

Republic of Iraq
Ministry of Higher Education
and Scientific Research
University of Diyala
College of Medicine



Human Norovirus Detection among Children with Gastroenteritis in Diyala Governorate

A Thesis

Submitted to the Council of College of Medicine – University of Diyala in
Partial Fulfillment of the Requirements for the Degree of Master of
Sciences in Medical Microbiology

By

Marwa Ghazzay Diraa

B.Sc. Biology (2016) - College of Science - University of Diyala

Supervised by

Professor

Dr. Areej Atiyah Hussein

(Ph.D. in Medical Microbiology)

Professor

Dr. Jalil Ibrahim Kadhim

(Board in FICMS)

2019 A.D.

1441 A.H.

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ
(يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ
دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ)

صدق الله العظيم

سورة المجادلة ، الآية (١١)

Dedication

- To the absent present, my elderly brother "Maad" may Allah have mercy upon his soul.
- To the most potent moral force in my life "my parents " who see the best of me and unconditionally teach, inspire as well as encourage me. No words can ever express my gratitude to you.
- To the sunshine of my life, these whom always there for me when I need them, pick me up when I fall, stick up for me when no one else will my sister "Maysam" and brothers "Mokhallad" and "Marwan", thank you for believing in me and encouraging me.
- To the stars of my sky my nephews especially "Bassam".

Marwa

Acknowledgment

First and foremost, I do praise God (Allah), without his love, grace and mercy none of my achievements would be possible. My Gratitude to my role model prophet "Mohammad" (peace be upon him, his relatives and companions) who said: (seek knowledge from the cradle to the grave).

I would like to acknowledge with deep appreciation and gratitude the invaluable help of my supervisors Professor Dr. Areej Atiyah Hussain and Dr. Jaleel Ibrahim whose hands always ready to help me, they have been tremendous mentors for me, encouraging my research and greatly appreciate our discussion and optimism, their advices and guidance on my thesis and writings have been priceless.

My appreciation goes to staff of the Department of Microbiology for facilitating higher education. Special thanks to Assist. Prof. Mohammad Jasim Shakir for his precious kindly help during sample processing.

I am indebted to the staff of Emergency Lab, Floors Lab staff and Emergency Doctors who have been helpful all the time. My appreciation to the biologist Massar Hadi Esmaeel at the College of Science-University of Diyala for helping me in performing ELISA technique. Also, I would like to thank all patients and their families who helped me by giving me samples and information that helped me in my study. Finally, special thanks are delivered to my dearest friends who support me during this stage of my study, especially Hala Luay Abd Al-Jabbar.

Marwa

Summary

Acute gastroenteritis remains a global public health problem. It causes significant morbidity and mortality among children worldwide. Human noroviruses are a major cause of gastroenteritis and severe diarrheal disease around the world.

The present study is designed to determine the rate of human norovirus infection among children with gastroenteritis in Diyala governorate using enzyme linked immunosorbent assay and immunochromatography, also, to evaluate genogroup 1(GGI) and genogroup 2(GGII) by nested polymerase chain reaction among study population and study the association between the rate of infection and different parameters such as age, body mass index, gender, the education level of the mothers, water source, type of feeding and clinical aspects.

A cross sectional study was carried out for patients with acute gastroenteritis who attended to the Emergency Department of Pediatrics in Al-Batool Teaching Hospital for Maternity and Pediatric in Baqubah city, during the period from 6th of September 2018 to 4th of March 2019. A total of 182 children under the age of five years old (115 males and 67 females) are admitted during the study period. The stool samples were collected from each participant and stored as frozen at -70 °C until used to an enzyme immune assay and immunochromatographic test for the qualitative identification of human norovirus genogroups I and II in human stool samples, as well as used nested polymerase chain reaction after RNA extraction among positive samples from study population.

The results of this study shows that, the rate of human norovirus infection was 6.04% (11 out of 182) samples by enzyme linked immunosorbent assay and immunochromatography techniques; infection

among females was (54.55%) higher than males (45.45%). The positive results 8 (72.73%) were in age group (1-12) months and 3 (27.27%) in age group (13-24) months, while no positive cases among other ages. All the positive patients for human norovirus were from Baqubah city. The education level of the mothers of the positive patients were highest rate with primary education 6(54.55 %) followed by 2 (18.18%) for each secondary and higher education.

The distribution of positive human norovirus infection regarding the type of feeding showed that 9 cases (81.82%) were used artificial milk and 2 cases (18.18%) were mixed feeding that drinking artificial and breast feeding, while there were no positive results recorded among children with breast feeding. Concerning the sources of water use, the highest infection rate was noticed among patients were used filtered and boiled water 6 cases (54.54%) followed by filtered water about 3 cases (27.28 %) and boiled tap water 2 cases (18.18 %).

The signs and symptoms of infection were fever 5(45.45%), nausea 7(63.63%), vomiting 10 (90.09%), weight loss 4(36.36%) and dehydration 6(54.54%). Plus, all patients had abdominal pain 11(100%).

Only two cases, (18.18%) that acquired the infection while they were hospitalized infections. However, three cases (27.27%) were have other cases with the same signs and symptoms in the same family, six cases (54.55%) were having non-sporadic infection.

The result of nested polymerase chain reaction demonstrated that only one case was positive for human norovirus genogroup 2(GGII).

Table of Contents

Contents		Page No.
Dedication		
Acknowledgment		
Summary		i
List of contents		III
List of tables		VII
List of figures		VIII
List of abbreviations		IX
Chapter one		
1.1	Introduction	1
1.2	Aims of study	4
Chapter two		
2	Review of literatures	5
2.1	Historical background of human norovirus	5
2.2	Classification	5
2.3	Characteristics	6
2.4	Genotyping	7
2.5	Transmission	8
2.6	Replication cycle	9
2.7	Risk factor for human norovirus infection	11
2.7.1	Age	11
2.7.2	Immunosuppressed	11
2.7.3	Transplant patients	11
2.7.4	Nosocomial infection	11
2.8	Pathogenesis	12
2.8.1	Histological alterations in the intestine	13

2.8.2	Physical and biochemical manifestations	13
2.9	Signs and symptoms	13
2.10	Immune response	14
2.11	Laboratory diagnosis	15
2.12	Treatment and prevention	17
2.13	Epidemiology	18
Chapter three		
3	Patients, Materials and Methods	21
3.1	Patients	21
3.1.1	Study design	21
3.1.2	Sample collection	21
3.1.3	Ethical approval	21
3.2	Materials	22
3.2.1	Laboratory apparatus and tools	22
3.2.2	Chemical materials	23
3.2.3	Kits	24
3.2.3.1	Enzyme immune assay	24
3.2.3.2	Quick qualitative immunochromatographic test	24
3.2.3.3	Viral RNA Mini Kit	25
3.2.3.4	cDNA synthesis from total RNA template	26
3.2.4	Agarose gel electrophoresis requirements	26
3.2.5	PCR requirements	27
3.3	Methods	28
3.3.1	Enzyme linked immune sorbent assay (ELISA)	28
3.3.1.1	Principle of the test	28
3.3.1.2	Procedure for detection human norovirus in stool samples	28
3.3.2	Immunochromatographic test	30

3.3.2.1	Principle of the test	30
3.3.2.2	Procedure for detection human norovirus in stool samples	
3.3.3	Steps of PCR	25
3.3.3.1	RNA extraction from stool samples	31
3.3.3.2	cDNA synthesis from total RNA template	32
3.3.3.2.1	Principle	32
3.3.3.2.2	Protocol	32
3.3.3.3	Gene amplification by nested polymerase chain reaction of 0human norovirus	33
3.3.3.4	Primers preparation	33
3.3.3.5	PCR program	34
3.3.3.6	Agarose gel electrophoresis	35
3.4	Statistical analysis	35
Chapter four		
4	Results	36
4.1	Rate of human norovirus infection.	36
4.2	Distribution of human norovirus according to demographic characteristic.	37
4.3	Distribution of positive human norovirus cases according to type of feeding.	38
4.4	Distribution of positive human norovirus cases according to water source.	38
4.5	Distribution of human norovirus according to clinical signs.	39
4.6	Body mass index and positive results with human norovirus	40
4.7	The rate of human norovirus as (sporadic infection).	40

4.8	Detection of GI and GII genes for Norovirus using nested PCR	41
Chapter five		
5	Discussion	44
5.1	Rates of human norovirus infections	44
5.2	Rate of human norovirus infection according to gender	46
5.3	Rate of human norovirus infection according to age	46
5.4	Distribution of human norovirus according residence	47
5.5	Distribution of human norovirus according to the mother's level of education	48
5.6	Distribution of positive human norovirus cases according to type of feeding.	49
5.7	Distribution of positive human norovirus cases according to water source.	50
5.8	Distribution of positive human norovirus cases according to signs and symptoms	51
5.9	Distribution of positive human norovirus cases according to BMI	52
5.10	Relation between positive human norovirus cases and sporadic infection	53
5.11	Detection of GI and GII genes for human norovirus	54
Chapter six		
6.1	Conclusion	57
6.2	Recommendations and future studies	58
References		
	References	59
Appendix		

List of tables

Table	Title	Page No.
3-1	The general apparatuses and tools were used in this study	22
3-2	Various chemical and materials that used in this study	23
3-3	Components of RIDASCREEN human norovirus ELISA kit	24
3-4	Components of immunochromatographic test	25
3-5	Kit contents of viral RNA extraction that used in this study	25
3-6	Kit Component for cDNA synthesis from total RNA template	26
3-7	Components of Go Taq® green master mix (2X)	27
3-8	Components of PreMix tubes	32
3-9	Steps of reaction for cDNA synthesis	32
3-10	Sequence of primer that used in this study to amplification of fragment of the GI and GII human norovirus gene	33
3-11	Component of nested polymerase chain reaction	34
3-12	Protocol of a nested reverse transcription-polymerase chain reaction of GI and GII of human norovirus gene	34
4-1	Distribution of human norovirus according to demographic factors	37
4-2	Distribution of positive human norovirus cases according to type of feeding	38
4-3	Distribution of positive human norovirus cases according to sours of water.	38
4-4	Distribution of positive human norovirus cases according to clinical signs	39
4-5	Body mass index and positive results with human norovirus	40

List of figures

Figure	Title	Page No.
2-1	A-Human norovirus gene map, B- Human norovirus virion particle	6
2-2	Phylogenic tree of human norovirus depends on amino acid sequencing of the major capsid protein VP1. Human norovirus host range is largely determined by genogroup	8
2-3	Human norovirus replication cycle	10
2-4	An aggregate of human noroviruses observed by negative staining of a stool sample collected from a patient with acute gastroenteritis. examination by immune electron microscopy	16
2-5	World map showing areas where GII.17 human norovirus strains have been detected, 1978-2015	19
4-1	Rate of human norovirus according to ELISA results	36
4-2	The rate of human norovirus as (sporadic infection).	40
4-3	Gel electrophoresis of the first round PCR with product size 380-390bp stained with ethidium bromide and illustrated under UV light. A- on gel is abbreviation including L (ladder), 1,8 (negative sample) for genogroup I. B- on gel is abbreviation including L (ladder), 1,8 (negative sample) for genogroup II.	41
4-4	Gel electrophoresis of the first round PCR with product size 380-390bp stained with ethidium bromide and illustrated under UV light. A- on gel is abbreviation including L (ladder), sample no. 9,10,11,12 (negative sample) for genogroup I. B- on gel is abbreviation including L (ladder), sample no. 9,10,11,12 (negative sample) for genogroup II	42
4-5	Gel electrophoresis of the second round PCR of human norovirus GII with product size 340bp stained with ethidium bromide and illustrated under UV light. Number 6 on gel is positive sample and sign L is abbreviation for ladder.	43

List of abbreviations

Abbreviation	Meaning
AGE	Acute gastroenteritis
BMI	The body mass index
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
ID	Infecting dose
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscope
GG	Genogroup
HBGAs	Histo-blood group antigens
HLADR	Human leukocyte antigen complex on chromosome 6 region 6p21.31
ICTV	International Committee on Taxonomy of Viruses
IFNs	Interferons
IL	Interlukin
MENA	Middle East and North Africa
MHC	Major histocompatibility complex
NK cells	Natural killer cells
NSP	Non-structural proteins
°C	centigrade
°F	Fahrenheit
ORF	Open reading frames
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase–polymerase chain reaction
RT-qPCR	Quantitative real time polymerase chain reaction
TNF- α	Tumor necrosis factor alpha
VLP	Viral like particle
VPg	Viral protein genome
WHO	World health organization

Chapter One

Introduction

1.1 Introduction.

Acute gastroenteritis (AGE) remains a global public health concern, causes significant morbidity and mortality among children worldwide (Arowolo *et al.*, 2019; Plants-Paris *et al.*, 2019).

The causes of acute gastroenteritis in children vary depending on multiple factors such as location, season, and the population studied (Dennehy, 2005). There is a wide range of infections that can cause acute gastroenteritis. These are viruses (rotavirus, norovirus, astrovirus, sapovirus, adenovirus), bacteria (*Shigella*, *Escherichia coli*, *Campylobacter*, *Salmonella*, *Vibrio cholerae*, *Yersinia enterocolitica*, *Aeromonas*), and protozoa such as *Cryptosporidium*, *Entamoeba histolytica*, *Giardia intestinalis*. In addition, *Clostridium difficile* may induce diarrhoea when the antibiotic treatment alters the intestinal microbial balance, and bacterial toxins may cause gastroenteritis without enteric infection such as *Staphylococcus aureus* (Kabayiza, 2014).

Viruses are the most frequently implicated pathogens causing pediatric acute gastroenteritis and viral diarrhea in pediatric patients in both outpatient, emergency department, and inpatient settings (Trang *et al.*, 2012). Enteric viruses, particularly rotaviruses and human noroviruses, are a leading cause of gastroenteritis worldwide. Rotaviruses primarily affect young children, human noroviruses affect people of all ages, and are a leading cause of foodborne disease and outbreaks of gastroenteritis worldwide (Krisztián *et al.*, 2018). Human noroviruses remain a major cause of gastroenteritis and severe diarrheal disease around the world (Karst and Tibbetts, 2016).

Human noroviruses which are small, non-enveloped, positive-stranded RNA viruses belong to Caliciviridae family is now comprised of five genera, including Norovirus, Sapovirus, Lagovirus, Nebovirus, and Vesivirusl (Green, 2013). The human norovirus genus can be subdivided in seven genogroups, of which genogroups GI, GII and GIV have been detected in humans, and can be further subdivided into more than 40 genotypes (Vinje, 2015).

Human norovirus causes ~20% of all acute gastroenteritis and ~200,000 deaths per year, primarily in young children. Most epidemic and all pandemic waves of disease over the past 30 years have been caused by type GII.4 human norovirus strains (Lias *et al.*, 2019).

The transmission of human norovirus occurs primarily via the fecal oral route, including direct person to person contact, consumption of contaminated food or water, or contact with contaminated environmental surfaces (CDC, 2011). Common symptoms of illnesses include increase in bowel movement frequency with or without vomiting, fever, abdominal cramping, headache, dehydration and myalgia (Sattar and Shashank, 2018). The illness is generally mild and short duration (1-2 days) (Bok and Green, 2012).

It is not well known whether human norovirus infections induce any lasting protective immunity (Simmons *et al.*, 2013). Extent immunity protects against exposure to different strains. This is important because noroviruses are highly genetically and antigenetically diverse this complexity that is a big challenge for the development of an efficient human norovirus vaccine (Rackoff *et al.*, 2013). Infections are notoriously difficult to prevent and control, owing to their low infectious

dose, high shedding titer, and environmental stability (Barclay *et al.*, 2014).

Globally, human norovirus resulted in a total of \$4.2 billion in direct health system costs and \$60.3 billion in societal costs per year. Disease amongst children <5 years cost society \$39.8 billion, compared to \$20.4 billion for all other age groups combined. Costs per norovirus illness varied by both region and age and was higher among adult ≥ 55 years, low- and middle-income countries and high-income countries had a similar disease incidence (Bartsch *et al.*, 2016).

In Iraq, several studies have been conducted in various provinces to determine the rate of human norovirus infections by using different techniques; such as study done by Al-Mashhadani *et al.*, (2008) in Kurdistan region, Thwiny and Hassan (2015) in Basrah, Al-Marsome *et al.*, (2016) detected the virus by enzyme linked immunoassay (ELISA) and polymerase chain reaction (PCR) in Basrah city and Mohamed *et al.*, (2016) in Mosul city detected the virus using (RT-qPCR) for NVGI and NVGII got the same rate, Al-Khoweledy (2016) diagnosed the virus in Al-Najaf Province using reverse-transcriptase-polymerase-chain-reaction (RT-PCR) technique and Al-Moussawi *et al.*, (2018) in Thi-Qar province with infection rates 30%, 8% , 28% , 37.9% and 17.5 % for each one respectively. To the best of our knowledge, there is no previous study done in Diyala governorate about human norovirus genotyping.

1.2 Aims of the study.

The study aims at.

- 1.** To detection the rate of human norovirus infections among children with acute gastroenteritis in Diyala governorate by enzyme linked immunosorbent assay and immunochromatography.
- 2.** To determine genogroup 1(GGI) and genogroup 2(GGII) by nested polymerase chain reaction among study population.
- 3.** To study the association between the rate of infections and different parameters such as age, gender, the education level of the mothers, water source, type of feeding and clinical aspects.