Application of Polymerase Chain Reaction (PCR), Bacteriological Culture, Immunoassay, and Microscopy for Detection and Identification of Gastrointestinal Pathogens in Children, Gaza – Palestine

Prepared by
Farid Hassan Abu ELAmreen

Supervisors

Prof. Fadel A. Sharif Dr. Abdalla A. Abed

Submitted in Partial Fulfillment of Requirements for the Degree of Master of Biological Sciences/Medical Technology Faculty of Science.

March 2006
Declaration

I hereby 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 substantial extent has been accepted for the award of any other degree of the university of other institute, except where due acknowledgment has been made in the text.

Signature  Name  Date

Farid  Farid Hassan Abu ELAmreen  March-2006

Copy right.

All Rights Reserved: No part of this work can be copied, translated or stored in any retrieval system, without prior permission of the authors.
Abstract

Acute gastroenteritis and diarrhea are common and costly problems that cause significant morbidity and mortality in children worldwide. In Palestine, diarrhea is one of the major causes of many outpatient visits and hospitalizations. In order to improve knowledge on the etiology of gastroenteritis and diarrhea in our patient population, stool specimens from 150 children less than 5 years of age suffering from acute gastroenteritis and diarrhea and admitted to the central pediatric hospital in Gaza strip (ElNasser pediatric hospital) were investigated for various common enteropathogens by conventional and molecular techniques. Enteropathogens were detected in 51.3% of the diarrheal samples. A single enteric pathogen was detected in 40.0% of the children, while, multiple pathogens were detected in 11.3% of the specimens. The most important cause of diarrhea revealed by this study was rotavirus as it represented 28.0% of the etiologic agents, detected by immunochromatographic assay.

*Shigella* was the most common bacterial pathogen as identified by PCR (6.0%), although bacteriological culture showed (4.0%) only, followed by *Campylobacter* (4.7%) identified by PCR only, *E.coli O157:H7* identified by PCR and culture (4.7%, 4.0%, respectively), *Salmonella* sp was found in only 2.0% of the specimens by both PCR and culture.

By microscopical examination *Entamoeba histolytica/dispar* was found in 15.3%, *Giardia intestinalis* in 1.33%, and *Strongyloids stercoralis* in 0.7% of the samples.

*Shigella* and *Salmonella* isolates were tested for their susceptibility to common antimicrobial agents and most of the isolates were resistant to ampicillin, and trimethoprim/sulfamethoxazole.

Findings from this study demonstrated that rotavirus, *E. coli O157:H7* and *Campylobacter*, which are not screened for during routine examinations of stool samples in Palestinian health laboratories in Gaza strip, were significant enteropathogens in the studied children. The detection of rotavirus will decrease the cost of hospitalization and prevent the unnecessary use of antibiotics. Moreover, the high detection rate of
rotavirus points toward the need for considering a childhood vaccine for this pathogen.

The results of the study highlight the value of using a combination of traditional and molecular techniques in the diagnosis of diarrheal disease in this population.

To the best of our knowledge, this is the first study in Gaza investigating several kinds of possible enteric pathogens in diarrhea in children less than 5 years of age.

**Key words**
Polymerase Chain Reaction; Gastroenteritis; Diarrhea; Rotavirus; Gaza; Enteropathogens.
تطبيقات تقنية PCR، المزارع البكتيرية، الطرق الميدانية والمجهرية في الكشف عن المسببات المعوية المرضية في أطفال غزة، فلسطين.

الملخص:

* يعتبر الإسهال والإسهالات المعوية الحادة من الأمراض الشائعة في فلسطين كسائر بلدان العالم والتي تؤدي إلى نسبة عالية من الوفيات كما تضيف شيئاً مادياً كبيراً ككلفة العلاج والإقامة داخل المستشفيات.*

* تهدف الدراسة إلى التعرف على الأسباب الميكروبية الموصلة إلى الإسهال والإسهالات المعوية حيث أجريت الدراسة في الفترة من بداية شهر مايو إلى نهاية شهر أغسطس 2005 في أكبر مستشفيات الأطفال في فلسطين (مستشفى النصر للأطفال - غزة) وشملت الدراسة 150 طفل دون سن الخامسة من العمر أدخلوا المستشفى نتيجة الإسهال والالتهابات المعوية.*

* تم جمع العينات اللازمة من الأطفال وفحصت باستخدام تقنية PCR واللبيولوجيا البكتيرية والفحوصات الميدانية والمجهرية.*

* خلال الدراسة تم عزل 94 ميكروب مما نسبته 51.3% من العينات حيث وجد أن 40% من المرضى مصابين بنوع واحد من الميكروبات و11.3% مصابين بأكثر من نوع واحد من الميكروبات.*

* وجدت الدراسة أن فيروس الروتا كان المسبب الرئيسي لالتهابات المعوية حيث شكل ما نسبته 28% باستخدام الفحص المناعي، ثم تلا ذلك بكتيريا الشيجلا عزلت بنسبة 40% باستخدام المزارع البكتيرية و6.0% باستخدام PCR تقنية O157:H7، وكتيريا الابيسيريشيباكولاي ونسبة 4.7% باستخدام تقنية PCR عزلت بنسبة 4.0% باستخدام المزارع البكتيرية و4.7% باستخدام تقنية PCR، وفي النهاية بكتيريا السلمونيلا عزلت بنسبة 2.0% باستخدام المزارع البكتيرية وتقنية PCR.*

* بالفحص المجهرية وجد أن الطلائع المعوية شكلت ما نسبته 17.33% من العينات الموجبة حيث كان هنالك الامتصاص الهستوينيكي الأعلى بنسبة 33.3% ثم الجاردية المعوية بنسبة 15.3% وسترونجيلويدس ستركورس بنسبة 7.8%.*

* وجدت الدراسة أن معظم بكتيريا الشيجلا والسلمونيلا كانت مقاومة لعدد من الخصائص الحيوية وخاصة الإمساحين والسلفاميثوكسيازول بنسبة 77.8% لكلهما.*

* توصي الدراسة بإدخال تقييد في مراكز الخدمات الصحية لدقة النتائج التي يتم الحصول عليها وسرعتها في التشخيص وذلك بالإضافة للفحوصات التقليدية.*

* كما توصي بإدخال الفحوصات اللازمة للتعرف على فيروس الروتا وبكتيريا الابيسبريشيباكولاي O157:H7 الذي لا يتم الكشف عنه بالفحص الروتيني العادي بالإضافة إلى تطعيم الأطفال ضد فيروس الروتا بسبب ارتفاع نسبة الإصابة به، وتوصي برفع الكشف عنه مما يقلل من انتشاره ويقلل من استخدام الكثير من مضادات الأمراض والحيوية لغير فعالة لعلاج مثل هذه الحالات.*

* يشار إلى أن هذه أول دراسة تجري في قطاع غزة في الكشف عن العديد من الميكروبات المسببة للاسهال والالتهابات المعوية عند الأطفال.*
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT (English)</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT (Arabic)</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>ix</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>x</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xi</td>
</tr>
</tbody>
</table>

## CHAPTER 1

### INTRODUCTION

... 1

## CHAPTER 2

### Literature review

2.1. Infectious diarrhea ................................................................. 4
2.2. Shigella .................................................................................. 5
2.3. Salmonella ............................................................................. 6
2.4. E.coli O157:H7 ...................................................................... 7
2.5. Campylobacter ....................................................................... 9
2.6. Rotavirus ............................................................................. 10
2.7. Parasitic Diarrhea .................................................................. 12
2.8. Enteropathogens .................................................................... 12
2.9. Detection and identification of enteropathogens by PCR .......... 22

## CHAPTER 3

### Materials and Methods

3.1. Materials ................................................................. 27
   3.1.1. PCR primers .......................................................... 27
   3.1.2. Bacterial Culture Media ........................................ 27
   3.1.3. Reagents and Materials ......................................... 28
   3.1.4. Enzymes ..................................................................... 28
   3.1.5. Commercial Kits ................................................... 28
   3.1.6. Apparatus and Equipments ...................................... 29
3.2. Study population .................................................................. 29
   3.2.1. Sample collection .................................................. 30
   3.2.2. Ethical Considerations .......................................... 30
   3.2.3. Data Analysis ...................................................... 30
3.3. Parasites detection .......................................................... 30
3.4. Rotavirus detection ........................................................... 31
3.5. Bacterial Detection by Culture ........................................... 32
3.6. Identification of E.coli, Salmonella, and Shigella ................. 32
   3.6.1. Colony morphology ................................................. 33
   3.6.2. Gram stain ............................................................. 33
   3.6.3. Biochemical tests .................................................... 33
      3.6.3.1. Oxidase test ................................................... 33
      3.6.3.2. Hy.enterotest ................................................ 34
      3.6.3.3. The API-20E test kit ....................................... 34
      3.6.3.4. Fluorescence production on MUG medium ........... 35
   3.6.4. Serological tests ..................................................... 36
      3.6.4.1. Anti-Shigella agglutination sera ....................... 36
<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6.4.2. Anti <em>Salmonella</em> agglutination sera</td>
<td>36</td>
</tr>
<tr>
<td>3.6.4.3. Anti <em>E. coli</em> O157:H7 latex test kit</td>
<td>37</td>
</tr>
<tr>
<td>3.7. Antimicrobial susceptibility testing by disk diffusion</td>
<td>38</td>
</tr>
<tr>
<td>3.8. Polymerase Chain Reaction (PCR)</td>
<td>39</td>
</tr>
<tr>
<td>3.8.1. Preparation of Fecal Specimens for PCR Assays</td>
<td>39</td>
</tr>
<tr>
<td>3.8.2. DNA Extraction from stool</td>
<td>39</td>
</tr>
<tr>
<td>3.8.3. Detection and measurement of extracted DNA</td>
<td>41</td>
</tr>
<tr>
<td>3.8.3.1. Agarose gel electrophoresis</td>
<td>41</td>
</tr>
<tr>
<td>3.8.3.2. Spectrophotometry</td>
<td>41</td>
</tr>
<tr>
<td>3.9. Detection of <em>Shigella</em> by PCR</td>
<td>42</td>
</tr>
<tr>
<td>3.9.1. Temperature cycling program</td>
<td>42</td>
</tr>
<tr>
<td>3.9.2. Expected <em>Shigella</em> PCR results</td>
<td>42</td>
</tr>
<tr>
<td>3.10. Detection of <em>Salmonella</em> by PCR</td>
<td>43</td>
</tr>
<tr>
<td>3.10.1. Temperature cycling program</td>
<td>44</td>
</tr>
<tr>
<td>3.10.2. Expected <em>Salmonella</em> PCR results</td>
<td>44</td>
</tr>
<tr>
<td>3.11. Detection of <em>E.coli</em> O157:H7 by PCR</td>
<td>44</td>
</tr>
<tr>
<td>3.11.1. Temperature cycling program</td>
<td>45</td>
</tr>
<tr>
<td>3.11.2. Expected <em>E.coli</em> O157:H7 PCR results</td>
<td>45</td>
</tr>
<tr>
<td>3.12. Detection of <em>Campylobacter coli/jejuni</em> by PCR</td>
<td>46</td>
</tr>
<tr>
<td>3.12.1. Temperature cycling program</td>
<td>47</td>
</tr>
<tr>
<td>3.12.2. Expected <em>Campylocacter</em> PCR results</td>
<td>47</td>
</tr>
</tbody>
</table>

CHAPTER 4

RESULTS.................................................................................................................................... 48
| 4.1. Age group distribution of the study population | 48 |
| 4.2. Prevalence of Enteropathogens | 49 |
| 4.3. PCR Results | 51 |
| 4.4. Comparison of Bacteriological Stool Culture, and PCR Assay | 53 |
| 4.5. Mixed Infections | 54 |
| 4.6. Occurrence of Enteropathogens among the different age groups | 55 |
| 4.7. Clinical features and infection | 55 |
| 4.8. Enteropathogens distributed by residence of the subjects | 56 |
| 4.9. Antimicrobial Susceptibility of *Shigella* and *Salmonella* isolates | 57 |

CHAPTER 5

DISCUSSION.............................................................................................................................. 59

CHAPTER 6

CONCLUSION and RECOMMENDATIONS.................................................................................... 73

CHAPTER 7

REFERENCES............................................................................................................................ 77

APPENDICES ......................................................................................................................... 88
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1. Sequence of the primers used in PCR</td>
<td>27</td>
</tr>
<tr>
<td>Table 3.2. Tests of Hy.enterotest used for differentiating the genera of Enterobacteriacea</td>
<td>34</td>
</tr>
<tr>
<td>Table 3.3. Shigella PCR reaction mixture for ipaH gene</td>
<td>42</td>
</tr>
<tr>
<td>Table 3.4. Salmonella PCR reaction mixture for 16S rDNA gene</td>
<td>43</td>
</tr>
<tr>
<td>Table 3.5. E.coli O157:H7 PCR reaction mixture using uidA gene</td>
<td>45</td>
</tr>
<tr>
<td>Table 3.6. Campylobacter c/j PCR reaction mixture for 16S rDNA gene</td>
<td>46</td>
</tr>
<tr>
<td>Table 4.1. Age group distribution of the study subjects</td>
<td>48</td>
</tr>
<tr>
<td>Table 4.2. Distribution of the specimens in terms of residence area of the subjects</td>
<td>49</td>
</tr>
<tr>
<td>Table 4.3. Enteropathogens identified in the 150 studied specimens</td>
<td>50</td>
</tr>
<tr>
<td>Table 4.4. Comparison between bacteriological stool culture, and PCR assay in detecting three bacterial enteropathogens</td>
<td>53</td>
</tr>
<tr>
<td>Table 4.5. Occurrence of mixed infections by enteric pathogens</td>
<td>54</td>
</tr>
<tr>
<td>Table 4.6. Occurrence of mixed infections by age group</td>
<td>54</td>
</tr>
<tr>
<td>Table 4.7. Enteropathogens encountered among the different age groups</td>
<td>55</td>
</tr>
<tr>
<td>Table 4.8. Clinical symptoms in relation to infections in 150 children with diarrhea</td>
<td>56</td>
</tr>
<tr>
<td>Table 4.9. Enteropathogens distributed by residence</td>
<td>56</td>
</tr>
<tr>
<td>Table 4.10. Antibiotic susceptibility of Salmonella and Shigella isolates</td>
<td>57</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1. Rotavirus particles</td>
<td>11</td>
</tr>
<tr>
<td>Figure 3.1. Interpretation of rotavirus results</td>
<td>32</td>
</tr>
<tr>
<td>Figure 3.2. Colony morphology of selected bacteria</td>
<td>33</td>
</tr>
<tr>
<td>Figure 3.3. Hy enterotest results for selected bacteria</td>
<td>34</td>
</tr>
<tr>
<td>Figure 3.4. The API-20E test kit</td>
<td>35</td>
</tr>
<tr>
<td>Figure 3.5. E.coli O157:H7 does not produce fluorescent product on MUG medium</td>
<td>36</td>
</tr>
<tr>
<td>Figure 4.1. Frequency of the identified enteropathogens</td>
<td>50</td>
</tr>
<tr>
<td>Figure 4.2. Gel electrophoresis for DNA extracted from stool samples by AccuPrep Stool DNA Extraction Kit</td>
<td>51</td>
</tr>
<tr>
<td>Figure 4.3. Specific amplification of Shigella specific invasion plasmid antigen H locus DNA by PCR</td>
<td>51</td>
</tr>
<tr>
<td>Figure 4.4. The 16S rDNA gene PCR assay specific for Salmonella species</td>
<td>51</td>
</tr>
<tr>
<td>Figure 4.5. PCR for identification of E.coli O157:H7 targeting uidA</td>
<td>52</td>
</tr>
<tr>
<td>Figure 4.6. 16S rDNA-based PCR assay specific for C. jejuni and C. coli</td>
<td>52</td>
</tr>
<tr>
<td>Figure 4.7. Interpretation of rotavirus results</td>
<td>53</td>
</tr>
<tr>
<td>Figure 4.8. Antibiotic resistance for Salmonella and Shigella isolates</td>
<td>58</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

µl  Micro liter
ADH  Arginine dehydrogenase
bp  Base pair
CDC  Center of Disease Control and Prevention
CFU  Colony forming unit
CIT  Citrate
DAEC  Diffusely adherent Escherichia coli
DNA  Deoxyribonucleic acid
dNTPs  Deoxynucleotide triphosphates
EAEC  Enteroaggregative Escherichia coli
EDTA  Ethylenediaminetetraacetic acid
EHEC  Enterohemorrhagic Escherichia coli
EIEC  Enteroinvasive Escherichia coli
ELISA  Enzyme-linked immunosorbent assay
EM  Electron microscope
ETEC  Enterotoxigenic Escherichia coli
g  Gram
GEL  Gelatin liquefaction
GN broth  Gram negative broth
H antigen  Flagellar antigen
H2S  Hydrogen sulfide
HE agar  Hektoen enteric agar
HUS  Hemorrhagic uremic syndrome
LA  Latex agglutination
LDC  Lysine Decarboxylase
mg  Milli gram
MgCl2  Magnesium chloride
MH agar  Muller Hinton agar
MUG  4-methylumbelliferyl-β-D-glucuronide
NCCLS  National Committee for Clinical Laboratory Standards
NSF  Non sorbitol fermenter
O antigen  Somatic antigen
O.D  Optical density
ODC  Ornithine Decarboxylase
ONPG  o-Nitrophenyl-beta-galactopyranoside
PAGE  Polyacrylamide gel electrophoresis
PCR  Polymerase chain reaction
PFGE  Pulsed field gel electrophoresis
RAPD  Random amplified polymorphic DNA
RFLP  Restriction Fragment Length Polymorphism
rpm  Round Per Minute
rRNA  Ribosomal RNA
RT-PCR  Reverse transcriptase PCR
SMAC agar  Sorbitol-MacConkey agar
SS agar  Salmonella shigella agar
STEC  Shiga toxin-producing Escherichia coli
SXT  Trimethoprim/sulfamethoxazole
Tris Base  Hydroxymethyl aminomethane
URE  Urease
URTIs  Upper respiratory tract infections
Vi antigen  Capsular antigen
VP  Voges Proskauer
WHO  World Health Organization
XLD agar  Xylose Lysine Deoxycolate agar
To my beloved parents and family

to my wife,

to my sons,

Osama, Mohamed, Hassan, and Mohanad

and

to my daughter

Lina
Acknowledgements

This work has been carried out at the Medical Technology laboratories in the Islamic University of Gaza, Palestine.

I would like to express my sincere thanks to all the people who directly or indirectly have contributed to this work. In particular, I would like to thank: Mr. Tarek Omar Aggad - Chairman & CEO of Arab Palestinian Investment Co. Ltd (APIC) and the staff of the Medical Supplies & Services Co. Gaza Palestine especially Dr. Sohail Obeed for their excellent funding of this work.

Professor Fadel A. Sharif, my great supervisor for being professional, encouraging and enthusiastic, for your great knowledge and tremendous intelligence, without your stimulating, critical discussions, comments, and great help, this work would not have been completed.

Dr. Abdalha A. Abed co-supervisor for your great help, very nice comments, discussions, and great support.

I would like to extend my thanks to all the staff at the Department of the biological sciences, particularly Dr. Abbood Kichaoi, for their support and help.

Dr. Randa Elkhodary always having an open door and encouraging my questions and ideas.

Miss. Najah Eliwa, Head manager of the AlShifa Hospital Central laboratory for her generous support, orientation, and very good comments.

Mr. Nasser Abu Shaaban, for all their helpful and friendly support in the lab work.

All the staff at the ElNasser Pediatric Hospital (physicians, nurses, and lab technicians), and special thanks to Dr. Abderahman Eisa the director of ElNasser Pediatric Hospital, and to Mr. Shaker Abu Shaaban the director of the ElNasser Pediatric Hospital laboratory.

All the staff at the Medical Microbiology Department of AlShifa Hospital Laboratory, Gaza, where I am working, for their support and help.

I am deeply grateful to Mr. Arafat Elhindawy, Mr. Hassan Eltiby for their helpful and friendly support.
For all of the laughs and good times, I would also like to thank my friends and colleagues, for all of their support and guidance and encouraging; good luck to all.

Finally, I want to say that my beloved family especially my brothers and sisters, my wife, they always stand beside me and give me encouragement all time and for their never-ending love and support. I am so proud of my family.
CHAPTER 1
INTRODUCTION

Gaza Strip is an elongated area located in an arid to semi-arid region. It is bordered by Egypt from the South, the green line from the North, Nagev desert from the East and the Mediterranean Sea from the West. The total surface area of the Gaza Strip is 360 km$^2$, and its population has been estimated to be 1.3 million for the year 2004, two thirds of them are mainly concentrated in eight refugee camps. 49.4% of the population in Gaza strip is under 15 years old (1,117).

Acute gastroenteritis, (infectious diarrhea) is one of the leading causes of illnesses and death in infants and children throughout the world, especially in developing countries. This is so in Asia, Africa and Latin America, where an estimated 2.5 million deaths occur each year in children less than 5 years of age (88,128). Diarrhea is also one of the leading causes of deaths among the population in Gaza Strip (16).

Gastroenteritis (human enterocolitis) is a disease characterized by fever and diarrhea. This disease is characterized by painful abdominal cramps and frequent defecation of blood and mucus, attributed to penetration and destruction of colonic epithelia by invasive microorganisms. Symptoms can include nausea, vomiting, diarrhea, fever, abdominal cramping and/or pain and a general feeling of tiredness. Approximately 9% of all hospitalizations of children younger than 5 years are due to diarrhea and dehydration (14).

Investigations on diarrheal diseases in young children demonstrated that Salmonella spp., Cryptosporidium, Campylobacter spp. and rotavirus were the major pathogens in Gaza Strip, and overcrowding was linked with an increased risk of diarrhea (1,151).

Worldwide, the most common pathogens that cause this disease are: Salmonella spp., Shigella spp., Campylobacter spp., E. coli O157:H7, Listeria monocytogenes, Vibrio cholera, Yersinia enterocolitica, Rotavirus, Cryptosporidium spp, Entamoeba histolytica, and Giardia intestinalis (lamblia). These pathogens can cause potentially serious diseases which may be fatal, especially in children. The common route of infection by these pathogens is the ingestion of contaminated foods and drinks (13).
Rotavirus is a major cause of severe gastroenteritis among children. This virus is transmitted by fecal-oral route and constitutes an important cause of nosocomial gastroenteritis. Timely diagnosis of rotavirus infection in patients with acute diarrhea helps determine appropriate treatment, prevents the unnecessary use of drugs and minimizes the spread of the disease (123,143).

The identification and diagnosis in Palestinian health laboratories is done only for *Salmonella* spp. and *Shigella* spp, through culture, biochemical and serological assays, while *Entamoeba histolytica, Giardia intestinalis* and helminthes are diagnosed by direct microscopic slide method. The other pathogens, however, are not routinely diagnosed (117).

Data from the health laboratories all over Gaza Strip show that the detection rate of *Salmonella* spp. is very low (about 0.4% in the year 2004) and even lower for *Shigella* spp. (0.4, 1.2 and 0.12%, in the respective years 2002, 2003 and 2004). Moreover, data concerning cases of *Campylobacter, E. coli O157:H7* and rotavirus and their relation to infection in Palestinian children are extremely scarce (115,116,117).

One of the modern techniques for identifying enteropathogens relies on PCR amplification assays with specifically designed nucleotide primers. PCR is suggested, by many investigators to be safer, more sensitive and more rapid than the ordinary culture methods for the diagnosis of bacteria or viruses (17).

In the present study both conventional and molecular diagnostic (PCR) techniques were used for analyzing stool samples collected from 0-5 years old children with acute diarrhea for the presence of the four common bacterial enteropathogens: *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., and *E. coli O157:H7*. Moreover, the occurrence of parasites and rotavirus in the samples were investigated by microscopic examination and an immunochromatographic method, respectively.
Microorganisms are present in the nature between heaven and earth. In stool there are microorganisms which are considered as beneficial, and those which are harmful. Our objectives were to perform a detailed molecular and microbiological investigation of some potential pathogens associated with diarrhea, to characterize the isolates, and to assess clinical symptoms and the epidemiological factors related to the diarrheal disease in children less than 5 years of age in Gaza, Palestine.

Specifically the aims of the present study were as follows:

1. To detect and identify bacterial enteric pathogens from fecal samples in children less than 5 years of age in Gaza, Palestine.
2. To assess the antibiotic susceptibility of isolated Shigella and Salmonella strains in these children.
3. To determine the role of Group A rotavirus in causing diarrhea in children in Gaza.
4. To determine the role of the common parasites in causing diarrhea in children in Gaza.
5. To investigate the sensitivity and performance characteristics of a direct PCR on stool samples as compared to conventional techniques for diagnosis of certain enteropathogenic bacteria.

Hence, four of the most relevant enteropathogens were selected for this study: Salmonella spp., Shigella spp., Campylobacter jejuni/coli. and E. coli O157:H7.
Chapter 2

Literature review

2.1. Infectious diarrhea

Diarrhea is one of the principal causes of morbidity and mortality among children in the developing world. In 1982, on the basis of a review of active surveillance data from studies conducted in the 1950s, 1960s and 1970s, it was estimated that 4.6 million children died annually from diarrhea (163).

In 1992, a review of studies conducted in the 1980s suggested that diarrheal mortality had declined to approximately 3.3 million annually (24).

Recent investigations show that diarrhea accounted for a median of 21% of all deaths of children aged under 5 years in developing areas and countries, being responsible for 2.5 million deaths per year. Increases in immunization coverage, better health care access, improvements in water and sanitation, and other socioeconomic changes affect both diarrheal mortality and childhood nutrition. Improvement of the techniques and methods used in the detection of enteropathogens may justifying the decreased rates of mortality (60,88).

Two converging factors highlight the growing need for clear guidelines for the diagnosis and management of infectious diarrhea.

First, there is increasing recognition of a widening array of enteric pathogens associated with illnesses of the gastrointestinal tract. Agents such as discussed before cause about 1.3 billion cases of diarrheal illnesses in the world each year. Many of these organisms are easily transmitted through food or water or from one person to another, and some are devastating particularly, to individuals with compromised immune systems (55,59).

With the rapid globalization and industrialization of our food supply and with a multiplicity of recognized pathogens and diagnostic tools, the challenges of determining optimal, cost-effective means for appropriate diagnosis, clinical management, and public health control of diarrheal illnesses are great (43).

The second factor arises from our having entered an era when health care is increasingly managed with an eye to cost containment. Critical to developing a cost effective approach to the evaluation and management of infectious diarrhea is the selective use of available diagnostic methods, therapies, and preventive measures. These must be targeted to the clinical scenarios in which
they will yield the greatest benefits, and certain factors must be taken into account: the patient’s history, exposure, and immune status, and the nature of the illness: its severity and duration and whether the process is inflammatory or hemorrhagic. Clear guidelines are needed for the application of diagnostic methods to identify enteric infections that require specific therapy or are responsive to control measures (59).

Oral rehydration, clinical and epidemiological evaluation, performance of selective fecal studies, administration of selective antimicrobial therapy, contraindicated antidiarrheals, and available immunizations will continue to evolve as improved understanding of pathogenesis and development and use of inexpensive, rapid tests improve diagnosis and management of infectious diarrheal illness, one of the most common clinical syndromes in our society (171,184).

### 2.2. **Shigella**

This bacterium was named as *Shigella* by the Japanese bacteriologist K. Shiga who used bacterial culture to investigate an epidemic of acute dysentery in Japan in 1898. *Shigella* is moderately-sized, gram-negative rod-shaped, facultative anaerobes, nonspore forming, closely related to *E. coli*. They are usually non-motile, oxidase negative, and do not ferment lactose. *Shigella* belongs to the *Enterobacteriaceae* family (186,129).

There are 4 known species of *Shigella* with multiple serotypes: A (*S. dysenteriae*, 12 serotypes); B (*S. flexneri*, 6 serotypes); C (*S. boydii*, 18 serotypes); and D (*S. sonnei*, 1 serotype), *S. flexneri* is the predominant species in endemic areas, accounting for approximately 50% of culture-positive cases (152).

*Shigella* is the primary causative agent of bacillary dysentery throughout the developing world. According to the World Health Organization, the annual number of *Shigella* episodes throughout the world was estimated to be 164.7 million, of which 163.2 million were in the developing countries (with 1.1 million deaths) and 1.5 million in industrialized countries. A total of 69% of all episodes and 61% of all deaths are attributable to shigellosis involved children under 5 years of age (89,186).
A definitive diagnosis of *Shigella* infection can be made by isolating the organism from stool and serotyping the isolate. Culture is also required to determine the antimicrobial sensitivity. The standard procedure for detection of *Shigella* spp. is based on isolation on selective culture media followed by identification by biochemical tests and agglutination assays (81). This process may take 48-72h or even longer to obtain results. Since *Shigella* are very fastidious organisms, appropriate collection, rapid transport to the laboratory and rapid plating of the sample are important for isolation. Such conditions are often difficult to attain in developing countries. Thus, rapid, highly sensitive and specific techniques based on genetic characteristics have been developed. PCR is the best known of these techniques and is often used as a test for the detection and identification of pathogenic microorganisms. Several PCR protocols for detection of *Shigella* in feces (73,190), food (100), and water (23) have been published. These protocols use primers directed at sequences located on the invasion plasmid of *Shigella* spp. (100,190).

2.3. *Salmonella*

Members of the genus *Salmonella* are gram-negative, facultatively anaerobic, rod-shaped, non-spore-forming bacteria. They are usually motile, oxidase negative, and do not ferment lactose, the genus *Salmonella* belongs to *Enterobacteriaceae* family (86). First described in 1880 and cultured in 1884, the bacterium has been named after Daniel E. Salmon, the pathologist who first isolated the organism from porcine intestines. The majority of *Salmonella* cause food poisoning and gastroenteritis in humans all over the world, but one species, S. typhi, frequently disseminates into the blood and causes a severe form of salmonellosis called typhoid fever (62).

Currently, the CDC (Center of Disease Control and Prevention) recognizes two species which are divided into seven subspecies: S. *enterica* (six subspecies) and *S.bongori* (one subspecies). The subspecies are divided into over 50 serogroups based on somatic (O) antigens present. The serogroups are further divided into over 2300 serotypes. *Salmonellae* can be serotyped according to their particular complement of somatic (O), capsular (Vi), and flagellar (H) antigens (32,152).
Conventional methods for detecting and identifying *Salmonella* involve cultural, biochemical and immunological assays, that rely on phenotypic characterization. These methods require selective enrichment and plating, which often take several days to complete the identification (172).

Although bacteriological assays have historically been the method of choice for the recovery of *Salmonella* from feces and environmental samples (35,38,167), the whole procedure takes at least 3 days to complete. These methods are generally time-consuming (10,30).

Several alternative analysis strategies have been proposed, and PCR in particular has been found to be a highly specific molecular diagnostic tool (67,142).

Multiplex PCR methods for detecting *Salmonella* have been published utilizing specific gene sequences as targets. Such methods are simple, inexpensive, and sensitive and enable the quick and precise detection of the most prevalent serotypes of *Salmonella* in human clinical samples (10,172).

PCR has become an important technique for more-rapid detection of pathogens in feces and environmental samples when an isolate is not required (35,38,167).

Few investigations have evaluated *Salmonella*-targeted PCR primers with intestinal bacteria. In determining which PCR primers were best suited for detection of *Salmonella* in stool samples, the 16S rDNA primer sets were approved for their applicability to gastrointestinal samples (194).

2.4. *E coli O157:H7*

*E. coli O157:H7* is one of hundreds of strains of the bacterium *Escherichia coli*. They are gram-negative, facultatively anaerobic, rod-shaped, non-spore-forming bacteria. They are usually motile, oxidase negative, ferment lactose, and belong to the *Enterobacteriaceae* family. Although most strains are harmless and live in the intestines of healthy humans and animals, this strain produces a powerful toxin and can cause severe illness.

*E. coli O157:H7* was first recognized as a human pathogen in 1982 (78), and it is increasingly recognized as an important cause of sporadic and outbreak-associated bloody diarrhea (145).
An estimated 73,480 illnesses, 62,458 hospitalizations and 61 deaths occur each year in the United States from this pathogen (113).

*E. coli* serotype O157:H7 is designated by its somatic (O) and flagellar (H) antigens. From both a clinical and public health standpoint, *E. coli* O157:H7 is by far the most important serotype in the world that cause bloody diarrhea. Children with gastrointestinal infections caused by *E. coli* O157:H7 are at risk for the hemolytic-uremic syndrome (HUS) that can be fatal as it causes acute kidney failure (189).

In many hospitals and private laboratories, a routine stool culture does not include testing for *E. coli* O157:H7. Sorbitol-MacConkey (SMAC) agar is the standard culture medium for *E. coli* O157:H7 (152). Unlike 80 to 90 percent of *E. coli* strains, *E. coli* O157:H7 does not ferment sorbitol rapidly. The colorless, sorbitol-negative colonies can then be assayed for the O157 antigen and H antigen with the use of commercially available antiserum (107).

SMAC medium stool culture is a simple, inexpensive, rapid, and reliable means of detecting *E. coli* O157:H7 (108).

Some laboratories also test *E. coli* O157 strains for the enzyme B-glucuronidase using agar medium containing the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) (138). When MUG is cleaved by this enzyme, a fluorescent product is produced that is detectable with long-wave ultraviolet light. Unlike approximately 92% of *E. coli*, *E. coli* O157:H7 and nonmotile *E. coli* O157 strains that produce Shiga-like toxins lack the enzyme and are MUG negative. For this reason the MUG assay is used in conjunction with testing for sorbitol fermentation and agglutination with *E. coli* O157 antiserum as a useful screening test for toxigenic strains of O157 (65). Culture may also be problematic due to the large numbers of other flora that either overgrow or mimic the non-sorbitol-fermenting *E. coli* O157:H7 (169).

Investigators have documented the use of conventional culture methods and PCR for the detection of *E. coli* O157:H7. The PCR for *E. coli* O157:H7 that is not readily detected by conventional culture has proved its ability to provide rapid same day results (65,102).
2.5. *Campylobacter*

*Campylobacter* species are microaerophilic, motile with a single polar flagellum, Gram negative, usually curved or spiral rod-shaped bacteria, 0.2-0.5µm wide and 0.5-0.8 µm long, non-fermenting, oxidase positive, and grow optimally at 37° or 42°C (77).

It is one of the most common causes of bacterial diarrhea in the world, fifteen species of *Campylobacter* have been described, two of these; *Campylobacter jejuni* and *Campylobacter coli*, account for the majority of human infections. *Campylobacter* species can cause mild to severe diarrhea, with loose, watery stools often followed by bloody diarrhea (162,166).

In most industrialized countries *Campylobacter* have become the most frequently reported cause of bacterial gastrointestinal illness. It affects all ages, but peak incidence appears to be in the ages 1 to 5 years. Campylobacteriosis occurs much more frequently in the summer months than in the winter (185).

Identification of *campylobacters* is well known to be problematic, principally because of their complex taxonomy, biochemical inertness, and fastidious growth requirements. Darting motility in a fresh fecal specimen observed by dark-field or phase-contrast microscopy or characteristic vibrio forms visible after Gram staining permit a presumptive diagnosis. The diagnosis is confirmed by isolating the organism from a fecal culture or, rarely, from a blood culture (74,152).

Conventional cultural methods for detecting *Campylobacter* spp. involve enrichment in selective broth, followed by isolation on selective differential agar. *Campylobacter* spp. have demanding growth requirements because they need to be incubated under microaerobic conditions (5% O2, 10% CO2, and 85% N2), at 37°C or 42°C for 48 hours. A further 24 to 48 h is required for full phenotypic identification, which makes the task of isolation laborious, costly and time consuming (47).

Culture methods are biased toward the detection of *C. jejuni* and *C. coli*. A number of the antimicrobial agents incorporated into the commonly used selective media (e.g., Preston agar, Skirrow agar, and Butzler agar) may inhibit growth of some *Campylobacter* species. Cephalexin, colistin, and polymyxin B can be inhibitory to some strains of *C. jejuni* and *C. coli*. As a result,
microbiological methods do not provide a true measure of the frequency and diversity of *Campylobacter* species associated with diarrhea and their feces (39). Recently, genetic methods have been used to identify *Campylobacter* species (48,85,150).

Molecular methods based on PCR amplification may provide an alternative to culture methods for the detection of *Campylobacter* in clinical specimens. The application of PCR based assays for the detection of *Campylobacter* species in clinical and food samples has been previously reported by several authors. These reports describe amplification of a number of DNA targets including e.g., 16S rDNA, 23S rDNA, *ceuE*, and *mapA* (54,85,94).

The application of PCR provides a more accurate description of the prevalence of *Campylobacter* species associated with diarrhea (119).

The 16S rDNA-based PCR assay specific for *C. jejuni* and *C. coli*. Regions were identified from an alignment of 16S rRNA gene sequences in which the sequences for *C. jejuni* and *C. coli* differed from those of all other Campylobacter species. A primer pair was designed for coidentification of the two species. The primers were tested against the DNAs of the type strains of all species in the genus *Campylobacter*. After PCR an amplicon of 854 bp was generated from all the tested strains of both *C. jejuni* and *C. coli* but not from all the tested strains of the other *Campylobacter* species. There was no reaction with any other non *Campylobacter* species (101).

### 2.6. Rotavirus

The wheel-like (Latin rota = wheel) particles of rotavirus were first identified as a human pathogen in 1973 by Bishop et al., when characteristic particles were observed in the cytoplasm of duodenal epithelial cells obtained from young children admitted to the hospital for treatment of acute diarrhea (20,79,83).

Rotavirus is a 65-70 nm RNA virus of the family *Reoviridae*, icosahedral, with segmented double-stranded (11 segments). It is classified into seven serogroups A-G, Group A subtypes 1, 2, 3, 4 constitute the main human pathogens. Group B-E infects mainly animals and birds (37).

The rotaviruses are composed of three protein shells surrounding the genome. This triple layered structure possesses capsomeres radiating from the inner to the outer capsid, like the spokes of a wheel figure 2.1. (103).
Figure 2.1. Rotavirus particles.

Viral pathogens account for approximately 70% of episodes of acute infectious diarrhea in children, and rotavirus is the most commonly implicated virus. Group A rotaviruses are responsible for 30–60% of all cases of severe watery diarrhea in young children (26,27,122).

The death rate due to rotavirus infection is responsible for 21% for low-income countries, 17% for low-middle income countries, 9% for high-middle income countries, and 1% for high-income countries (137).

In temperate climates, rotavirus is the main cause of winter gastroenteritis (79), in Israel and Jordan, however, hospitalization due to rotavirus diarrhea have been shown to occur more frequently in the summer (42,132), whereas in the tropics it is found all the year round, with less-defined seasonal variation (131).

In Gaza Palestine, Saltan et al. (1994) and Simhon et. al (1990) investigated the etiology of diarrheal diseases in children and they found that rotavirus represented 6.8% and 6.9%, respectively (151,161).

Rotavirus was the most common organism detected in children with diarrhea in various countries e.g., 14% in Southern Israel (42), 26.6% in Zliten, Libya (7), 28.6% in Alexandria, Egypt (140), 34.6% in Saudi Arabia (46). In Jordan, Youssef et al. (2000) and Meqdam et al. (1997) recorded the incidence of rotavirus as 33% and 40%, respectively (114,192).
Rotavirus is responsible for many cases of gastroenteritis in children as seen in about 42% in Argentina, 35% in Iran, 46% in Vietnam [57,118,131] and in 30-50% of the hospitalized patients in Europe, and 16% to 36% in Turkey [9]. It is not possible to distinguish diarrhea caused by rotavirus clinically, because diarrhea, vomiting, fever, and dehydration are not absolutely associated with rotavirus infection [87,131,195]. The most commonly used tests in diagnosing rotavirus infections are electron microscopy, latex agglutination (LA), enzyme-linked immunosorbent assays (ELISA), polyacrylamide gel electrophoresis (PAGE), immunochromatographic methods and molecular tests [9,29,34]. Simple to use ELISA, immunochromatographic tests and latex agglutination kits have been developed. These antigen detection systems have become the tests of choice in the clinical settings [29,56,75]. The sensitivity and specificity rates for immunochromatography were found to be as high as ELISA, and RT-PCR. These methods may thus be used as a reliable test for diagnosis of rotavirus infection [29,141,160].

2.7. Parasitic Diarrhea

Several species of protozoa and helminthes can cause diarrheal disease. The exposure to enteric parasites is more common in tropical and developing countries. Some of the more common causes of parasitic diarrhea are *Entamoeba histolytica*, *Giardia intestinalis*, *Cryptosporidium sp*, and other intestinal parasites [152].

2.8. Enteropathogens

Wierzba et al. (2006) in Egypt studied 714 patients with diarrhea, they found that 23% had rotavirus-associated diarrhea, 14% were *ETEC*-associated diarrhea, 1.0% *Campylobacter*-associated diarrhea and *Shigella*-associated diarrhea represented 2%. Rotavirus-associated cases presented with dehydration, vomiting, and were often hospitalized. Children with *Shigella-* or *Campylobacter*-associated diarrhea were reported as watery diarrhea and rarely dysentery. *ETEC* did not have any clinically distinct characteristics.

Helms et al. (2006) in Denmark studied foodborne bacterial infection and hospitalization among 52,121 patients. They found that (14.4%) were
hospitalized with a diagnosis of gastroenteritis. A total of (17.7%) of the hospitalized patients with infections due to S. enterica and (10.8%) of the infections were due to Campylobacter species.

Nguyen et al. (2005) studied the prevalence of enteric pathogens in 884 children aged less than 5 years (627 with diarrhea, 257 ages matched controls) in Hanoi, Vietnam. A multiplex PCR was successfully developed to identify diarrhoeagenic E. coli strains from fecal sample in a single reaction. Of the 884 processed fecal samples, 172 strains of diarrhoeagenic E. coli were identified with the significantly higher prevalence in the diarrhea group compared to the healthy ones. In addition, 19 Shigella strains were isolated. Imipenem, ciprofloxacin, nalidixic acid, cefotaxime, and cefuroxime were active against E. coli and Shigella strains, while high frequencies of resistance to ampicillin, chloramphenicol, and trimethoprim-sulphamethoxazole were shown. Of the 884 samples, group A rotavirus was identified in 45% of the children with diarrhea, and 3.5% in the healthy ones showing a significant difference. Within the diarrhea group, the highest prevalence was seen in children aged 13-24 months, and in males more than in females. More than 20% of rotavirus-infected children had association with diarrheagenic E. coli or Shigella spp.

Diniz-Santos et al. (2005) studied the epidemiological and microbiological aspects of acute bacterial diarrhea in 260 positive stool cultures of children between 0 and 15 years of age during two years at a pediatric tertiary care facility in Salvador, Brazil. Bacterial strains had been presumptively identified by culturing in selective media and by biochemical testing and their antimicrobial susceptibility patterns were automatically detected by the MicroScan Walkaway System. Most of the patients (42.7%) were between one and four years of age. Shigella was the commonest pathogen, being found in 141 (54.3%) of the cultures, while Salmonella was found in 100 (38.4%) of the cultures and enteropathogenic E. coli in 19 (7.3%). Salmonella was the main causal agent of diarrhea in children younger than five years old, whereas Shigella was the most frequent pathogen isolated from the stools of children between five and 15 years old. The peaks of incidence corresponded to the periods of school vacations. Shigella specimens presented a very high resistance rate to
trimethoprim-sulfamethoxazole (90.1%) and to ampicillin (22.0%), while *Salmonella* presented very low resistance rates to all drugs tested.

**Nimri et al. (2004)** in Jordan investigated the polymicrobial infections in 220 children with diarrhea in a rural population. Potential pathogenic agents isolated from 143 (65%) children were identified by molecular and standard microbiological methods. Co-infections with two or more agents were detected in 50 (35%) cases. *E. coli*, *Shigella* spp, *Giardia* and *Entamoeba histolytica* were found to be predominant.

**Amar et al. (2004)** in the UK detected gastrointestinal pathogens by microscopy, bacteriological culture, immunoassays and PCR in 92 fecal samples of patients with community-acquired diarrhea. Conventional techniques detected a single potential etiological agent in 15% of the samples, whereas results of PCR detected evidence of at least one agent in 41% of the samples. Overall, the detection rates for the different pathogens were as follows: adenovirus serogroup F, 1%; *Campylobacter* spp., 7.6%; *Salmonella* spp., 4%; enteroaggregative *E. coli*, 9.8%; enteropathogenic *E. coli*, 6.5%; enterotoxigenic *Clostridium perfringens*, 3%; *Cryptosporidium* spp., 13%; and *Giardia* spp., 11%. Results for the detection of *Salmonella* spp., *Campylobacter* spp. and *C. perfringens* were similar by both techniques, whereas *Cryptosporidium* and *Giardia* spp. were detected 22 times more often by PCR than by conventional microscopy. The results of this small study clearly demonstrate the advantages of PCR-based methods compared to conventional techniques for the detection of gastrointestinal pathogens.

**Nimri and Meqdam. (2004)** in Jordan investigated the enteropathogens associated with cases of gastroenteritis in a rural population in 180 children. All samples were examined for parasites and bacterial pathogens by culture and PCR. Pathogens and potential enteropathogens were identified in 140 (77.8%) of the patients, with more than one pathogen being recovered from 67 (37.2%) of the patients. Potentially pathogenic parasites were observed in 90 (50%) patients; those that were associated significantly with diarrhea were *Giardia lamblia, Blastocystis hominis, Cryptosporidium* spp., *Entamoeba histolytica* and
Cyclospora cayetanensis. Pathogenic bacteria were isolated from 72 (40%) patients, and, of these, 62.5% were resistant to at least one antibiotic, and 30.6% of these were multiresistant. Diarrheagenic Escherichia coli strains were found in 14.3% of the specimens. The most common enteropathogenic bacteria found were Shigella spp., Campylobacter jejuni and Yersinia enterocolitica.

Vargas et al. (2004) in Tanzania investigated the etiology of diarrhea in 451 children less than five years of age, the stool specimens were divided into 348 from the dry season and 103 from the rainy season. Overall, diarrheagenic Escherichia coli (35.7%) were the predominant enteropathogen, with enterotoxigenic E. coli, enteroaggregative E. coli, and enteropathogenic E. coli being the most prevalent. Moreover, enteroaggregative E. coli (63% versus 35.5%), Shigella spp. (24% versus 12%), and rotavirus (23% versus 4%) were more prevalent in the dry season than in the rainy season and enterotoxigenic E. coli (51.6% versus 20%) and Giardia intestinalis (14% versus 1%) were more prevalent in the rainy season.

Schnack et al. (2003) in Brazil investigated the prevalence of enteropathogens associated with diarrheal disease in 94 infants < 5 years old. Cryptosporidium (85.1%) topped the list of parasite isolates, followed by Entamoeba histolytica (56.4%) and Giardia intestinalis (4.3%). Four samples contained enteropathogenic Escherichia coli (4.3%). Samonella and Shigella, however, were not detected, and only one sample contained rotavirus (1.1%).

Battikhi (2002) in Jordan conducted on epidemiological study on 1400 Jordanian patients suffering from diarrhea for bacterial pathogens and Rotavirus over a four- year period (1997-2000). Pathogenic bacteria were identified in 343 patients (24.5%), most often from children. Salmonella spp. was the most frequently isolated organism in 10.7% of the patient's cultures, followed by enteropathogenic Escherichia coli (EPEC) in 3.9%, Shigella spp. in 0.8% and Campylobacter spp. in 0.9%. Resistance to ampicillin was observed in 42.2% of the Salmonella, 77.0% of the Shigella, and 31.0% of the EPEC isolates. Cotrimoxazole resistance was observed in 34.0% of the Shigella and 13.0% of
the EPEC isolates and 77.0% of Campylobacter isolates. Rotavirus was identified in 373 (26.6%) of the samples.

Souza et al. (2002) in Sao Paulo (Brazil) studied the etiologic profile of acute diarrhea in 154 children aging less than 5 years. Intestinal pathogens were detected in 112 (72.8%) cases. The association of two or more intestinal pathogens occurred in 47 (30.5%) cases. The pathogens identified were, rotavirus: 32 (20.8%), bacteria: 53 (34.4%), both: 25 (16.2%), and 2 (1.4%) with Giardia intestinalis (in one case associated with rotavirus and in another one associated with bacteria). Altogether, there were 105 bacterial isolates; 90 were Escherichia coli (EPEC 27, DAEC 24, ETEC 21 and EAEC 18), 12 were Shigella sp, 2 were Salmonella sp, and one was Yersinia sp. Children with mixed infections (viral and bacterial) had increased incidence of severe vomiting, dehydration and hospitalization.

Ballal and Shivananda (2002) in India investigated the occurrence of rotavirus and enteric pathogens in infantile diarrhea. Rotavirus in the feces has been detected by Latex agglutination and accounted for 19.6% of the diarrhea with maximum incidence (65%) in the 7-12 months age group. Bacterial etiological agents continued to play a significant role (69.6%) in diarrheal diseases. Enteroaggregative E. coli was common in the age group 25-36 months, shigellosis in the 37-60 months group and Salmonella typhimurium enteritis in the 7-12 months of age. The other pathogens isolated were vibrio cholerae (4.98%), species of Aeromonas (15.92%), along with Cryptosporidium (6.47%) and Candida albicans (3.98%).

Khan et al. (2002) in Bangladesh investigated the trend in isolation of Vibrio cholerae, Shigella, and Salmonella in neonates with diarrhea. The study population included 240 neonates who were admitted with acute diarrhea and other medical complications to the inpatient department of large Hospital, Dhaka, Bangladesh, in 2001. A single enteric pathogen was detected in 71 (29.5%), and multiple pathogens were detected in 12 (5%) of the neonates.
Enteropathogens identified were as follows: *V. cholerae* O1 (17.5%), *Shigella* spp. (9.1%), *Salmonella* spp. (3.3%), *Aeromonas* spp. (3.7%), and *Hafnia alvei in* (0.8%) of the neonates.

Urio et al. (2001) in Gaborone, Botswana investigated the *Shigella* and *Salmonella* strains isolated from 221 children under 5 years, and their antibiotic susceptibility patterns. They isolated *Shigella* from (21%) and *Salmonella* (3%). *S. boydii* (13%) was the most prevalent *Shigella* species followed by *S. flexneri* (6%) and *S. sonnei* (2%). *Salmonella* species were *S. typhimurium* and *S. paratyphi*. Antibiograms of the predominant isolates showed that most *Shigella* species were resistant to ampicillin but susceptible to chloramphenicol. The *Salmonella* species were susceptible to chloramphenicol, colistin-sulphate, gentamicin, cotrimoxazole, and ampicillin.

El-Sheikh and, El-Assouli. (2001) investigated the prevalence of viral, bacterial and parasitic enteropathogens among young children with acute diarrhea in Jeddah, Saudi Arabia, in 576 fecal samples collected from children <5 years old suffering from acute diarrhea and attending hospitals and outpatient clinics. One or more enteropathogens were identified in 45.6% of the stool specimens. Mixed infections were detected in 12.2% of the diarrheal cases. Rotavirus was detected in 34.6% of the specimens of the hospitalized patients and in 5.9% of the specimens of the outpatients. Other etiologic agents recognized included *E. coli* (13%), of which 3.8% were enteropathogenic *E. coli* (*EPEC*) and 1.9% enterohaemorrhagic *E. coli*. Other detected pathogens were: *Klebsiella pneumoniae* (4%), *Giardia intestinalis* (3.1%), *Salmonella* sp. (3%), *Shigella flexneri* (2.6%), *Entamoeba histolytica* (2.2%), *Trichuris trichiura*, *Hymenolepis nana*, and *Ascaris lumbricoides* (0.7% each), and *Candida albicans* (0.5%).

Orlandi et al. (2001) in Brazil, investigated the prevalence of enteropathogens associated with diarrheal disease in 130 infants living in the poor urban areas of Porto Velho, Rondonia. 80% of diarrheal cases were observed in the groups under 2 years of age. Rotavirus (19.2%) was the most frequent enteropathogen associated with diarrhea, followed by *Shigella flexneri* (6.15%) and *S. sonnei*
infections (3.1%), enteropathogenic *E. coli* (2.3%), enteroinvasive *E. coli* (0.8%) and *Yersinia enterocolitica* (0.8%). Mixed infections were frequent, associating rotavirus, *EPEC* and *Salmonella* sp. with *Entamoeba histolytica* and *Giardia intestinalis*.

**Ono et al. (2001)** in Nepal, studied the seasonal distribution of enteropathogens detected from diarrheal stool samples. A total of 334 diarrheal fecal samples (from 210 males and 124 females) collected in Kathmandu, Nepal, were studied for various kinds of enteropathogens. Overall, 33% (111/334) of the fecal samples were positive for one or more enteropathogens. Enteropathogen detection rates in summer, winter, spring, and autumn were 61% (40/66), 52% (45/87), 31% (25/81), and 25% (25/100), respectively. Altogether eight species of bacteria, three types of viruses, and five species of protozoan parasites were detected with considerable seasonal variations. Among the bacterial isolates, enteropathogenic *Escherichia coli* topped the list followed by *Vibrio* sp. Only one sample had *Shigella* (*S. sonnei*). Rotavirus type A was the most frequently detected among the enteric viruses, followed by human enterovirus and human adenovirus, respectively. Among the enteric protozoan parasites, *Giardia intestinalis* was the most frequently detected followed by *Cryptosporidium parvum*. Detection of bacterial and protozoan pathogens showed a slightly high tendency in the summer season compared with that in the other seasons, whereas the detection of viruses was significantly higher in the winter season.

**Youssef et al. (2000)** in Jordan investigated the incidence of enteric pathogens in 265 children with gastroenteritis by PCR & conventional methods; they detected enteropathogens in 66.4% of the examined patients. A single enteric pathogen was detected in 50.9% of the children, and multiple pathogens were detected in 15.5%. The prevalence of enteropathogens identified was as follows: rotavirus (32.5%), enteropathogenic *Escherichia coli* (12.8%), enteroaggregative *E. coli* (10.2%), enterotoxigenic *E. coli* (5.7%), *Shigella* spp. (4.9%), *Entamoeba histolytica* (4.9%), *Salmonella* spp. (4.5%), *Campylobacter jejuni/coli* (1.5%), *Cryptosporidium* spp. (1.5%), enteroinvasive *E. coli* (1.5%), *Giardia intestinalis* (0.8%) and *Yersinia enterocolitica* (0.4%). No *Vibrio*
*cholerae*, Shiga toxin-producing *E. coli*, *microsporidia*, *adenovirus* or small round viruses were detected.

**Wasfy et al. (2000)** in Egypt investigated the incidence, isolation and antibiotic susceptibility of *Salmonella*, *Shigella*, and *Campylobacter* from acute enteric infections in 6,278 patients, presenting to the Abbassia Fever Hospital. Their results showed that *Salmonella* predominated, totalling 465 isolates, followed by *Shigella* with 258 isolates, and *Campylobacter* with 146 isolates. Of the *Shigella* isolates, 124 were *Shigella flexneri*, 49 were *S. sonnei*, 47 were *S. dysenteriae* (mainly serotype 1, 2, and 3), and 38 were *S. boydii*. *Campylobacter* spp. comprised 92 *Campylobacter jejuni* and 54 *C. coli* isolates. Isolation of *Salmonella* was highest during the months of February-March, June-July, and October-November, while that of *Shigella* was maximal on July to October. Isolation of *Campylobacter* increased during May-June and again during August-October. Although *Salmonella* was sensitive to amikacin, aztreonam, ceftriaxone, and nalidixic acid, it was, however, resistant to erythromycin, streptomycin, ampicillin, chloramphenicol, and tetracycline. *Shigella* (>80%) was sensitive to amikacin, ceftriaxone, cephalexin, sulphamethoxazole-trimethoprim (except *S. sonnei*), aztreonam, and nalidixic acid. Resistance (>50%) was noted only for ampicillin, chloramphenicol, and tetracycline. *C. jejuni* and *C. coli* were resistant to cephalexin, aztreonam, and streptomycin. Some of the above antibiotics were employed to characterize the Egyptian isolates, but did not have any clinical utility in the treatment of diarrhea. Significant differences (*p*<0.05) were observed in the resistance profiles of *Shigella* and *Salmonella* between late 1980s and early 1990s. The results suggest the use of fluoroquinolones or a third-generation cephalosporin as an empirical treatment of enteric diseases. However, alternative control strategies, including the aggressive development of broadly protective vaccines, may be more effective approaches to curbing morbidity and mortality due to acute enteric infections.

**Shubair et al (2000)** studied the Palestinian intestinal parasites and diarrhea, in children. The intestinal parasites were found to be prevalent in Gaza, with an overall prevalence of 24.5%. *Giardia intestinalis* (62.2%) was the most common
parasite, followed by *Ascaris lumbricoides* (20.0%), then *Entamoeba histolytica* (18.0%).

**Shallow et al. (2000)** in USA studied the regional variation in the incidence of laboratory-confirmed bacterial foodborne illnesses. In this study 12,125 cases were identified. The incidence per 100,000 population was highest for *Campylobacter* (15.7%), followed by *Salmonella* (14.4%), and *Shigella* (7.9%). Lower incidences were reported for *E. coli* O157 (2.1%), *Yersinia* (0.4%), *Listeria* (0.3%) and *Vibrio* (0.2%). The incidence of *Campylobacter* and *Salmonella* among infants proved particularly high, although substational regional variations were observed.

**Albert et al. (1999)** in Bangladesh investigated the enteropathogens associated with childhood diarrhea, in 814 cases of diarrhea, 0 to 5 years of age. A potential enteric pathogen was isolated from 74.8% of diarrheal children. The study identified these pathogens as being significantly associated with diarrhea; the study identified the enteropathogenic *E. coli, Aeromonas* spp., *V. cholerae* O139, enterotoxigenic *Bacteroides fragilis, Clostridium difficile*, and *Cryptosporidium parvum*, as being significantly associated with diarrhea. *Plesiomonas shigelloides*, *Salmonella* spp., diffusely adherent *E. coli*, enteroaggregative *E. coli, Entamoeba histolytica*, and *Giardia lamblia* were not significantly associated with diarrhea. The major burden of diseases due to most pathogens occurred in the first year of life, and infections with multiple pathogens were common.

**Yoshida et al. (1998)** in Japan studied the bacteriological and virological etiologies of sporadic acute gastroenteritis in 1,564 samples, 722 (46.2%) were enteropathogen positive cases, and mixed infection was observed in about 15% of the positive cases. Among 13 different kinds of enteropathogens identified, the most prevalent one was pathogenic *E. coli* (20.7%), followed by *Campylobacter* spp. (10.0%), rotavirus (8.8%), *Salmonella* spp. (3.9%), adenovirus (1.9%), ECHO virus (0.9%), *Vibrio parahaemolyticus* (0.8%), poliovirus (0.7%), *Aeromonas* spp. and *Coxsackie B virus* (both 0.6%). In addition, *Shigella sonnei* (3 cases), *S. paratyphi-A* (1 case) and
enterohemorrhagic *E. coli* O157: H7 (2 cases) were also detected. A higher detection ratio was recorded in February, August and November, reflecting respectively by month a higher frequency of Rotavirus and food-poisoning causing enteric bacteria.

**Rohner et al. (1997)** in Switzerland studied the etiological agents of infectious diarrhea. A total of 13,965 specimens from 7,124 patients (1.96 specimens per patient) were cultured, yielding 369 (2.6%) *Salmonella* spp., 408 (2.9%) *Campylobacter* spp., and 79 (0.6%) *Shigella* spp. The cumulative positivity rate of 6.1% decreased to 2.7% when patients received antimicrobial agents. The positivity rate for 5,912 specimens obtained from patients hospitalized for <3 days was 12.6%, whereas it dropped to 1.4% for patients hospitalized for >3 days. Of 3,837 stool samples originating from pediatric patients, 8.8% were positive, and 5.1% of 10,128 samples from adults were positive. Rotaviruses were detected in 190 of 1,601 (11.9%) samples.

**Lerman et al. (1994)** in Israel studied the epidemiology of acute diarrheal diseases in children. A total of 284 diarrheal fecal samples were studied for various enteropathogens, they isolated enteropathogens in 40% of the diarrheal episodes from which stool cultures were obtained. The identification rates of the various enteropathogens were: diarrheagenic *Escherichia coli*, 11%; *Shigella* spp., 10%; *Giardia lamblia*, 10%; *Salmonella* spp., 4%; *Staphylococcus aureus*, 3%; *Campylobacter jejuni*, 1%. Children less than 12 months of age had a lower incidence of acute diarrheal diseases during the months they were being breast-fed than children that were fed with formula during the same period. And also in Israel **Finkelstein et al. (2002)** evaluated the impact of a serious bacterial etiology in clinical dysentery in hospitalized children and determined if children at high risk could be identified on the basis of clinical or laboratory parameters. The study population included 60 children admitted to the hospital with clinical dysentery over a 16-month period. Stool cultures were positive for *Shigella* spp. in 18 children (30%), and *Salmonella* spp. in 15 children (25%), *Campylobacter jejuni* was identified in one patient (2%). There were no significant differences in clinical characteristics or laboratory parameters between children with positive and negative stool cultures.
Na'was and, Abo-Shehada. (1991) in Jordan carried out a study on the bacterial and parasitic causes of acute diarrhea in Northern Jordan in 200 patients. One or more bacterial or parasitic enteropathogens were isolated from 79 patients (39.5%). Prevalence rates for these pathogens were as follows: enterotoxigenic Escherichia coli, 9%; enteropathogenic E. coli, 9%; Salmonella spp. 7%; Campylobacter spp, 5.5%; Yersinia enterocolitica, 4.5%; Shigella spp, 4%; Aeromonas spp, 3.5%; enterotoxigenic Clostridium perfringens, 2%; Vibrio spp, 2%; and Plesiomonas shigelloides, 0.5%. Both Giardia intestinalis and Entamoeba histolytica were detected in 2% of the stool samples examined.

2.9. Detection and identification of enteropathogens by PCR

The use of PCR for detecting and identifying enteric pathogens is rapid and easy while the conventional identification methods are not only time consuming, but also require an experienced laboratory technician to isolate and identify bacterial colonies accurately. Studies in several parts of the world have shown that the sensitivity and specificity of a direct PCR method for the detection of enteric pathogens in stool samples is quite high when compared with conventional methods (90,91,93,98,177).

Naravaneni and Jamil (2005) in India employed PCR-based techniques for the rapid detection of food-borne pathogens by using molecular techniques. Salmonella and Escherichia coli were undertaken. Suitable primers were designed based on specific genes fimA of Salmonella and gene afa of pathogenic E. coli for amplification. This study has established that fimA and afa primers were specific for detecting Salmonella and pathogenic E. coli, respectively, in the environmental samples. Thus a rapid, sensitive and reliable technique for the detection of Salmonella and pathogenic E. coli was developed.

Lukinmaa et al. (2004) in Finland studied the application of molecular genetic methods in the diagnosis and epidemiology of food-borne bacterial pathogens. Salmonella enterica, Campylobacter and Yersinia species, Shiga toxin-producing Escherichia coli (STEC), Listeria monocytogenes and Clostridium
perfringens were the bacterial pathogens constituting the greatest burden of food-borne disease in Finland. Several molecular genetic methods have been applied to diagnose, discriminate and survey these bacteria. PCR, PCR-RFLP (Restriction Fragment Length Polymorphism) and PFGE (Pulsed Field Gel Electrophoresis) are the most widely and successfully used. However, these methods are unable to replace conventional and internationally standardized phenotyping methods.

Iijima et al. (2004) in Japan improved the detection rate of diarrheagenic bacteria in human stool specimens by a rapid real-time PCR assay. A rapid laboratory system has been developed and evaluated for its ability to simultaneously identify major diarrheagenic bacteria, including *Salmonella enterica*, *Vibrio parahaemolyticus*, *Campylobacter jejuni* and Shiga toxin-producing *Escherichia coli*, in stool specimens by real-time PCR. Specific identification was achieved by using selective TaqMan probes, detecting two targets in each pathogen. A positive result was scored only when both targets of a pathogen were amplified and the difference between threshold cycles for detection was less than five. Diagnosis of enteric bacterial infections using this highly sensitive method, including DNA extraction and real-time PCR, requires only 3 h. Forty stool specimens related to suspected food poisoning outbreaks were analyzed: 16 (40%) of these samples were found to be positive for diarrheagenic bacteria using a conventional culture method; while 28 (70%) were positive using the real-time PCR assay. Of the 12 PCR-positive but culture-negative cases, 11 patients had consumed pathogen-contaminated or high-risk food. Analysis of fecal samples from 105 outpatients who complained of diarrhea and/or abdominal pain identified 19 (18%) patients as being positive for diarrheagenic bacteria using the culture method. An additional six (6%) patients were found to be positive by PCR analysis.

Li et al. (2004) in China studied the detection and identification of enteric pathogens using PCR. A set of conventional PCR assays were applied to detect and identify *Salmonella*, *Shigella*, and *E. coli O157:H7* directly from pure cultures and fecal samples. *Shigella* primers were derived from *ipaH* gene coding for invasive plasmid relative antigen and which exists on both plasmid
and the genome. The primers of *Salmonella* were designed from the 16S rDNA sequence. The primers of *E. coli* *O157:H7* were taken from *eaeA* gene. The detection system included common PCR, semi-nested PCR and RAPD (Random amplified polymorphic DNA). This method was more sensitive, specific and efficient and its processing was rapid and simple. For example, the method could be used to specifically detect and identify *Salmonella*, *Shigella*, and *E. coli O157:H7*, and its sensitivity ranged from 3 to 50 Colony Forming Unit (CFU), and its detection time was 4 hours. This PCR method, therefore, can serve as a routine and practical protocol for detecting and identifying pathogenic microorganisms from clinical samples.

**Hong et al. (2004)** in China improved an application of oligonucleotide array technology for the rapid detection of pathogenic bacteria of foodborne infections.

A rapid and accurate method for detection of common pathogenic bacteria in foodborne infections was established by using oligonucleotide array technology. Nylon membrane was used as the array support. A mutation region of the 23S rDNA gene was selected as the discrimination target from 14 species of bacteria causing foodborne infections and two unrelated bacterial species. A pair of universal primers was designed for PCR amplification of the 23S rDNA gene. Twenty-one species specific oligonucleotide detection probes were synthesized and spotted onto the nylon membranes. The 23S rDNA gene amplification products of 14 species of pathogenic bacteria were hybridized to the oligonucleotide array. Hybridization results were analyzed with digoxigenin-linked enzyme reaction. Results indicated that nine species of pathogenic bacteria (*Escherichia coli*, *Campylobacter jejuni*, *Shigella dysenteriae*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Proteus vulgaris*, *Bacillus cereus*, *Listeria monocytogenes* and *Clostridium botulinum*) showed high sensitivity and specificity for the oligonucleotide array. Two other species (*Salmonella enterica* and *Yersinia enterocolitica*) gave weak cross-reaction with *E. coli*, but the reaction did not affect their detection. After redesigning the probes, positive hybridization results were obtained with *Staphylococcus aureus*, but not with *Clostridium perfringens* and *Streptococcus pyogenes*. The oligonucleotide array can also be applied to samples collected in clinical settings of foodborne
infections. The superiority of oligonucleotide array over other tests lies on its rapidity, accuracy and efficiency in the diagnosis, treatment and control of foodborne infections.

Fukushima et al. (2003) in Japan studied the use of duplex real-time SYBR (SYNERGY BRANDS) Green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. The 17 species of food- or waterborne pathogens examined were enteroinvasive *Escherichia coli*, enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Campylobacter jejuni*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Aeromonas* spp., *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus*. The detection levels were approximately $10^5$ food or waterborne pathogenic bacteria per g of stool. The protocol for processing stool specimens for less than $10^4$ food- or waterborne pathogenic bacteria per g of stool required an overnight enrichment step to achieve adequate sensitivity. However, the rapid amplification and reliable detection of specific genes of greater than $10^5$ food or waterborne pathogenic bacteria per gram samples should facilitate the diagnosis and management of food- or waterborne outbreaks.

Leushner and Kelly (2001) in Canada studied the detection of pathogenic enteric bacteria in stool by multiplex PCR. They detected 4 species (the most common causes of bacterial diarrhea) i.e., *Salmonella*, *Shigella*, *Campylobacter* spp., and *E. coli* O157:H7 by multiplex PCR in the same reaction tube.

Sharma and Carlson (2000) in the US studied the simultaneous detection of *Salmonella* strains and *Escherichia coli* O157:H7 with fluorogenic PCR and single-enrichment-broth culture. A multiplex fluorogenic PCR assay for simultaneous detection of pathogenic *Salmonella* strains and *Escherichia coli* O157:H7 was developed and evaluated for use in detecting very low levels of these pathogens in meat and feces. Two sets of primers were used to amplify a junctional segment of virulence genes *sipB* and *sipC* of *Salmonella* and an intragenic segment of gene *eae* of *E. coli* O157:H7. Fluorogenic reporter probes
were included in the PCR assay for automated and specific detection of amplified products. The assay could detect <10 CFU of *Salmonella enterica* serovar Typhimurium or *E. coli O157:H7* per g of meat or feces artificially inoculated with these pathogens and cultured for 6 to 18 h in a single enrichment broth. Detection of amplification products could be completed in <4 hour after enrichment.

Vidal et al. (2004) in Chile employed multiplex PCR for the diagnosis of enteric infections associated with diarrheagenic *Escherichia coli*, Vanniasinkam et al. (1999) in Australia used PCR for the detection of *Campylobacter* spp. in clinical specimens, Gilbert et al. (2003) in USA developed a triplex PCR assay for the specific detection of *Campylobacter jejuni*, *Salmonella* spp., and *Escherichia coli O157:H7* from food, Matar et al. (2002) in Lebanon conducted a multiplex-PCR-based detection and genotyping of diarrhoeagenic *Escherichia coli* in diarrhoeal stools, Kumar et al. (2003) in India investigated the *Salmonella* spp. in tropical seafood by PCR, Alvarez et al (2004) in Spain, developed a multiplex PCR technique for the detection and the epidemiological typing of *Salmonella* in human clinical samples. More studies have shown the importance of PCR for detection of many enteropathogens e.g., Kulkarni et al., (2002) and Lawson et al. (1999) studies on *Campylobacter*, in Dutta et al., (2001) and Islam et al (1998) investigations on *Shigella*, Feder et al., (2001) and Kurowski et al. (2002) reports on *Salmonella*, Beutin et al., (2004) and Cubbon et al., (1996) work on *Escherichia coli O157:H7*. Actually, several studies have shown that PCR techniques are far more sensitive and specific than culture methods in detecting various enteropathogens and efficient and its processing was rapid and simple.
Chapter 3
Materials and Methods

3.1. Materials

3.1.1. PCR primers
Oligonucleotide primers for the PCR were designed from the published nucleotide sequences.

Table 3.1. Sequence of the primers used in PCR

<table>
<thead>
<tr>
<th>Organism</th>
<th>Primer name</th>
<th>Sequence 5' to 3'</th>
<th>Annealing temp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shigella sp</td>
<td>ipaH (F)- H8/15</td>
<td>5'-GTTCCTTGACCGCCTTTCCGATAC-3'</td>
<td>59°C</td>
<td>(73)</td>
</tr>
<tr>
<td></td>
<td>ipaH (R) - H8/3</td>
<td>5'-GCCGCTAGCCACCCTTA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella sp</td>
<td>16S rDNA (F)</td>
<td>5'-TGTTGTGGTAAATAACCGCA-3'</td>
<td>58°C</td>
<td>(194)</td>
</tr>
<tr>
<td></td>
<td>16S rDNA (R)</td>
<td>5'-CACAAATCCATCTCTGGA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td>UidA PT2</td>
<td>5'-GCGAAAACGTGGAGATTGGG-3'</td>
<td>55°C</td>
<td>(33)</td>
</tr>
<tr>
<td></td>
<td>UidA PT3</td>
<td>5'-TGATGCTCCATACTTCTGGA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campylobacter c/j</td>
<td>16S rDNA CCCJ609F</td>
<td>AATCTAATGGCTTAACCATTA</td>
<td>58°C</td>
<td>(101)</td>
</tr>
<tr>
<td></td>
<td>16S rDNA CCCJ1442R</td>
<td>GTAACCTAGTTTAGTATCCGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F: forward, R: reverse, (All primers were synthesized by Operon Biotechnologies, Germany).

3.1.2. Bacterial Culture Media
- *Salmonella Shigella* agar (Difco, USA).
- Xylose Lysine Deoxycolate agar (Difco, USA).
- Hektoen enteric agar (Difco, USA).
- Selenite cysteine broth (Himedia, India).
- Gram negative (GN) broth (Himedia, India).
- Sorbitol-MacConkey agar (Himedia, India).
- Violet red with MUG agar (Himedia, India).
- Muller Hinton agar (Sanofi Diagnostic Pasteur, France).
- Muller Hinton broth (Sanofi Diagnostic Pasteur, France).
3.1.3. Reagents and Materials

- Gram stain (Industrias Aulabor S.A. Spain)
- Oxidase test (Hy.laboratories Ltd, Israel)
- Agarose Molecular Biology grade (Promega, USA)
- DNA molecular weight marker 100 (Promega, USA)
- dNTPs: (dATP, dCTP, dGTP and dTTP) (Promega, USA)
- EDTA disodium salt (Promega, USA).
- Absolute Ethanol (Sigma, USA)
- Ethidium bromide (Promega, USA).
- Absolute Isopropanol (Sigma, USA)
- Pipettes Research (Eppendorf)
- Tris base[hydroxymethyl aminomethane (Promega, USA)
- Acetic acid (Sigma, USA)
- DNAse, RNAse free Water (Promega, USA).
- Microfuge tubes - 1.5 mL capacity.
- Microfuge tubes for PCR - thin wall 0.2 mL and 0.5 mL capacity
- PCR buffer, MgCl₂ (Euroclone - Italy).
- Antibiotic disks (Oxoid Ltd, UK)
- Bacterial positive control

3.1.4. Enzymes

- Taq DNA polymerase, (Euroclone, Italy).

3.1.5. Commercial Kits

- Rotavirus detection (RotaStick one step test kit - Novamed Ltd., Jerusalem)
- The API-20E test kit (bioMerieux, Inc., France)
- Hy.enterotest (Hy.laboratories Ltd, Israel)
- Anti-Shigella agglutination sera (BIORAD, France)
- Anti Salmonella agglutination sera (BIORAD, France)
- Anti E. coli O157:H7 Latex Test Kit (Plasmatic, UK)
- AccuPrep Stool DNA Extraction Kit (Bioneer, Korea)
3.1.6. Apparatus and Equipments

- Thermal Cycler (Eppendorf Mastercycler Personal).
- L.G. Microwave Oven.
- Hoefer Shortwave UV Light Table (Transilluminator).
- Digital Camera.
- Vortex Mixer.
- Power Supply (Biorad).
- Micro Centrifuge.
- Freezer, Refrigerator.
- Electrophorosis Tank.
- Petri dishes.
- Incubator.
- Spectrophotometer UV-Vis.
- Water Bath.
- Weight Mod.
- Microscope.
- Computer.

3.2. Study population

During the peak of diarrheal season (May-August of 2005), 150 children up to 5 years of age who were admitted with acute diarrheal diseases to ElNasser Pediatric Hospital Gaza, were enrolled in the study. Diarrhea was defined as the passage of three or more loose or watery stools in the preceding 24 hours.

A questionnaire was completed for each patient containing the following information: age, gender, address, clinical data, fever, vomiting, dehydration status and previous hospitalization.

Vomiting was defined as the forceful expulsion of gastric contents occurring at least once in a 24 hours period. Fever was defined as an under arm measured temperature > 37.2°C, and dehydration level was assessed following the recommendations of WHO Program for Control of Diarrheal Diseases and was done by the pediatricians (59,129).

After the informed consent was obtained from the parents of the subjects, a pediatrician filled out the information relevant to clinical symptoms and illness onset on a standardized questionnaire, the rest of the questionnaire was filled
by the parents of the children, refer to Appendix A for a complete listing of the items on this questionnaire.

### 3.2.1. Sample collection

Fecal samples (one per each subject), from children with diarrhea were collected as soon as the children were admitted to the ElNasser pediatric Hospital Gaza by the help of their parents. Each stool specimen was collected in a special container, kept at 4°C, and processed within 3 hours for collection. The residual of each sample, after the first culture on the media, immunoassay and microscopy, was kept at -70°C for further work e.g., DNA extraction, and PCR analysis.

### 3.2.2. Ethical Considerations

An authorization to carry out the study was obtained from Helsinki (Declaration of Helsinki the most widely accepted guideline on medical research involving human subjects) using an agreement letter prepared from the Islamic university of Gaza. Parents gave their consent for participation in the study and all the information that were obtained about the subjects as well as the parents were kept confidential; refer to Appendix B for more details.

### 3.2.3. Data Analysis

The data was entered, sorted and analyzed by a personal computer using SPSS 8.0 statistical package, differences in proportions were assessed by a Chi-square test, P values <0.05 were considered statistically significant.

### 3.3. Parasites detection

A smear of fecal specimens with 0.9% saline was examined microscopically for the presence of leukocytes, red blood cells and parasites. In addition, stool samples were examined in iodine wet mounts. Slides were scanned by light microscopy at 100 X and 400 X magnifications.
3.4. Rotavirus detection

Stool samples were analyzed for group A rotavirus using RotaStick one step test kit for determination of rotavirus in human feces (Novamed Ltd., Jerusalem) following the manufacturer's instructions.

3.4.1. Principle of the procedure

The principle of the kit depends on a rapid immunochromatographic test for the qualitative screening of human fecal samples for detecting the presence of rotavirus antigen.

In brief, 0.1 g of stool specimen was added to 0.6 ml of buffer solution in a test tube. The content of test tube was then mixed vigorously by vortex to suspend the specimen. After sedimentation of large particles to the bottom of the tube (2-5 min), the dipstick test strip was placed vertically into the sample tube and removed after 10 seconds or when the fluid had reached the middle of the test area of the dipstick. The test strip contains a mobile monoclonal rabbit origin anti-Rotavirus antibody conjugated to colloidal gold particles. Then, a polyclonal rabbit origin anti-rotavirus antibody is immobilized in the test area of the strip. If the stool sample extract contains rotavirus antigens, these form antigen antibody complexes with the gold particles. These complexes migrate along the dipstick to the immobilized capture antibody, and a positive test band becomes visible. An immobilized anti-rabbit antibody captures the remaining conjugate, forming a control band, which indicates proper performance of the test procedure. The test is judged positive when, in addition to the control band, a clearly distinguishable purple band becomes visible in the test window. This second band works as an internal control and indicates proper performance of the test. Figure 3.1 below illustrates the interpretation of results.
Figure 3.1. Interpretation of rotavirus results

N = Negative: only one pink/purple band appears in the control window, no band is visible in the test window, 
P = Positive: in addition to the control band a clearly distinguishable pink/purple band also appears in the test window, and 
I = Inconclusive: If no control band is visible (with or without a visible band in the test window) the test is inconclusive. The test should be repeated using a new device.

3.5. Bacterial Detection by Culture

All collected stool specimens were tested for Salmonella spp., Shigella spp., and diarrheagenic Escherichia coli (E. coli O157:H7).

Fresh stool samples were plated onto Salmonella Shigella (SS) agar medium, Hecktoen enteric (HE) agar, Xylose Lysine Deoxycolate (XLD) agar and Sorbitol MacConkey agar (SMAC). Approximately 1 g of each sample was inoculated into 10 ml of selenite cysteine broth and 10 ml gram negative (GN) broth, and incubated for 18 to 24 h at 37°C.

The SS agar, HE agar, XLD agar and SMAC agar plates were incubated for 18 to 24 h at 37°C.

Approximately 0.5 ml of selenite cysteine broth, and GN broth were subcultured onto SS agar, HE agar and XLD agar after 18 to 24 h of incubation.

Suspected colonies on the primary or subculture plates were further identified by standard laboratory procedures (31,106,175).

3.6. Identification of E.coli, Salmonella, and Shigella

Conventional methods based on culture, biochemical tests, and serotyping were used to identify E. coli O157:H7, Salmonella, and Shigella. E.coli, Salmonella, and Shigella can also be detected by PCR due to the presence of specific DNA segments or genes in each species. Therefore
agglutination with antisera specific to each species was further verified by PCR, using species specific PCR primers.

3.6.1. Colony morphology
Distinctive shape, texture and color of bacterial colony (Figure 3.2).

**SS agar**: *Salmonella* species appear on SS agar as colorless colonies with black centers owing to H2S production, *Shigella* species show same type of colonies but without any blackening.

**HE agar**: *Salmonella* colonies are blue-green, typically with black centers due to H2S production, *Shigella* appears greener than *Salmonella*, with the color fading to the periphery of the colony and without black centers.

**XLD agar**: *Salmonella* species show transparent red colonies with black centers owing to H2S production, *Shigella* species show same type of colonies but without any blackening.

**SMAC agar**: *E. coli* O157:H7 colonies appear pale yellow due to their inability to ferment sorbitol.

![Figure 3.2. Colony morphology of selected bacteria. A: Salmonella on XLD, B: E coli O157:H7 on SMAC, and C: Shigella on SS agar.](image)

3.6.2. Gram stain
Upon Gram staining, *E.coli, Salmonella*, and *Shigella* spp appear as Gram negative bacilli (short rods)

3.6.3. Biochemical tests
These tests serve to determine aspects of bacterial metabolism; nutritional and survival strategies of bacteria.

3.6.3.1. Oxidase test
All suspected bacteria are oxidase negative.
3.6.3.2. Hy.enterotest

To differentiate the genera of Enterobacteria Hy.enterotest was used which includes the following tests:

**Table 3.2. Tests of Hy.enterotest used for differentiating the genera of Enterobacteriacea**

<table>
<thead>
<tr>
<th>Reaction site</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stopper</td>
<td>Indole</td>
</tr>
<tr>
<td>Upper layer</td>
<td>Slant, ONPG</td>
</tr>
<tr>
<td>Butt</td>
<td>Glucose, H2S</td>
</tr>
<tr>
<td>Lower layer</td>
<td>Motility, Urea</td>
</tr>
</tbody>
</table>

The reactions of selected bacteria are shown in Figure 3.3, which we can use to differentiate between the bacteria.

**Figure 3.3. Hy enterotest results for selected bacteria**
A: unreacted tube, B: *E.coli*, C: *salmonella*, D: *Shigella*, E: *proteus*

3.6.3.3. The API-20E test kit

The API-20E test kit used for the identification of enteric bacteria (bioMerieux, Inc., France) provides an easy way to inoculate and read tests relevant to
members of the Family *Enterobacteriaceae* and associated organisms. A plastic strip holding twenty mini-test tubes (Figure 3.4) is inoculated with a saline suspension of a pure culture (as per manufacturer's directions). This process also rehydrates the dessicated medium in each tube. Few tubes are completely filled (CIT, VP and GEL as seen in the photos below), and some tubes are overlaid with mineral oil so that anaerobic reactions can be carried out (ADH, LDC, ODC, H₂S, URE).

After incubation in a humid chamber for 18-24 hours at 37°C, the color reactions are read (some with the aid of added reagents), and the reactions (plus the oxidase reaction done separately) are converted to a seven-digit code. The code is fed into the manufacturer's database gives back the identification, usually as genus and species.

![Figure 3.4. The API-20E test kit](image)

### 3.6.3.4. Fluorescence production on MUG medium

*E. coli* O157 strains were also tested for the presence of glucuronidase enzyme using agar medium containing the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). When MUG is cleaved by this enzyme, a fluorescent product is produced that is detectable with long-wave ultraviolet light (Figure 3.5). Unlike approximately 92% of *E. coli* strains, *E. coli* O157:H7 and nonmotile *E. coli* O157 strains that produce Shiga-like toxins lack the enzyme and are MUG negative. The MUG assay is usually used in conjunction with testing for sorbitol fermentation and agglutination in *E. coli* O 157 antiserum (138).
3.6.4. Serological tests

3.6.4.1. Anti-Shigella agglutination sera

These sera should be used only to study the antigenic characteristics of non-motile Enterobacteria which present the biochemical characteristics of *Shigella*. Each of the polyvalent sera contains a number of serotypes which belong to the sub-groups:

1) Sub-group **A**: polyvalent sera *anti-Shigella dysenteriae*
2) Sub-group **B**: polyvalent serum *anti-Shigella flexneri*
3) Sub-group **C**: polyvalent sera *anti-Shigella – boydii*
4) Sub-group **D**: mixed anti-*Shigella* sonnei serum

3.6.4.2. Anti *Salmonella* agglutination sera

*Salmonella* species are serotyped according to their O (somatic) antigens, Vi (capsular) antigen and H (flagellar) antigens. The antigenic formula of *Salmonella* serotypes are listed in the Kauffman-White scheme and are expressed as follows: O antigens; Vi when present; H antigens phase 1; H antigens phase 2 (when present).

In the present study we defined *Salmonella* according to their O and H antigens.

Each the polyvalent sera contain:

1) Polyvalent O Antisera (*Salmonella* O Antiserum Poly A-I).
2) Polyvalent H Antisera (*Salmonella* H Antiserum Poly a-z)
(Difco Laboratories, USA)
The technique used for both *Salmonella* and *Shigella*. On a clean glass slide a drop of serum was deposited. Using a platinum loop a small amount of bacteria, from an 18 hours old culture on nutrient agar, was gradually mixed with the serum to form a light uniform suspension. Agglutination is observed in the corresponding serum after a maximum of one minute.

3.6.4.3. Anti *E. coli* O157:H7 latex test kit

The Plasmatic *E. coli* test kit is a latex agglutination test for the rapid identification of *E. coli* sero-group O157. The test is best used in conjunction with Sorbitol MacConkey Agar. *E. coli* O157 strains cannot ferment sorbitol and will therefore give colourless colonies. The majority of other *E. coli* strains is capable of fermenting sorbitol and therefore, gives characteristic pink colonies. The non-sorbitol fermenting (NSF) colonies were further tested by the latex agglutination method.

**Principle of the method**

This method depends on latex particles coated with an antiserum against *E. coli* O157 antigen. When the latex particles are mixed with fresh colonies of *E. coli* O157 the bacteria will bind the antiserum, causing the latex particles to visibly agglutinate (Positive). Bacteria which are not O157 serotype will not bind to the antiserum and will not result in agglutination (Negative)

**Sample preparation**

Non-sorbitol fermenting (NSF) colonies may be taken directly from the Sorbitol MacConkey plate or NSF isolates may be subcultured onto non-selective media for testing. At least 10 different NSF colonies should be tested, to ensure that any mixed strains or contaminants are not detected. Use of control latex will ensure that the tested colonies do not autoagglutinate.

**The method**

Using the provided pipette, one drop of saline was placed onto two separate circles of a reaction slide, using a sterile loop, colonies of the organism to be investigated were picked and emulsified in the drop of saline in one of the circles on the slide. The same is repeated with the second drop. One drop of test latex reagent was then added to one of the drops of saline and one drop of control latex reagent to the other, using the loop, the test reagent
and control reagent were thoroughly mixed. The slide was rotated for two minutes while observing for agglutination.

**Interpretation of results**

A positive result is indicated by agglutination with the test reagent, whilst the control reagent should appear milky and smooth.

### 3.7. Antimicrobial susceptibility testing by disk diffusion

Antibiotic susceptibility of bacteria was determined using the disk diffusion method as described by the National Committee for Clinical Laboratory Standards (NCCLS).

Approximately 4 mm thick Mueller-Hinton agar was prepared from a dehydrated base according to the manufacturer’s recommendations.

The inoculums for antimicrobial susceptibility testing of bacterial isolates were prepared from fresh pure cultures. The cell suspensions of the bacterial isolates were prepared in sterile Mueller-Hinton broth. A cell suspension equal to a density of a 0.5 McFarland turbidity standard was used for inoculation.

When the proper density is achieved, a cotton swab was dipped into the bacterial suspension excess fluid was removed by pressing and rotating the swab against the wall of the tube.

The swab was used to inoculate the entire surface of the Mueller-Hinton agar plate three times, rotating the plate 60 degrees between each inoculation.

The inoculum was allowed to dry before placing the disks on the plates. Drying usually takes only a few minutes, and should take no longer than 15 minutes. After the plate was dry, the antimicrobial disks were placed on the plates.

Sterile forcep was used to place the disks on the Mueller Hinton agar and taped them gently to ensure that they adhere to the agar. Diffusion of the drug in the disk begins immediately; therefore, once a disk contacts the agar surface, the disk should not be moved.

The plates were incubated in an inverted position for 18–24 hours at 37°C. After overnight incubation, the diameter was measured of each zone of inhibition with a ruler or a caliper. In all measurements, the zones of inhibition were measured as the diameters from the edges of the last visible colony, and the results were recorded in millimeters (mm). Inhibition zone diameters were compared to the reference list of the manufactures.
Salmonella, and Shigella species were tested against the following antibiotics: Ampicillin, Piperacillin, Cephalexin, Cefuroxime Ceftazidim, Ceftriaxone, Amikacin, Gentamicin, Doxycycline, Sulfamethoxazol/Trimethoprim, Ciprofloxacin, Nalidixic acid, Chloramphenicol, Cefaclor and Meropenem. (72,175,187).

3.8. Polymerase Chain Reaction (PCR)

3.8.1. Preparation of Fecal Specimens for PCR Assays
Processing of specimens is the most critical step in PCR assays. Although it is not essential to isolate DNA in great purity, it is necessary to remove or inactivate substances that may inhibit PCR assays. Stool specimens are known to contain potent PCR inhibitors, most of which have not been identified.
A washing step was used in order to decrease the concentration of PCR inhibitors. The fecal debris, which is inhibitory to PCR, was then removed by low-speed centrifugation. The kit extraction eliminates other contaminants (biological, proteins polysaccharide, and bile acids) in stool suspensions that may inhibit PCR (111,144,181).
The stored (−70 °C) fecal samples were thawed before processing. To maximize the bacterial populations present in the fecal specimen, approximately one gram feces specimen was added to 5 ml of sterile 0.85% NaCl and mixed by inverting and vortexing the tubes. The samples were then centrifuged at low speed (2000) rpm for 2 min to pellet larger fecal debris. The supernatant was transferred to a new tube and then centrifuged at 12000 rpm for 5 min to pellet the bacterial cells. The cells in the pellets were washed three times with normal saline and once with water, resuspended in 0.1 ml of distilled water, and their DNA was extracted using AccuPrep Stool DNA Extraction Kit (Bioneer, Korea) according to the manufacturer’s protocol except that in the final step only 100 µl (instead of 200 µl) of heated elution buffer (70 °C) was used.

3.8.2. DNA Extraction from stool
The AccuPrep Stool DNA Extraction Kit can quickly and conveniently extract up to 5µg of DNA from about 100 to 200 mg of stool. In the presence of chaotropic salt, DNA is bound to glass fibers fixed in a column. Proteins and other contaminants are removed through washing steps, and the DNA isolated and eluted in the final elution step. The process does not require the use of
dangerous organic solvents or ethanol precipitation steps. Also, DNA is efficiently extracted regardless of the condition of the stool.

The kit contains the following Reagents:
Proteinase K, lyophilized
Stool Lysis buffer (SL)
Binding buffer (ST)
Washing buffer 1 (W1)
Washing buffer 2 (W2)
Elution buffer (E)

Procedure of Extracting DNA from Stool
20 ul proteinase k (25 mg/ml) was added to 1.5 mL microcentrifuge tube, washed stool sample pellets were then added, followed by 400 ul Stool Lysis buffer (SL) and the mixture was mixed by light vortexing for about 30 seconds, complete mixing is essential for maximum lysis. The tubes were then incubated at 60 °C for 10 min.

After 10 mins incubation, the tubes were centrifuged 12,000rpm for 5 mins, then the supernatant was transferred to a new tube, 400 ul Binding buffer (ST) was added, and the tubes were incubated again at 60 °C for 10 min.

After 10 mins incubation 100µl absolute isopropanol was added, mixed by lightly vortexing for about 5 seconds, and the mixture was transferred into the binding column. The lid was closed and the column was centrifuged for 1 min at 8,000 rpm, or until the liquid has completely passed.

The binding column was transferred to a new 2 ml collection tube, after centrifugation, 500µl Washing buffer 1 (W1) was added to the column, and the column was centrifuged again for 1min at 8,000 rpm.

After centrifugation, the binding column was transferred to a new 2 ml collection tube, 500µl Washing buffer 2 (W2) was added, and centrifuged for 1min at 8,000 rpm.

The column was further centrifuged at 13,000 rpm for 1 min to completely remove alcohol. The binding column was transferred to a 1.5 ml collection tube, 100µl of heated (70°C) elution buffer was added, and the column was let to stand for 5 min to allow the buffer to permeate the column. The DNA was then eluted by spinning down at 8,000 rpm for 1 min.
The eluted DNA solution can directly be used, or stored at 4°C or –20°C for longer storage periods.

3.8.3. Detection and measurement of extracted DNA

3.8.3.1. Agarose gel electrophoresis

The quality of the isolated DNA was determined by running 5 μl of each sample on 1.0% ethidium bromide stained agarose gels and the DNA was visualized on a short wave U.V. transilluminator, the results were documented by photography.

3.8.3.2. Spectrophotometry

The optical density (O.D.) at 260 nm of diluted fractions of the isolated DNA samples was measured by a spectrophotometer and the DNA concentration was calculated by considering 1 O.D.\(_{(260\text{ nm})}\) = 50 ug/ml DNA and taking into account the dilution factor.
3.9. Detection of *Shigella* by PCR

5 uL (~ 200 ng) of the prepared DNA template was added to 45 uL of PCR reaction mixture (Table 3.3) in 0.2 ml thin walled microfuge tube. Microfuge tubes were then placed in a thermal cycler and PCR amplification was done according to the program described below.

Upon completion of PCR, the products were analysed by 2% agarose gel electrophoresis, otherwise, the amplicons can be stored at 4°C until analysis. Presence of *Shigella* specific DNA should yield a band of 700 bp size.

**Table 3.3. *Shigella* PCR reaction mixture for ipaH gene**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>PCR reaction mixture</th>
<th>Initial concentration</th>
<th>Volume (μl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer</td>
<td>10 X</td>
<td>1X</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25 mM</td>
<td>2.0 mM</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>dNTPs</td>
<td>20 mM</td>
<td>0.1 mM</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Primer 1 ipaH (F)-H8/15 *</td>
<td>100 μM</td>
<td>1.0 μM</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Primer 2 ipaH (R)-H8/3 *</td>
<td>100 μM</td>
<td>1.0 μM</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5 U/μL</td>
<td>2.0 U</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Template DNA</td>
<td>2 ug</td>
<td>200 ng</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>34.35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td></td>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Sequence of the primers is given in Table 3.1.

### 3.9.1. Temperature cycling program

The thermal cycler program was set as follows:

**Step 1:** Denaturation for 3 minutes at 95°C

**Step 2:** 35 cycles of:

Step 2.1. Melting for 60 seconds at 95°C

Step 2.2. Annealing for 60 seconds at 59°C

Step 2.3. Extension for 90 seconds at 72°C

**Step 3:** Final elongation for 10 minutes at 72°C

### 3.9.2. Expected *Shigella* PCR results

The amplicon (PCR product) generated from *Shigella* spp. *ipaH* gene sequences by this PCR method is a double stranded DNA fragment of 700 bp
length. Therefore, a positive PCR test should yield a 700 bp DNA fragment which would appear as an intense band on an ethidium bromide-stained agarose gel. The molecular size of the band can be verified by comparing its migration to that of a DNA molecular size marker (e.g., 100 bp ladder DNA) run on the same gel.

A negative PCR test normally will not produce any visible bands in the ethidium bromide-stained agarose gel.

A 700 bp band should appear for the positive control. Absence of a positive control band invalidates the test and the samples should be re-analyzed.

Any test sample showing a distinct band of 700 bp was considered as positive.

3.10. Detection of *Salmonella* by PCR

5 uL (~ 200 ng) of the prepared DNA template was added to 45 uL of PCR reaction mixture (Table 3.4) in 0.2 ml thin walled microfuge tube.

Microfuge tubes were then placed in a thermal cycler and PCR amplification was done according to the program described below.

DNA was amplified according to reaction conditions published for each primer pair. The annealing temperatures for each primer and the number of cycles are shown below.

*Table 3.4. *Salmonella* PCR reaction mixture for 16S rDNA gene*

<table>
<thead>
<tr>
<th>Reagents</th>
<th>PCR reaction mixture</th>
<th>Initial concentration</th>
<th>Volume (μl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer</td>
<td>10 X</td>
<td>5.0</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25 mM</td>
<td>4</td>
<td>2.0 mM</td>
<td></td>
</tr>
<tr>
<td>dNTPs</td>
<td>20 mM</td>
<td>0.25</td>
<td>0.1 mM</td>
<td></td>
</tr>
<tr>
<td>Primer 1 16S-5*</td>
<td>100 μM</td>
<td>0.5</td>
<td>1.0 μM</td>
<td></td>
</tr>
<tr>
<td>Primer 2 16S-3*</td>
<td>100 μM</td>
<td>0.5</td>
<td>1.0 μM</td>
<td></td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5 U/μL</td>
<td>0.4</td>
<td>2.0 U</td>
<td></td>
</tr>
<tr>
<td>Template DNA</td>
<td>2 μg</td>
<td>5</td>
<td>200 ng</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>34.35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Sequence of the primers is given in Table 3.1.
Upon completion of PCR, the products were analysed by 2.0 % agarose gel electrophoresis, otherwise, the amplicons can be stored at 4°C until analysis. Presence of *Salmonella* specific DNA should yield a band of 574 bp size.

### 3.10.1. Temperature cycling program

The thermal cycler program was set as follows:

**Step 1:** Denaturation for 3 minutes at 95°C

**Step 2:** 35 cycles of:

- Step 2.1. Melting for 60 seconds at 95°C
- Step 2.2. Annealing for 60 seconds at 58°C
- Step 2.3. Extension for 90 seconds at 72°C

**Step 3:** Final elongation for 10 minutes at 72°C

### 3.10.2. Expected *Salmonella* PCR results

PCR products were analyzed by electrophoresis with 2.0% (w/v) agarose gel containing ethidium bromide. The gel was visualized with UV illumination and photographed. DNA molecular size standards (100 bp ladder, Promega) were included in each agarose gel.

PCR amplification product was considered positive if the amplicon was the same size (in base pairs) as the product for *Salmonella* (574 bp), and negative if no amplicon was detected and non target if amplicon was detected but its size was different from that obtained for *Salmonella*.

### 3.11. Detection of *E.coli* O157:H7 by PCR

5 uL (~ 200 ng) of the prepared DNA template was added to 45 uL of PCR reaction mixture (Table 3.5) in 0.2 ml thin walled microfuge tube. Microfuge tubes were then placed in a thermal cycler and PCR amplification was done according to the program described below.

DNA was amplified according to reaction conditions published for each primer pair. The annealing temperatures for each primer and number of cycles are given below.

Upon completion of PCR, the products were analyzed by 2.0% agarose gel electrophoresis. Otherwise, the amplicons can be stored at 4°C until analysis. Presence of *E.coli* O157:H7 specific DNA should yield a band of 252 bp size.
Table 3.5. *E. coli* O157:H7 PCR reaction mixture using uidA gene

<table>
<thead>
<tr>
<th>Reagents</th>
<th>PCR reaction mixture</th>
<th>Initial concentration</th>
<th>Volume (μl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer</td>
<td>10 X</td>
<td>5.0</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25 mM</td>
<td>4</td>
<td>2.0 mM</td>
<td></td>
</tr>
<tr>
<td>dNTPs</td>
<td>20 mM</td>
<td>0.25</td>
<td>0.1 mM</td>
<td></td>
</tr>
<tr>
<td>Primer 1 PT2*</td>
<td>100 μM</td>
<td>0.5</td>
<td>1.0 μM</td>
<td></td>
</tr>
<tr>
<td>Primer 2 PT3*</td>
<td>100 μM</td>
<td>0.5</td>
<td>1.0 μM</td>
<td></td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5 U/μL</td>
<td>0.4</td>
<td>2.0 U</td>
<td></td>
</tr>
<tr>
<td>Template DNA</td>
<td>2 μg</td>
<td>5</td>
<td>200 ng</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>34.35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td></td>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Sequence of the primers is given in Table 3.1.

3.11.1. Temperature cycling program

The thermal cycler program was set as follows:

**Step 1**: Denaturation for 3 minutes at 95°C

**Step 2**: 35 cycles of:

- Step 2.1. Melting for 60 seconds at 95°C
- Step 2.2. Annealing for 60 seconds at 55°C
- Step 2.3. Extension for 90 seconds at 72°C

**Step 3**: Final elongation for 10 minutes at 72°C

3.11.2. Expected *E. coli* O157:H7 PCR results

A positive PCR test should yield a 252 bp DNA fragment which would appear as an intense band on an ethidium bromide-stained 2.0% agarose gel. The molecular size of the band can be verified by comparing its migration to that of a DNA molecular size marker (100 bp ladder DNA) run on the same gel.

A negative PCR test normally will not produce any visible bands in the ethidium bromide-stained agarose gel.

The PCR products were visualized with UV illumination and the results were documented by photography.
3.12. Detection of *Campylobacter coli/jejuni* by PCR

The 16S rDNA-based PCR assay was used for detecting for *C. jejuni* and *C. coli*. A primer pair was designed for co-identification of the two species (*C. jejuni* and *C. coli*). The sequence of the forward primer, termed CCCJ609F and the sequence of the reverse primer, termed CCCJ1442R are indicated in Table 3.1.

The primer pair was employed in a PCR with an annealing temperature of 58°C, generating an amplicon of 854 bp either *C. jejuni* or *C. coli*, but not from other strains of *Campylobacter*.

5 uL (~ 200 ng) of the prepared DNA template was added to 45 uL of PCR reaction mixture (Table 3.6) in 0.2 ml thin walled microfuge tube.

Microfuge tubes were then placed in a thermal cycler and PCR amplification was done according to the program described below.

Upon completion of PCR, the products were analysed by 2.0 % agarose gel electrophoresis. Otherwise, the amplicons can be stored at 4°C until analysis. Presence of *Campylobacter c/j* specific DNA should yield a band of 854 bp.

Table 3.6. *Campylobacter c/j* PCR reaction mixture for 16S rDNA gene

<table>
<thead>
<tr>
<th>Reagents</th>
<th>PCR reaction mixture</th>
<th>Initial concentration</th>
<th>Volume (μl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer</td>
<td>10 X</td>
<td>5.0</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25 mM</td>
<td>4</td>
<td>2.0 mM</td>
<td></td>
</tr>
<tr>
<td>dNTPs</td>
<td>20 mM</td>
<td>0.25</td>
<td>0.1 mM</td>
<td></td>
</tr>
<tr>
<td>Primer 1 CCCJ609F*</td>
<td>100 μM</td>
<td>0.5</td>
<td>1.0 μM</td>
<td></td>
</tr>
<tr>
<td>Primer 2 CCCJ1442R*</td>
<td>100 μM</td>
<td>0.5</td>
<td>1.0 μM</td>
<td></td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5 U/uL</td>
<td>0.4</td>
<td>2.0 U</td>
<td></td>
</tr>
<tr>
<td>Template DNA</td>
<td>2 ug</td>
<td>5</td>
<td>200 ng</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>34.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td></td>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Sequence of the primers is given in Table 3.1.
3.12.1. Temperature cycling program
The thermal cycler program was set as follows:

**Step 1:** Denaturation for 3 minutes at 95°C

**Step 2:** 35 cycles of:
Step 2.1. Melting for 60 seconds at 95°C
Step 2.2. Annealing for 60 seconds at 59°C
Step 2.3. Extension for 90 seconds at 72°C

**Step 3:** Final elongation for 10 minutes at 72°C

3.12.2. Expected *Campylocacter* PCR results
The amplicon (PCR product) generated from *Campylobacter c/j* spp. 16S rDNA gene should yield a fragment of 854 bp length. Therefore, a positive PCR test should yield a 854 bp DNA fragment which would appear as an intense band on an agarose gel. The molecular size of the band can be verified by comparing its migration to that of a DNA molecular size marker (e.g., 100 bp ladder DNA) run on the same gel.

A negative PCR test does not normally produce any visible bands in the agarose gel.

A 854 bp band should appear for the positive control. Absence of a positive control band invalidates the test and the samples should be re-analyzed.

Any test sample showing a distinct band at 854 bp was considered as positive.
CHAPTER 4
RESULTS

The study focused on detection and identification of some potential enteric pathogens causing diarrhea (viral, bacterial, and parasites) in 150 children less than 5 years of age in Gaza, Palestine. The results of the study can be summarized as follows:

4.1. Age group distribution of the study population

Among the study population a clear higher incidence of diarrhea was observed in the age groups from 0 to 2 years old subjects. Infants below 12 months of age were particularly affected, accounting for 95 (63.3%) of the cases and the 13-24 months old constituted 31 (20.7%) of the cases Table 4.1.

Table 4.1. Age group distribution of the study subjects

<table>
<thead>
<tr>
<th>Age group (months)</th>
<th>Number of subjects</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-12</td>
<td>95</td>
<td>63.3%</td>
</tr>
<tr>
<td>13-24</td>
<td>31</td>
<td>20.7%</td>
</tr>
<tr>
<td>25-36</td>
<td>8</td>
<td>5.3%</td>
</tr>
<tr>
<td>37-48</td>
<td>7</td>
<td>4.7%</td>
</tr>
<tr>
<td>49-60</td>
<td>9</td>
<td>6.0%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>150</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>

Most of the children 59.3% (89 of 150) who had diarrhea and were admitted to the hospital came from the Gaza region (Table 4.2), and 38.0 % (57 of 150) were from Northern Gaza strip and the rest were from Mid zone and Southern Gaza strip.
Table 4.2. Distribution of the specimens in terms of residence area of the subjects.

<table>
<thead>
<tr>
<th>Area</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern Gaza strip</td>
<td>57</td>
<td>38.0 %</td>
</tr>
<tr>
<td>Gaza</td>
<td>89</td>
<td>59.3 %</td>
</tr>
<tr>
<td>Mid zone and Southern Gaza strip</td>
<td>4</td>
<td>2.7 %</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>100 %</td>
</tr>
</tbody>
</table>

4.2. Prevalence of Enteropathogens

Using a combination of traditional and molecular diagnostic techniques, we detected one or more enteropathogen in 77 cases (51.3%) of the diarrhea specimen examined. The total number of various pathogens identified was 94. Table 4.3 shows the distribution of pathogens found in stools of the 150 children with diarrhea. Rotavirus was the most frequently encountered pathogen; it was present in 42 cases (28.0% of the diarrheal cases) as detected by an immunochromatographic method (Figure 4.7). *Shigella* species were found in 9 cases (6.0%) by PCR (Figure 4.3) and in only 6 cases (4.0%) by bacteriological culture, with higher frequency of *S. flexneri* (3/6) in relation to *S. sonnei* (2/6), and *S. boydi* (1/6). *Salmonella* spp. was found in 3 cases (2.0 % of diarrheal cases) as revealed both by PCR (Figure 4.4) and bacteriological culture. *E coli O157:H7* was found in 7 cases (4.7%) by PCR (Figure 4.5), and in 6 (4.0%) of the diarrheal cases by bacteriological culture while *Campylobacter* was evident in 7 (4.7%) of the diarrheal cases as revealed by PCR (Figure 4.6).

Microscopical parasite examination showed that 23 cases (15.3%) of the diarrheal specimens contained *E. histolytica/dispar*. *G. intestinalis* was found in 2 cases (1.33%), and *Strongyloides stercoralis* was found in only one case (0.7%).
Table 4.3. Enteropathogens identified in the 150 studied specimens

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Number and percentage of positive samples by</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR</td>
<td>Culture</td>
<td>Immuno-</td>
<td>Microscopical</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>chromatography</td>
<td>Examination</td>
<td></td>
</tr>
<tr>
<td>Salmonella spp</td>
<td>3 (2.0)</td>
<td>3 (2.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shigella spp</td>
<td>9 (6.0)</td>
<td>6 (4.0)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.coli O157:H7</td>
<td>7 (4.7)</td>
<td>6 (4.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campylobacter j/c</td>
<td>7 (4.7)</td>
<td>ND**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotavirus</td>
<td>42 (28.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.histolytica/dispar</td>
<td></td>
<td>23 (15.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giardia intestinalis</td>
<td></td>
<td>2 (1.33)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strongyloides stercolaris</td>
<td></td>
<td>1 (0.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 3 cases of the Shigella spp were S. flexneri, 2 were S. sonni, and 1 was S. boydi, no cases of S. dysenteriae were encountered.

** Not determined.

Figure 4.1. Frequency of the identified enteropathogens
4.3. PCR Results

The following Figures (Figures 4.3 to 4.6) represent the PCR results for *Shigella sp*, *Salmonella sp*, *E. coli* O157:H7, and *Campylobacter c/j*, respectively. Figure 4.2 represents the quality of the isolated DNA.

**Figure 4.2.** Gel electrophoresis for DNA extracted from stool samples by AccuPrep Stool DNA Extraction Kit (11 stool sample).

**Figure 4.3.** Amplification products of *Shigella* specific invasion plasmid antigen H locus DNA by PCR. Amplified products of each reaction were analyzed by electrophoresis on a 2.0 % agarose gel. Lane 1: 100-bp DNA ladder; lane 2: positive control, lane 4 negative control and lane 3: PCR amplified DNA extracted from a stool sample positive for *Shigella*, showing the 700 bp product.

**Figure 4.4.** The 16S rDNA gene PCR assay specific for *Salmonella species*.
In Figure 4.4 Ethidium bromide-stained agarose gel (2%) showing PCR products (574 bp product) for *Salmonella* spp. Lanes 1 and 15: 100-bp DNA ladder; lane 2: positive control, lanes 3 and 14: negative control, lanes 4 to 13 tested sample, and Lane 13, a stool samples positive for *Salmonella* showing the 574 bp product.

**Figure 4.5.** PCR for identification of *E.coli* O157:H7 targeting *uidA*. All positive samples yielded an amplicon size of 252 bp. Lane 1: 100-bp DNA ladder; Lane 2: a negative control; Lane 3: a blank, lane 4: a positive control, Lane 5: an amplicon (252 bp) from DNA extracted directly from a stool sample.

**Figure 4.6.** 16S rDNA-based PCR assay specific for *C. jejuni* and *C. coli*. PCR assay yields an 854 bp product, resolved on a 2 % agarose gel. Lane 1: 100 bp DNA ladder; lane 2: negative control, lane 3 positive control; lanes 4 to 6 stool samples showing positive samples in lanes 5 and 6 with the 854 bp product.
4.4. Comparison of Bacteriological Stool Culture, and PCR Assay

Table 4.4 presents a comparison of the detection rate of three bacterial enteropathogen by conventional culture, and PCR techniques. *Shigella spp* was detected in 4.0% and in 6.0% of the samples when analyzed by the culture and PCR, respectively. By the use of PCR we found that 3 of samples were *Shigella spp* positive by PCR but not by culture. *E coli O157:H7* was detected in 4.0% and in 4.7% of the samples when analyzed by the bacteriological and PCR, respectively. By use of PCR we found one sample was *E coli O157:H7* positive by PCR while it was not resolved by culture. *Salmonella spp* was detected in 2.0% of the specimens both by culture, and PCR. In total we found 4/19 (21.1%) enteropathogen positive specimens by PCR that the bacteriological stool culture revealed negative.

**Table 4.4.** Comparison between bacteriological stool culture, and PCR assay in detecting three bacterial enteropathogens.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Assay compared</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture positive</td>
<td>PCR positive</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td><em>E coli O157:H7</em></td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>15</td>
<td>19</td>
</tr>
</tbody>
</table>

The presence of Rotavirus in stool specimens was examined using the Rotastick immunochromatographic method, Figure 4.7 below illustrates the interpretation of the results.

**Figure 4.7.** Interpretation of rotavirus results
(a: negative result, b: positive result, C: control window, T: test window)
4.5. Mixed Infections

Mixed infections were relatively frequent and as indicated in Tables 4.5, five of the cases were positive for both parasite and rotavirus, one case contained both *Campylobacter* and rotavirus; one case showed *Shigella spp* and rotavirus; one case of infections by *Salmonella* and rotavirus, one case of *Campylobacter* and parasites, five specimens were positive for *Shigella* and parasites, and two of the specimens were infected with *E. coli* O157:H7 and parasites.

**Table 4.5.** Occurrence of mixed infections by enteric pathogens

<table>
<thead>
<tr>
<th>Patterns of infection</th>
<th>Mixed infection</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percentage</td>
<td></td>
</tr>
<tr>
<td>Rotavirus + parasite</td>
<td>5</td>
<td>3.3%</td>
<td></td>
</tr>
<tr>
<td>Rotavirus + <em>Campylobacter</em></td>
<td>1</td>
<td>0.7%</td>
<td></td>
</tr>
<tr>
<td>Rotavirus + <em>Shigella</em></td>
<td>1</td>
<td>0.7%</td>
<td></td>
</tr>
<tr>
<td>Rotavirus + <em>Salmonella</em></td>
<td>1</td>
<td>0.7%</td>
<td></td>
</tr>
<tr>
<td>Parasite + <em>Campylobacter</em></td>
<td>2</td>
<td>1.4%</td>
<td></td>
</tr>
<tr>
<td>Parasite + <em>Shigella</em></td>
<td>5</td>
<td>3.3%</td>
<td></td>
</tr>
<tr>
<td>Parasite + <em>E. coli</em> O157:H7</td>
<td>2</td>
<td>1.4%</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>17</td>
<td>11.3%</td>
<td></td>
</tr>
</tbody>
</table>

In Table 4.6 the age groups 25-36, and 37-48 months were more susceptible to infection with enteropathogens, evidently, there were 8 children in the 25-36 age group who were infected with 8 enteropathogens, and 7 children in the age group 37-48, who had been infected with 10 enteropathogens.

**Table 4.6.** Occurrence of mixed infections by age group

<table>
<thead>
<tr>
<th>Age group (months)</th>
<th>Number of subjects</th>
<th>Total Positive</th>
<th>Mixed infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-12</td>
<td>95</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>13-24</td>
<td>31</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>25-36</td>
<td>8</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>37-48</td>
<td>7</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>49-60</td>
<td>9</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>150</td>
<td>94*</td>
<td>17</td>
</tr>
</tbody>
</table>

*The total number of enteropathogens identified in 77 positive cases.
4.6. Occurrence of Enteropathogens among the different age groups

We found that 75.5% of positive diarrheal cases occurred in the age groups below 2 years (53.2% in the age group 0-12 months, and 22.3% in the age group 13-24 months). Rotavirus was the highest among infants in the age group 13-24 months old 13/31 (41.9%), followed by the 0-12 month’s age group with 25/96 (26.0%). The other pathogens were distributed among all the age groups, but their relative frequencies cannot be defined in view of the low number of diarrheal cases. Occurrence of enteropathogens among the different age groups enrolled in the study is provided in Table 4.7.

Table 4.7. Enteropathogens encountered among the different age groups

<table>
<thead>
<tr>
<th>Enteropathogen</th>
<th>Age groups</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-12</td>
<td>13-24</td>
<td>25-36</td>
<td>37-48</td>
<td>49-60</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>25 (60.0%)</td>
<td>13(31.0%)</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Salmonella</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Shigella</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>E.coli O157:H7</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Campylobacter</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Parasites</td>
<td>12</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Total positive and percentage</td>
<td>50 (53.2%)</td>
<td>21 (22.3%)</td>
<td>8 (8.5%)</td>
<td>10 (10.6%)</td>
<td>5 (5.4%)</td>
</tr>
<tr>
<td>Total samples</td>
<td>150</td>
<td>95*</td>
<td>31</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

* Each mixed infection is considered as a single isolate.

4.7. Clinical features and infection

Clinical signs and symptoms recorded by qualified pediatricians at admission are summarized in Table 4.8. Fever was found to occur more frequently in Salmonella, Shigella (100% of the cases) Campylobacter (85.7%), parasites (88.5%), rotavirus (73.8%), and in E.coli O157:H7 (42.8%) infected subjects. Vomiting was a common finding in infections with Salmonella, rotavirus, E.coli O157:H7, parasites, Campylobacter, and in Shigella (100, 92.9, 85.7, 80.8, 71.4 and 66.7 %, respectively).
The dehydration status of the diarrheal children was observed more common in rotavirus infection, (14.3%), followed by Shigella (11.1%), and E.coli O157:H7 (11.1%).

**Table 4.8. Clinical symptoms in relation to infections in 150 children with diarrhea**

<table>
<thead>
<tr>
<th>Enteropathogen</th>
<th>Clinical symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fever n (%)</td>
</tr>
<tr>
<td>Rotavirus (n = 42)</td>
<td>31 (73.8)</td>
</tr>
<tr>
<td>Salmonella (n = 3)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>Shigella (n = 9)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>E.coli O157:H7 (n = 7)</td>
<td>3 (42.8)</td>
</tr>
<tr>
<td>Campylobacter (n = 7)</td>
<td>6 (85.7)</td>
</tr>
<tr>
<td>Parasites (n = 26)</td>
<td>23 (88.5)</td>
</tr>
</tbody>
</table>

4.8. Enteropathogens distributed by residence of the subjects

Most 59.6 % (56/94) of the fecal samples tested positive for enteropathogens were from Gaza region while 40.4 % were from outside Gaza (39.4 % from Northern Gaza strip, 1.0% from Mid zone and Southern Gaza strip) Table 4.9. Moreover, the distribution of rotavirus by residence was statistically significant (p<0.05)

**Table 4.9. Enteropathogens distributed by residence**

<table>
<thead>
<tr>
<th>Enteropathogen</th>
<th>Area</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Northern Gaza Strip</td>
<td>Gaza</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>9</td>
<td>32</td>
</tr>
<tr>
<td>Salmonella</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Shigella</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>E.coli O157:H7</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Campylobacter</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Parasites</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Total Positive (%)</td>
<td>37 (64.9%)</td>
<td>56 (62.9%)</td>
</tr>
<tr>
<td>Total tested sample</td>
<td>57</td>
<td>89</td>
</tr>
</tbody>
</table>
4.9. Antimicrobial Susceptibility of *Shigella* and *Salmonella* isolates

The susceptibility of isolated *Salmonella* and *Shigella* species to various antimicrobial drugs was determined by the disk diffusion method following the recommendations of the NCCLS. The antimicrobial agents used and the susceptibility results of the isolates are shown in Table 4.10.

**Table 4.10. Antibiotic susceptibility of *Salmonella* and *Shigella* isolates**

<table>
<thead>
<tr>
<th>Organism</th>
<th>AM</th>
<th>PI</th>
<th>CN</th>
<th>CM</th>
<th>CAZ</th>
<th>RO</th>
<th>EC</th>
<th>ME</th>
<th>AK</th>
<th>GN</th>
<th>DO</th>
<th>C</th>
<th>SXT</th>
<th>NA</th>
<th>CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Shigella flexneri</em></td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Shigella sonni</em></td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Shigella sonni</em></td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Shigella boydii</em></td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella</em> spp</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td><em>Salmonella</em> spp</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella</em> spp</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td><strong>Percentage of resistance</strong></td>
<td>77.8</td>
<td>44.4</td>
<td>33.3</td>
<td>22.2</td>
<td>11.1</td>
<td>11.1</td>
<td>22.2</td>
<td>0.0</td>
<td>0.0</td>
<td>22.2</td>
<td>88.8</td>
<td>33.3</td>
<td>77.8</td>
<td>33.3</td>
<td>11.1</td>
</tr>
</tbody>
</table>

**S:** sensitive, **R:** resistance

Ampicillin (AM), Pipracillin (PIP), Cephalexin (CN), Cefuroxime (CM), Ceftazidim (CAZ), Ceftriaxone (CRO), Cefaclor (CEC), Meropenem (MEM), Amikacin (AK), Gentamicin (GN), Doxycycline (DO), Chloramphenicol (C), Trimethoprim/sulfamethoxazole (SXT), Nalidixic acid (NA), and Ciprofloxacin (CIP).

Antimicrobial susceptibility testing of *Salmonella* and *Shigella* strains showed that 77.8% of the isolates were resistant to Ampicillin, 44.4% to Pipracillin, 33.3% to Cephalexin, 22.2% to Cefuroxime, 11.1% to Ceftazidim, 11.1% to Ceftriaxone, 22.2% to Cefaclor, 22.2% to Gentamicin, 88.8% to Doxycycline, 33.3% to Chloramphenicol, 77.8% to Septrin, 33.3% to Nalidixic Acid and 11.1% to Ciprofloxacin. All isolates were sensitive to Meropenem, and Amikacin (Figure 4.8).
It’s important to notice here that Doxycycline and Ciprofloxacin are contraindicated for children.

Figure 4.8. Antibiotic resistance for Salmonella and Shigella isolates.
Chapter 5
DISCUSSION

Diarrhea remains one of the most common illnesses of children and one of the major causes of infant and childhood mortality in developing countries. Considering the usually scanty resources available in the third world countries, a reduction in diarrhea-related mortality may be possible by identifying high risk subjects and targeting them for intensive intervention. In the present study, we were able to identify most of the etiologic agents significantly associated with early childhood diarrhea.

The aim of this study was to determine the prevalence of enteropathogens, including bacteria, rotavirus, and parasites, causing diarrhea among children less than five years old in Gaza, Palestine.

To our knowledge, this is the first study in Gaza investigating several kinds of possible enteric pathogens in diarrhea in children less than 5 years of age. The study emphasizes the role of rotavirus, Salmonella, Shigella, Campylobacter coli/jejuni (c/j), E coli O157:H7 and Parasites in the study group.

This new knowledge on the etiology of diarrhea in the examined patients will help us plan future studies on various aspects of diarrheal diseases in this population.

Out of the 150 diarrheal patients enrolled in the study, 63.3% were less than 1 year of age, and 83.9% were less than 2 years of age. This shows a strong tendency of diarrhea to occur among children less than 2 years of age. This finding is consistent with other studies conducted in the middle east countries and various other developing countries where the major burden of diseases due to most pathogens occurred in the first and second years of life (1,46,127,164).

Prevalence of enteropathogens: According to the current study, the percentage of examined diarrheal cases with a known etiology is 51.5%. When compared to other studies, this percentage is still lower than that reported by Nimri and Meqdam (2004) in Jordan where 77.8% of their samples had a potential enteric pathogen, Souza et al. (2002) in Sao Paulo where 72.8% of their samples were positive, Albert et al. (1999) in Bangladesh where 74.8% of the samples had enteropathogens, and Youssef et al. (2000) in Jordan where 66.4% of the samples showed enteric pathogens.
However, our percentage is higher than that reported by Battikhi (2002) in Jordan where he recorded 24.5% as enteropathogens positive, Na'was and Abo-Shehada (1991) in Jordan where they found 39.5%, and Khan et al., (2002) in Bangladesh where 29.5% of the samples had potential enteric pathogens.

Meanwhile our finding is nearly congruent with that of other studies, such as that of El-Sheikh and El-Assouli (2001) in Saudi Arabia where they reported that 45.6% of their samples had potential enteric pathogens, and Yoshida et al. (2000) in Japan where, 46.2% of their samples contained enteropathogens.

These variations could be explained by differences in the study population (e.g., genetics of the different ethnic groups), endemicity of enteropathogens, socioeconomic and specific practices prevalent among the different populations. In principal, enteric pathogens in feces infect humans via contaminated fluids, and foods, and personal contact; factors that are associated with the living standards of people.

**Rotavirus is the most common:** In the 150 investigated children with diarrhea, group A rotavirus was the most frequently identified enteric pathogen, where it was found in 28.0% of the children with diarrhea versus 17.3%, of those with bacteria. However, parasites played another important role and represented 17.3% of the enteropathogens. These figures are in agreement with those reported in many studies on diarrhea (192). Moreover, mixed infections were relatively frequent, as 11.3% of the specimens were found to contain more than one enteropathogen (68,70).

The high incidence of rotavirus recorded in the present study conforms to other recent investigations, which showed an increasing rate of this virus as a causing agent of diarrhea, even in developing countries (41). For example, rotavirus was the most frequent enteropathogen (15 to 40%) encountered in three studied regions neighboring Palestine namely, Jordan, Egypt, and the occupied territories (42,188,192).

Timely diagnosis of rotavirus infection in patients with acute diarrhea helps determine appropriate treatment, prevents the unnecessary use of antibiotics and minimizes the spread of the disease (123,143).
In the current study the prevalence of rotavirus infection was significantly higher in the children less than 2 years of age than in the older ones. The highest prevalence was seen in children from 13 to 24 months of age 13/31 (41.9%), followed by those less than 1 year of age 25/95 (26.3%), and the prevalence decreased in the older children. This result is similar to that published by other authors (19,80,165).

There is a difference in the age distributions of rotavirus infections in developing and developed countries. In the former, the highest rates occur during the first year of life. However, in developed countries the peak rates of infection occur in the second year of life. This could lead to recommending early application of rotavirus vaccine to children in developing countries (131). Moreover, our study indicated that there was a trend of decreasing rates of rotavirus infection in the older children. This might partly be explained by the fact that older children acquired protective immunity during previous, probably subclinical, exposures to rotavirus and they therefore become more resistant to infection with this agent (76,105). Our results emphasize the importance of considering rotavirus vaccines, which have been undergoing field trials for several years (19,80,165).

Although, many recent studies on rotavirus were done in countries neighboring Palestine, no studies on this virus have been carried out in Gaza since 1994 (151).

While numerous studies have demonstrated rotavirus to be a common cause of diarrhea among young children living in developing nations, most of the studies have been hospital-based, which potentially detect only the more severe cases of diarrhea, and thus may underestimate the true incidence of rotavirus associated diarrhea (19,80). Compared with the community-based study of rotavirus conducted on Palestinian children in Gaza by Sallon et al. (1994) and Simhon et.al. (1990), rotavirus was isolated nearly four times less frequently than in the present study.

Interestingly, incidence of rotavirus disease was observed to be similar in both industrialized and developing countries, suggesting that adequate control may not be achieved by improvements in water supply, hygiene, and sanitation. Thus, the development, trial, and widespread use of rotavirus vaccines were recommended to prevent severe and fatal rotavirus disease (137).
The current study was done during the late spring and summer seasons, rotavirus infections however, are known to be more common in the winter season, consequently the true prevalence of rotavirus may be well over than 28.0% if the study has been done during the cold season, and this further potentiates the importance of rotavirus etiology in diarrhea (49,105,154).

**Parasites:** *E histolytica/dispar* was the second most common enteric pathogen found in the present study, the isolation rate was 15.3%, which indicates the importance of this pathogen among the diarrheal infectious factors. Meanwhile our finding is nearly congruent with that of other studies, for example Shubair et al. (2000) found that the prevalence of *Entamoeba histolytica* among school children in Gaza was (18.0%), and also in Gaza, AL-Zain and Al-Hindi (2005) found that *Entamoeba histolytica/dispar* was evident in 11.4% in the less than 4 years old children. The isolation rate was (1.33%) for *Giardia intestinalis* and (0.7%) for *Strongyloides stercoralis* (11,159).

**Bacterial enteropathogens:** *Shigella* was the third most common enteric pathogen identified in the present study, the isolation rate was 6.0%, followed by *Campylobacter cij* (4.7%), *E coli O157:H7* (4.7%), and *Salmonella* (2.0%).

The enteropathogens thus identified reflect the importance of these microorganisms in the epidemiology of childhood diarrhea in our region and suggest that either these enteropathogens are more common in our environment or that they are associated with more severe presentations, as patients with more severe disease are more likely to seek medical help.

The infections with bacterial, parasitic and some viral pathogens peaked during the summer time when there are suitable conditions such as humidity and high temperature which facilitate the bacterial growth and dissemination.

Intestinal nematodes do not seem to play a significant role in diarrheal diseases in the study area. The low rate of nematodes isolated from the investigated patients suggests a low incidence of those pathogens in the Gaza region particularly for patients at this age.
Mixed infections: Mixed infections were relatively frequent, among the children with diarrhea where 17 out of the 150 (11.3%) studied children had infection with two or more pathogens. Several previous studies have shown mixed infections in stool samples from children with diarrhea, including various combinations of different types of enteropathogens (46,164). In our study, most of the mixed infections were rotavirus with parasite (3.3%), and Shigella with parasites (3.3%). These findings are in agreement with other studies conducted in developing countries that reported one or more of these pathogens (21,129,136,192). Improvement of the techniques and methods used in the detection of enteropathogens may justify the high rates of detection of mixed infections.

Children 3- 4 years old are more susceptible to infection: According to our results the age groups 25-36, and 37-48 months were more susceptible to infection with enteropathogens, evidently, there were 8 children in the 25-36 age group who were infected with 8 enteropathogens, and 7 children in the age group 37-48, who had been infected with 10 enteropathogens. The highest levels of incidence of most enteropathogens in these two age groups could be due to several reasons such as: the increased prevalence of enteropathogens in the environment, increased exposure of those children to environmental pathogens, the unaware consumption of contaminated foods and drinks, the relative ease of infection by enteropathogens through the fecal-oral route of transmission and the bad hygiene practices of children particularly at those ages. However, the parental and the social habits of increased attention to younger children may partially explain the bias observed in the enteropathogen distribution of the younger age groups. Looking at children up to 5 months of age, the prevalence of enteropathogens were 9 cases with rotavirus, 2 cases with parasites, one case with Campylobacter, and there was no Shigella, Salmonella or E coli O157:H7 recorded. The low rate of occurrence of bacterial enteropathogens in early infancy (the first 5 months of life) may be due to breastfeeding, the breastfed infants are less
likely to develop bacteria-associated diarrhea, is well documented in the literature (4,97,176).

In addition to the age distribution of enteropathogenic infections, many studies have indicated a higher ratio of infected males as compared to females. In our study the demographic information, epidemiological data and clinical symptoms show that among the 150 children, the male/female ratio of children with respect to diarrhea was 1.24, no reasonable explanation has yet been given for this distribution, but it is possible that the cultural or behavioral norms in our study area are contributing factors. For example, it is a common practice in many families to preferentially seek medical care for boys. The genetics of males, however, may be another contributing factor in the higher susceptibility of males to get enteropathogenic infections (44,68,130).

**Enteropathogens in Gaza region:** Most of the patients (89/150; 59.3%) admitted with acute gastroenteritis were from the Gaza region, 38.0% (57/150) were from Northern Gaza Strip, and 2.7% (4/150) were from Mid zone and Southern Gaza Strip. It is important to note that there is no pediatric hospital in Northern Gaza Strip. The low number of cases admitted from the mid zone and Southern Gaza Strip is due to the continuous closures and separation of Gaza from the Southern area by the Israeli occupation during the study period, and the presence of some hospitals in that area.

Our results show that the prevalence of enteropathogens identified in diarrhea specimens was comparable in Northern Gaza Strip (64.9%), and Gaza region (62.9%).

These high percentages and as pointed earlier, may indicate that either the enteropathogens are quit prevalent in those environments or that they are associated with more severe presentations.

The present study revealed that rotavirus was the microorganism associated most frequently (36.0%) with gastroenteritis in the Gaza region, followed by parasites and bacteria (13.5% each).

Many authors showed agreement with our results that rotavirus infects children in urban areas more than the rural areas (112,136).
Therefore, the reason of increased rotavirus prevalence in Gaza region might be due to nosocomial infections in neonatal nurseries, particularly present more in urban areas than rural ones, especially when mothers spend most of their time outside the home (worker female), and where the management of rotavirus infection is difficult.

**Enteropathogens in Northern Gaza Strip:** In Northern Gaza Strip we found that parasites and bacteria were the most common pathogens (24.6% each) followed by rotavirus (15.8%). Differences in the frequency of enteropathogens in both regions may be due to the different living standards, educational level, and the agricultural practices. Another important factor is the contaminated drinking water which represents a potential source of infection (132). Furthermore, the practice of dumping solid wastes in the streets and raising animals in the living area, as it is common to see children playing in and around waste disposal sites in Gaza Strip, can certainly cause serious health problems (5). Additionally, flies, other insects and rats are important vehicles in spreading infectious agents from rotting and contaminated wastes (82).

**Why do children attend hospitals for investigation?** The reasons for children enrolled in this study to attend the hospital for examination were: (i) diarrhea only, (ii) diarrhea with vomiting, (iii) diarrhea and fever, and (iv) diarrhea together with vomiting and fever. Although most of the children received oral rehydration fluid before hospitalization, some of them were still dehydrated. Dehydration was more common in children with rotavirus diarrhea. The most common risks with diarrheal illnesses are dehydration and, in developing countries, malnutrition. Thus, the critical initial treatment must include rehydration, which can be accomplished with an oral glucose or starch containing electrolyte solution in the vast majority of the cases (129).

**Clinical signs of gastroenteritis:** It is not possible to distinguish diarrhea caused by most enteropathogens clinically, because diarrhea, vomiting, fever, and dehydration are not absolutely associated with any of these enteropathogens infections.
Watery stools were predominant (67.3%), followed by mucous stools (21.4%). Other types of stool accounted for 11.3% of the cases. Children singly infected with *Shigella, or Salmonella* had fever with average temperature significantly higher than that of those infected with rotavirus or other enteropathogens. Fever has been shown to be a common symptom in diarrhea caused by *Shigella* and *Salmonella* by other authors (15,51). Our study supports the conclusions from other studies that enteropathogens induce a clinical illness characterized by vomiting, diarrhea, fever, and dehydration, or various combinations of these symptoms (19,129,164,192).

**Antimicrobial agents**: Antibiotic therapy is not appropriate in the management of simple gastroenteritis even when a bacterial cause is suspected because most cases of acute diarrhea are of viral etiology or self-limited. Antibiotics are recommended for treating bloody diarrhea to shorten the duration of illness, decrease morbidity and mortality, and reduce the duration of bacterial shedding (59,148). Antimicrobial resistance among the major bacterial causes of bloody diarrhea is increasing worldwide (186). Despite this, a recent multicenter study from Europe showed that 44% of physicians would use antibiotics (43). Furthermore, some studies suggest that the empiric use of antibiotics in children with *E coli* O157:H7 infections may increase the risk of hemolytic uremic syndrome (HUS) (30,139).

In spite of the relatively small size of the study sample, we believe that our data provide useful information about the antimicrobial resistance of *Shigella* and *Salmonella* isolates. In order to help practitioners to choose an adequate antimicrobial drug to start empirical therapy in a patient with severe diarrhea without knowledge of a specific pathogen, we assessed the antimicrobial resistance patterns of *Shigella* and *Salmonella* spp that could be isolated.

Antimicrobial susceptibility patterns among the 9 bacterial (3 *Salmonella*, 6 *Shigella*) isolates tested from persons with diarrhea were shown in Table 4.10. The antibiograms of *Salmonella* and *Shigella* isolates, showed that the most frequent patterns of resistance were exhibited towards doxycycline (88.8%), ampicillin and trimethoprim/sulfamethoxazole (SXT) (77.8% each), cefatazidim,
ceftriaxone, and ciprofloxacin had the low resistance pattern, whereas all the isolates were sensitive to Meropenem, and Amikacin.

*Shigella* is becoming more resistant to the commonly used antibiotics especially in the developing countries (3,64,149). However, the prevalence of resistance to the same antibiotics is lower in developed countries (15,36). The reason could be the more appropriate usage of antibiotics in the developed as compared to the developing countries.

Several studies have indicated the high resistance of *Salmonella, Shigella* to ampicillin, SXT and doxycyclines which is in agreement with our study (110,129,168).

Multiple antibiotic resistant enteric pathogens have been reported in many developing countries, especially Pakistan, India, Bangladesh, and The Philippines (147).

In most pediatric hospitals the empirical antimicrobial drugs used in the treatment of gastroenteritis and diarrhea patients is the intravenous ampicillin, and the physicians usually prescribe SXT after discharge from the hospital.

The high resistance of our isolates to SXT and ampicillin observed may be because in the primary heath care in Gaza strip, SXT is the most commonly used antimicrobial drug for the treatment of gastroenteritis and diarrhea, while amoxycillin is the most frequently prescribed antibiotic for the treatment of upper respiratory tract infections (URTIs).

As in the developing countries, ampicillin, chloramphenicol and SXT are widely used to treat diarrhea because of their low cost and ready availability. Most children in our community treated with antibiotics had been purchased from private drug outlets.

Our study results show that *Shigella* isolates were completely sensitive to nalidixic acid. Other studies have also found little resistance to nalidixic acid among *Shigella* in many countries (4,28).

The use of nalidixic acid is recommended by the World Health Organization for the management of acute bloody diarrhea in children, and it may remain an important option for the treatment of acute infectious diarrhea, especially in services that cannot afford far more expensive drugs (184).
However, in areas where nalidixic acid has been introduced as the drug of choice to treat presumptive shigellosis, a marked increase in corresponding resistance has been observed (4,15).

Misuse of antibiotics has resulted in increased resistance to most of the commonly used drugs for treatment. A call to regulate the use of antimicrobials may be necessary.

According to our study, ampicillin, trimethoprim/sulfamethoxazole, and doxycycline should not be used for treatment of diarrhea in children. Moreover, it should be taken into consideration that in spite of the low bacterial resistance to amikacin and meropenem, if they were widely used, a rapid emergence of resistance would likely to occur.

According to the results of this study and in very severe cases, with evidence of dissemination of disease, intravenous amikacin or meropenem, cefatazidim, ceftriaxone, and ciprofloxacin is the best choice.

It is worth noting here that it has been recommended by the NCCLs in 1999 that, aminoglycosides and first generation cephalosporins may appear active in vitro but are not effective clinically and should not be reported as susceptible (NCCLs 1999) (126).

In conclusion, the current study highlights the necessity for continuous monitoring of antibiotic resistance in diarrhea related bacterial pathogens.

Some of the above antibiotics were employed to characterize the isolates, but did not have any clinical utility in the treatment of diarrhea.

**Bacterial detection methods:** *Shigella* species were identified in 6 cases (4.0%) by bacteriological culture, *Shigella flexneri* was found with the highest prevalence (50%) among the isolated *Shigella spp*, emphasizing the importance of this pathogen in the epidemiology of childhood bacterial diarrhea in Gaza, and indicating that either *S. flexneri* is more common in our environment or it is more associated with severe diarrheal presentations.

*Shigella flexneri* was the predominant species isolated among *Shigella spp* during this study, followed by *S. sonnei*. This finding is consistent with other reports from developing countries such as India, Bangladesh, Brazil, Tanzania, Egypt, and Thailand. Our results are in good agreement with this trend (148,170).
The prevalence of *Shigella* recorded in this study is similar to that reported by other investigators in many neighboring countries such as Saudi Arabia, Jordan, Lebanon, Kuwait, and Israel (96,120,121,155,193), with the exception of one study from Saudi Arabia which reported that over 17% of the tested specimens were positive for *Shigella* (8).

Our percentage is however higher than the detection rate of Gaza strip health laboratories for *Shigella* which was 0.2 % (117).

Three cases (2%) proved positive for *Salmonella* both by culture and PCR. The detection rate of Gaza strip health laboratories for *Salmonella* is only 0.4 % (117).

Other reports from Gaza and neighboring countries indicate that the detection rate of *Salmonella* ranges from 2% to 18% (8,121,151,155).

The very low detection rate of *Salmonella* and *Shigella* by Gaza strip health laboratories might be due to the low sensitivity of the culture methods employed, since they use only one or two selective agar media (usually SS agar and Selenite-F enrichment broth) for isolation of these bacteria.

Selenite-F broth is not suitable, particularly for isolation of *S. paratyphi A* and *S. choleraesuis*. Additionally, some strains of *Shigella* fail to multiply at all in this enrichment broth (174). Moreover, many studies have pointed that SS agar, could inhibit the growth of many strains of *Shigella* (106,180).

Another factor that may explain the low recovery of *Salmonella* or *Shigella* from stool specimens cultures in Gaza strip health laboratories is the delay in ordering stool cultures since physicians sometimes ask for stool cultures after > 4 days of hospitalization and after children treatment had failed.

The recovery rate of *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. from stool culture has been shown to decrease by more than the quarter when patients have been hospitalized for > 3 days (146).

Additionally, antibiotic therapy prior to specimen collection and delay for > 3 h in delivering some of the specimens to the laboratory for processing could be responsible for the low yield of bacterial cultures.

In this study at least three selective media and two enrichment broths were employed, and in addition to the usual biochemical tests and serotyping, the bacteriological screening was reinforced by a molecular approach i.e., PCR assays.
*E. coli* O157:H7 was found in 6 (4.0%) diarrheal cases by bacteriological culture. This microorganism is not routinely analyzed in our clinical laboratories. *E. coli* O157:H7 has been associated with 10-15% of bloody diarrheas by several investigators (145,157).

Children with gastrointestinal infections caused by *E. coli* O157:H7 are at risk for the hemolytic-uremic syndrome (HUS) which can be fatal as it may lead to acute kidney failure. Furthermore, some studies suggest that the empiric use of antibiotics in children with *E. coli* O157:H7 infections may increase the risk of HUS (52,189). Consequently, the identification of the causative agent of diarrhea may be life-saving.

**PCR is more sensitive and specific than conventional culture methods:** Identification of *Campylobacter* is well known to be problematic, principally because of their complex taxonomy, biochemical inertness, and fastidious growth requirements (74,152).

As a result of the fastidiousness and diversity of *Campylobacter*, it is clear that no one medium will provide an accurate measure of their occurrence. Furthermore, inexperienced personnel can easily overlook *Campylobacter* colonies, and the species may be missed. For this reason, the use of PCR-based detection methods is becoming more attractive.

*Campylobacter c/j* was detected in 4.7% (7/150) of the stool samples when analyzed by PCR assay. It was isolated in Gaza two times more frequently than in the present study (151).

Culture methods are biased toward the detection of *Campylobacter spp.* since a number of antimicrobial agents is commonly incorporated into the selective media (e.g., Preston agar, Skirrow agar, and Butzler agar). These drugs may inhibit the growth of some *Campylobacter* species. Cephalothin, colistin, and polymyxin B can be inhibitory to some strains of *C. jejuni* and *C. coli* (39). As a result, microbiological methods do not provide a true measure of the frequency and diversity of *Campylobacter* species associated with diarrhea.

The etiological agents could not be determined in 73 (48.7%) of the 150 investigated specimens. The causative agents might be bacterial, viral or other infectious agents not considered in this research. Chemicals, oral antibiotics,
stress, food allergies or dietary indiscretion are other factors that may cause diarrhea. Actually the problem of recognizing an associated pathogen in all diarrhea specimens is usually difficult, even during the onset of outbreaks (59,129).

*Shigella spp* was detected in 4.0% and in 6.0% of the stool samples when analyzed by the bacteriological culture and PCR assay, respectively. By the use of PCR we found that 3 of the samples were *Shigella spp* positive by PCR and not by culture. *E coli O157:H7* was detected in 4.0% and in 4.7% of the samples when analyzed by conventional culture method and PCR, respectively. By use of PCR we found one sample of *E coli O157:H7* that was positive by PCR while it was not resolved by culture. *Salmonella spp* was detected in 2.0% of the specimens by both culture, and PCR.

In total we found that 4/19 (21.1%) enteropathogen positive specimens by PCR that the bacteriological stool cultures failed to detect.

PCR is a selective, sensitive, and specific assay that can detect a small number of culturable as well as non-culturable organisms. Such detection is especially important for *Shigellae*, since they can produce disease by as few as 10 to 100 organisms. In our study, the use of PCR technique improved the rate of detecting *Shigellae* in stool samples from 6 to 9 positive samples; which is 33.3% (3/9) higher than the conventional culture method. Moreover, the time requirement of this technique is quite shorter when compared to that of the culture technique.

The assay was extremely reliable, being able to detect 100% of culture-confirmed bacterial infections in the study specimens. Furthermore, it also detected 4 culture negative clinically important gastroenteritis cases, indicating the high level of efficiency of the assay system. Thus, PCR may be judged as superior for its rapidity and sensitivity in the detection of *Shigellae*. Moreover, since this method is applied without cultivation of the organism on synthetic media, nonculturable populations of *Shigellae* can also be detected by this method.

The PCR assay can be chosen as an alternative to the culture technique, and can further be used for identifying asymptomatic carriers who serve as potential reservoirs of *Shigellae*, and silently transmit the disease within the community.
Relative to microbiological isolation, PCR was found to be more sensitive for detecting *Campylobacter* in many studies; the advantages of PCR over conventional isolation methods witnessed in this study are congruent with the conclusions of Lawson et al. who found that direct PCR of human feces provided unique data in detection of *Campylobacter* (94,101).

Our method requires approximately 4 h, from manipulating samples to reading the results. This time includes 1 h for DNA extraction from the stool specimens and 2-3 h for the PCR assay as compared to 48-72 hours of standard stool culture. It detected pathogens in culture negative stool specimens derived from patients who had been linked to gastroenteritis or a food poisoning outbreak. Hence, we conclude that this PCR-based method contributes to improvement of the rapid diagnosis of enteric bacterial infections, whilst yielding higher detection rates of causative agents (71).

Other advantages include its high sensitivity, specificity and ease of operation, furthermore, no radio-isotopes or their dangerous concomitants are involved. Two problems are associated with the detection of bacteria in stool by PCR: stool matrices may inhibit the PCR, and the PCR cannot distinguish the DNA of living cells from that of dead cells. To overcome these two problems, several investigators have used DNA isolation and purification methods using a modified procedures such as a ready kits for isolation, and various enrichment culture methods (63,69,124,182).

We believe that enrichment culture methods may be helpful in distinguishing living cells from dead cells. Therefore, we applied a short enrichment culture step (3-4 hours) with BHI (brain heart infusion broth) medium prior to PCR detection of *Salmonella*, *Shigella*, and *E. coli* O157:H7 (181).

Bacterial DNA was prepared by a simple DNA extraction method with the use of a ready-to-use kit in order to reduce the potential inhibitory effects of PCR by stool matrices.

Finally the applications of the techniques such as PCR, immunoassay, and culture will improve the quality of detection of the pathogens. This will make the clinical diagnosis better and help the pediatricians in treatment of children with diarrhea.
The present study focused on detection and identification of some potential enteric pathogens causing diarrhea (viral, bacterial, and parasites) in 150 children less than 5 years of age in Gaza, Palestine. The results of the study can be summarized as follows:

1. Analysis of data according to age showed a significant association with diarrhea for all enteropathogens in the first two years of life except for *Shigella*, which had an overall association with diarrhea in older age group.

2. We found that 75.5% of positive diarrheal cases occurred in the age groups below 2 years (53.2% in the age group 0-12 months, and 22.3% in the age group 13-24 months). Rotavirus was the highest (13/31, 41.9%) among infants in the age group 13-24 months old.

3. We detected one or more enteropathogen in 77 cases (51.3%) of the diarrhea patients examined.

4. Mixed infections were relatively frequent mainly and were in the form of rotavirus with parasite, and *Shigella* with parasite.

5. Rotavirus was confirmed as the most common pathogen in childhood diarrhea (28.0% of the diarrheal cases), especially in children less than 2 years. This fact, in combination with the severity of the infections, warrants consideration of a rotavirus vaccine in the childhood immunization program. Timely diagnosis of rotavirus infection in patients with acute diarrhea helps determine appropriate treatment, prevents the unnecessary use of antibiotics, minimizes the spread of the disease and decreases the cost of hospitalization.

6. The current study was done during the late spring and summer seasons, rotavirus infections however, are known to be more common in the winter season, consequently the true prevalence of rotavirus may be well over than 28.0% if the study has been done during the cold season, and this further potentiates the importance of rotavirus etiology in diarrhea.
7. *Shigella* species were found in 9 cases (6.0%) by PCR and in only 6 cases (4.0%) by bacteriological culture, with higher frequency of *S. flexneri* (3/6) in relation to *S. sonnei* (2/6), and *S. boydii* (1/6).

8. *Salmonella* spp. was found in 3 cases (2.0 % of diarrheal cases) as revealed both by PCR and bacteriological culture.

9. *E. coli O157:H7* was found in 7 cases (4.7%) by PCR, and in 6 (4.0%) of the diarrheal cases by bacteriological culture.

10. *Campylobacter* was evident in 7 (4.7%) of the diarrheal cases as revealed by PCR only.

11. By the use of PCR we found that 4/19 (21.1%) of enteropathogens positive specimens by PCR that the bacteriological stool culture revealed negative. PCR seem to be safer, more sensitive and more rapid than the conventional culture methods for the diagnosis of bacteria. PCR-based method contributes to improvement of the rapid diagnosis of enteric bacterial infections, whilst yielding higher detection rates of causative agents. Our findings highlight the value of using a combination of traditional and molecular techniques in the diagnosis of diarrheal disease in this population.

12. Our study also emphasizes the importance of *E. histolytica/dispar* in causing diarrhea at the study age.

13. The age groups 25-36, and 37-48 months were more susceptible to infection with enteropathogens (especially *Shigella* and parasites). The development of a polyvalent vaccine that covers strains of *S. flexneri* and *S. sonnei* would be predicted to provide protection against *Shigella* infections occurring in Palestine.

14. Fever, vomiting, diarrhea and dehydration were the most common clinical signs of the studied enteropathogens. We recommend visiting a Health Care Professional if your child has diarrhea as well as vomiting or fever. The dehydration status of the diarrheal children was observed more common in rotavirus infection, (14.3%), followed by *Shigella* (11.1%), and *E. coli O157:H7* (11.1%).

15. The antibiograms of *Salmonella* and *Shigella* isolates, showed that the most frequent patterns of resistance were exhibited towards Doxycycline (88.8%), Ampicillin and trimethoprim/sulfamethoxazole (77.8% each),
cefatazidim, ceftriaxone, and ciprofloxacin had the low resistance pattern, whereas all the isolates were sensitive to Meropenem, and Amikacin. Ampicillin, trimethoprim/sulfamethoxazole, and doxycycline should not be used for treatment of diarrhea in children. Amikacin or meropenem, cefatazidim and ceftriaxone are the best choice for treatment of infectious diarrhea. The current study highlights the necessity for continuous monitoring of antibiotic resistance in diarrhea related bacterial pathogens

16. We recommend the use of both Selenite cysteine broth and GN broth for pre-enrichment of stool samples beside the use of a minimum of two selective agar media other than SS agar (such as HE and/or XLD) to solve the very low detection rate of *Salmonella* and *Shigella* by Gaza strip health laboratories. Additionally, antibiotic therapy prior to specimen collection and delay for >3 h in delivering some of the specimens to the laboratory for processing should be minimized as they may be responsible for the low yield of bacterial cultures. We recommend physicians to request *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. cultures as early as patients are hospitalized.

17. We recommend that hospital and private laboratories in Gaza strip to detect all diarrheal stool samples for rotavirus and to culture routinely stool specimens on sorbitol-MacConkey agar, the standard culture medium for *E. coli* O157: H7. We also strongly encourage health care providers to determine the etiology of diarrhea before prescribing antibiotics. Children with gastrointestinal infections caused by *E. coli* O157:H7 are at risk of developing hemolytic-uremic syndrome (HUS) which can be fatal as it may lead to acute kidney failure.

18. Improved hygiene, health education, drinking clean water, breast feeding (which helps in providing passive immunity in the newborn for at least 6 months) are highly recommended in order to minimize the infection rate of many enteropathogens. Furthermore, the practice of dumping solid wastes in the streets and raising animals in the living areas should be minimized.
Finally, diarrhea is still a health problem in children not only in Gaza, but also nationwide in Palestine. Some enteric pathogens such as rotavirus, *Entamoeba histolytica/dispar*, and *Shigella*, are the major causes of diarrhea. This study has highlighted many of the enteric pathogens that cause diarrhea in children in Gaza. The application of techniques such as PCR and immunoassay will improve the quality of detection of the pathogens. This will make the clinical diagnosis better and help the pediatricians in treatment of children with diarrhea. The findings from the study will also help the policy makers to improve the health care program to provide better services to children.

To our knowledge, this is the first study in Gaza investigating several kinds of possible enteric pathogens in diarrhea in children less than 5 years of age. Based on the preliminary data presented by this study, further work is needed in order to provide a broader picture of the burden of rotavirus and other enteropathogens in children less than 5 years old all over Gaza strip.
CHAPTER 7
REFERENCES


13. American Medical Association; Centers for Disease Control and Prevention; Center for Food Safety and Applied Nutrition et al. (2001). Diagnosis and management of foodborne illnesses: a primer for physicians. MMWR Recomm Rep. 26;50(RR-2) 1-69


18. Ballal M, and Shivananda PG. (2002). Rotavirus and enteric pathogens in
infantile diarrhoea in Manipal, South India. Indian J Pediatr. 69(5):393-6.


<table>
<thead>
<tr>
<th>No.</th>
<th>Author(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>------------</td>
<td>-------------------------------------------------------------</td>
<td></td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>152. Samuel B. (1996). Medical Microbiology. The University of Texas Medical Branch at Galveston–Tx, USA</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Reference</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>186. WHO Guidelines for the control of Shigellosis, including epidemics due to Shigella dysenteriae type 1; <a href="http://www.who.int/child-adolescenthealth/new_publications/child_health/isbn_94_4_159233_0.pdf">http://www.who.int/child-adolescenthealth/new_publications/child_health/isbn_94_4_159233_0.pdf</a></td>
<td></td>
</tr>
</tbody>
</table>
APPENDICES

Appendix A

<table>
<thead>
<tr>
<th>Questionnaire</th>
</tr>
</thead>
<tbody>
<tr>
<td>Questionnaire number: …………………………………………………………………………</td>
</tr>
<tr>
<td>Date of admission to the hospital: ……………………………………………………………</td>
</tr>
<tr>
<td>Date and time of the sample collection: ……………………………………………………</td>
</tr>
<tr>
<td>Ward: ……………………………………………………………………………………………</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Personal Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name: ……………………………………………………………………………………………</td>
</tr>
<tr>
<td>ID No: ……………………………………………………………………………………………</td>
</tr>
<tr>
<td>Age: ………………………………………………………………………………………………</td>
</tr>
<tr>
<td>Gender: …………………………………………………………………………………………..</td>
</tr>
<tr>
<td>Address: ………………………………………………………………………………………..</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever: …………………………………………………………………………………….. °C</td>
</tr>
<tr>
<td>Vomiting: No ……………………………./day</td>
</tr>
</tbody>
</table>

Type of diarrhea:
- ☐ Mucoid
- ☐ Watery
- ☐ Bloody

<table>
<thead>
<tr>
<th>Diarrhea</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ……………………………./day</td>
</tr>
</tbody>
</table>

Dehydration: …………………………………………………………………………………………… |

Previous: ☐ Hospital
Primary clinic: ☐
Private: ☐

Medications: …………………………………………………………………………………………… |

By physician or pharmacy: …………………………………………………………………………… |

<table>
<thead>
<tr>
<th>Macroscopic exam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood: ……………………………………………………………………………………………</td>
</tr>
<tr>
<td>Worms: ……………………………………………………………………………………………</td>
</tr>
<tr>
<td>Mucus: ……………………………………………………………………………………………</td>
</tr>
<tr>
<td>Others: ……………………………………………………………………………………………</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Father</th>
<th>Mother</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>Education</td>
<td></td>
</tr>
<tr>
<td>Job</td>
<td></td>
</tr>
<tr>
<td>No of children</td>
<td></td>
</tr>
</tbody>
</table>

88
Appendix B
Security
Appendix D

50x TAE buffer

Composition:
Tris base ................. 242 g
glacial acetic acid .......... 57.1 ml
EDTA ..........................18.6 g
H2O to .................... 1000 ml
pH 8.0

Ethidium bromide (stock solution)
Ethidium bromide 10 mg/ml in water.

DNA loading buffer
bromphenol blue 0.25 g
xylene cyanol 0.25 g
glycerine 30 ml
H2O 70 ml

Iodine solution
Potassium iodide (KI) ............. 1 g
Powdered iodine crystals ....... 1.5 g
Distilled water .................. 100 ml
Appendix E

*Shigella* polyvalent (BIO-RAD, France):

1) Sub-group A : 2 polyvalent sera
   - Serum A1 : *anti-Shigella dysenteriae*: 1, 3, 4, 5, 6
   - Serum A2 : *anti-Shigella-dysenteriae*: 2, 7, 8

2) Sub-group B: 1 polyvalent serum
   - *Anti-Shigella flexneri* serum, it agglutinates the 6 serotypes of this subgroup.

3) Sub-group C : 3 polyvalent sera
   - Serum C1 : *anti-Shigella – boydii*: 1, 2, 3, 4
   - Serum C2 : *anti-Shigella - boydii*: 8, 10, 14
   - Serum C3: *anti-Shigella- boydii*: 5, 7, 9, 11, 15

4) Sub-group D: 1 mixed *anti-Shigella sonnei* serum
   - It agglutinates, the 2 phases of this species.