective medium (MacConkey and others) will be further explored to improve its performance in obtaining pure cultures of FGNs bacteria.

1.7 Hypothesis and work plan

In this study, modifying MacConkey medium with blood and or other nutrients and agents should satisfy the two requirements of a Gram negative selective medium for growing fastidious Gram negative bacteria; adding blood to MacConkey medium should allow some, most, or all FGN bacteria to grow while inhibiting all or most Gram positive bacteria and saprophytic fungi.

Chapter Two

2. Review of the Literature

2.1 Culturing Bacteria

The process of culturing bacterial on artificial media is influenced by several essential factors involving nutrients (carbon, nitrogen, inorganic phosphates and sulfur, metals, water, and vitamins), oxygen or other atmosphere, moisture, pH, and temperature.[56, 57].

2.2 Culture media

Culture media are of primary significance for the most part of microbiological tests: to obtain pure cultures that are used for several applications and evaluation such as identification, antibiotic sensitivity, and storage. Despite rapid advances in molecular and microbiological methods, culture media remain important in research, taxonomy, identification, and storage of bacteria; microbiology is incomplete without culture media[57].

2.3 History of culture media

The start of microbiological culture media discover in nineteenth century when the science of bacteriology was just started. During this time, bacteriologists try, with constant success, to grow microorganisms also directly using the food, material on which the bacteria had first been seen. Perhaps, the initial to cultivate bacteria on a growth medium, with a degree of reproducibility, was the French chemist and microbiologist Louis Pasteur (1922–1985). Pasteur formed a medium composed of yeast, ash, candy sugar, and ammonium salts in 1860. This medium contained the necessary supplies for microbial growth: nitrogen (ammonium salts), carbon (sugar), and vitamins (ash).

Robert Koch (1843–1910) discovered the broth media which mainly composed of fresh beef serum or meat extracts (so-called bouillons). In fact, Koch's work was so groundbreaking that the nickname "The Father of Culture Media," in many microbiological textbooks, is not lost[57].

2.4. Types of culture media

Many different general purpose, enrichment, selective agar or broth media are used for routine diagnostic bacteriology. In general there are many few outstanding media; Nutrient agar, Muller-Hinton, Brain Heart Infusion, Blood agar, Chocolate agar, MacConkey, and others that are usually applied in processing different clinical specimens[31, 57].

2.4.1 Brain-Heart Infusion

Brain-heart infusion (BHI) is a medium of rich nutrition used to grow different micro organisms. The media contains infusion from different animal tissue sources, added peptone (protein), phosphate buffer, and a few concentration of carbohydrate mainly dextrose. Majorly BHI broth is often used to culture patient's blood to isolate and establish bacterial identification[31].

2.4.2 Columbia Nalidixic Acid (CNA) with Blood

Columbia agar base, rich formulated nutrition containing three different peptone and 5% defibrinated sheep blood(whole blood remove fibrin to prevent clotting).several antibiotics are added to the media as (colistin (C) and nalidixic acid (NA)) which inhibit the growth of most Gam negative bacteria and allow Gram positive bacteria to grow, thus conferring a selective property to this medium. Colistin damages the cell membranes of Gram negative bacteria and nalidixic aid blocks DNA replication in bacteria[31]**.**

2.4.3 Chocolate Agar (CA)

Chocolate agar is essentially the same as blood agar but during preparation their blood cells are lyses at high temperature around 70°C. The cell lyses release of intracellular nutrients such as haeme (heme) or hydroxide hematin (haematin) also known as the X factor and the coenzyme nicotin amide adenine dinucleotide (NAD or factor V) into the agar. These two factors are essential for growth of some FGN. Also, lyses of red blood

cells gives the agar chocolate-brown color from which the medium gets its name. The media is suitable for growth of *Neisseria gonorrhoeae* and *Haemophilus* spp. Neither of these microorganisms is able to grow on sheep blood agar[31].

2.4.4 Gram-Negative (GN) Broth

A selective Gram negative (GN) broth is used for the cultivation of gastro intestinal pathogens like *Salmonella* spp. and *Shigella* spp. from stool specimens and rectal swabs. The broth contains different ingredients, such as sodium citrate and bile salt that inhibit Gram positive bacteria and enhance reduplication of Gram-negative, non- enteric pathogens while mannitol is used as the primary carbon. But this broth is not used by many non-pathogenic enteric micro organisms[31].

2.4.5 Hektoen Enteric (HE) Agar

Hektoen enteric (HE) agar consists of bile salts and dyes such as "bromthymol blue and acidfuchsin", HE allows selective growth of *Salmonella* spp. and *Shigella* spp. But delays growth of most nonpathogenic Gram negative bacilli found in the gastro intestinal tract (GIT).

HE classified as differential media because most of non-enteric bacterial growth will appear as orange to *Salmonella* colored colonies. [31].

2.4.6 MacConkey Agar

MacConkey agar is most repeatedly used as selective and differential agar. This medium contains crystal violet dye to inhibit the growth of Gram positive bacteria and fungi, and allows many types of Gram negative bacilli to grow. The pH indicator, neutral red, provides this medium with a differential capacity. Lactose fermenter bacteria appear as pink to red colored colonies, while non lactose fermenters stay colorless and translucent [31].

2.4.7 Phenyl ethyl alcohol (PEA) agar

Phenyl ethyl alcohol(PEA) agar is basically sheep blood agar with added phenyl ethyl alcohol to suppress the growth of Gram negative bacteria and allow the growth of Gram positive *enterococci*, *streptococci*, and *staphylococci*[31].

2.4.8 Modified Thayer-Martin Agar (MTM)

Modified Thayer-Martin(MTM) agar is an enrichment and selective medium for the isolation of *Neisseria gonorrhoeae,* and *Neisseria meningitidis,* The additionof4-5different antibiotics such as colistin,vancomycin, nystatin, trimethoprim, ansamycin, provide selective power to suppress growth of certain bacteria on this media [31].

2.4.9 Thioglycollate Broth

Thioglycollate broth is the enrichment broth most repeatedly used in diagnostic bacteriology. The broth contains many different nutrients, such as casein, yeast and beef extracts, and vitamins which enhance the growth of most bacteria whether Gram-negative or Gram positive[31].

2.4.10 Xylose-Lysine-Desoxycholate (XLD) agar

As with HE agar, xylose-lysine-desoxycholate (XLD) agar is selective and differential medium for *Shigella* spp. And *Salmonella* spp. The salt, sodium desoxycholate, blocks many Gram negative bacilli and Gram positive bacteria[31].

2.5 Bacterial Identification

It is important to show the identity of bacteria growing on a given medium in order to be able to assign bacteria to media that can support their growth.

Traditional identification of fastidious Gram-negative bacteria is difficult and is a time consuming, often requiring special media and phenotypic tests that require special skills and knowledge[29].

Now a days ,two fully automated identification systems for distinguishing FGN bacteria are used; The Vitek 2 *Neisseria-Haemophilus* (NH) (bioMérieux, Marcy L'Etoile, France) and Phoenix (Becton Dickinson, Cockneyville, MD, USA). BioMérieux has developed a card for the identification of 26 taxa of fastidious Gram-negatives, covering *Neisseria, Haemophilus*, and the HACEK bacteria, for use in the Vitek 2 system. The card contain 30 biochemical tests that are monitored up to 8 hours[29].

Chapter Three

3. Materials sand Methods

3.1Materials

Sterile Petri-dishes (plastic),15-ml and 50-ml conical sterile disposable tubes (screw caps), microfuge tubes, sterile disposable bacteriological loops. Dehydrated media; blood agar base, MacConkeyagar, bile esculin agar, colombia charcoal, nutrient broth, agar, yeast extract. defibrinated sterile sheep blood, crystal violet, reverses osmoses water (RO), vitamin cocktail, glycerol, line's and Dent's selective antibiotic mixes. Autoclave, water bath, incubator, *Helicobacter* antigen detection kit, flasks, beakers, centrifuge, micro centrifuge, micropipettes and tips, and light compound microscope.

3.2Bacterial species

Fastidious Gram negative (FGN) used in this study were: *Campylobacter jejuni* ATCC 29428culti- loop, thermo scientific (Remel Europe, Ltd) and *Helicobacter pylori ATCC* 43504, and *Haemophilus influenzae (clinical sample from* Palestinian Ministry of Health*)* were used in this study. Other locally isolated bacteria, *Staphylococcus aureus*, MRSA (Methicillin resistant staphylococcus aureus*) a*nd *Bacillus atropheus.*

3.3 Sheep blood collection, treatment, and antibiotic stocks

Glass marbles (0.5 to 1 cm in diameter) were purchased from local toy stores, washed and placed in 500-ml large-mouth screw cap bottles with 250 ml water and autoclaved. Water was drained off while hot (70° C) and allowing beads and bottle to dry before tightening the lid. Governmental slaughter house (Al-Biereh and Nablus, Palestine) have kindly collected sheep blood into the supplied bottles. Collected blood was shaken and defibrinated within 15 minutes of collection, immediately 45 ml- aliquots were treated with

250 µL of 200x Skirrow's antibiotic which prepared from (10mg /ml vancomycin,5mg/ml tri methoprim, 2.5 IU/ml polymyxin B, 100mg /ml cyclohiximide) or Line's modified Skirrow's (10mg /ml vancomycin,5mg/ml tri methoprim, 2.5 IU/ml polymyxin B, 100mg /ml cyclohiximide, and10 mg /ml rifampicin prepared as 400X concentrates in 70% ethanol.Theantibiotic stock was filter sterilized using mini pore filter 0.45 µm in diameter and kept at 4°C (Line, John Eric (Watkinsville, GA), Garrish, johnKennedy (Hull, GA), Glass moyer person, and Kirsten Elizabeth, USA patent 6,368,847). The treated aliquots of blood were kept for 24h at 4°C before use to allow antibiotics to kill possible contaminating organisms. Treated blood was then used for media preparation. Agar plates were used within 10-15 days[44].

3.4 Vitamin mix used as media enrichment (1000X concentrate;Table 3.1)

One tablet of multi vitamin weighting 0.626 g (Al-Quds Pharmaceutical Company, Palestine) and another tablet weighing 1.293 g (Spring Valley high potency B)were crushed into fine powder and dissolve in 50ml of RO water, liquid phase was collected, the remaining solids were dissolved in 10 ml of absolute ethanol and pooled with the 50 ml extract, mixed and filtered through Wattman filter paper, then sterilized by filtering through 0.45 µm syringe fitted filter., collected into a sterile 50-ml tube, aseptically aliquot into 1ml fractions into screw cap microfuge tubes and kept frozen at-20°C. It was used at concentration of 2 µL per ml of mediumor2 ml/liter of medium added to chocolate agar at 50° C just before pouring agar plates.

Table 3.1: **Vitamin and minerals mix used as media enrichment (1000X concentrate). *IsoVitaleX was included for comparison[58]**

Vitamins and minerals	Stock (60 ml)		BBL-IsoVitaleX* Per L	Per Liter
A	800		Thiamine phosphate	100 mg
B1/Thiamine	55 mg		B1/Thiamine	3 mg
B12	13.5 µg		B12	10 mg
B2	13.5 µg		Adenine	1000 mg
B3/niacin	50mg		Guanine	30 mg
B6	14.5 mg		p-aminobenzoate	13 mg
Ferric Ammonium Citra	3 mg		Ferric nitrate	20 mg
C	25 mg		Nicotinamide	250 mg
D2	400 IU		L-glutamine	10g
E	0.75 mg		L-Cystine	1.1 g
Folate	150 µg		Cysteine	25.9 g
H/ biotin	25 µg		Glucose	100 g
Nicotinamide	60 mg		Adenine	1000 mg
Pantothonate	56 mg			
Ca3(PO4)2	40 mg			
Mg Phosphate	20 mg			
Mn.Glycerophosphate	4 mg			

3.5 Stock solutions

3.5.1 Crystal violet stock solution (CV)

26.5 mg of crystal violet were dissolved in 14 ml distilled water, which produced a solution containing 1.9 mg/ml,and appropriate amount of it, 0.526 ml,0.395 ml,0.263 and0.132 ml,was added to one liter of prepared media before autoclavingto obtain 1 mg, 0.75 mg, 0.5 mg and 0.25mg final concentration of CV per liter of media respectively. The original crystal violet concentration in MacConkey(1mg / liter) was used as the upper limit reference in preparing new media. The four different concentration of crystal violet 1mg, 0.75 mg, 0.5 mg, 0.25mg /L were used to prepare blood or chocolate agar with 7% sheep blood.

3.5.2. Glycerol stock

Pure RO water and glycerol were mixed (1: 1 V/V) and autoclaved then used aseptically to supplement different media, usually diluting the stock to a final concentration of 1%.

3.6 Preparation of Blood Agar

The blood agar base (or another dehydrated medium) was prepared as instructed by the manufacturer (Difco-BBL, Swedesboro, NJ, USA or HIMEDIA, Mumbai, India). 28 g dehydrated medium was Suspend in 1L (litter) of RO water and then autoclave for 18 min at 15 lbs pressure at 121°C for 18 min. Then the agar was allowed to cool to 48 °C in a water bath. After that 8-10% (vol/vol) sterile defibrinated sheep blood that has been warmed to room temperature was added to agar. Also, at this stage, any supplements such as vitamins, and glycerol were aseptically added to their proper concentration from filter (0.45 µm) sterile concentrated stocks, and mix gently before pouring into plates. Aseptically, dispensing 15- 20 ml amounts of medium into 90 mm Petri plates and bubble formation was avoided. The agar was allowed to solidify at room temperature; the nit was tested for sterility by incubating sample plates aerobically or micro- aerobically at 37° C for three days. After that the Petri plates were labeled with the date of preparation, and stored in an inverted position in plastic sleeves at 4-8°C. The plates were used within 2 weeks and their validity was tested up to four weeks of preparation.

3.7 Preparation of chocolate agar (CA)

Blood agar base was prepared as instructed by the manufacturer (Difco™ Blood Base Agar).28 grams of dehydrated medium were suspended in 1L of RO water, mixed, heated with frequent agitation and boiled for one minute. Sterilized by autoclaving at 15 lbs pressure at 121 degrees Celsius (121°C) for 18 min. After cooling to70°C in a water bath. Defibrinated sheep blood was warmed to room temperature, 80 to 100 ml were then added per liter of Blood base agar then re-incubated at 70°C with occasional shaking until it turned to chocolate color.

Medium was allowed to cool to 45-50 °C in water bath.15- 20 ml of cooled medium was aseptically dispensed into 15×90mm sterile Petri plates avoiding the formation of air bubbles. Labeled with the name and date of medium, plates were allowed to stand at

room temperature until completely solidified. Plates were stored in plastic bags at 4 °C in up-side-down position.

3.8 Modifications of Blood Agar and Chocolate Agar

3.8.1 Selective chocolate with Line's antibiotics (CAL): As with BA and CA preparation, (CAL) was obtained when 2.5 ml of Line's antibiotic mixture were added to one liter of medium after autoclaved and cooled to50°C.

3.8.2 Chocolate MacConkey Yeast Glycerol Vitamin medium (CHMYG-V)

The chocolate MacConkey yeast glycerol media was prepared as instructed by the manufacturer (Difco™ MacConkey Agar) in which 50 gram of MacConkey medium and 5 gram of yeast extract medium were suspended in 1L (litter) purified/distilled water. The mixture was mixed thoroughly and then heated with frequent agitation until boiling for one minute to completely dissolve all components. After that, it was sterilized by autoclaving at 15 lbs. pressure and 121°C for 18 minutes. The agar was allowed to cool to 70 °C on water bath before 8-10% (vol/vol) sterile defibrinated sheep blood that has been warmed to room temperature was added to it (heated blood turns a chocolate color and facilitate the growth of bacteria).Then 10 ml of glycerol and 2ml of vitamin were added to the prepared medium after it was cooled to 45-50 °C on water bath. The, they were mixed gently before pouring into plates which were allowed to solidify at room temperature. The final product was tested by incubating two plates at 37°Cfor 48 hrs. The Petri plates were labeled with the date of preparation, and then stored in an inverted position in plastic bags to prevent loss of moisture at 4-8 °C.

3.8.3 Preparation of 70% Chocolate MacConkey Yeast Glycerol Vitamin medium

Preparation of the 70% chocolate-MacConkey-yeast-glycerol-vitamin medium based on manufacturer information (Difco™ MacConkey Agar). Preparation was as following; 35 g of dehydrated MacConkey agar were suspended in 1L RO water. Then, 10 g of Blood Base agar and 5 g of yeast extract were added, mixed heated with frequent agitation and boiled for one minute. Sterilized by autoclaving at 15 lbs pressure at 121 degrees Celsius (121°C) for 18 min. Defibrinated sheep blood was warmed to room temperature, 80 to 100 ml were

then added per liter of Blood base agar at 70°C with occasional shaking until it turned chocolate in color. Medium was allowed to cool to 45-50°C in water bath, 2 ml of vitamin mix and 10ml of autoclaved (50%) glycerol were aseptically added to the agar solution.

After gently mixing medium 15- 20 ml amounts were dispensed into 15×90 mm sterile Petri plates aseptically. Air bubble formation was avoided. Plates were left to stand at room temperature until solidified. Labeled with the medium name and date of medium preparation, plates were allowed to stand at room temperature until completely solidified. Plates were stored in plastic bags at 4°C in up-side-down position.

3.8.4 Preparation of Chocolate MacConkey Salt Agar

The chocolate MacConkey salt agar was prepared as instructed by the manufacturer (Difco-BBL, Swedesboro, NJ, USA or HIMEDIA, Mumbai, India). In which 50gram of MacConkey medium was suspended in 1L (liter) purified/distilled water and then heated with frequent agitation until boiling for one minute to completely dissolve all components. After that, it was sterilized by autoclaving at 15 lbs. pressure and 121°C for 18 minutes. The agar was allowed to cool to 70 °C on water bath before 8-10% (vol/vol) sterile defibrinated sheep blood that has been warmed to room temperature was added to it (heated blood turns a chocolate color and facilitate the growth of bacteria). Then, 4 grams of salt were added to the media where they mixed gently before pouring into plates which were allowed to solidify at room temperature. The final product was tested by incubating two plates at 37°Cfor 48 hrs. The Petri plates were labeled with the date of preparation, then stored in an inverted position in plastic bags to prevent loss of moisture at 4-8 °C.

3.9 Selective Media with Crystal Violet

Blood agar and Chocolate agar media were modified by the addition of different crystal violate concentrations to the media in the absence of bile salts. This was achieved by adding crystal violet solution in a concentration of 1 mg, 0.75 mg, 0.50 mg, or 0.25 mg to one liter of the prepared blood or chocolate agar before autoclaving. After that, it was sterilized by autoclaving at 15 lbs. pressure and 121°C for 18 minutes. The agar was allowed to cool to 70 °C on water bath before 8-10% (vol/vol) sterile defibrinated sheep blood that has been warmed to room temperature was added to it (heated blood turns a chocolate color and facilitate the growth of bacteria). Also 2 ml of vitamin mix was added

to the medium aseptically after autoclaving at 50°C. After that, they were mixed gently before pouring into plates which were allowed to solidify at room temperature. The final product was tested by incubating two plates at 37°Cfor 48 hrs. The Petri plates were labeled with the date of preparation, and then stored in an inverted position in plastic bags to prevent loss of moisture at 4-8 °C.

3.10 Media containing blood base agar & MacConkey agar at (3:1 ratio) & different crystal violet concentrations (MacConkeyNM 6, 7, 8, and 9)

30 g blood base agar was mixed with 12.5MacConkeydehydrate and 5g yeast extract. Then crystal violet stock was added to a final concentration of 1mg, 0.75 mg, 0.5 mg, or 0.25 mg per liter. After that, it was sterilized by autoclaving at 15 lbs. pressure and 121°C for 18 minutes. The agar was allowed to cool to 70°C on water bath before 8-10% (vol/vol) sterile defibrinated sheep blood that has been warmed to room temperature was added to it (heated blood turns a chocolate color and facilitate the growth of bacteria). Then the media were cooled to 50°C where 2ml of vitamin stock and 10ml of glycerol were added to it (Table 3.2). After that, they were mixed gently before pouring into plates which were allowed to solidify at room temperature. The final product was tested by incubating two plates at 37°Cfor 48 hrs. The Petri plates were labeled with the date of preparation, then stored in an inverted position in plastic bags to prevent loss of moisture at 4-8°C.

Table 3.2: NM media 1-9 per liter of medium have 25% bile salt concentration and variable crystal violet concentrations

Medium	macConky	added crystal violet (mg)	total crystal violet (mg/liter)	blood base agar
NM 1	0.0	0.0	1.007	100%
NM 2	0.0	0.0	0.75	100%
NM 3	0.0	0.0	0.503	100%
NM 4	0.0	0.0	0.251	100%
NM 5	0.00	0.0	0.00	100%
NM 6	25%	0.750	1.00	75%
NM 7	25%	0.5	0.75	75%
NM 8	25%	0.25	0.50	75%
NM 9	25%	0	0.25	75%

3.11 Preparation of Bile Esculin Chocolate Agar with Sheep Blood

Preparation of 100% Bile Esculin Chocolate Agar according to manufacturer instructions (HIMEDIA, Mumbai, India). The medium was prepared by dissolving 64 g of dehydrated medium in 1L of RO, 80-100 ml of defibrinated sheep blood were added to the autoclaved medium at 70°C, mixed for 30 min and cooled to 45-50°C, then 2ml of vitamin stock and 10 ml of glycerol were added before pouring into Petri plates.

Half strength medium (50%) was prepared by dissolving 32 g and 7.5 g agar in 1L of RO water, sterilized by autoclaving at 15 lbs. pressure and 121°C for 18 minutes. The agar was allowed to cool to 70°C in a water bath, 80-100 ml of defibrinated sheep blood were added, mixed for 30 min and cooled to 45-50°C, then 2ml of vitamin stock and 10 ml of glycerol were added before pouring into Petri plates. Plates were labeled with the date of preparation, and stored in an inverted position in plastic sleeves at 4-8°C.

Media were tested for sterility by incubating sample plates aerobically or micro-aerobically at 37° C for three days.

3.12 MacConkey Agar

MacConkey agar was prepared as instructed by the manufacturer (Difco-BBL, Swedesboro, NJ, USA or HIMEDIA, Mumbai, India), in which 50 g of dehydrated medium was suspended in 1L of RO water then heated until boiling, after that, it was autoclaved at 121°C for 18 minute. When the agar temperature decreased to about 50°C, it was poured into sterile Petri- dishes in aseptic environment.

13.3 Methods of identification and characterization of FGN

3.13.1 Gram staining

Gram stain is one of the most usually used stains in the field of microbiology. It is one of the differential stains that are used to characterize bacteria depending on its cell wall thickness into Gram positive bacteria for those having thick cell wall and Gram negative bacteria for those having thin cell wall. In Gram stain, bacteria smear was prepared by emulsifying 1 or 2 colonies from fresh cultured plate with 2 to 3 drops of sterile normal saline on a glass slide using an inoculating needle, then the slide was left to air dry before

heat fixing by carefully passing the slide through the Bunsen burner. Then the slide was flooded with crystal violet stain for about 1 minute, before it was washed gently with water for 2 seconds. After that, the slide was flooded with a mordant (Gram's iodine) for one minute, then was washed gently with water for 2 seconds. This was followed by washing the slide with acetone alcohol decolorized for 20 seconds. In the next step, the slide was flooded with counter stain safranin for one minute, and then was washed gently with tap water before blot drying it. Finally, a drop of mineral oil was placed onto the slide which then examined under oil immersion lens of the light microscope. Gram positive bacteria will appear as purple cells and Gram negative bacteria will appear as red cells[59, 60]

3.13.2 Catalase test

This test is used to differentiate between bacteria that produce the enzyme catalase from those which lack this enzyme. In this test, 1 to 2 colonies of the tested bacteria were emulsified with one drop of 3% hydrogen peroxide (H_2O_2) on a clean glass slide. Catalase positive bacteria was detected by appearance of bubbles due to breakdown of hydrogen peroxide to oxygen and water, while absence of such bubbles was used as an indication of catalase negative bacteria[60-62]

3.13.3 Oxidase test

The oxidase test is used to differentiate those bacteria that produce cytochrome c oxidase. In this test, a filter paper was soaked with the substrate tetramethyl-p-phenylene diamine dihydrochloride and then the colony to be tested was taken with platinum or wooden loop and smeared on to the filter paper. The development of a blue to purple color within few second was considered as oxidase positive test while absence of such color was considered as oxidase negative test [61, 63].

3.13.4 Urease test

Urea is a diamide of carbonic acid. It is hydrolyzed with the release of ammonia and carbon dioxide. Urea test broth was prepared by adding20 g of urea, 9.5 g of Na_2HPO_4, and 9.1 g of KH_2PO_4, 0.1g of yeast extract and 0.01 g of phenol red. The pH was made to 6.8±0.2 at 25°C. 200µl (micro liter) of broth was inoculated with few colonies of the tested bacterium and then was incubated for 24 hour at 37°C. Urease positive test was indicated

by development of red to pink color, while no change in the color of the medium was reported as urease negative test[61]

3.14 Preparation of agarose gel for electrophoresis

0.4 gram of agarose powder (from Hy. Labs) was added to 24.5 ml RO water and 500 micro liters TAE Buffer (tris acetate EDTA) in a flask. The PH of the mixture was adjusted to 8.The agarose was boiled until the solution becomes clear and then 2 micro litters of ethedium bromide was added before the solution was cooled to about 45-55°C. The ends of the casting tray were closed with two layers of tape and the comb was sited in the gel casting tray where the agarose solution was poured. After that and when the agarose gel became solid, the comb was pulled out and the tapes were removed. Finally the gel was placed in the electrophoresis chamber[64]

3.15 DNA extraction from bacteria by boiling method

About 3 to 4 of the suspected and purified colonies were suspended in 0.5 ml sterile free nuclease water, and then were mixed by vortex thoroughly. After that, they were Incubated at 100° C for 15 minutes, and then centrifuged at maximum speed for 15 minutes to precipitate cell-debris and proteins (DNA will remain soluble in the supernatant).The procedure was performed in a BSL-2 biohazard hood in the specimen preparation area[64]

3.16 DNA detection and amplification

PCR was performed based on published primers, a gift from the Ministry of Health, Central Public Health Laboratories, Ramallah city, Palestine.

The standard operation procedure (SOP) about Real-time PCR (qRTPCR) protocol for detection of *Campylobacter jejuni* and *Haemophilu sinfluenzae* using ABI 7500 Real-Time Machine.[65, 66]

The followings are the primers used for *Campylobacter jejuni* and *Haemophilus influenzae*:

The sequence of the primers and probe selected for *Campylobacter jejuni*is[65]:

Cj-F1 Forward:	5'-TGCTAGTGAGGTTGCAAAAGAATT- 3'
Cj-R1 Reverse:	5'-TCATTTCGCAAAAAAATCCAAA – 3'
Cj- probe:	5'-FAM-ACGATGATTAAATTCACAATTTTTTTCGCCAAA–TAMRA-3'

The sequence of the primers and probe selected for *Haemophilus influenzae[66]*

hpdF:	5'- GGTTAAATATGCCGATGGTGTTG - 3'
hpdR:	5'- TGCATCTTTACGCACGGTGTA – 3'
Probe:	5'-6-FAM- TTGTGTACACTCCGT"T"GGTAAAAGAACTTGCAC- BHQ-1-3'

Chapter Four

4. Results

4.1. Isolation of fastidious GN bacteria from sewage, chicken stool, cat stool, five throat swab pool, soil sample, stool sample which show positive *H. pylori* antigen test, gastro biopsy and other stool sample (Alarabi center)

50mlof sewage sample was collected from sewage treatment plant (Al-Biereh, Palestine). Within 1 hour, 3 ml were centrifuged at 12,000 rpm for 3min in microfuge tubes, then the supernatant was discarded, and the pellets were washed in 1 ml of 10 mM phosphate buffered saline (PBS) (pH 7.2) for three times. The suspended pellet (1ml PBS) was streaked on different media; blood agar, chocolate agar, CHMYG, chocolate MacConkey, 70% CHMYG, blood MacConkey, chocolate macConkey Line, chocolate macConkey and then incubated in candle jar for 4 days. Six bacteria isolates were obtained on CHMYG from sewage sample named Ode1-6; these were subculture onto fresh CHMYG plates to insure purity. The other samples in section 4.1 were streaked on different media and the result shown in (Table 4.2)

4.2 Identification of bacterial colonies which were grown on CHMYG medium

Gram stain, catalase, oxidase tests were done for each one of the sixth colony, all of them have spiral shape except ode3 which have diplococcic morphology. Also, all of them were catalase positive except Ode6 which was catalase negative. On the other hand, all of them were oxidase negative except Ode1 and Ode4 which were oxidase positive (Table 4.1).

Table 4.1: Ode1-6 Sewage isolates obtained on CHMYG medium (section 8.3.2).

	Microscopic examination (1000xoil)	Catalase	Oxidase	CHMYG
Ode1	fine spiral	+	+	+
Ode2	Spiral	+	Neg	+
Ode3	Diploccoci	+	Neg	+
Ode4	Short spiral	+	+	+
Ode5	Spiral may be encapsulated	+	Neg	+
Ode6	Spiral	Neg	Neg	+

4.3 Characterization of Ode1

This isolate from sewage sample was Gram negative bacterium that formed punctiform colonies with spiral/curved rod cells that turned coccid after 5 days of sub culturing onto CHMYG medium (Figure 4.1), it grew well in candle jar but poorly when aerobically incubated. It failed to grow on MacConkey agar which indicated it belongs to the FGN bacteria.

From its morphology, ode1 was suspected to be *H. Pylori*. So DNA extraction was done for ode1 colonies, and then PCR for DNA amplification with the universal method followed by gel electrophoresis and partial sequence of 16 S genes was obtained with G7 and QUGPF4.Rn2 primers (Barghouthi, 2011). The sequencing results (Figure 4.3) showed an unknown bacterium based on sequence analysis using on line nucleotide BLAST.

PCR results showed that it was not *Helicobacter* since it produced negative results when tested with two pairs of *H. pylori* specific primers (*ribosomal and flagellar genes*) (Figure 4.2)

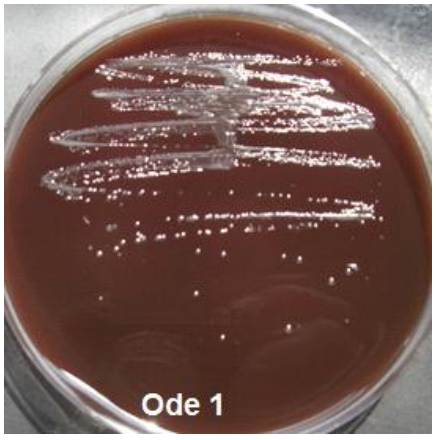

Figure 4.1: Characterization of Ode1

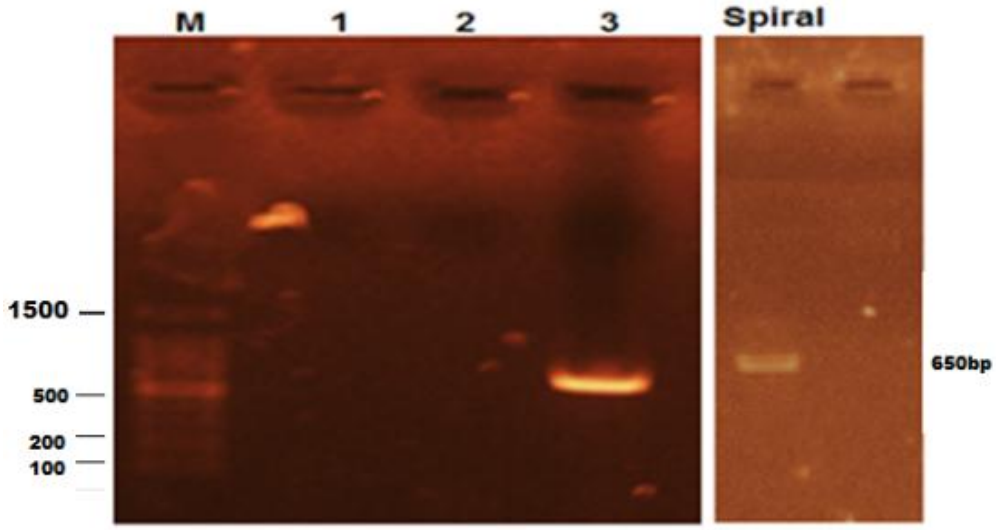

Figure 4.2: PCR detection of Ode1. Lanes 1 *H. pylori* 16S ribosomal specific PCR, Lane 2 Flagellar PCR, Lane 3 G7 (F4.Rn2) amplicon; Spiral amplicon was used for sequencing.

Microbacterium oxydans strain AN-07 16S ribosomal RNA gene, partial sequence
Sequence ID: KU937308.1Length: 1368Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
843 bits(456)	0.0	530/563(94%)	16/563(2%)	Plus/Plus

```
Query  10   CCGCCCTGCAGA-GGTGACCGGCC-CACTGGGACTGAGACACGTGTCTCCAGACTCCTAC  67
            ||| |||| ||| |||||||||||| |||||||||||||||||| | | ||||||||||||
Sbjct  231  CCGGCCTG-AGAGGGTGACCGGCCACACTGGGACTGAGACACG-G-C-CCAGACTCCTAC  286

Query  68   GGGAGGCAGCAGTGGGGTAATATTGCACAATGGGCGCAACCCTGATGCAGCAACGCCGCG  127
            |||||||||||||||| |||||||||||||||||||| ||||||||||||||||||||||
Sbjct  287  GGGAGGCAGCAGTGGGG-AATATTGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCG  345

Query  128  TGAGGGAAGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAGTGACG  187
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  346  TGAGGGACGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAGTGACG  405

Query  188  GTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCG  247
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  406  GTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCG  465

Query  248  CAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTG  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  466  CAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTG  525

Query  308  TGAAATCCGGAAGCTCAACCG-CGGCCTGCAGTGGATACGGTCAGACTAGAGTGCGGTAG  366
            |||||||||||| ||||||||  |||||||||||||| ||||| ||||||||||||||||||
Sbjct  526  TGAAATCCGGAGGCTCAACCTCCGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAG  585

Query  367  GGGAGATTGGAATTCCTGGTGTAGCGGTGAAAATGCGCAGATATCAGGAGGAACACCGAT  426
            ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  586  GGGAGATTGGAATTCCTGGTGTAGCGGTGGAAATGCGCAGATATCAGGAGGAACACCGAT  645

Query  427  GGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGAAACGAAAAGCGTGGGTAGCAAA  486
            |||||||||||||||||||||||||||||||||||| | ||||| | ||||| ||||||
Sbjct  646  GGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGG-GTGGGGAGCAAA  704

Query  487  CAGGATTACATACCCTGGTAGTCCAACCCCCGTAAAACGTTTGGGGAACTAATTGTGGGG  546
            |||| ||| |||||||||||||||| |||| ||||| |||| |||||||| ||||||||
Sbjct  705  CAGGCTTAGATACCCTGGTAGTCCA-CCCC-GTAAA-CGTT--GGGAACTAGTTGTGGGG  759

Query  547  TTCCATTTCAACGAATTTCCGTG  569
             |||||||| ||| ||| |||||
Sbjct  760  -TCCATTTCCACGGATT-CCGTG  780
```

Figure 4.3: Ode1 DNA sequence: Nucleotide BLAST with SpiralF.R4 ~ 650bp PCR. Spiral F (5'-AGGTAATGGCTTACCAAGGCTATGACG). Sequencing data was not conclusive; it indicated a gram positive Micro bacterium spp.

4.4 Selectivity of CHMYG (Section 3.8.2)

This medium was subjected to further testing to show its ability to support the growth of several types of bacteria and to screen the patterns of growth of heavily contaminated samples that may contain *Helicobacter, Campylobacter,* and other FGN bacteria.

The medium was also evaluated for its ability to inhibit the growth of Gram positive bacteria such as *Staphylococcus aureas, Staphepidermedis, Streptococcous pyogen* , both aerobically and micro-aerobically over a three-day incubation period. Control plates of BA, CA, showed good growth, however no growth was observed on MacConkey agar or CHMYG (Table 4.2).

One type of fungus continued to grow on all blood containing media that was inhibited when cycloheximide (50 µg/ml was incorporated in the CHMYG medium)

Table 4.2: Modified MacConkey agar support growth

Isolates	BA	CA	MacC	70%CHMYG	Blood macC	Ch.m line	Choc-mac	chmyG
Sewage sample	+	+	neg	+	+	neg	+	+
Chicken stool	+	+	neg	+	+	neg	+	+
Cat stool	+	+	neg	+	+	neg	+	+
5 Throat swab Pool	+	+	neg	+	+	neg	+	+
Soil sample	+	+	neg	neg	neg	neg	neg	neg
H.pylori Ag positive stool	+	+	neg	+	+	+	+	+
H.pylori ATCC	+	+	neg	+	+	+	+	+
Stool (Alarabi Center,P1)	+	+	neg	+	+	+	+	+
Ode1	+	+	neg	+	+	+	+	+
gastro, biopsy	+	+	neg	+	+	+	+	+

4.5 The selectivity of the newly developed media was investigated by culturing FGN bacteria on it as *Campylobacter jejuni*, *Heamophilus influenzae*, *Helicobacter pylori* .

4.5.1 Campylobacter jejuni

Campylobacter jejuni ATCCC 29428 was cultured on different types of media prepared in this study (Table 4.3), it was able to grow on all chocolate media with or without vitamins. These include CHMYG-V, CHMY-V (no glycerol), and their diluted form of 70% CHMYG, and 70% CHMYG-V. Also it grew on CHMYG-V with Dent's antibiotic supplement in a candle jar at 37° C for three days.

Table 4.3: *Campylobacter jejuni* growth on different media

***Campylobacter jejuni* 29428**	**RESULT**
CHMY-V	+
CHMYG-V	+
70% CHMYG	+
70%CHMYG-V	+
CHMYGVIT- Dent's	+
Chocolate Agar	+
Chocolate Agar-V	+
MacConkey Agar	No growth

Best results were obtained with CHMYG-V medium where the bacterium remained spiral not coccoid for more than 13 days as judged by microscopic examination of colonies (Figure 4.5). On Chocolate agar coccoid forms were abundant after 5 days of subculture. *Campylobacter jejuni* ATCC 29428 was grown on blood agar (BA) with no selection where as the contaminating fungus were able to grow on MacConkey forming much smaller colonies (Figure 4.4)

Figure 4.4: Fungal overgrowth. Fungus can quickly over grow *Campylobacter jejuni ATCC*29428 on blood agar (BA) with no selection.

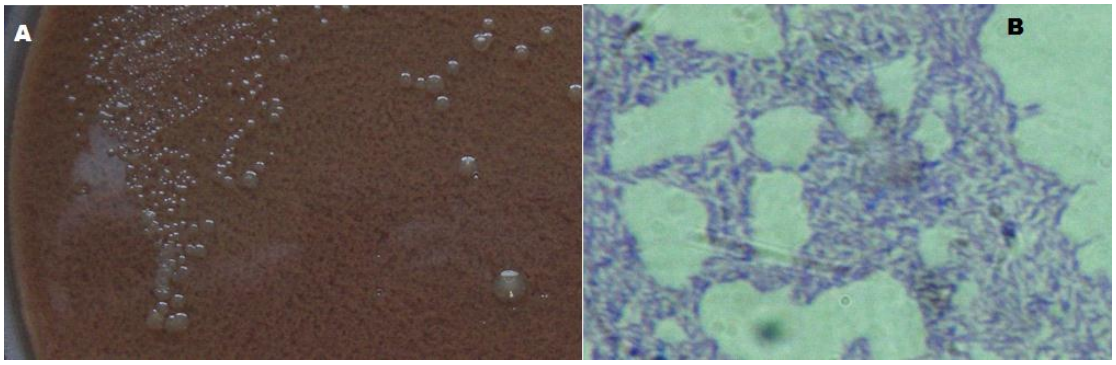

Figure 4.5: *Campylobacter jejuni ATCC* 29428. *Campylobacter jejuni* grown on the CHMYG-V in a candle jar. A: 3-days old colonies, B: Simple crystal violet stains of bacterial smear taken from A on day 4.

4.5.1.1 Identity confirmation of *Campylobacter jejuni*

Real time PCR (performed by The Ministry of Health Laboratories)was used to confirm that the DNA extracted from colonies grown on CHMYG or CHMYG-V was positive for *Campylobacter jejuni* (Figure 4.6).

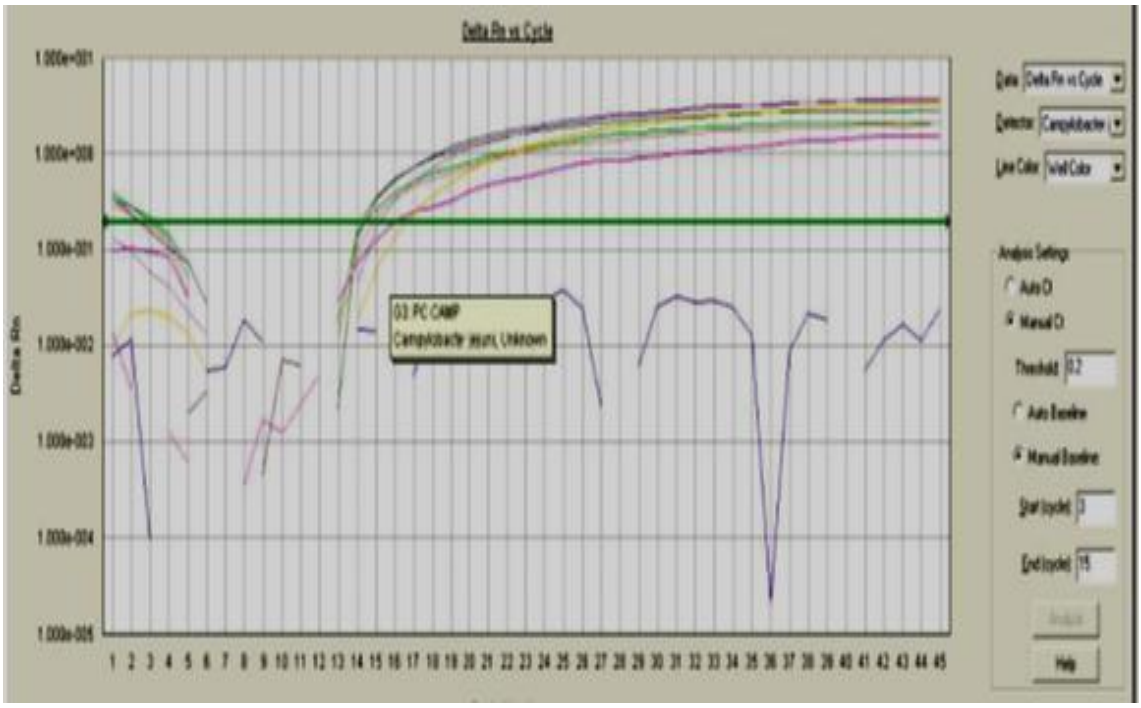

Figure 4.6*:* Real Time PCR for *Campylobacter jejuni*.

*4.5.2 Haemophilus influenza*e

A clinical isolate of *H. influenzae* was obtained from the ministry of health as a frozen glycerol vial at -70°Candwas cultured onto different types of media (Table 3.2) the cultured plates were incubated under 5-10% CO_2at 35°Cusing candle jar for three days. After the incubation period, there has been a marked growth on Chmygvit, whereas no growth could be detected on the surface of other types of media.

When *Haemophilus influenzae* growth on chocolate agar and CHMYG-VIT agar were compared together, it was noticed that small, translucent, well isolated colonies of Haemophilus *influenza* combined with few fungal contamination on the surface of chmyg-vit media compared to few colonies of *Haemophilus influenza*e with high degree of fungal contamination on the surface of chocolate vitamin media (Figure 4.8). The identity of suspected *H. influenzae* which was grown on CHMYG- VIT agar was confirmed by RT-PCR (Figure 4.9)

Fungal contamination was a significant problem in this work, as fungal growth was easily spread over chocolate and blood agar which was the main cause of loosing most of bacterial isolate such as *H. influenzae* (Figure 4.7).

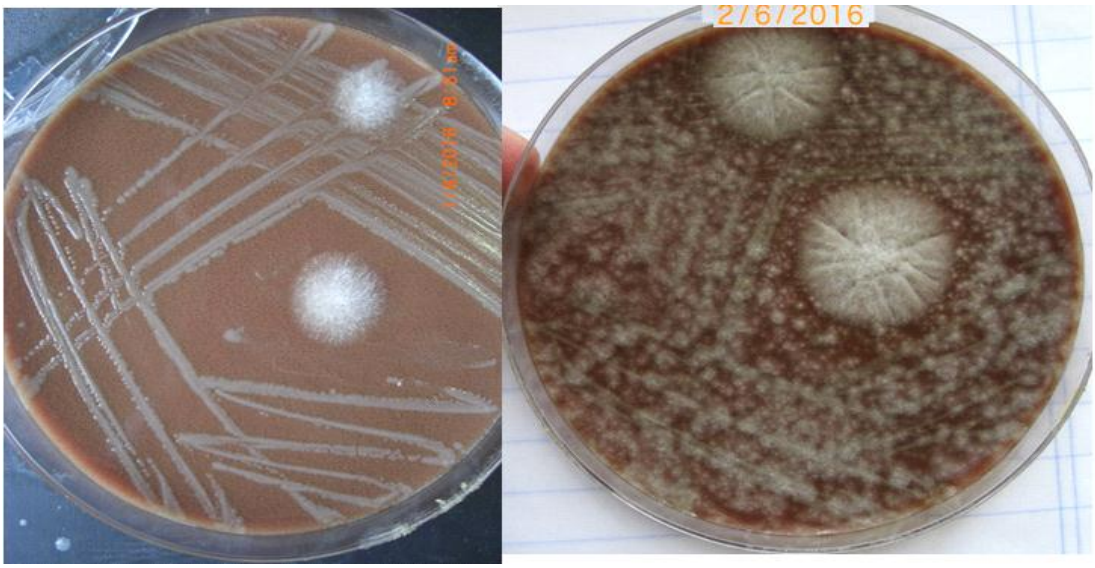

Figure 4.7: Contamination of Chocolate agar. Fungal contaminated *H. influenzae* grown on regular chocolate agar, and the loss of the isolate due to fungal over growth on the following day.

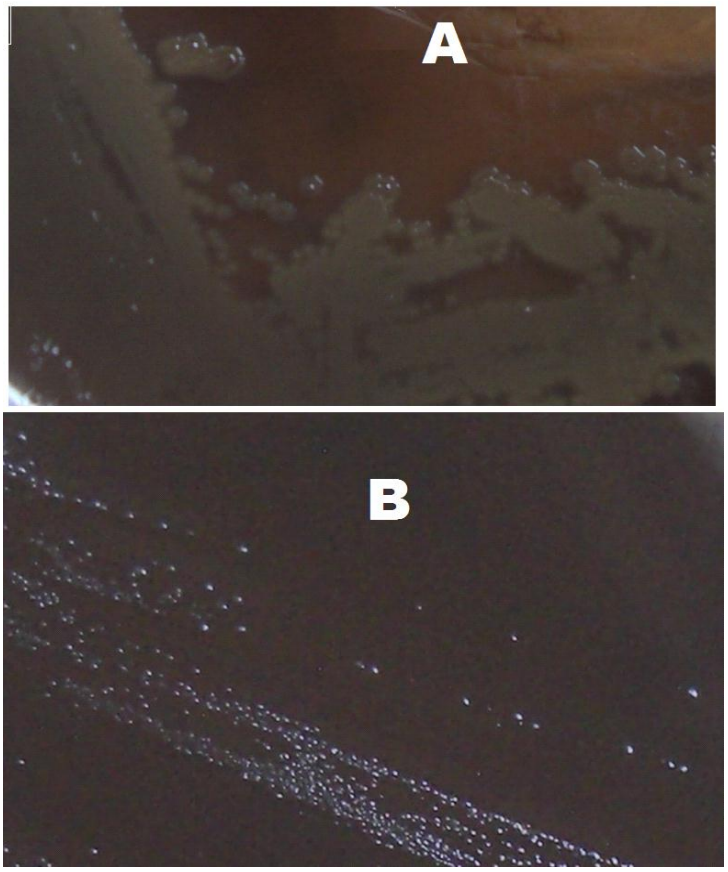

Figure 4.8*:* Growth of *Haemophilus influenza*e on CHMYG-V. Chocolate-Vitamin (A) and CHMYG-V medium (B).

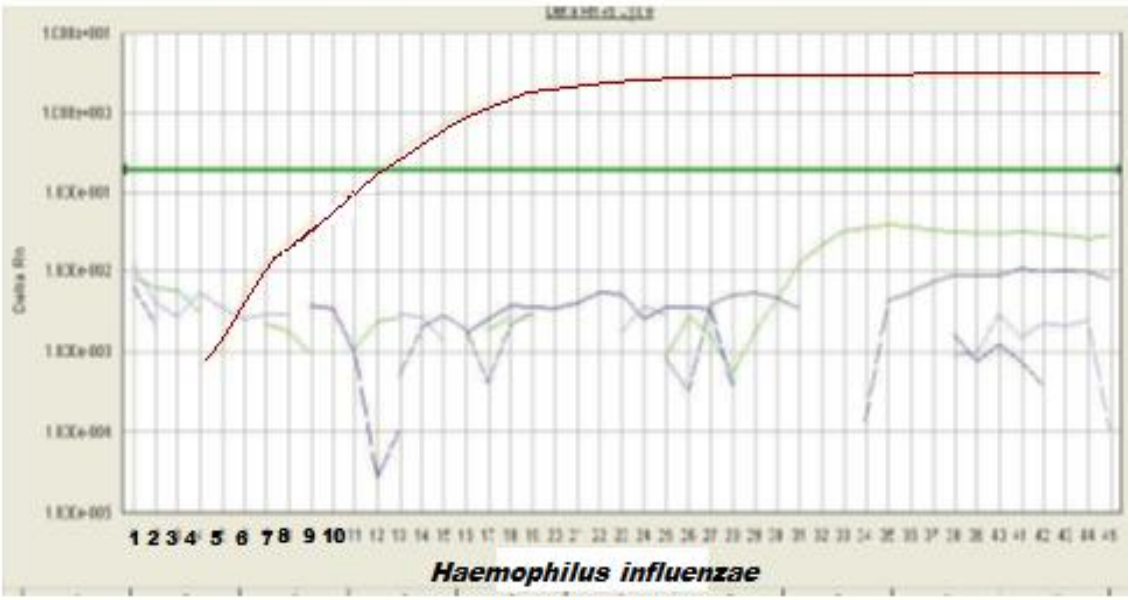

Figure 4.9: *Haemophilus influenzae* Real time PCR (RT-PCR)

4.5.3 *Helicobacter pylori* ATCC 43504

Helicobacter pylori Culture was extremely difficult on BA, CA, and other readymade media. It was assumed that this strain is particularly sensitive to MacConkey components, but upon reduction of MacConkey concentration to 70 % successful growth was obtained for it (Figure 4.10).

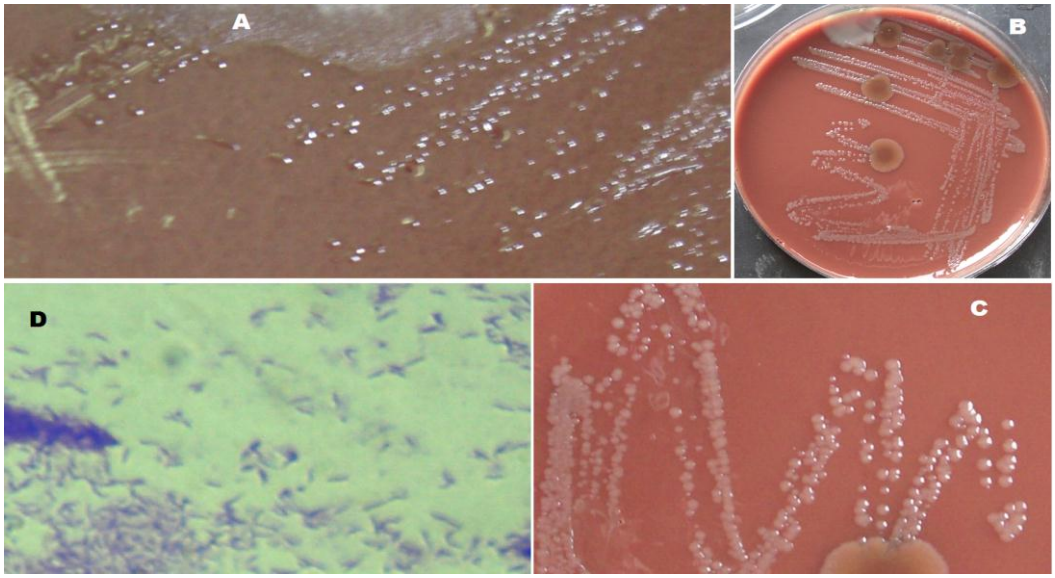

Figure 4.10*:* Spiral and Coccoid forms of *Helicobacter pylori.* *H. Pylori ATCC* 43504(A) Grown on 70% MacConky (section 3.8.3). (Band C) subcultured on Chocolate Agar (CA) then, examined microscopically at 1000x oil (D).

H.pylori ATCC 43504 was streaked onto the surface of the new media (Table 3.2) and the plates were incubated under 10%CO_2at 37°C for three days. After incubated period, good growth was seen on the surface of the media. The isolated bacteria were smeared on a slide and stained by Gram stain. Then microscopic examination of the smear under oil-immersion lens strongly suggested its identity as *H. pylori* by observing its weak Gram negative red color and its S- shape (Figure 4.11). The results are summarized in Table 4.4 showing that NM 2-4 provided better support for the growth of *H. pylori*.

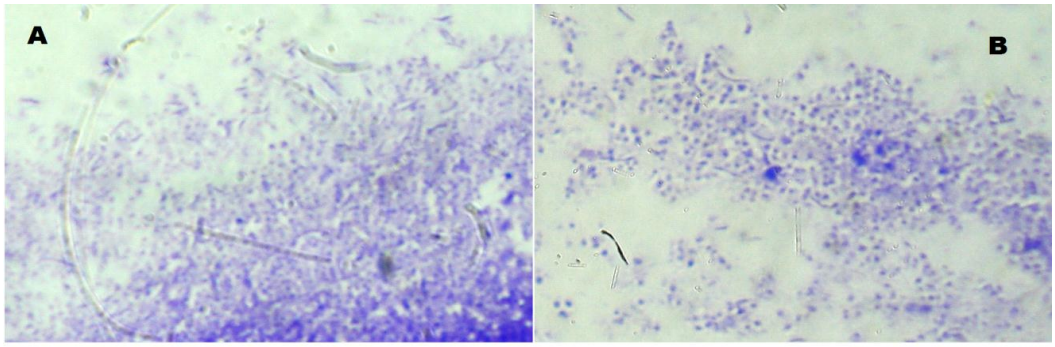

Figure 4.11: Spiral and coccoid forms: 4-day old culture of H. *pylori* grown on A: 70% CHMYG (3.8.3) and B: CH-V.

The identity of the growing colonies which are suspected as *Helicobacter pylori* was confirmed using H.P antigen strip test as shown in Figure 4.12.

Figure 4.12: Antigen test of *H. Pylori*. Positive antigen test of colonies grown on NM; containing up to 70% MacConkey agar. Full strength MacConkey was not suitable for this *ATCC* 43504.

In summary: the newly developed media could be promising selective media for isolating FGN particularly *Heamophilus influenzae*, *Helicobacter pylori*, *Campylobacter jejuni*. The new media number 3 and 4 (NM3, 4) showed the highest selectivity to isolating FGN as shown in Table 4.4.

Table 4.4: Growth profiles of all three bacteria on NM 1-9 media (see Table 3.2)

Media	***Heamophilus influenzae***	**Helicobacter pylori**	***Campylobacter jejuni***
NM1	++	++	++
NM2	+++	+++	+++
NM3	++++	++++	++++
NM4	++++	++++	++++
NM5	+	+	+
NM6	++	++	++
NM7	+	+	+
NM8	+	+	+
NM9	+	+	+
Ch-V	++	++	+
70%CHMYG-V	++	++	++

Chapter Five

Discussion

5.1 Formulation of MacConkey based blood agar medium

The fact that fastidious Gram negative pathogenic bacteria belong to different genera and species paused several obstacles in formulating a selective medium that may satisfy the growth requirements for some, most, or all fastidious Gram negative bacteria (FGN; Table1.1).

5.1.1 Incorporation of MacConkey Agar

Since MacConkey medium has proven its efficiency in growing most non-fastidious Gram negative bacteria, while selecting against and inhibiting the growth of most Gram positive and saprophytic fungi, it was a first choice as a base medium to be modified to accommodate the growth of fastidious Gram negative bacteria. Because most currently applied selective media used to isolate FGN incorporate a number of antibacterial and antifungal antibiotics which increases the risk of evolving antibiotic resistant pathogens, replacement of such selective media with MacConkey (i.e. bile salts and crystal violet, or their alike) is suggested. To enrich MacConkey, blood or serum must be added in order to satisfy most FGN pathogenic bacteria known to grow on blood agar (BA) and/or chocolate agar (CA). In addition, MacConkey agar inhibits swarming motility of *Proteus* and possibly gliding as well as swarming of other Gram negative bacteria. It is known that the selectivity of MacConkey II known as cystine lactose electrolyte deficient(CLED) which is an improved form of MacConkey is due to its content of bile salts and crystal violet[67].

5.1.2 Carbon source in MacConkey

Since the carbon source utilized by this diverse group of known and unknown FGN bacteria cannot be predicted, a common intermediate of the glycolytic pathway was used to fulfill or support the requirement for carbon and energy sources. Glycerol was added to the medium at a moderate single concentration throughout this study.

However, there is no strong evidence that *H. pylori* can use glucose as a carbon and energy source and the amino acids alone cannot supply as carbon and energy sources for *H. pylori.* In the lack of glucose the adding of single amino acids at high concentration did not enhance growth. On the other hand, the growth of *H. pylori* was induced in the presence of glucose and several amino acids .In the lack of glucose none of the 20 amino acids trigger the growth when added at high concentration[68, 69].

5.1.3 Addition of sheep blood

Two forms of the medium were used at the beginning; a blood agar and chocolate agar. However since it was observed that chocolate agar has outperformed blood agar in several ways, less contamination which may have been due to heating at 70 °C for 30 min to lyses the sheep red cells. The shelf-life of chocolate agar seemed longer than blood agar as observed for two-week old blood agar plates often failed to support the growth of *Helicobacter* whereas the chocolate counterpart was capable of supporting the growth of *Helicobacter pylori*. It is also known that *Haemophilus influenzae* is routinely cultured on chocolate agar supplemented with vitamins and iron in the form of a vitamin mix (Bio-X, IsovitaleX; Table 3.1). Shelf life for Chocolate Agar with IsoVitaleX and Bacitracin, about one week when stored at 2-8° C[51].

5.1.4 Manipulation of Crystal Violet and Bile Salt Concentration

The formulation of the NM media (Table 3.2) was the results of failing growth of both *H. pylori* and *Haemophilus influenzae* on media formulated with full strength MacConkey, but not *Campylobacter jejuni*. Manipulation of bile salt concentration (section 3.8.3) and crystal violet in the media labeled NM 1-9 (Table 3.2) showed that bile salt had negatively impacted the growth of all three bacteria, Crystal violet also affected bacterial growth when supplied at high concentration as in medium NM1 (Table 4.4). Improved growth for all three species was observed in NM2-NM4 which

had no bile salts and had reduced crystal violet. Slow growth was observed for the three FGN (*Campylobacter jejuni*, *Haemophilus influenzae*, *H. pylori*) at 50% bile esculin but no growth on 100% bile esculin.

5.2 Conclusion

This work has unraveled the relationship between three important pathogenic bacteria and their sensitivity to bile salts and crystal violet. This work has achieved its goals by formulating a selective medium that preferentially supports the growth of fastidious Gram negative bacteria over Gram positive bacteria. It is recommended that 70% CHMYG-V be used for the initial isolation of these bacteria which should be maintained on medium NM3 later on to avoid devastating frequent contamination such as that shown in Figure 4.1 which was observed several times when plain BA or CA were used. This phenomenon was avoided when MacConkey base was used in any reported form; although it did not prevent the contamination, it did control spreading of the fungi all over the plate. These media allowed minimizing the use of antibiotics as selective agents against fungi and Gram positive bacteria.

Future work should explore the application of more non-specific selective agents and focus on controlling *Pseudomonas* contamination which presented a real challenge during this work.

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تشكيل أوساط غذائية انتخابية للبكتيريا المتطلبة سالبة الغرامية

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إشراف: د. سمير البرغوثي

الملخص:

لاختبار قدرة الاوساط الغذائية لدعم نمو البكتيريا المتطلبة (FGN) بصورة انتخابية بحيث تسمح للبكتيريا المتطلبة سالبية الصبغة الجرامية بينما تثبط نمو كامل او معظم موجبة الجرامية والفطريات. لهذا الغرض تم اختيار ثلاثة اصناف من البكتيريا المتطلبة. في هذه الدراسة تم اختبار العديد من الخلطات الغذائية وذلك بادخال تعديلات واضافات على الوسط الغذائي (مكونكيMacConkey agar) وتحويله الى شوكولاتة مكونكي بعد اضافة 8–10% من دم الاغنام وخلاصة الخميرة والجلسريين والفيتامينات. تم تجنب اضافة المضادات الحيوية الا انه يمكن اضافتها دون تأثير سلبي على آداء الأوساط الجديدة. وقد تمت اضافة خلطات المضادة الحيوية المعروفة باسماء دنت، سكيرو، ولاين للأوساط الجديدة في هذه الدراسة .

بينت النتائج أن الاوساط التي هيئت بتخفيض تركيزات املاح الصفراء (bile salt) و الصبغة البلورية البنفسجية وزودت بالفيتامينات وخلاصة الخميرة والجلسريين تمكنت من دعم نمو كل من بكتيريا القرحة والبكتيريا اللولبية المعوية والبكتيريا الرئوية:

Campylobacterjejuni, Haemophilus influenzae. Helicobacter pylori بينما تمكنت هذه الاوساط من تخفيض كميات التلوث ولم تمنعه بالمطلق مقارنة باطباق دم الاجار وشوكولاتة الاجار العادية.

كشفت هذه الدراسة وللمرة الاولى أن البكتيريا المتطلبة هي حساسة لوجود املاح الصفراء (bile salts) والصبغة البلورية البنفسجية لأن نموها بدى أفضل في غياب هذه المكونات أو عند تخفيض تركيزاتها الموجودة في الاوساط الجاهزة من شركة ديفكو (Difco).

تم استنتاج أن الاوساط المكونة من 70% مكونكيووسطين جديدين وهما (NM2 و NM4) جميعها مفيدة في انماء البكتيريا المتطلبة وخصوصا من حيث ابطال الحاجة لاضافة المضادات

الحيوية لاجل السيطرة على موجبة الجرامية و الفطريات. هذا البحث يحفز على انتاج اوساط انتخابية جديدة للجراثيم الممرضة.