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Isolation and Identification of Rotavirus and Norovirus from Children under Five Years of Age

A Thesis

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

(شَهِدَ اللَّهُ أَنَّهُ لَا إِلَهَ إِلَّا هُوَ وَالْمَلَائِكَةُ وَأُولُوا الْعِلْمِ قَائِمًا بِالْقِسْطِ لَا
إِلَهَ إِلَّا هُوَ الْعَزِيزُ الْحَكِيمُ)

صَدَقَ اللَّهُ الْعَلِيِّ الْعَظِيمِ

من سورة آل عمران
الآية (18)

Dedication

*The light of my life... prophet Mohammed and the
holy household (peace be upon them).*

.....Iraq

My wonderful country

*To dear father who removed the thorns from my
path to pave my way to science ...*

*To dear mother who makes me happy when I see
her The kindest heart...*

To my dear wife

To My brothers and sister

To my friends

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Initially, I thank Almighty God for giving me the strength and the will to complete this work

*My deepest affection and gratitude to my supervisor, **Assist Prof. Dr. Basim A. Jarullah** for his supervision assistance and considerable scientific advice*

I extend my thanks to my family and my wife.

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Summary

The objective of this study is to determine the frequency of the two most important enteric viruses (Rotavirus group A and Norovirus) in stool specimens of children aged less than five years and their association with demographic and some parameters including age, sex, feeding type and clinical symptoms .

A total of 100 fecal specimens are collected from infants and children under five years of age in Thi-Qar Province south of Iraq during Five Months (From November 2017 To March2018): 60 stool specimens are collected from the city of Nasiriyah (including Mohammed Al Mousawi children's Hospital ,Bint Al-Huda Teaching Hospital and Private Clinics) and 40 stool specimens are collected from Al-Nasiriyah, Sayed Dakhil, Al-Shatrah, SuqAlshyuokh, Al-Rifai, Al-Garraf and Al-Batha (including 63 males and 37 females). The frequency of male children patients with diarrhea caused by Rotavirus and Norovirus were higher than their female .

The samples are categorized into eight groups according to the age of the children: 0-1 years, 1-2 years, 2-3yearss, 3-4 years, 4-5 years,. Age specific frequency in children patients with diarrhea, caused by Rotavirus and Norovirus is high in aged 1-2 years .

According to diarrhea suffered children which revealed that 35% are caused by Rotavirus while 9% diagnostic as Norovirus on the others hand there was 10% of total 100 diarrheal patients considered mixed infections.

The highest rate of detection of Rotavirus gastroenteritis is found in December (18 cases, 40%) and the lowest in the month march (1 cases, 2.2%). The highest rate of detection of Norovirus gastroenteritis was

found in December (8 cases, 42.1%) and the lowest in the month march (0 cases, 0%).

According to results the percentage of infection with Rotavirus show that 16 (35.6%), 22 (48.9%) and 7 (15.6%) children are fed on breast feeding, bottle feeding and mixed feeding respectively. However, in case Norovirus the percentage of infection with Norovirus, show 5 (26.3%), 10 (52.6%) and 4 (21.1%) children are fed on breast feeding, bottle feeding and mixed feeding respectively.

The clinical signs of patient include diarrhea, vomiting, fever and abdominal pain. The percentage of most predominant signs for Rotavirus were diarrhea (100%) compare to other signs, while the percentage most predominant signs for Norovirus were vomiting and diarrhea (100%) compare to other signs.

RT-qPCR is performed for detection of Rotavirus and Norovirus based capsid gene. Also RT-PCR technique is performed on some positive isolates in RT-qPCR method that used for Rotavirus and Norovirus genotyping by using DNA sequencing analysis.

In Rotavirus phylogenetic tree analysis, results are show that the local Rotavirus isolate (IQ-C1) are closed related to NCBI-Blast Rotavirus (JQ069617.1) (EF472951.1) that related to (genotype G9P8-G9) with NCBI- BLAST Homology Sequence identify (99-100%) and submitted into NCBI-Genbank and provided accession numbers IQC1(MH463489), IQC2(MH463490). In Norovirus phylogenetic tree analysis results are show the local Norovirus isolate (IQ-C1) are closed related to NCBI-Blast Norovirus (HQ005296.1) (HM748972.2) that related to (genotype GII.4) with NCBI- BLAST Homology Sequence

identify (99-100%) and submitted into NCBI-Genbank and provided accession numbers IQC1(MH490799) ,IQC2(MH490800)

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LIST OF ABBREVIATIONS

SYMBOL	DEFINITION
AGE	Acute gastroenteritis
bp	Base pair
C°	Degree centigrade
cDNA	Complementary Deoxy Ribonucleic Acid
DNA	Deoxy Ribonucleic Acid
dsRNA	Double stranded Ribonucleic Acid
ELISA	Enzyme Linked immunosorbent assays
EM	Electron Microscope
GI,GII,GIII	Genogroup I,II,III
GI.1,GII.2,GII.4	Genogroup I,II, genotype 1,2,4
HRV	Human Rotavirus
HuNoV	Human Norovirus
mRNA	Messenger Ribonucleic Acid
NLV	Norwalk like virus
NSP	Non structural protein
NV	Norwalk Virus
ORE	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RdRp	RNA-dependent RNA Polymerase
RNA	Ribonucleic Acid
rpm	Round per minute
RT-PCR	Reverse Transcription Polymerase chain reaction
RT-qPCR	Reverse Transcription Real-Time Polymerase chain reaction
RV	Rotavirus
SG	subgroup
USA	United states of America
VP	Viral protein
WHO	World Health Organization
μl	Microliter

Chapter One

Introduction

1.1 Introduction

Diarrheal diseases are a major cause of morbidity and mortality among young children in developing and developed countries (Wilhelmi *et al.* , 2003 ; Okitsn- Negshi *et al.* , 2004). It is established that in Africa, Asia, and Latin America 744 million to one billion cases of acute gastroenteritis and 2.4 to 3.3 million deaths occur annually among children of less than 5 years of age (Okitsn- Negshi *et al.* , 2004 ; Santos and Hoshino , 2005).

Although at least 25 different bacteria and protozoa can cause childhood diarrhea. More than 75% of cases are caused by viruses, and seven groups of diarrheal viruses (rotavirus, norovirus, sapovirus, adenovirus, astrovirus, parechovirus, and Aichi virus) are considered as the common etiologic agents of acute gastroenteritis in humans (Wilhelmi *et al.* , 2003 ; Pham *et al.* , 2007). Rotavirus

(RV) is the main cause of acute viral gastroenteritis in infants and young children worldwide, and in the young animals of a large variety of species (Santos and Hoshino, 2005 ; Parashar *et al.* , 2006).

Before the early 1970s, no virus had been confirmed as a causative agent of acute gastroenteritis, only bacterial or parasitic etiologic agents could be detected in 10–30% of children with diarrhea. Rotavirus was first described as a human pathogen in 1973 by Bishop and colleagues (Bishop *et al.*, 1973).

Extensive work on the genetics of these viruses has been performed, and both genotypes and genogroups (which include multiple genotypes) have been established..

Rota Virus classification is currently based on all 11 gene segments of the virus; the genes encoding the spike protein VP4 (P-protease sensitive) and the coat protein VP7 (G-glycoprotein) specify the P and G genotypes, respectively. These proteins are located in the outer

capsid of the virus and confer antigenic properties. Group A RVs can be classified into 27 G genotypes and 35 P genotypes which fall within five P genogroups (liu *et al.*, 2012). Rotavirus infections spread easily and usually occur in the winter and early spring between about November and April. Rotavirus infection often spread in settings where many children are together such as day care centers (Atchison *et al* ., 2010).

Globally, rotavirus infection is present all the year around which suggests that low level transmission could maintain the chain of transmission. Before rotavirus is discovered, the disease was called ‘winter diarrhoea and winter gastroenteritis (Pitzer *et al* ., 2009). In temperate climates, seasonality of rotavirus infection has been well demonstrated (Atchison *et al* ., 2010), where rotavirus is most frequently detected in the winter and rarely identified in the summer months (Pitzer *et al* ., 2009).

Human Norovirus cause acute gastroenteritis and outbreaks of the so-called winter vomiting disease. The incubation period is 1-2 days and the symptoms typically include a rapid onset of nausea, vomiting, diarrhea, sometimes together with headache, myalgia, and low grade fever.

Norovirus (NoVs) can be classified into five genogroups; three of which infect humans, i.e., genogroup I (GI), GII, and GIV (Kroneman *et al.*, 2013). The most commonly found NoV genogroup is GII with the GII.4 genotype predominating worldwide The infectious dose is 10-100 particles and the virus is normally shed in large numbers in feces up to 72 h after the last symptom (Persson, 2013).

Noroviruses are currently recognized as the most important food borne pathogens with respect to the number of outbreaks and individuals affected (Koopmans and Duizer, 2004;Thomas *et al* ., 2013). Norovirus infections are responsible for 10-15% of severe gastroenteritis cases in children under the age of 5 years (Hall *et al* ., 2011).The highest rate of

Norovirus infection is observed in children under 24 months (40%) which is in accordance with the studies reported from UK(Payne *et al.*, 2013) and China(Ozawa *et al.* , 2007).

They reported that 48.6% of the patients were positive for rotavirus and 16.5% for Norovirus and 3.8% had rotavirus-norovirus coinfection (Tran *et al.* , 2010). In a study of Babylon in Iraq it reveal that 50.5% (52/103), 29.1% (30/103) and 20.4% (21/103) were positive for Rotavirus, Norovirus and Adenovirus respectively(Jafar *et al.* , 2013). The detection of viruses by sensitive molecular diagnostic methods based on reverse transcription polymerase chain reaction (RT-PCR) The genome sequencing techniques to detect and characterize Norovirus has markedly enhanced our understanding of the epidemiology of NoV infections (yan *et al.*, 2003). These techniques have demonstrated that Norovirus are genetically and antigenically diverse (Zheng *et al.* , 2006). Based on sequence similarity and phylogenetic analysis, Norovirus can be classified into genogroup (G), genotype, and genocluster. Norovirus are presently classified into five distinct genogroups,GI–GV. Human NoVs belong to GI, GII, and GIV, with most strains relevant to human disease belonging to GI and GII (Green, 2007).

NoVs GIII and GV so far have been found in bovine and murine species, respectively. Within genogroups, Norovirus are subdivided further into genotypes, of which at least 29genotypes have been reported. The GI genogroup is currently divided into eight genotypes (GI/1–GI/8), GII contains at least 17 genotypes (GII/1–GII/17), GIII has two genotypes (GIII/1–GIII/2), and GIV and GV each contain one genotype (Zheng *et al.*, 2006)

1.2.Aim of the study

Isolation and Identification of Rotavirus and Norovirus from Children under Five Years of Age

- 1- comparative study of Rotavirus and Norovirus infection as causative agents of diarrhea in children
- 2- Determining the prevalence of rotavirus and norovirus among a group of children suffering from diarrhea.
- 3-Detecting molecular characterisation of rotavirus and norovirus infection among children under 5 years age.
- 4-Genotype and phylogenetic tree of two viruses .

Chapter Two

Literature Review

2. Literatures Review

2.1. Historical background

Infectious diarrhea can be caused by a wide range of viruses, bacteria, or Parasites (O'Ryan *et al.*., 2005). Studies have shown that diarrhea can be caused by a single pathogen or a mixture of two or more pathogens (Moyo *et al.*., 2011; Kotloff *et al.*., 2013). Viruses have been suspected of being important causes of gastroenteritis since the 1940s, but the a etiology remained unknown in most cases (Parashar *et al.*, 1998). In 1972, Norwalkvirus became the first viral agent identified to cause diarrhea, using immune electron microscopy (EM) after an outbreak of diarrhea (Kapikian *et al.*, 1996).

One year later rotaviruses were identified as a cause of diarrhea in children (Flewett *et al.*, 1973). In 1975, astroviruses were detected in the stools of children with acute diarrhea (Madeley and Cosgrove, 1975). This was followed by the discovery of enteric adenoviruses (Madeley and Cosgrove, 1975) and human caliciviruses of two different genogroups now called Norovirus (NoV) and Sapovirus (SV) (Chiba *et al.*, 2000). Other viruses which have been linked with gastroenteritis in humans are coronaviruses, picobirnaviruses, pestiviruses and toroviruses which produce diarrhea in animals, and they are emerging as causes of viral gastroenteritis in humans, according to several studies (Török *et al.*, 1997).

2.1.1. History of Rotavirus

Rotaviruses were discovered in animals in the 1963 (Adams and Kraft, 1963). Ten years after the virus was first described in humans when it was found by electron microscopy in duodenal biopsies from children with acute gastroenteritis (Bishop *et al.*, 1973). After this,

rotavirus was also identified in feces by electron microscope (EM) by several studies (Flewett *et al.*, 1973; Kapikian *et al.*, 1974). Using EM, the viral particles observed resembled a short-spiked wheel, which led to the name rotavirus (from the Latin Rota, meaning wheel) (Flewett *et al.*, 1974).

Rotavirus is the leading cause of severe diarrhea in children worldwide (Parashar and Glass, 2003). It is estimated to cause 36% of diarrhoea hospitalizations among children aged less than 5 years, according to WHO rotavirus surveillance networks. Rotavirus diarrhea is estimated to cause 453,000 deaths in children younger than five years, more than half occurring in developing countries of Africa and Asia (Tate *et al.*, 2012).

2.1.2. History of Norovirus

Norovirus is a major cause of viral gastroenteritis in different ages and in different countries (Koopmans *et al.*, 2000). The infection can be deadly in some age groups, such as infants and the elderly. Norovirus is the most common cause of diarrhea in adults and the second most common cause of acute gastroenteritis in childhood.

Causes of the global virus Norovirus 700 million disease and 0.2 million deaths per year, this leads to economic losses of 60 billion dollars, including the cost of health care and other losses resulting from infection (Bartsch *et al.*, 2016). That is the gastroenteritis Bacterial source of major concern all over the world. In 1929 with the first documented version of the report suggested that Dr. Zahorsky the term "winter vomiting disease" winter vomiting disease on the cases resulting from infection with this type of viruses due to increased occurrence during the winter months (Robilotti *et al.*, 2015).

Noroviruses are among the leading cause of sporadic cases and outbreaks of acute gastroenteritis across all age groups (De Wit *et al.*, 2001; Walker *et al.*, 2012).

It is estimated to cause 18% of all cases of sporadic acute gastroenteritis in children younger than 5 years older children and adults were associated with Noro(Ahmed *et al.*, 2014). In developing countries, Noroviruses are estimated to cause more than 1.1 million hospitalizations and 200,000 deaths in children <5 years of age each year (Patel *et al.*, 2008).

When Norovirus infections occur in outbreaks, they are usually difficult to control because of the prolonged shedding of the virus, environmental stability and resistance to disinfection, low infectious dose, and contribution of virus-induced vomiting to the spread of the infectious agent (Treanor *et al.*, 2014).

2.2. Structure and classification

2.2.1. Rotavirus structure and classification

The RVs are non-enveloped double-stranded RNA viruses belonging to the *Reoviridae* family, The genome contains 11 gene segments that encode the structural proteins VP1-8 and the non-structural proteins NSP1-6 (Than and Kim , 2013). The RV capsid is formed by three protein layers (Figure 2-1).

The outer capsid of the RV virion consists of two proteins, VP7 and VP4, both of which are immunogenic antigens and induce type-specific neutralizing antibodies. The VP7 proteins form the shell of the RV virion, and VP4 proteins spike outwards from the capsid and act in the attachment of the virion. Trypsin cleavage of VP4 into VP5 and VP8 enhances viral penetration to the target cell and infectivity of the virus (

Crawford *et al.*, 2001). The inner capsid of the RV virion is formed by VP6 which is a highly conserved and abundant protein inducing heterotypic cross-protective immunity in humans (Esquivel *et al.*, 2000). The innermost layer of the virion core consists of the proteins VP1, VP2 and VP3.

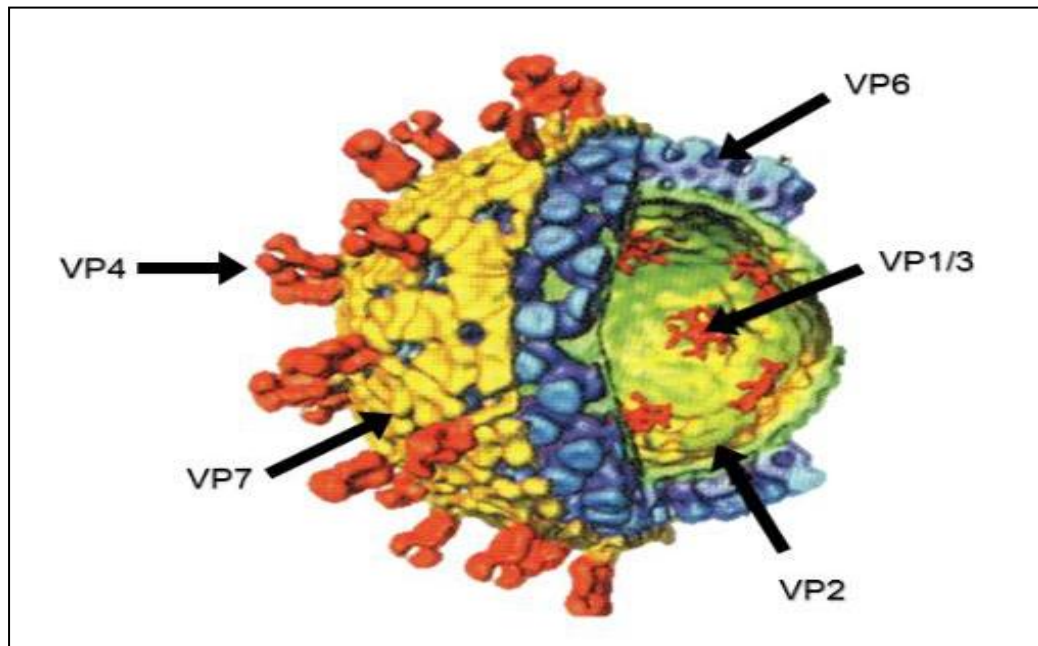


Figure 2.1. The Protein structure of Rotavirus Capsid. Modified from (Desselberger *et al.*, 2009)

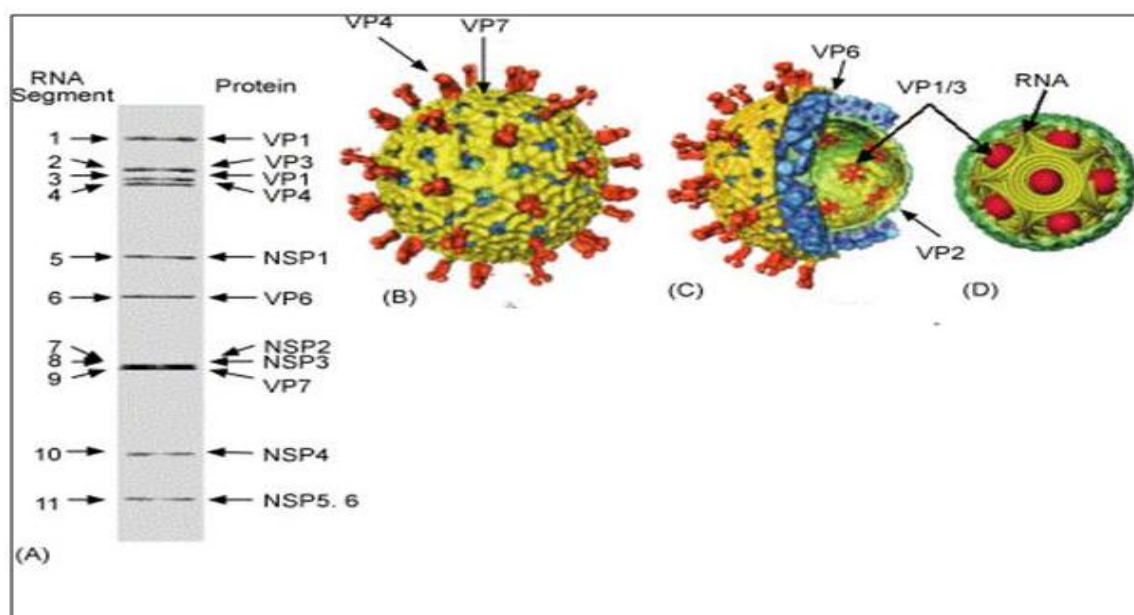


Figure 2-2. Architectural features of rotavirus. (a) RNA-PAGE showing, 11 dsRNA segments comprising the rotavirus genome. The gene segments are numbered on the left and the proteins they encode are indicated on the right. (b) Cryo-EM reconstruction of the rotavirus triple-layered particle. The spike proteins VP4 and the outermost VP7 layer are indicated by arrows. (c) A cutaway view of the rotavirus triple layered showing the inner VP6 and VP2 layers and the transcriptional enzymes anchored to the VP2 layer at the five-fold axes. (d) Schematic depiction of genome organization in rotavirus. The genome segments are represented as inverted conical spirals surrounding the transcription enzymes inside the VP2 layer. (Jayaram *et al.*, 2004).

Rotavirus has three important antigenic specificities: serogroup, subgroup and serotype (Collins *et al.*, 2010) (Fig 2-3). At present seven serogroups (A to G) of Rotavirus have been identified of which groups A, B and C infect human (Fig. 2-3) (Collins *et al.*, 2010).

Human group A rotavirus that is the dominant (>90%) Rotavirus serogroup can be classified into four antigenic subgroups (SG) (Fig. 2-3) specificities based on VP6 reactivity with monoclonal antibodies (MAbs) (Greenberg *et al.*, 1983). More recently, based on VP6 phylogenetic analysis of strains isolated in UK and USA, only two lineages (termed

genogroups)are distinguished and there is certain correlation between genogroups and SG (genogroup I:SGI; genogroup II: SGII, SG I + II and SG non-I, non-II) (Kerin *et al.*, 2007).

Furthermore, RNA polyacrylamide gel electrophoresis (RNA-PAGE) can be used to classify virus strain in short and long electropherotypes (Spencer *et al.*, 1986). which correlates with subgroup specificity (Nakagomi *et al.*, 1989). Group A rotavirus is traditionally classified in serotypes by virus neutralization assay using MAbs against VP7 (G-serotypes) and VP4 (P-serotypes) proteins. The most common Gserotypes found worldwide are G1, G2, G3, and G4 (Gentsch *et al.*, 2005) and the most common P serotypes areP1A, P1B, P2A and P2B (Collins *et al.*, 2010) (fig 2-3).

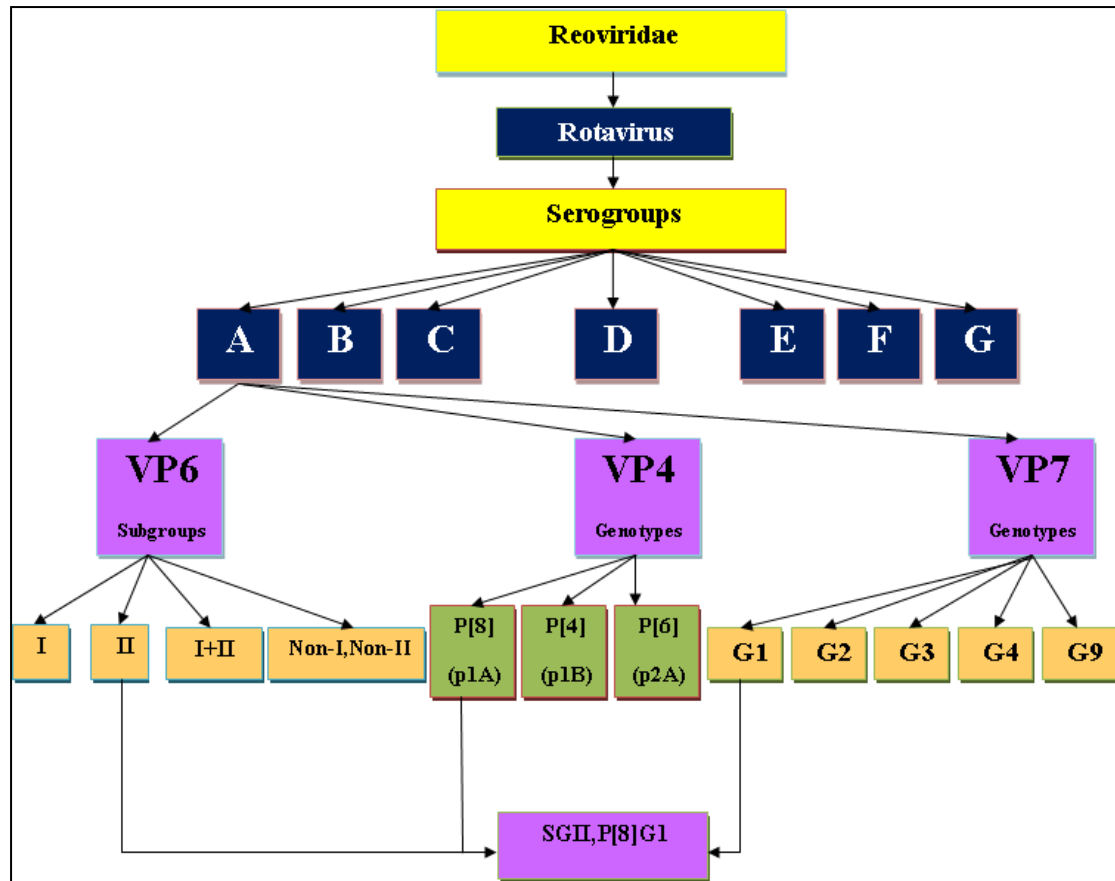


Figure 2-3. Rotavirus classification. Subgroup specificity is based in the reaction with one, both or neither of two MAbs, 255/60 (SGI) and 631/9 (SGII) VP4 serotypes are indicated in parenthesis, P[6] genotypes might be also P2B. VP7 serotypes correlate with genotype. Only the most common G and P genotypes are shown in the figure Adapted from. (Greenberg *et al.*, 1983).

2.2.2. Norovirus Structure and Classification

Norovirus are small, non enveloped, round viruses with a linear, positive sense, single stranded RNA encoding three open reading frames (ORFs) . Of these ORF1 encodes non-structural proteins including the viral polymerase, and ORF2 and ORF3 encode the major and minor structural capsid proteins VP1 and VP2 (Jiang *et al.*, 1993).

The VP1 consists of the N-terminal region, the shell (S) region and protruding (P) regions. The virus capsid is formed by 90 VP1 dimers which are arranged as shown in (Figure 2.4) NoVs do not grow in cell cultures in vitro, and NoV particles cannot be produced by standard methods. However, when expressed in baculoviruses and produced in insect cells, the NoV capsid protein spontaneously forms a virus-like particle (VLP) that is stable and antigenic and can be used as an antigen in serological assays and vaccine research (Treanor and Dolin, 2005; Blazevic *et al.*, 2011)

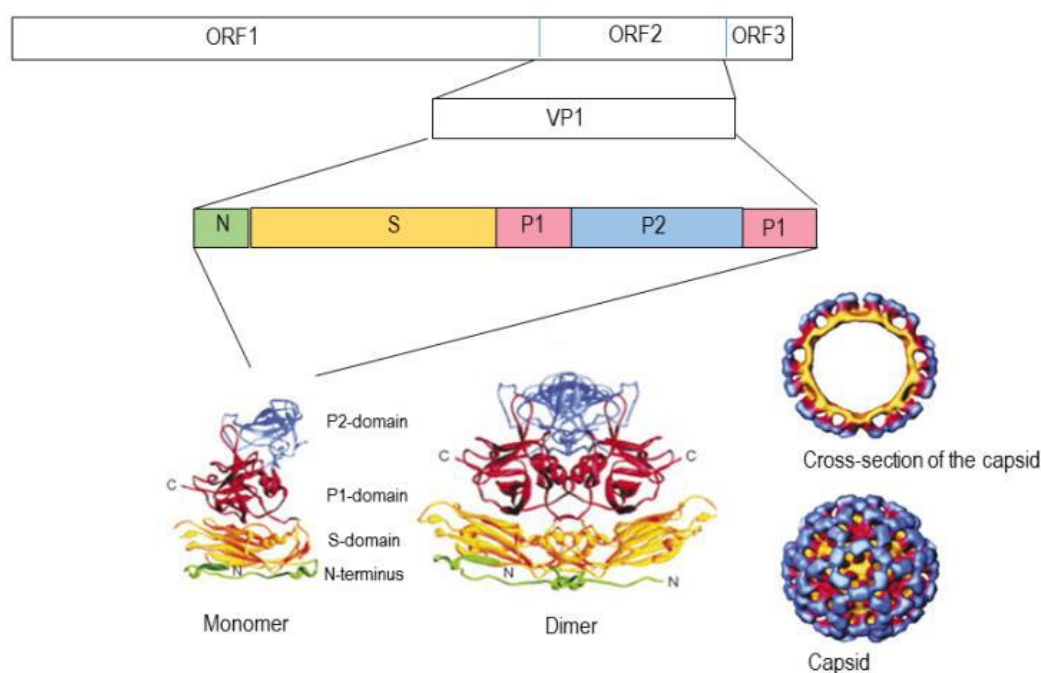


Figure 2-4. The arrangement of norovirus genome, capsid protein VP1 and structure of the norovirus capsid. Modified from (Hutson *et al.*, 2004).

Genogroups, genotypes and variants NoVs belong to human caliciviruses (HuCVs). Of the family *Caliciviridae*, the genus *Norovirus* can be divided into at least five genogroups (Kroneman *et al.*, 2013)

of which genogroups 1 (GI) and 2 (GII) are the common human pathogens. Also genogroup 4 (GIV) viruses are able to infect humans, however they are rare as Acute gastroenteritis (AGE) causative agents (Patel *et al.*, 2009).

The Norovirus genogroups are further divided into genotypes indicated by numbers, e.g. GI.1 (Kroneman *et al.*, 2013). More than 31 genotypes of noroviruses are known, and of these, particularly genotype GII.4 seems to be under constant genetic variation with new variants (or strains) emerging every two or three years in the 2000s (Kroneman *et al.*, 2013)

The mechanisms NoVs use to increase the variation are mutation and recombination.

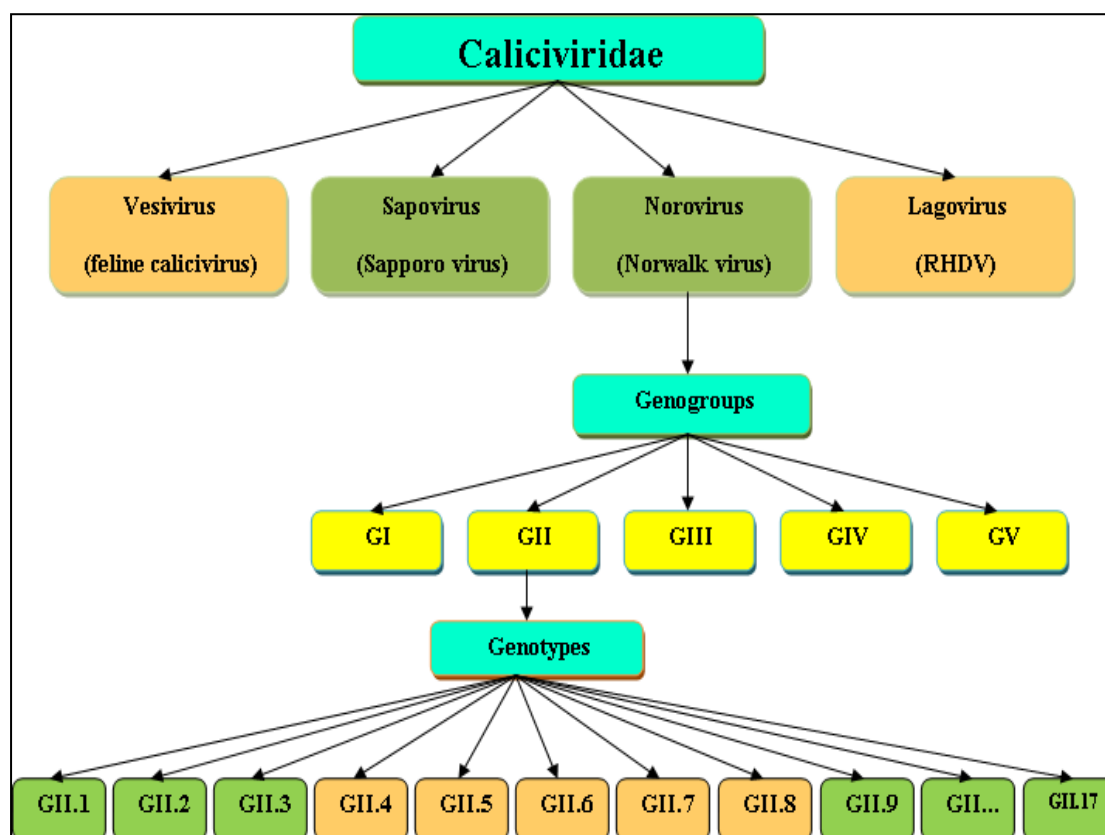


Figure 2-5. Schematic classification of the Norovirus based on phylogenetic analysis of the capsid gene adapted from (Zheng *et al.*, 2006).

2.3.Replication

2.3.1. Rotavirus replication

Rotaviruses infect the mature endothelial cells at the tip of the villi that are present in the small intestinal lumen. This Replication can be divided into several steps: attachment, entry, un coating, genome translation, replication, virus assembly and release. Prior to infection, the VP4 spike protein is proteolytically cleaved by trypsin--like proteases present in the small intestine into VP5 (body of the spike protein) and VP8 (head of the spike protein) (Arias *et al.*, 1996).

It has been shown that proteolytical cleavage of VP4 drastically increases the infectivity of a rotavirus particle. Before cleavage the VP4 spikes are flexible, but proteolysis induces a conformational change and forms a rigid dimeric VP5 spike, while the third VP5 lies flat to the virion surface (Crawford *et al.*, 2001).

The VP8 remains non--covalently attached to the VP5 protein (Dormitzer *et al.*, 2004)it has been demonstrated that for some rotavirus strains VP8 is able to bind to terminal silica acids (Monnier *et al.*, 2006)recently, this was expanded to a much broader range of glycans, suggesting that VP8 is a strong determinant for host range restriction (Hu *et al.*, 2012; Ramani *et al.*, 2013)much in a similar way as the VP1 protein does in noroviruses(Tan and Jiang , 2014). After initial cell binding, rotaviruses probably utilize the endocytosis pathway to enter the cytoplasm (Wolf and Greenberg, 2011)

Once in the endosome calcium depletion probably triggers the VP7 trimmers to dissociate (Chemello *et al.*, 2002) which results in its release from the viral particle and allowing VP5 to refold in a collapsed trimmer state while penetrating the endosomal membrane (Trask *et al.*, 2010; Trask *et al.*, 2012)

The dissociation of VP7 from the virion induces an upwards and outwards movement of VP6 and VP2, thereby expanding the five-fold axis channel and allowing passage of rotavirus transcripts (Trask *et al.*, 2012). The resulting double-layered particles contain only the VP2 and VP6 protein layers and have become transcriptionally active (Lawton *et al.*, 1997). Subsequently, nascent plus-stranded RNAs can both serve as mRNA for protein synthesis or as genomic RNA for progeny viruses. During replication, viral proteins accumulate in cytoplasmic inclusion bodies (viroplasms) that are likely induced by interacting NSP2, NSP5 and likely NSP6 proteins (Hu *et al.*, 2012; Criglar *et al.*, 2014). In the viroplasm rotaviral RNA, VP1-3 and VP6 self--

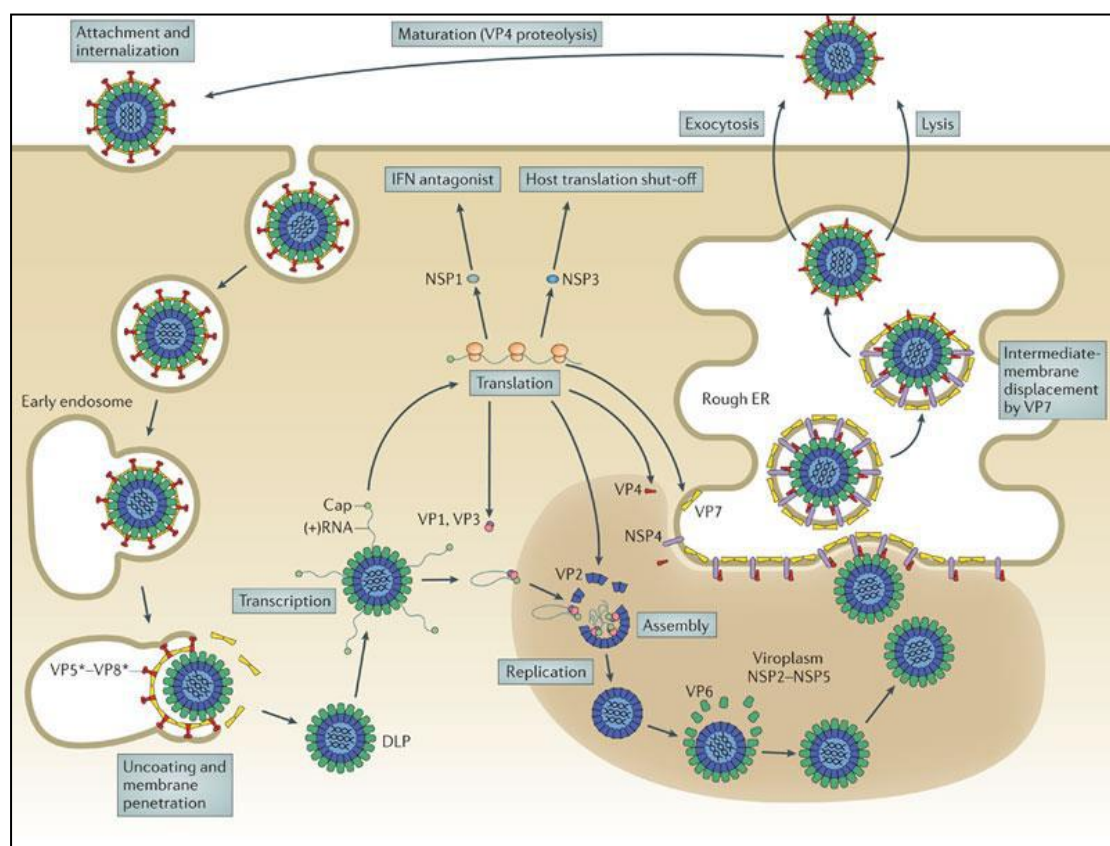


Figure 2-6. Rotavirus replication cycle. Initial cell attachment occurs through VP8 and the rotavirus particle is endocytosed. In the endosome low calcium concentrations

triggers the un coating of VP7 and membrane penetration of VP5. In the cytoplasm the double layered particle (DLP) becomes transcriptionally active and capped rotavirus mRNAs are produced for each of the eleven gene segments. Rotaviral mRNAs are translated into proteins and new DLPs are formed in the viroplasm, which is induced by NSP2 and NSP5. The DLPs are translocated to the endoplasmic reticulum as the VP6 layer interacts with the membrane--bound NSP4 proteins. In the endoplasmic reticulum VP4 and VP7 are assembled onto the DLP and mature virions are formed. Release from the cell occurs either via lysis or exocytosis. Adapted from (Trask *et al.*, 2012).

Assemble into new double layered particles only where after dsRNA can be produced (Lu *et al.*, 2008). The assembly of the outer capsid layer (VP4 and VP7) occurs exclusively in the endoplasmic reticulum (Stirzaker and Both , 1989) . Double layer particles are recruited into the endoplasmic reticulum via interaction with NSP4. NSP4 is a multifunctional protein that is attached to the endoplasmic reticulum membrane and possesses both VP6--binding and VP4--binding domains (O'Brien *et al.*, 2000).

Consecutive interaction between membrane--bound NSP4s and VP6s on the surface of the double layered particle is thought to result in budding of the double layered particle into the endoplasmic reticulum. Once in the endoplasmic reticulum, a cascade of processes, which are poorly understood, is initiated involving the assembly of VP4 and VP7 onto the double layered particle and the removal of the transient envelope (Trask *et al.*, 2012)

During this maturation process, VP4 is probably assembled before VP7 as the foot of the VP4 trimer is too large to be inserted after the assembly of VP7. Putatively, VP7 is also involved in the removal of the transient membrane as it has been shown that VP7 disrupts membranes *in vitro* (Charpilienne *et al.*, 1997).

Release from the cell is possible through two mechanisms: cell lysis (Musalem and Espejo ,1985)and secretion from the apical cell surface (Jourdan *et al.*, 1997).However, the relative importance of the secretory pathway is currently unknown (Jourdan *et al.*, 1997) .

2.3.2.Norovirus Replication

The Norovirus Replication represents of a typical positive-strand RNA virus Replication. This Replication can be divided into several steps: attachment, entry, un coating, genome translation, replication, virus assembly and release (Figure 2.7). The first step is viral attachment/binding in which an initial contact between the virus particle and the host cell is made particularly, attachment occurs between a viral component and a host cell component, Viral receptors can be classified as attachment or entry receptors and they can be host proteins, lipids, or carbohydrates. Studies in the NoV field have discovered that Human NoV use carbohydrates, such as silica acid, as an important requirement during attachment (Rydell *et al.*, 2008;Bally *et al.*, 2012).

Attachment receptors, which can also be co-receptors, in many cases do not possess a strong interaction, but they can still assist in virus attachment to host cells (Grove and Marsh, 2011).However, do not allow the virus to productively infect cells. Hence, entry receptor molecules are essential for a productive viral entry and infection (Donaldson *et al.*, 2010; Esseili *et al.*, 2012). Interestingly, in the early 2000s, mechanisms of Human Norovirus entry and infection into the host has been mostly known based on observations from studies with volunteer individuals that linked the ABO blood type of individuals to susceptibility of infection (Atmar *et al.*, 2011;Parmar *et al.*, 2012).

After successful attachment of the viral particle to the host cell, internalization is initiated by the interaction of the virus with its viral entry receptor(s). Non-enveloped viruses are internalized by different

mechanisms depending on the virus, and do not possess the mechanism of its viral membranes fusing with host cell membranes that is observed for enveloped viruses. Once an interaction with the host entry receptor is made, the endocytic machinery further internalizes the viral particle. In general, this means that an invagination of the cellular membrane forms a vesicle-like structure known as an endosome that traffics into the cell to become an early/late endosome and finally a lysosome (Hanover, 1985).

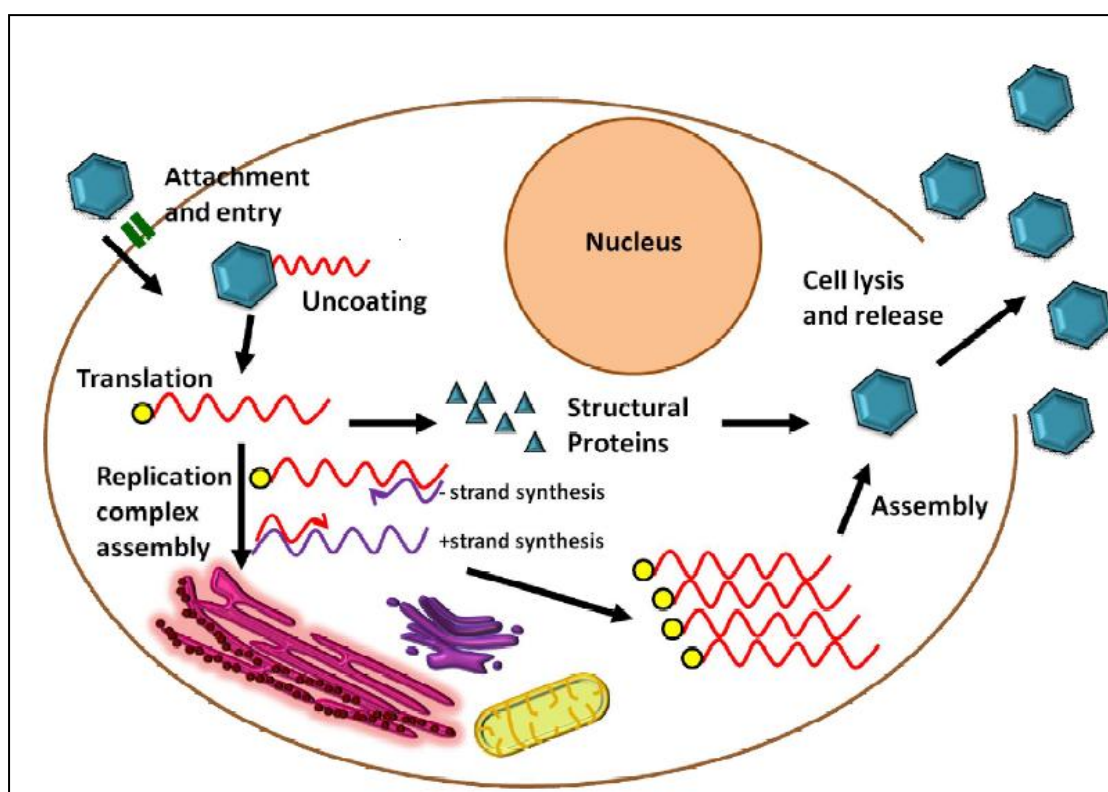


Figure 2-7. Representative calicivirus life cycle. Schematic of the life cycle of a positive-strand, non-enveloped RNA virus. Viral positive-sense RNA is shown in red and negative-sense RNA in purple. Yellow circles represent the virus protein genome-linked, VPg (Bally *et al.*, 2012).

The invaginations observed with Norovirus are, the beginning of virus entry into the host cell. Thus, that NoV enters the cells via one or more endocytic mechanisms (Marsh and Helenius, 2006). Interestingly,

another study using a NoV replica system, showed that host cholesterol is required during NoV replication (Chang, 2009). Uncoating occurs through multistep processes that are triggered by virus-host cell interactions. The protein shell, or capsid, contains the viral genome and protects it from environmental stresses, and only when time and important triggers have initiated, the genome will be released to the site of replication.

Once viruses have disrupted or penetrated the endosomal or cellular membrane upon entry, replication in the cytosol or nucleus can begin (Helenius, 2007). Uncoating can occur by either of two ways,

1- The virus capsid undergoes a conformational change triggered by environmental or host interactions that will eventually create a membrane pore in which the viral genome can exit and be transported to the replication site.

2- During the conformational change the virus particle releases peptides that can distort the endosomal membrane integrity preventing continuous enclosing of the virus particle and the capsid is transported to the cytosol for another self-conformational change that will release the genome into either the cytosol or nucleus for further replication (Helenius, 2007).

The next step of Replication involves the translation of the genome. NoVs can initiate translation with the help of a viral protein, VPg, that is linked to the genome at the 5' end (Daughenbaugh *et al.*, 2003; Karst *et al.*, 2003). For Human NoV, the VPg interacts directly with the host translational machinery (Thackray *et al.*, 2007). Once translated, the viral genome contains a long poly-protein of nonstructural proteins in which the viral protease (3CL-protease or NS6) can cleave the poly protein into the individual proteins. (Wobus *et al.*, 2004; Yokota *et al.*, 2009).

After translation, host membranes undergo remodeling to generate membranous structures, otherwise known as virus replication factories or membranous web that carry membranes from ER and the Golgi apparatus (Yokota *et al.*, 2009). Some membrane structures during replication contain double membrane characteristics, related to mitochondria (Tan and Jiang, 2005).

Based on these observations, it has been suggested that autophagy may play a role during viral replication. However, whether autophagy is important during NoV replication in general is still not clear (Glass *et al.*, 2009). The NoV replication machinery can be generally first, the viral protein RdRp, can bind to the 3' end of the viral genome either by an interaction with the conserved three-dimensional structure of the 3' untranslated region and/or the VPg (Belliot *et al.*, 2008).

Then, the RdRp begins to polymerize a matching anti-sense viral genome that once the VPg-linked 5' end is reached. the RdRp disassociates itself from the anti-sense genome. Next, the RdRp produces more positive-strand genomes using the matching anti-sense genome. Finally, after these additional positive-strand viral genomes are linked to the viral VPg by a covalent bond, they will then undergo translation or become packaged into new virus progeny (Goodfellow *et al.*, 2005).

The final step of the viral replication involves packaging the newly replicated viral genome into the viral capsid for successful assembly of progeny. Once assembly of the virus is finalized, the progeny is released from the infected cell to infect a new cell and begin the viral life cycle all over again. Since non-enveloped viruses lack a lipid component to fuse with the cell membrane and exit the cell, they require the cell's membrane be destroyed or lysed (Bok *et al.*, 2009). At the end, the newly synthesized and released progeny must find a new permissive cell so that, once found,

the initiation of the whole viral life cycle can take place again(Savill *et al.*, 2002). However, since not much is known about NoVs, future studies need to verify the mechanism of how assembly and release are accomplished (Savill *et al.*, 2002).

2. 4.Pathogenesis

2.4.1.Pathogenesis of Rotavirus

Rotaviruses infect cells in the villi of the small intestine.they multiply in the cytoplasm of enterocytes and damage their transport mechanisms.one of rotavirus-encoded proteins, NSP4, is a viral enterotoxin,damaged cells may slough into the lumen on the intestine and release large quantities of virus. which appear in stool, Viral excretion usually lasts 2-12 days in otherwise healthy patients but may be prolonged in those with poor nutrition. diarrhea caused by rotaviruses may be due to impaired in sodium and glucose absorption as damaged cells on villa are replaced by non absorbent immature crypt cells. It may take 3-8 weeks for normal function to be restored (Jawetz *et al.*, 2007).

2.4.2.Pathogenesis of Norovirus.

The human of the Norovirus infect the gastrointestinal tract, specifically the epithelium. This inflammation prevents the absorption of nutrients and liquids and causes severe vomiting and diarrhea in infected individuals. The mechanism of virus-induced diarrhea and vomiting is unknown (Treanor and Dolin, 2005).

The pathogenesis of human noroviruses has been described in human volunteers and in animal models, broadening and blunting of intestinal villa at the duodena jejuna junction, crypt cell hyperplasia, cytoplasmic vacuolization and infiltration of inflammatory cells into the lamina propriety were observed in biopsies from infected volunteers. These histological changes have been seen in symptomatic and

asymptomatic persons .Histopathologic changes resolve in 2 weeks in healthy subjects (Morotti *et al.*, 2004).

2.5.Clinical Signs and Symptoms

2.5.1.Clinical Signs and Symptoms of Rotavirus

The symptoms of RV AGE consist of watery diarrhea, vomiting and fever. Because of the wide spectrum of symptom severity, the causative agent of an individual AGE episode cannot be reliably differentiated by its symptoms (Aupiais *et al.*, 2009). In the bigger picture, RV AGE is often more severe compared to AGE caused by other viruses: the risk of dehydration is higher, fever is seen more often, and RV AGE more often leads to hospitalization (Giaquinto *et al.*, 2007;Aballéa *et al.*, 2013).

Clinically, RV AGE caused by different genotypes cannot be distinguished, although it has been proposed that G1P[8] and G9P[8] cause more severe symptoms on average than other RV genotypes (Linhares *et al.*, 2006). Diarrhea with or without vomiting is the predominant feature in RV AGE. In a Finnish community-based prospective study of AGE in children less than two years of old, Pang et al showed that there was a difference between AGEs caused by different viruses: diarrhea was more pronounced in RV AGE, whereas vomiting was seen more often in NoV AGE (Pang *et al.*, 2000).

The incubation period of RV AGE is typically one to three days, with an average of two days (Lee *et al.*, 2013). The symptoms begin acutely, most often with vomiting followed by watery diarrhea. Sometimes stomachache precedes the major symptoms. Vomiting lasts on average for two to three days and diarrhea for four to five (Räsänen, 2016), however there is a great deal of variation. Fever is often seen and 26 febrile seizures are seen in children with a predisposition for them.

Bloody diarrhea is rare (Payne *et al.*, 2013). Antigenemia and viremia can be seen, also in immune competent children, but systemic complications are rare (Blutt *et al.*, 2003; Chiappini *et al.*, 2005).

Many children, however, are infected by RV without marked clinical symptoms. Especially infants less than six months of age seem to have protection by maternal antibodies, and if infected during these first months, the infection often remains silent (Than *et al.*, 2013). Also in older children RV infection can sometimes be passed with nonspecific symptoms or no clinical symptoms at all (Räsänen, 2016), usually if the child has already had a primary RV infection (Franco *et al.*, 2006).

2.5.2 Clinical Signs and Symptoms of Norovirus

The incubation period of NoV AGE is 24-30 hours on average with variation from 12 to 72 hours (Lee *et al.*, 2013; Huhti, 2015). The major symptoms are nausea, vomiting with a sudden onset, abdominal cramps, non-bloody diarrhea (Glass *et al.*, 2009; Patel *et al.*, 2009). Also fever and generalized soreness of the body may occur, reflecting the name “stomach flu” sometimes used (Dolin *et al.*, 2004; Lopman *et al.*, 2004).

The symptomatic period of NoV AGE is reported to be 34 on average 24-48 hours (Chan *et al.*, 2015; Huhti, 2015). with a wide variation from asymptomatic infections to chronic symptoms seen immune compromised persons. However, the studies consider mainly adults, and the symptomatic period in children is often longer than the average mentioned above (Rockx *et al.*, 2002; Glass *et al.*, 2009). Also asymptomatic NoV infections exist (Chan *et al.*, 2015). In studies from developing countries, 12-49% of children were found to have NoV present in stools while not presenting with clinical symptoms (Garcia *et al.*, 2006; Bucardo *et al.*, 2010).

In a study from England NoV was found in up to 25% of previously healthy and asymptomatic children under three years of old and in less than 5% of adults (Phillips *et al.*, 2010). On the other hand, especially in immune compromised patients or in neonates, NoV infections can be atypical and severe (Payne *et al.*, 2013) and cause also severe intestinal infection, including necrotizing enter colitis, or systemic and extra intestinal symptoms such as encephalopathy (Karst, 2010).

The emergence of a new NoV GII.4 variant causing AGE symptoms with high fever and intestinal hemorrhage in children was reported in Taiwan in 2013 (Chen *et al.*, 2015). Also in otherwise healthy children, NoV AGE often is more severe and symptoms prolonged compared to adults (Payne *et al.*, 2013). It can lead to dehydration and imbalance in bodily salts and a need for hospital treatment, especially in young children. In general, diarrhea is seen more often in children than in adults. As with AGEs of other etiologies, NoV AGE cannot be reliably distinguished from other viral AGEs by symptoms only in an individual patient (Payne *et al.*, 2013). Assessed by the 20-point severity score, the NoV AGE cases in children seen in the hospital are often classified as severe (Sakai *et al.*, 2001; Patel *et al.*, 2009). but, on average, NoV AGE cases are not quite as severe as RV AGE cases (Payne *et al.*, 2013) .

2.6.Epidemiology

2.6.1.Epidemiology of Rotavirus

Rotaviruses mainly infect young children and before the age of five, every child has experienced at least one rotavirus infection. Although asymptomatic rotavirus infections are thought to occur regularly, the main driver of rotavirus epidemics are children which experience their first rotavirus gastroenteritis (Pitzer *et al.*, 2009). Rotavirus is extremely contagious and has a basic reproduction number

(the number of people one infected person can infect during its infectious period, also referred to as R_0) of approximately 25 and belongs to the most infectious human viruses currently known (Pitzer *et al.*, 2011).

In temperate climates, rotavirus incidence is highly seasonal and yearly epidemics occur every winter. The onset of rotavirus epidemics is closely related to the birth rate and countries with higher birth rates tend to have an earlier start of yearly epidemics because of a larger susceptible population making it easier to maintain transmission chains (Pitzer *et al.*, 2009). Due to the decreased size of the susceptible population, a delayed onset of yearly rotavirus epidemics was also observed in countries where rotavirus vaccines were introduced in the national immunization program (Steenland *et al.*, 2013). In tropical and subtropical climates rotavirus infections occur year round (Levy *et al.*, 2008).

In some tropical areas additional incidence peaks were observed during the season (Rahman *et al.*, 2007). In humans there are six major genotypes that are circulating worldwide and that are of epidemiological importance: G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8]. The G9P[8] and G12P[8] genotypes have only started to circulate worldwide in the 1990s and 2000s, respectively and were probably introduced into the human population after interspecies reassortment (Zeller *et al.*, 2011).

The prevalence of these six genotypes generally varies from season to season and between different geographical regions (Bányai *et al.*, 2012). Besides these six major genotypes, other genotypes can be of regional importance. For example, P[6] rotaviruses circulate frequently in sub Saharan countries (Mwenda *et al.*, 2014; Seheri *et al.*, 2014). Recently it has been suggested that differences in histo-blood group antigens between African and non-African people may be related to differences in P[6] prevalence (Imbert-Marcille *et al.*, 2013).

In fact, histo--blood group compatibility is probably the most important determinant for host range restriction of rotaviruses and could explain why animal rotaviruses do not cause large scale rotavirus epidemics in humans (Liu *et al.*, 2012). It is not yet fully understood how rotaviruses relate to each other from one season to the next. Large scale sequencing datasets have revealed the persistence of genetically closely related rotaviruses through multiple rotavirus seasons, suggesting that during the summer months rotaviruses are locally maintained in asymptomatic adults (McDonald *et al.*, 2011; McDonald *et al.*, 2012). However the introduction of new genotype variants has been observed which indicates that there is some degree of viral migration between different geographical locations. The recent spread of G9 and G12 rotaviruses only reconfirms these observations (Zeller *et al.*, 2011).

2.6.2. Epidemiology of Norovirus

For decades, probably the majority of cases of what was formerly the clinical syndrome “winter vomiting disease” – sharply beginning and short lasting illness with vomiting and diarrhea – have actually been NoV AGE (Patel *et al.*, 2009). In children, the clinical features of NoV AGE are often not so typical, and it takes a long time before it is realized that NoV also causes seasonal AGE in children. NoVs are generally recognized as the major causative agents of viral gastroenteritis in the world (Ahmed *et al.*, 2014; Huhti, 2015). They affect people of all ages and everywhere in the world. However, it is not easy to evaluate the exact real incidence or total burden of NoV AGE because laboratory studies are not commonly done, except in outbreaks (Huhti, 2015).

2.7. Predominating genotypes

2.7.1. Predominating genotypes of Rotavirus

The five major circulating RV genotypes in Europe, North America and Australia, G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8], are responsible for more than 90% of the RV AGE cases in these areas (Gentsch *et al.*, 2009; Dóro *et al.*, 2014). The predominant genotype in these parts of the world in the long-term perspective is G1P[8] (Van Damme *et al.*, 2007; Hull *et al.*, 2011). However, the epidemiologic situation is not constant: natural variation occurs. Especially genotype G9P[8] RVs have taken local predominance for single seasons in the 2000s (De Rougemont *et al.*, 2009; Hemming and Vesikari, 2013).

Such temporary predominance can also be seen with other major genotypes (Antunes *et al.*, 2009; Gómez *et al.*, 2014). Even though the variation in scale of the circulating RV genotypes is greater in Africa and Asia G1P[8] and G9P[8] are usually found to be the dominant RV types there as well (Seheri *et al.*, 2014; Yu *et al.*, 2015). The sixth relatively common RV genotype globally is G12P[8], which emerged in the 1990s and 2000s (Matthijnssens *et al.*, 2010; Dóro *et al.*, 2014). Of the other genotypes, G5P[8] in South America and G8P[6] in Africa, for example, are frequently seen in circulation (Gentsch *et al.*, 2005).

The presenting RV genotypes in seven European countries (Belgium, France, Germany, Italy, Spain, Sweden and United Kingdom) were studied during the same AGE season. In all these countries, the five main genotypes accounted for 98% of the RV AGE cases, but the predominant RV genotype varied from country to country even within Europe: in Belgium, Spain, United Kingdom and Sweden it was G1, in France and Italy G9, and in Germany G4 (Van Damme *et al.*, 2007).

2.7.2.Predominating genotypes of Norovirus

In seasonal NoV AGE in AGE outbreaks, GII.4 has been the predominating genotype since the 1990s (Huhti *et al.*, 2014;Wollants *et al.*, 2015). This genotype is constantly under active evolution, and, as a consequence, new variants of NoV GII.4 have appeared and spread, causing epidemics throughout the world every few years (Glass *et al.*, 2009; Ramani *et al.*, 2014).

Also other genotypes of NoVs are seen in seasonal AGE. the majority of them also belonging to genogroup GII. In a recent report about seasonal AGE in Belgium over the course of ten years, NoVs were detected in 18.7% of all AGE stool samples, and GII.4 predominated them by causing 86.6% of all NoV-positive AGE cases. In this ten-year follow-up, only 3.8% of seasonal NoV AGE was caused by genogroup GI NoVs (Wollants *et al.*, 2015).

In Finnish RV vaccine trials in 1994-1995 and 1998-2004, almost 5000 stool specimens were collected from young children with AGE. Among these cases, NoV was found in 25% of the samples, genotypes GII.4, GII.7, GII.3, and GII.b being the types seen more frequently than single cases; genotype GI.3 was found in 1-4% cases in 1998-2003; and other genogroup GI NoVs were found occasionally (Huhti *et al.*, 2014). In AGE outbreaks, the variation of causative NoV types is greater than in seasonal AGE (Matthews *et al.*, 2012; Vega *et al.*,2014).

In waterborne outbreaks and outbreaks caused by use of contaminated water in the source of the food (as in the case of imported frozen raspberries planted with contaminated water), the causative NoV most often belongs to the GI genogroup (Riera-Montes *et al.*, 2011; Bernard *et al.*, 2014). In AGE outbreaks in institutional or crowded settings such as elderly homes or cruise ships, the causative virus is often

the predominant NoV type circulating in the population, often a variant of GII.4 (Kroneman *et al.*, 2011; Bok and Green, 2012).

2.8. Seasonality

2.8.1. Seasonality of Rotavirus

In temperate climates, RV AGE seasons typically occur during the winter months, beginning in late autumn or winter and lasting until the spring (D'Souza *et al.*, 2008; Than and Kim, 2013). This seasonal pattern is seen in both the northern and southern hemispheres, most clearly in Europe, North America and Oceania. the RV seasonal peak occurs in the winter months of each. In tropical countries, the seasonal pattern is not seen or is not as definable. On the other hand, there are countries of temperate climate with a year-round occurrence of RV AGE and tropical countries with RV seasonality (Patel *et al.*, 2013). The reason for seasonality is not completely understood: there is probably no single explaining factor, but the seasonality is the result of multiple co-predictors including the temperature, humidity and yearly rainfall, altitude, population density and behavioral factors, and perhaps the economic income of the country (Patel *et al.*, 2013).

The economic status of countries does not explain the seasonal or year-round occurrence of AGE because in countries with similar income levels, seasonality exists or is lacking, depending on the country's distance to the equator (Patel *et al.*, 2013).

2.8.2. Seasonality of Norovirus

In moderate-climate countries, the NoV season traditionally begins in winter and lasts until spring between October and March in the northern hemisphere (Ahmed *et al.*, 2013; Wollants *et al.*, 2015). About three-quarters of NoV cases occur in these cool months. The explanation for the seasonality of NoV AGE is not known, but such factors as rainfall,

cool temperatures and the emergence of new variants have been proposed (Marshall and Bruggink, 2011; Ahmed *et al.*, 2013). It seems reasonable to think that there is also an association between AGE seasons and people's behavior: holiday seasons versus school and day care seasons with crowding indoors and public vehicles (Ahmed *et al.*, 2013).

2.9. Transmission

2.9.1. Rotavirus Transmission

The main route of transmission of RV AGE is fecal-oral (Than and kim, 2013; Räsänen, 2016), however it can also occur via respiratory droplets containing mucus and viruses (Aballéa *et al.*, 2013; Räsänen, 2016). The spreading of the infection is very difficult to control. The virus is shed in large numbers, up to 111 infectious viral particles/mL in stools of an infected person and, on the other hand, the contagious dose of RV is very small: about 10 viral particles (Räsänen, 2016). Besides these, knowing that RVs are highly resistant to environmental factors such as temperature and pH (Payne *et al.*, 2013), it is easy to understand why RV AGE is very easily transmitted, especially at day-care centers, where young children prone to infection are in close contact with each other, or in a hospital ward treating children with and without AGE. Shedding of viruses in stools has been demonstrated for about two or three weeks by RT-PCR (Mukhopadhyaya *et al.*., 2013; Corcoran *et al.*, 2014), but in immune compromised persons the shedding can last as long as months (Corcoran *et al.*, 2014)

2.9.2. Norovirus Transmission

NoVs are highly transmissible. It has been estimated that the infectious dose may be as low as 18 viral particles (Teunis *et al.*., 2008; Payne *et al.*, 2013). In a symptomatic person, the viral load in stools may peak two to five days after infection at as much as 111 viruses per gram

(Hall *et al.*, 2011). Fecal-oral spread from person to person or via contaminated surfaces is the most important route of transmission (Seitz *et al.*, 2011; Chan *et al.*, 2015). The concentration of viral particles is also high in fomites and they can spread by droplets (Glass *et al.*, 2009).

As NoVs can also be transmitted by aerosols generated by vomiting or flushing the toilet and spread outside a patient's room, the airborne route of transmission is also possible (Glass *et al.*, 2009; Lopman, 2011). NoVs are very stable in the environment and resistant to chemical disinfection substances (Lewis *et al.*, 2012). They survive in aerosols (Glass *et al.*, 2009) and can survive in a viable form on surfaces for about two weeks and more than two months in water (Hall *et al.*, 2011; Seitz *et al.*, 2011). Transmission often occurs through contaminated food or water. Regardless the route of transmission, the viruses always have to reach the gastrointestinal tract to reproduce. It is not yet clear how long the shedding of viable viruses can continue or what the significance is of prolonged shedding in NoV epidemiology. Even in healthy adults, NoVs can be detected in stools for an average of four weeks after an AGE episode (Teunis *et al.*, 2015).

In small children, elderly people and immune compromised persons, the shedding can last for much longer (200), even more than a year (Atmar *et al.*, 2011; Chan *et al.*, 2015). However, asymptomatic shedding seems to be much less contagious than shedding in symptomatic patients (Sukhrie *et al.*, 2012; Chan *et al.*, 2015), and its significance in transmitting NoV should be studied further. In addition, the window of time in which detectable viruses are found in stools is not automatically equal to the time of shedding of contagious viruses; sensitive PCR methods can also detect RNA of non-viable viruses in stools (Knight *et al.*, 2013).

2.10. Diagnosis

2.10. 1.Diagnosis of Rotavirus Gastroenteritis

The presence of RV is commonly detected in stools by the ELISA antigen test or RT-PCR. ELISA tests are commercially available and fast to perform. The sensitivity of ELISA tests is good (Pang *et al.*, 1999; Lewis *et al.*, 2012). However false positives can also occur. Compared to the ELISA test, RT-PCR finds more RV-positive stool specimens. However the additional cases are mild ones; the sensitivity of the two methods is about the same in finding severe cases (Pang *et al.*, 1999).

This suggests that the sensitivity of ELISA is good enough to be used at the hospital level and in vaccine efficacy studies, and the studies conducted using ELISA are comparable to the ones using RT-PCR. However, the ELISA test cannot be used if the size of the stool sample is very small or if the stool is soaked into a diaper. Furthermore, ELISA cannot determine the RV genotype. Compared to ELISA, RT-PCR is better in detecting especially RVs of genotype G2 and G4 (Pang *et al.*, 1999), and perhaps of more uncommon strains as well. The RT-PCR method is highly sensitive, and the test can be done from a minimal amount of stool, such as from diapers or a rectal swab. The genotype of RV can be determined by sequencing the VP7 and VP4 coding regions (Pang *et al.*, 1999; Lewis *et al.*, 2012)).

Until lately, RT-PCR testing has mainly been used in research, but along with rapid-PCR techniques, the test has become more commonly available, also in clinical practice (Pang *et al.*, 1999; Lewis *et al.*, 2012).

2.10.2.Diagnosis of Norovirus Gastroenteritis

In the 1970s, the method for detecting NoVs was EM or immune-EM ,Antigen-detecting tests using monoclonal antibodies have not proven to be ideal in detecting NoVs (Jiang *et al.*, 2000; Kroneman *et al.*, 2013). Challenges in antigen testing include the high antigenic variety and constant evolution in NoV strains and the high viral load needed for a positive test signal in ELISA.

NoVs are detected from stools or, less commonly, from fomites or food and environmental samples by RT-PCR. The virus genogroup, genotype and variant can be detected by further sequencing the partial genome. In the 1990s, several primer pairs were needed for detection, and the technique was laborious (Jiang *et al.*, 1992;Kroneman *et al.*, 2013).

In the 2000s, detection has based on primers targeting a conserved region of the genome open reading frame 1 (ORF1) which codes the viral RNA polymerase (Maunula *et al.*, 2005). The NoV genome ORF2 region C coding viral capsid can be used in determining the virus genotype and variant. Nowadays, it is recommended that both polymerase and capsid genotypes are determined, because recombination is common (Kroneman *et al.*, 2013).

Lately, rapid multiplex PCR tests for diarrheal pathogens have been developed and become commercially available.

2.11.Vaccines

2.11.1 Rotavirus Vaccines

Since there is a significant disease-related morbidity and mortality associated with rotavirus, the development of an effective vaccine is a priority. Several rotavirus vaccines candidates have been developed and tested (Parashar *et al.*, 1998).

The mechanisms and duration of protection against rotavirus infection are incompletely understood, however, it is suspected that clinical protection likely involves mucosal (intestinal) and systemic antibody response as well as cell-mediated immunity. Evidence suggests that intestinal (jejunal) IgA is possibly one of the more important mechanisms for long-term protection against rotavirus infection (Franco *et al.*, 2006). With regard to potential protection from systemic antibodies, immune response has been hypothesized to partly depend on neutralizing epitopes VP7 and VP4 on the viral capsid (Franco *et al.*, 2006; Ward, 2009). The antigen composition of the current and candidate rotavirus vaccines are typically described on the basis of these VP7 (G-type) and VP4 (P-type) proteins on the vaccine strain. The current and previous candidate rotavirus vaccines can be categorized in two groups on the basis of their development (Jiang *et al.*, 2010)

(1) First generation vaccines: these early rotavirus vaccines are single-strain animal strains that were naturally attenuated in that they did not cause clinical disease in humans but conferred protection against subsequent infection with human rotavirus strains.

(2) Second generation vaccines: these are vaccines developed through attenuation of human rotavirus strains. A live attenuated human rotavirus vaccine based on the G1 P1A serotype was first developed in Cincinnati, Ohio by tissue culture passage of a wild-type human rotavirus isolate (Dennehy, 2007). The rotavirus strain used in this candidate vaccine was chosen because it is the most common of the human rotavirus strains, G1 P1A. Greater than 70% of rotavirus infections in North America, Europe, and Australia are attributed to the G1 P1A strain (Van Damme *et al.*, 2007). This vaccine was further developed by Avanti Immunotherapeutics and later licensed to Glico Smith Kline Biological, who did further

research on this vaccine and continued through the licensure process to create RIX4144 (RotaRix[®]).

2.11.2 Norovirus Vaccines

Currently there is no licensed norovirus vaccine. Promising vaccine candidates are under development. Studies have shown that both orally and nasally administered virus-like particles (VLPs) can be well tolerated and are immunogenic in phase 1 trials (El-kamary *et al.*, 2010).

A nasally administered investigational GI.1 norovirus VLP vaccine is also shown to confer 47% protection against norovirus gastroenteritis in healthy adults in a human experimental challenge model (Atmar *et al.*, 2011). Recently a bivalent intramuscular norovirus virus-like particle (VLP) vaccine is reported to be well tolerated and immunogenic in adults (Treanor *et al.*, 2014).

Chapter Three

Materials and Methods

3. Materials and Methods

3. 1. Materials

3.1.1. Equipments and Instruments

Table (3-1): The Equipment and Instruments that Used in this Study with their Companies and Countries of Origin:

No.	Equipments & instrument	Company
1	Electrophoresis	Bioneer/ Korea
2	Eppendorf tubes	Bioneer/ korea
3	Exispin vortex centrifuge	Bioneer/ korea
4	Gloves	CYAN/ Belgium
5	High Speed Cold centrifuge	LabNet /USA
6	Micropipettes 5-50, 0.5-10, 100-1000µl	CYAN/ Belgium
7	Nanodrop	THERMO/ USA
8	PCR T100 Thermal cycler	BioRad /USA
9	Real-Time PCR	BioRad /USA
10	Refrigerator	Concord /Lebanon
11	Test Tubes	Cypress/Belgium
12	U.V transilluminator	Wised/Korea
13	Vortex	CYAN/ Belgium
14	Water Bath	Plymouth/USA

3.1.2. Kits

Table (3-2): The Kits Used in this Study with their Companies and Countries of Origin:

No.	Kit	Company	Country
1	AccuZol™ Total RNA extraction kit	Bioneer	Korea
	Trizol 100ml		
2	NEXpro™ qRT-PCR Master Mix (probe)	Genes Laboratories	Korea
	qRT-PCR Master Mix: MMLV (RT), HotstartTaq DNA Polymerase, ultrapure dNTPs, MgCl ₂ .		
3	NEXpro™ RT-PCR Master Mix	Genes Laboratories	Korea
	MMLV Reverse Transcriptase		
	HotstartTaq DNA Polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris-HCl pH 9.0, KCl, MgCl ₂		
	Stabilizer and Tracking dye		

3.1.3. Primers and Probes

The primers and probe that used for direct detection of Rotavirus and Norovirus were designed in this study by NCBI-Genbank and Primer3 plus design. The primers were provided by (Macrogen. Company, Korea) as following table:

Table (3-3) RT-Real-Time PCR Primers and Probe

Primer	Sequence		Amplicon	Reference
Rotavirus A VP6 gene Primer	F	ACGCTCCAGCAAATATTCAGC	145 bp	This Study
	R	AACCATGTAGTTGCGCCATC		
Rotavirus A VP6 gene Probe	FAM-TGTCCAGCTAAGGCGTGCGC-BHQ-1			This Study
Norovirus Capsid gene Primer	F	TGTAGCGGGCCAACAAAATG		This Study
	R	GCGCGCTCCATAGTATTTCAC		
Norovirus Capsid gene Probe	FAM-ACAAGCCCCTGGTGGAGAGTT-BHQ-1			

Rotavirus A GenBank: HM534677.1, NorovirusGenBank: HQ005298.1

Table (3-4) RT-PCR Primers for DNA Sequencing

Primer	Sequence		Amplicon	Reference
Rotavirus A VP6 gene	F	GCGTGCGCTAACTACAGCTA	369bp	This Study
	R	GCATCCGCAAGCACTGATTC		
Norovirus Capsid gene	F	TTCCTGCGGAGAACTGTGAC	540bp	This Study
	R	GTAAACTCTCCACCAGGGGC		

3.1.4. Chemicals

Table (3-5): The Chemicals with Their Companies and Countries of Origin Used in This Study:

No.	Chemical	Company and Origin
1	Absolute ethanol	BDH (England)
2	Chloroform	BDH (England)
3	Isopropanol	BDH (England)
4	DEPC water	Bioneer/ Korea
5	RNase free water	Bioneer/ Korea

3.2. Method

3.2.1 Study Population

A total of 100 fecal specimens were collected from infants and children under five years of age in Thi-Qar Province south of Iraq during Five Months (From November 2017 To March2018): 60 stool specimens were collected from the city of Nasiriyah(including Mohammed Al Mousawi children's Hospital ,Bint Al-Huda Teaching Hospital and Private Clinics) and 40 stool specimens were collected from Al-Nasiriyah, Sayed Dakhil, Al-Shatrah, SuqAlshyuokh, Al-Rifai, Al-Garraf and Al-Batha .The fecal specimens including 63 males and 37 females suffering from acute gastroenteritis.

3.2.2. Stool Samples Collection

Stool suspension from 10% to 50% of each specimen was prepared in 1 ml NaCl after vigorously mixing the stool suspension was clarified by centrifugation at 8000 rpm for 15 min at 4 °C. The resulting supernatants were collected and stored at -20 °C until use for nucleic acid extraction.

Table (3-6): Characteristics of the Study Population.

Variable (Parameter)	Diarrheal Group (N=100)
Sex	
Male	63
Female	37
Age Groups (years)	
0-1	28
1-2	43
2-3	15
3-4	10
4-5	4
Feeding Type	
Breast Feeding	34
Bottle Feeding	50
Mixed Feeding	16
months	
November	24
December	31
January	21
February	14
March	10
Symptom	
Diarrhea	100

Vomiting	70
Fever	62
Abdominal Pain	20
Total	100

3.2.3. Study Design

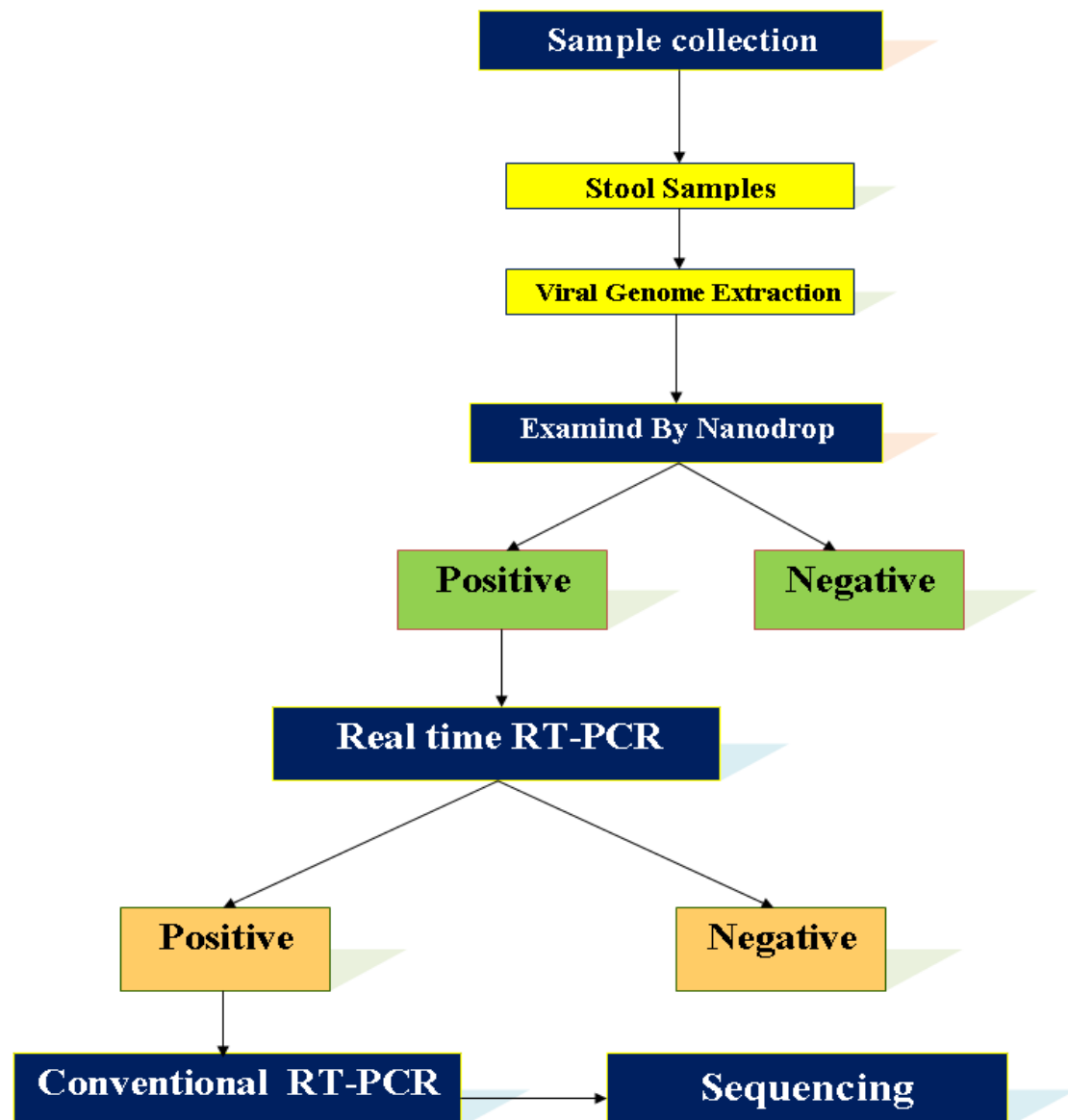


Figure (3-1)Study Design

3.2.4. Viral RNA Extraction

Viral RNA was extracted from stool samples by using AccuZolTM Total RNA extraction kit (Bioneer, Korea) and done according to company instructions as following steps:

1. The stool sample of 250 μ l was transferred by sterile pipette into sterile and clean 1.5ml Eppendorf tube, then 1ml Accuzol reagent an mixed by vortex.
2. Chloroform (200 μ l) was added to each Eppendorf tube and shaken vigorously for 30 seconds.
3. The mixture was incubated on ice for 5 minutes.
4. After that, the mixture was centrifuged at 12,000 rpm, 4C $^{\circ}$, for 15 minutes.
5. Supernatant was transferred to a new Eppendorf tube, and 500 μ l isopropanol was added.
6. The mixture Mixed by inverting the tube 4-5 times and incubated at 4C $^{\circ}$ for 10 minutes.
7. The mixture was centrifuged at 12,000 rpm, 4C $^{\circ}$, for 10 minutes.
8. The supernatant was discarded.
9. 80% Ethanol was added into each tube and mixed by vortex, then centrifuged at 12,000 rpm, 4C $^{\circ}$ for 5 minutes.
10. The supernatant was discarded and the RNA pellet left to dry at room air for 5 minutes.
11. After that, DEPC water (50 μ l) was added to RNA pellet tubes and mixed by vortex to dissolved the RNA pellet.
12. The extracted RNA sample was kept at -20 freezers.

3.2.5. Estimation of RNA extraction from stool samples

The extracted RNA from stool samples were estimated by using Nanodrop spectrophotometer that are used to measurement the RNA concentration and purity at absorbance 260/280 nm at ratio 2 as pure RNA, and done as following steps:

- 1- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).
- 2- A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipeted 2µl of free nuclease water and put on the surface of the lower measurement pedestal to blanking of Nanodrop.
- 3- After that, the pedestals are cleaned and pipette 1µl of total RNA sample for measurement.

3.2.6. Reverse Transcription Real-Time PCR (qPCR)

RT-qPCR was performed for detection of Rotavirus and Norovirus based capsid gene and this technique was carried out according to method described by (Kim *et al.*, 2012) as following steps:

3.2.6.1. RT-qPCR master mix preparation:

Real-Time PCR master mix was prepared by using d RT-qPCR detection kit NEXpro™ qRT-PCR Master Mix (probe) and done according to the company instructions as in the following table:

Table (3-7): RT-qPCR Master Mix Reaction Components:

RT-qPCR Reaction Components	Volume
Total RNA template	5 μ L
Forward primer (10pmol)	1 μ L
Reverse primer (10pmol)	1 μ L
Probe (20pmol)	1 μ L
RT-qPCR Master mix 2X	10
DEPC water	2 μ L
Total	20 μ L

The qPCR master mix reaction components that mentioned in table (3-7) above were added into Real-Time PCR strips. tubes Then all strips tubes mixed by vortex and centrifuged for 3000rpm for 3 minutes in Exispin centrifuge, after that transferred into Real-Time PCR thermocycler.

3.2.6.2. RT-qPCR thermocycler conditions:

RT-qPCR thermocycler conditions was set according to primer annealing temperature and qPCRTaqMan kit instructions as in the following table(3-8):

Table(3-8): qPCR Thermo Cycler Conditions

Step	Condition	Cycle
cDNA step	50° C 15 min	1
Pre-Denaturation	95° C 5 min	1
Denaturation	95°C 20 sec	45
Annealing	60° C 30 sec	45
Extension	72° C 60 sec	45
Final extension	72° C 5 min	1
Detection (scan)	60° C 30 sec	45

3.2.6. 3.Real-Time PCR Data analysis:

RT-qPCR data analysis was performed by calculation the threshold cycle number (CT) value that presented the positive amplification in Real-Time PCR cycle number.

3.2.7 .Conventional RT-PCR:

RT-PCR technique was performed on some positive isolates in RT-qPCR method that use for Rotavirus and Norovirus genotyping by using DNA sequencing analysisas following steps:

3.2.7.1. RT-PCR master mix preparation

PCR master mix was prepared by using (NEXpro™ RT-PCR Master Mix kit) and this master mix done according to company instructions as following table:

Table (3-9)master mix components conventional RT-PCR

RT-PCR Components	Volume
RNA template	5 μ l
Forward primer (10pmol)	1.5 μ l
Reveres primer (10pmol)	1.5 μ l
PCR Master Mix	12 μ l
Total volume	20 μ l

After that, these PCR master mix component that mentioned in table above placed in standard Accu Power RT-PCR Pre Mix Kit that contain all other components which needed to PCR reaction such as (RT enzyme, Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl₂, stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (MyGene. Korea).

3.2.7.2.PCR ThermoCycler Conditions

PCR thermocycler conditions were done by using convential PCR thermocycler system as following table:

Table (3-10)Thermo Cycler Conditions Conventional RT-PCR

PCR step	Temp.	Time	repeat
cDNA synthesis	50°C	1hour	1
Initial Denaturation	95°C	5min	1
Denaturation	95°C	30sec.	30 cycle
Annealing	55°C	30sec	
Extension	72°C	30 sec	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

3.2.7.3.conventional RT-PCR product analysis

The RT-PCR products was analyzed by agarose gel electrophoresis following steps:

- 1- 2% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

- 2- Then 3µl of ethidium bromide stain were added into agarose gel solution.
- 3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of RT-PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.
- 4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.
- 5- RT-PCR products were visualized by using UV Transilluminator.

3.2.8.DNA sequencing method

DNA sequencing method was performed for confirmative detection and genetic relationship analysis Rotavirus A and Norovirus by using NCBI- BLAST and phylogenetic tree analysis. The sequencing of the Rotavirus A and Norovirus RT-PCR product of capsid genes was purified from agarose gel by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). As the following steps:

1. The specific RT-PCR product was excised from the gel by clean, sharp scalpel. Then, transferred into a 1.5mL micro centrifuge tube.
2. 400µl Binding Buffer II was added to gel fragment. Then, incubated at 60°C for 10 minutes and mixed until the agarose gel is completely dissolved.

3. Add the above mixture to the EZ-10 column and let stand for 2 minutes. Centrifuge at 10,000rpm for 2 minutes and discard the flow-through in the tube.

4. 750µl Wash Solution was added to each tube and centrifuged at 10000rpm for one minute. Then, solution discarded.

5. After that, the step 4 was repeated. Then, centrifuged at 10000rpm for an additional minute to remove any residual wash Buffer.

6. The column was placed in a clean 1.5ml microcentrifuge tube and added 30µl of Elution Buffer to the center of the column and incubated at room temperature for 2 minutes. Then, the tube was centrifuged at 10000rpm for 2 minutes to elute PCR product and store at -20°C.

After that, the purified RT- PCR product samples were sent to Bioneer Company in Korea for performed the DNA sequencing by AB DNA sequencing system.

Phylogenetic analysis was performed based on NCBI-Blast Alignment identification and Neighbor Distance Phylogenetic tree analysis (Mega version 6).

3.2.9. Statistical analysis

The statistical analysis produced in all samples of study descriptive statistics analyzed by *chi_square* (P-value 0.01) was considered to be significant.

Chapter Four

Results

4.Results

4.1.Distribution of Rotavirus and Norovirus detected in Stool Specimens of Diarrheal Group.

According to diarrhea suffered children as in Table and figure (4-1) which revealed that 35% were associated with Rotavirus while 9% diagnostic as Norovirus on the other hand, there was 10% of total 100 diarrheal patients considered mixed infections This result showed there was statistical significant differences ($P < 0.01$).

Table (4-1):Distribution of Enteric Viruses Detected in Stool Specimens of Diarrheal Group.

Type Of Virus	Patients Group	
	No. of +ve cases/ No. of tested cases	% Positive
Rotavirus	35/100	35
Norovirus	9/100	9
Mixed infection Rota+Noro	10/100	10

Chi Square(cal) =26.78,DF=2,Chi table =5.99, Significant ($P < 0.01$)

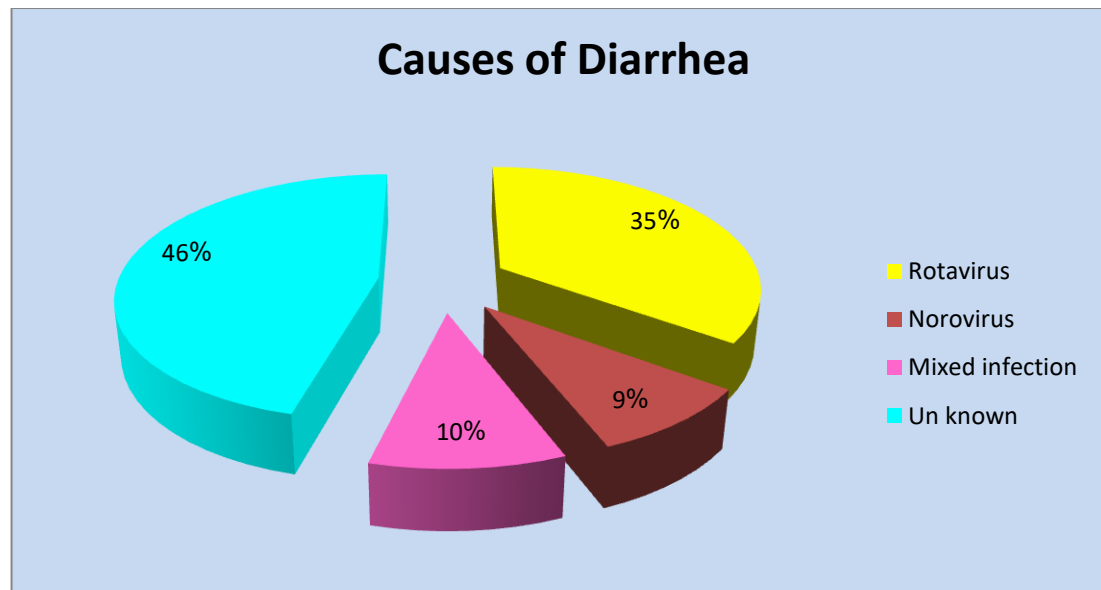


Figure (4-1): Distribution of causes diarrhea Detected in Stool Specimens of Diarrheal Group.

4.2. Distribution of Rotavirus infection among Group of Children under 5 years old according to sex

Table and figure (4-2) demonstrate of rotavirus-positive in diarrheal group of children according to gender .The study found that from one hundred children with diarrhea, the percentage of male is higher than female . The patient group by rotavirus shows that (68.9%)of them are males and (31.1%) are females This result shows there is statistical significant differences between male and female ($P < 0.01$).

Table(4-2): Distribution of Rotavirus infection among Children under 5 years old according to sex.

Variable	Diarrheal Group	
	No. of +ve cases	% of +ve cases
Male	31	68.9
Female	14	31.1
Total	45	100

Chi-Square=14.27, Df=1 Chi table =3.84, Significant (P<0.01)

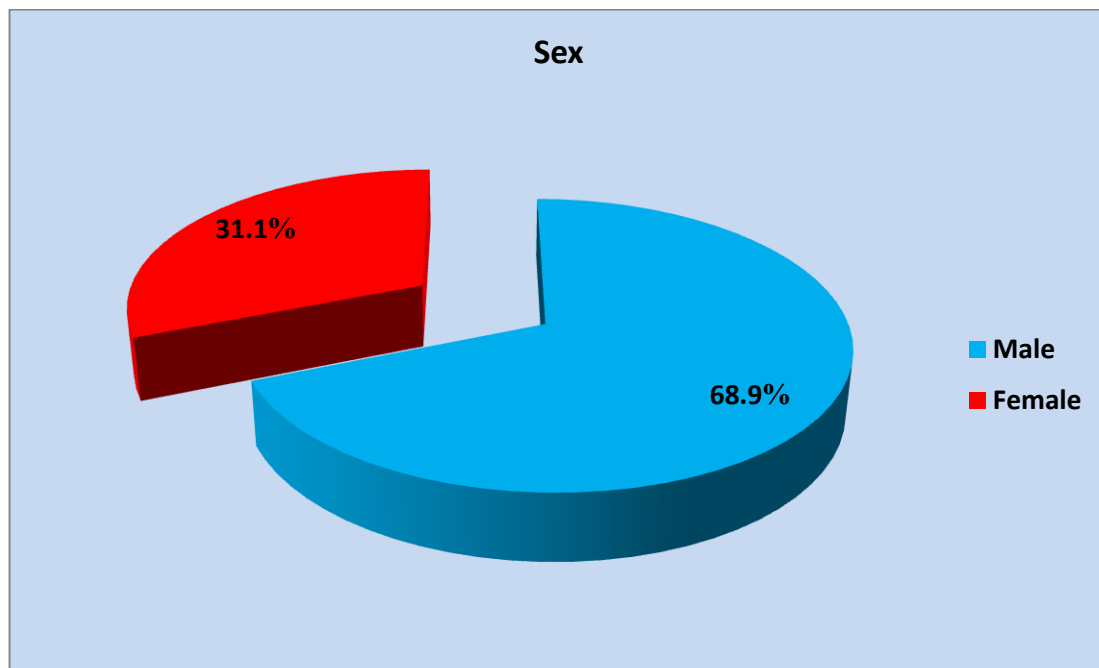


Figure (4-2): Distribution of Rotavirus infection among Children Under 5 years old according to sex.

4.3. Distribution of Norovirus infection among Children under 5 Years Old according to sex.

Table and figure (4-3) demonstrate of Norovirus-positive in diarrheal group of children according to gender. The study found that from one hundred children with diarrhea, the percentage of male is higher than female . The patient group by norovirus show that (57.9%) of them are males and (42.1%) are females This result show non statistical significant differences between male and female.

Table(4-3): distribution of norovirus infection among children under 5 Years Old according to sex .

Variable	Patients Group	
Sex groups	No. of +ve cases	% of +ve cases
Male	11	57.9
Female	8	42.1
Total	19	100

Chi-Square=2.49 Df=1, Chi=3.84 table, Not Significant (NS)

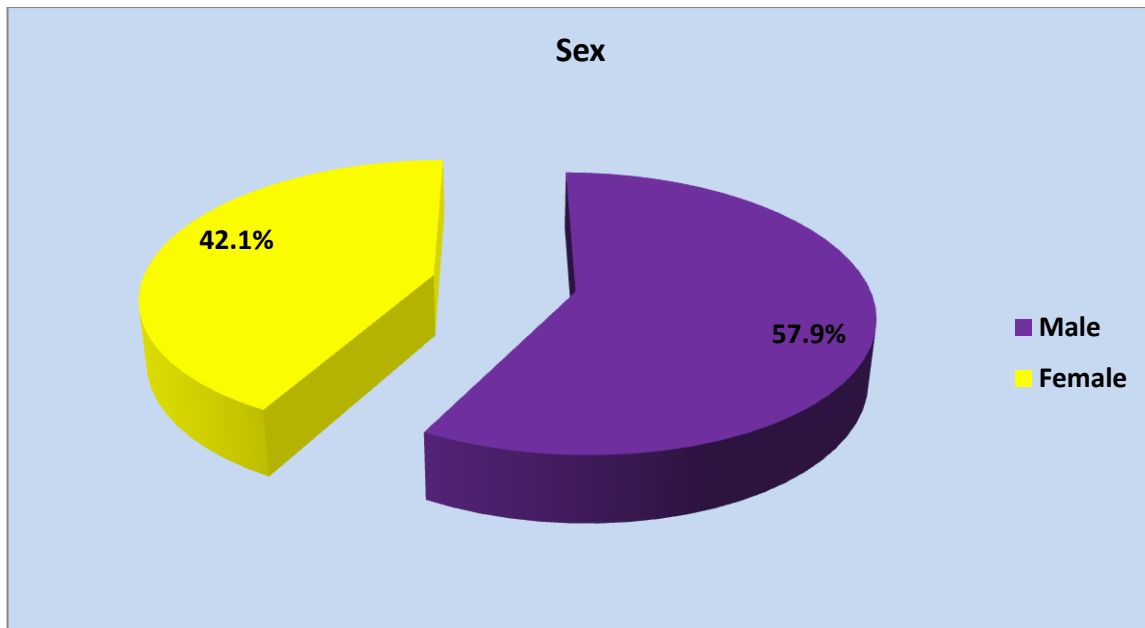


Figure (4-3): distribution of norovirus infection among children under 5 Years Old according to sex .

4.4. Distribution of Patients with Rotavirus According to the Feeding Types.

Table (4-4) we can see the distribution of patients with rotavirus and Norovirus according to the feeding types, show 16 (35.6%) child were fed on breast feeding, 22 (48.9%) child are fed on bottle feeding and 7 (15.6%) child were feeding on mixed feeding while Norovirus show 5 (26.3 %) child are fed on Breast feeding, 10 (52.6 %) child are fed on bottle feeding and 4 (21.1%) child are fed on mixed feeding. There are statistical significant differences between three types of feeding ($P < 0.01$).

Table (4-4): Distribution of Patients with Rotavirus and Norovirus According to the Feeding Types.

Type of virus	Feeding Type		
	Breast feeding	Bottle feeding	Mixed feeding
	No. of +ve cases (%)	No. of +ve Cases (%)	No. of +ve cases (%)
Rotavirus*	16 (35.6)	22 (48.9)	7 (15.6)
Norovirus**	5 (26.3)	10 (52.6)	4 (21.1)

*Chi-Square=16.89, DF=2, Chi-Square table=5.99, Significance (P<0.01)

**Chi-Square=17.17, DF=2, Chi Table =5.99, Significant (P<0.01)

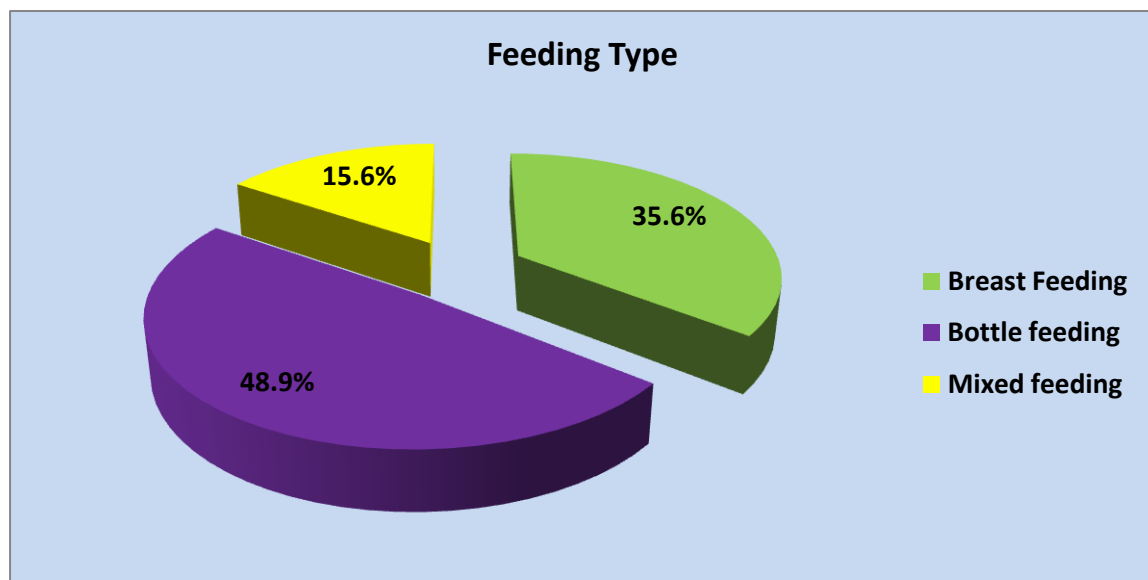


Figure (4-4): Distribution of Patients with Rotavirus According to the Feeding Type.

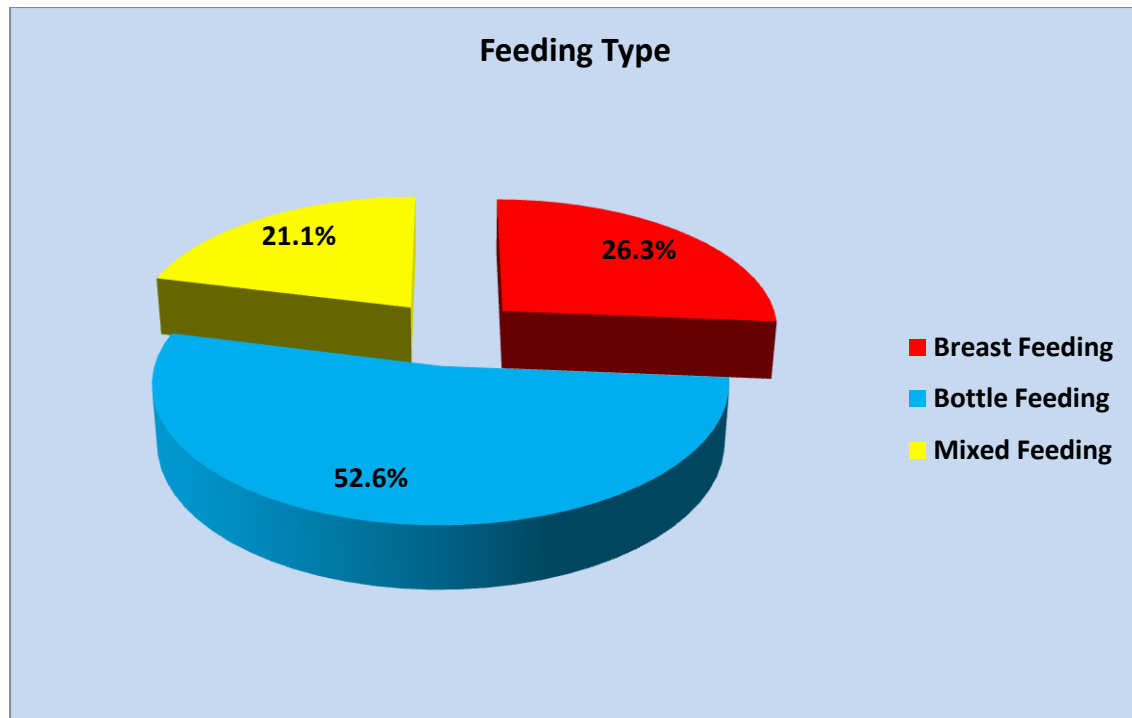


Figure (4-5): Distribution of Patients with Norovirus According to the Feeding Type.

4.5. Age Distribution of Rotavirus-Positive in Diarrheal Group of Children under 5 Years Old

Table (4-5) shows that more age groups were between (1-2 years) 29 cases, the percentage (64.4 %) of the patients infected with rotavirus while the age groups (4-5 years) the less percentage of the patients infected with rotavirus were 0 case, percentage (0%) there are statistical significant differences between the age groups ($P < 0.01$).

Table(4-5) Age Distribution of Rotavirus-Positive in Diarrheal Group of Children under 5 Years Old.

Variable	Diarrheal Group	
Age groups (Years)	No. of +ve cases	% of +ve cases
0-1	9	20
1-2	29	64.4
2-3	6	13.3
3-4	1	2.2
4-5	0	0
Total	45	100

Chi-Square=26.37, Df=7, Chi table =14.07, Significant (P<0.01)

4.6. Age Distribution of Norovirus-Positive in Diarrheal Group of Children under 5 Years Old

Table (4-6) shows that more age groups are between (1-2 years) 14 cases the percentage(73.7) of the patients infected with Norovirus while the age groups(3-4 years) and (4-5 years) the less percentage of the patients infected with norovirus were (0) case, the percentage (0%)for each of them there is statistical significant differences between the age groups.

Table(4-6) Age Distribution of Norovirus-Positive in Diarrheal Group of Children under 5 Years Old.

Variable	Diarrheal Group	
Age groups (months)	No. of +ve cases	% of +ve cases
0-1	3	15.8
1-2	14	73.7
2-3	2	10.5
3-4	0	0
4-5	0	0
Total	19	100

Chi-Squares=16.61, Df=4, Chi table =9.49, Significant (P<0.01)

4.7. Distribution of Rotavirus in Diarrheal Group of Children under 5 Years According to the Months of the Year.

According to the months of the year distribution, it has been observed that rotavirus infection is detected throughout the 5 months, the months of the year distribution of rotavirus infection is as follows: in December (18 cases) the percentage 40%, in January,(10 cases) the percentage 22.2%, in November (9 cases) the percentage 20%,in February (7cases) the percentage 15.6% and(1case) 2.2% in march, **(Table 4-7 and Figure4-6)**, The highest rate of detection of rotavirus is founded in December (18 cases, 40%) and the lowest in the month march (1cases, 2.2%) there are statistical significant differences between the months of the year (P<0.01).

Table(4-7)Distribution of Rotavirus in Diarrheal Group of Children under 5 Years According to the Months of the Year.

Month	Diarrheal Group	
	No. of +ve cases	% of +ve cases
November	9	20
December	18	40
January	10	22.2
February	7	15.6
March	1	2.2
Total	45	100

Chi-Square=37.04, DF=4 Chi table =9.49, Significant (P<0.01)

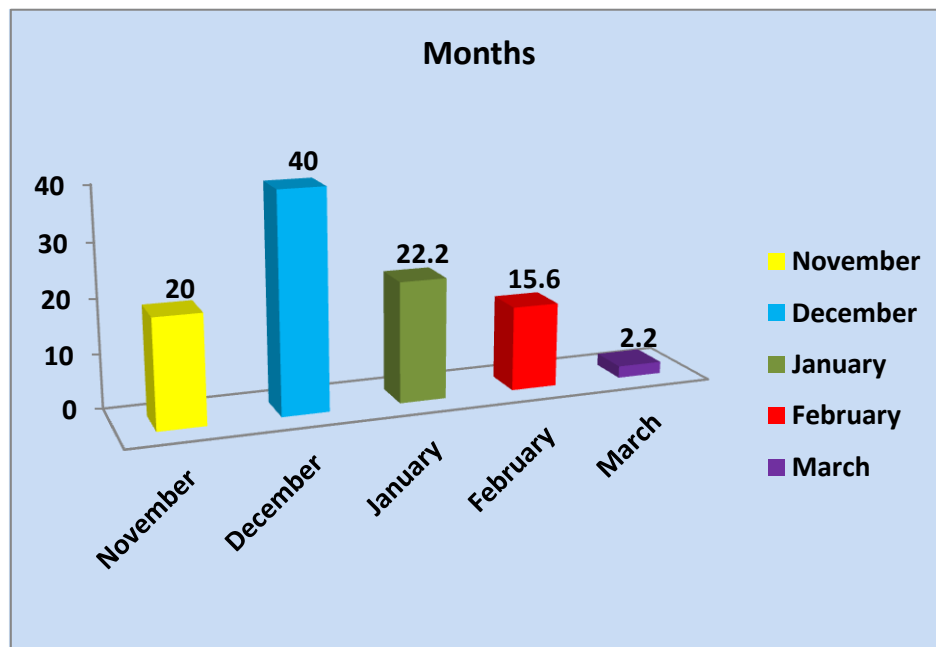


Figure (4-6):Distribution of Rotavirus in Diarrheal Group of Children under 5 Years According to the Months of the Year .

4.8. Distribution of Norovirus in Diarrheal Group of Children under 5 Years According to the Months of the Year.

According to the months of the year distribution, it has been observed that norovirus infection was detected throughout the 4 months. The months of the year distribution of norovirus infection were as follows: in December (8 cases) the percentage 42.1%, in January (6 cases) the percentage 31.6%, in November (4 cases) the percentage 21.1% and in February (1 case) the percentage 5.3%. While in March (0 case) the percentage 0% (Table 4-8 and Figure 4-7). The highest rate of detection of norovirus is found in December (8 cases, 42.1%) and the lowest in the month March (0 cases, 0%). There are statistically significant differences between the months of the year ($P < 0.01$).

Table(4-8): Distribution of Norovirus in Diarrheal Group of Children under 5 Years According to The Months of the Year.

Month	Diarrheal Group	
	No. of +ve cases	% of +ve cases
November	4	21.1
December	8	42.1
January	6	31.6
February	1	5.3
March	0	0
Total	19	100

Chi-Square=26.189, Df=4, Chi Table =9.49, Significant ($P < 0.01$)

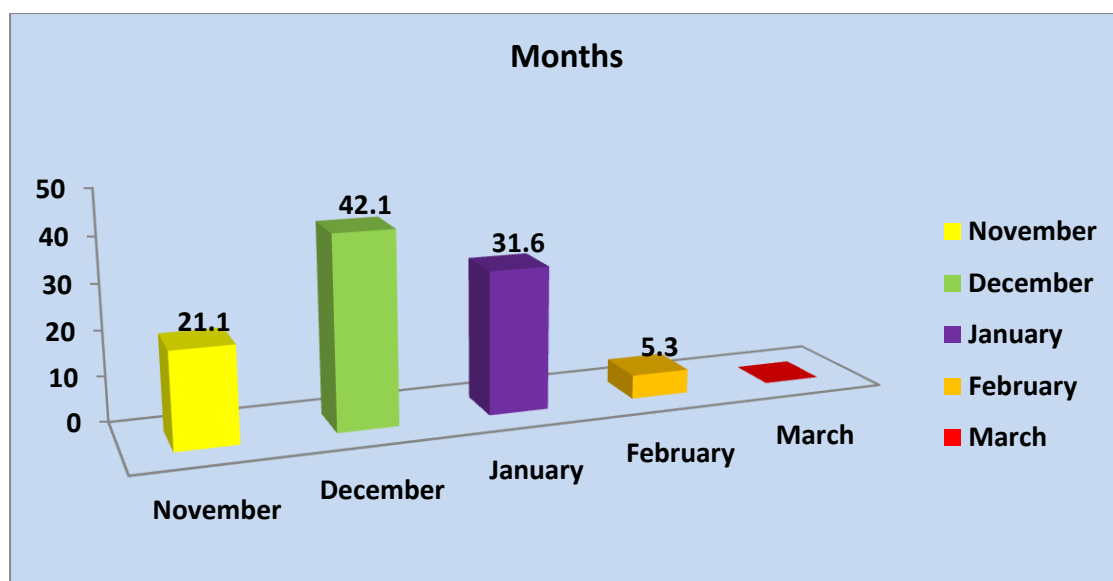


Figure (4-7): Distribution of Norovirus in Diarrheal Group of Children under 5 Years According to the Months of the Year.

4.9. Distribution of Patients with Rotavirus and Norovirus According to the Clinical Symptoms.

Diarrhea is observed in all children (100%) and analysis of rotavirus positivity in relation to other gastroenteritis symptoms such as vomiting, fever, and abdominal pain in association with the diarrhea, Rotavirus-positive patients were more likely to exhibit vomiting (84.4%) and fever (60%) followed by abdominal pain (13.3%) (**Table 4-9**) while Norovirus Diarrhea is observed in all children and analysis of norovirus positivity in relation to other gastroenteritis symptoms such as vomiting, fever, and abdominal pain in association with the diarrhea. In Norovirus infection ; vomiting is the most common feature and is observed in 100% of patients while the fever is 21.1% and abdominal cramp (5.3%) was not frequent in these patients There were statistical significant differences between the clinical symptoms ($P < 0.05$)

Table (4-9) Distribution of Patients with Rotavirus and Norovirus According to the Clinical Symptoms.

Type of virus	No. Of Cases	Symptoms			
		Diarrhea	Vomiting	Fever	Abdominal pain
		No. %	No. %	No. %	No. %
Rotavirus*	45	45 100	38 84.4	27 60	6 13.3
Norovirus**	19	19 100	19 100	4 21.1	1 5.3

*Chi-Square=6.95, Df=3, Chi table =7.81, Significant (P<0.05)

**Chi-Square=23.27, DF=3, Chi table =7.81 Significant (P<0.01)

4.10. Distribution of Rotavirus in Diarrheal Group of Children under 5 Years According to the Different Cities in Thi-Qar Province.

The results of the study show that the children living in the city of Nasiriyah are the highest percentage of infection with rotavirus reached (73.3%) while no infection are recorded in children living in both Al_Rifai and Al- Garraf (**Table 4-10**). There is statistical significant differences between the different cities in Thi-Qar province (P<0.01).

Table (4-10) Distribution of Rotavirus in Diarrheal Group of Children under 5 Years According to the Different Cities in Thi-Qar Province.

City	Diarrheal Group	
	No. of +ve cases	% of +ve cases
Al-Nasiriyah	33	73.3
SayedDakhil	3	6.7
Al-Shatrah	4	8.9
SuqAlshyuokh	4	8.9
Al-Rifai	0	0
Al-Garraf	0	0
Al-Batha	1	2.2
Total	45	100

Chi-Square=55.24, Df=6, Chi Table =12.59, Significant (P<0.01)

4.11. Distribution of Norovirus in Diarrheal Group of Children under 5 Years According to the Different Cities in Thi-Qar Province.

The results of the study show that the children living in the city of Nasiriyah are the highest percentage of infection with norovirus reached (68.4%) while no infection are recorded in children living in both Al_Rifai and Al- Garraf (**Table4-11**) there is statistical significant differences between the different cities in Thi-Qar province (P<0.05).

Table (4-11) Distribution of Norovirus in Diarrheal Group of Children under 5 Years According to the Different Cities in Thi-Qar Province.

City	Diarrheal Group	
	No. of +ve cases	% of +ve cases
Al-Nasiriyah	13	68.4
SayedDakhil	2	10.5
Al-Shatrah	2	10.5
SuqAlshyuokh	1	5.3
Al-Rifai	0	0
Al-Garraaf	0	0
Al-Batha	1	5.3
Total	19	100

Chi-Square=15.26, Df=6, Chi table =12.59, Significant (P<0.05)

4.12 Graphical Representation of Real Time PCR of Rotavirus and Norovirus in Patients Samples

The ability to monitor reaction progress has a number of advantage over end point analysis. As a result, Real-Time PCR has proven to be a powerful tool for genetic analysis (Kubista *et al.*, 2006 ; Lee and Chen, 2008). DNA detection simultaneous to amplification is preferentially achieved by the use of target sequence-specific oligo nucleotides linked to two different molecules, a fluorescent reporter molecule and a quenching molecule (Barbeau *et al.*, 2004). These probes bind the target

cDNA between the two PCR primers and are degraded or released by the DNA polymerase during DNA synthesis. In case of degradation the reporter and quencher molecules are released and separated, which results in the emission of an increased fluorescence signal from the reporter. Different variation of this principle of reporter and quencher are used by the different commercially available assays. The fluorescence signal, intensified during each round of amplification, is proportional to the amount of RNA in the starting sample. Quantification in absolute numbers is achieved by comparing the kinetics of the target amplification with the amplification kinetics of an internal control of a defined initial concentration (Macky, 2007).

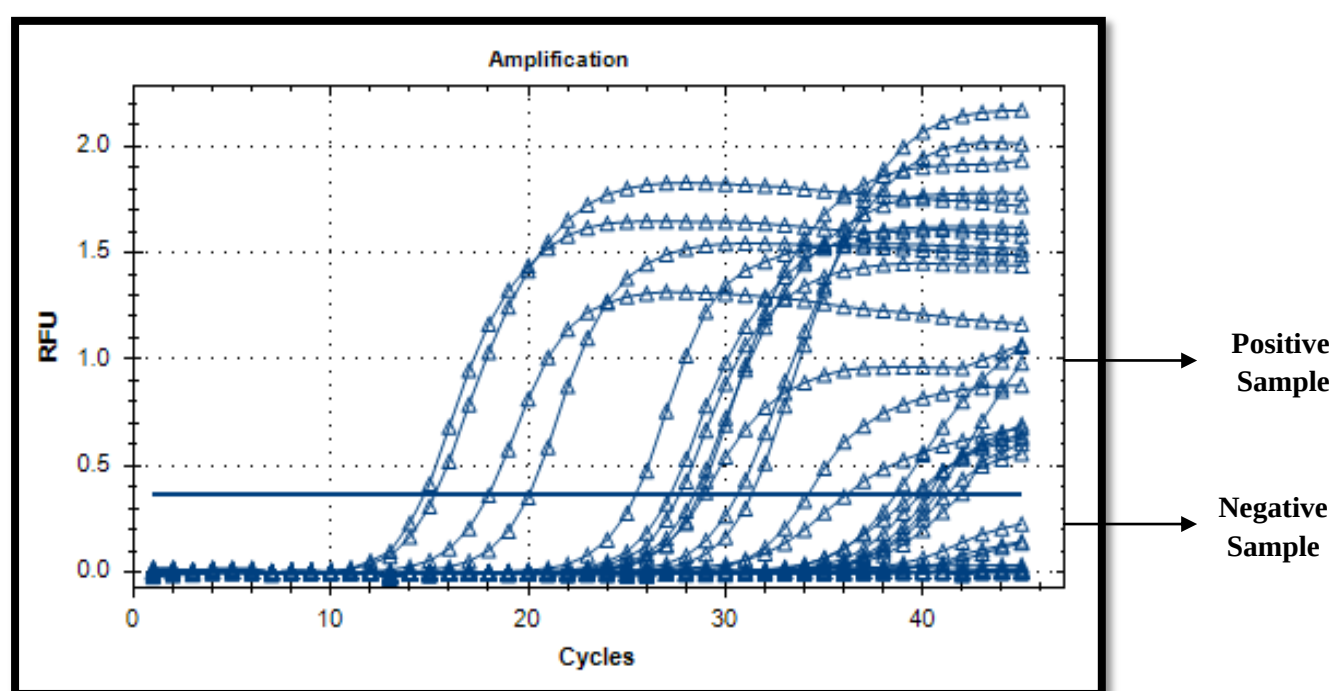


Figure (4-8) The Graph of Positive RV Obtained by RT-qPCR Thermo Cycler Template Samples Arise in Fluorescence of the Signal of Each Cycle Indicates Amplification.

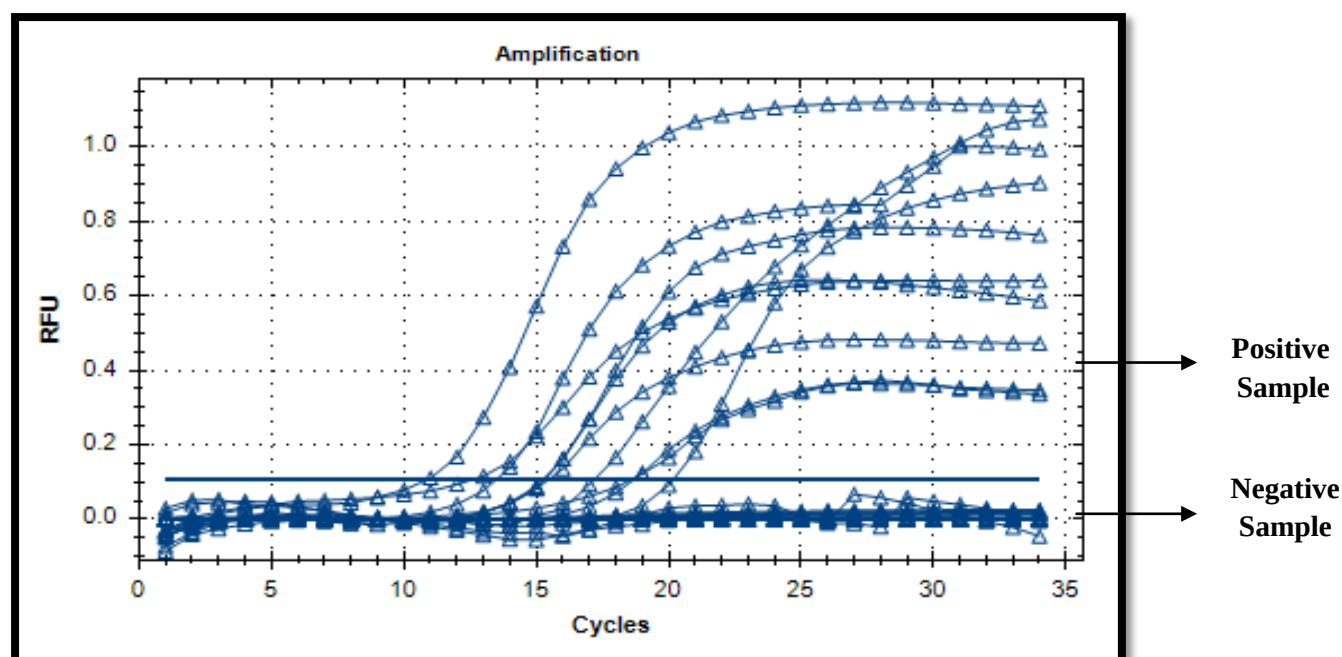


Figure (4-9):The Graph of Positive NV Obtained by RT-qPCR Thermo Cycler Template Samples Arise in Fluorescence of the Signal of Each Cycle Indicates Amplification.

4.13. Rotavirus Purification of Reaction Products RT-PCR

The RNA extracted from the sample is amplified by the results of Real Time RT-PCR technique. The previously mentioned results are amplified. The amplification of the gene is carried out with a PCR results, and its presence is detected by Gel-Electrolysis of PCR products on Gel agarose at concentration 2% When the base pair size is 369bp.

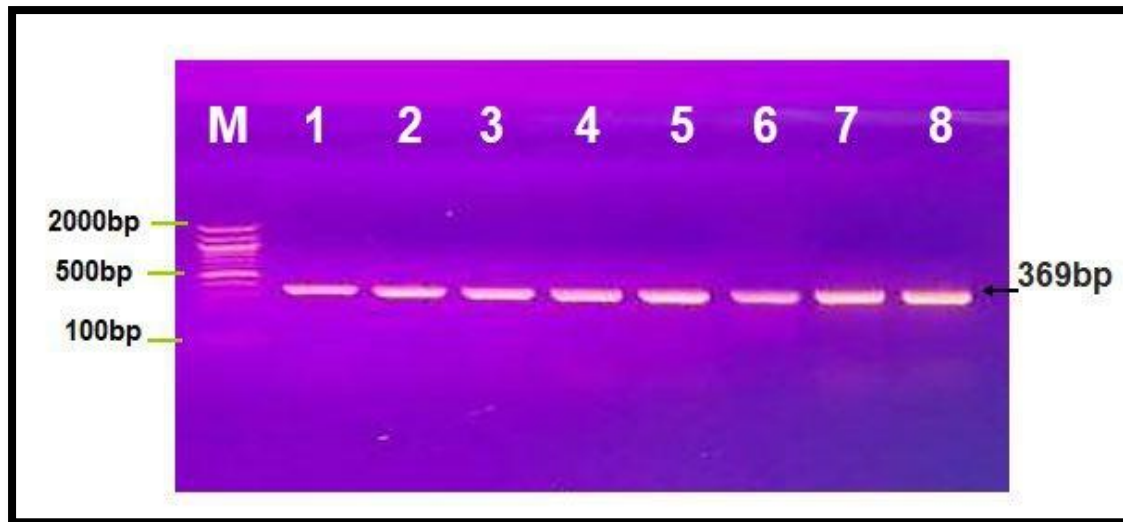


Figure (4-10): Agarose Gel Electrophoresis Image That Show the RT-PCR Product Analysis of VP6 Gene Rotavirus from in RNA Extracted from Stool Patients Samples, Where Ladder(**M**) (100-2000bp), Some Positive Samples (**1....8**) at (369bp) PCR Product Size.

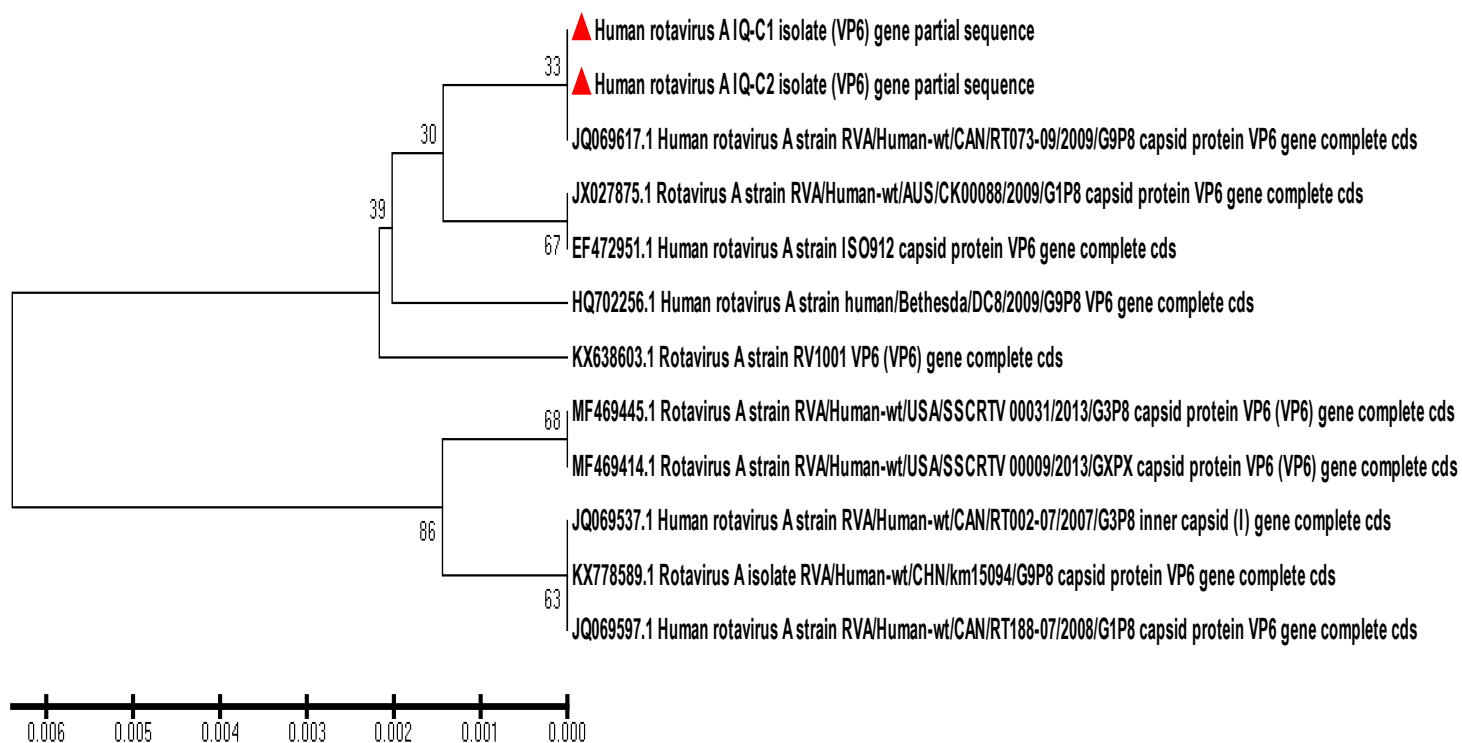


Figure (4-11): Phylogenetic Tree Analysis Based on VP6 Gene Partial Sequence That Used for Rotavirus A Confirmative Detection and Genetic Relationship Analysis . the Phylogenetic Tree is Constructed Using Un Weighted Pair Group Method with Arithmetic Mean (UPGMA Tree) in (MEGA 6.0 Version). The Local Rotavirus A Isolate (IQ-C1 And IQ-C2) Were Show Closed Related to NCBI-Blast Rotavirus A(JQ069617.1) **Genotype9P8**, Whereas ,NCBI-Blast Rotavirus A are Shown Less Different and Out of Tree.

4.14. Norovirus Purification of Reaction Products RT-PCR

The RNA extracted from the sample is amplified by the results of Real Time RT-PCR technique. The previously mentioned results are amplified. The amplification of the gene is carried out with a PCR results, and its presence is detected by Gel-Electrolysis of PCR products on Gel agarose at concentration 2% When the base pair size is 540bp.

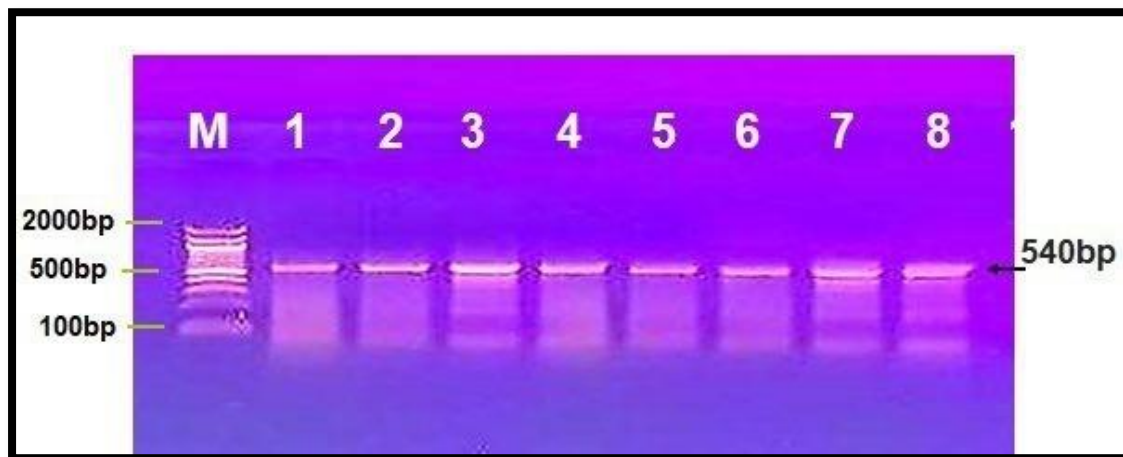


Figure (4-12):Agarose Gel Electrophoresis Image That Show the RT-PCR Product Analysis of Capsid Gene Norovirus from in RNA Extracted from Stool Patients Samples, Where Ladder(**M**) (100-2000bp), Some Positive Samples (**1...8**) at (540bp) PCR Product Size.

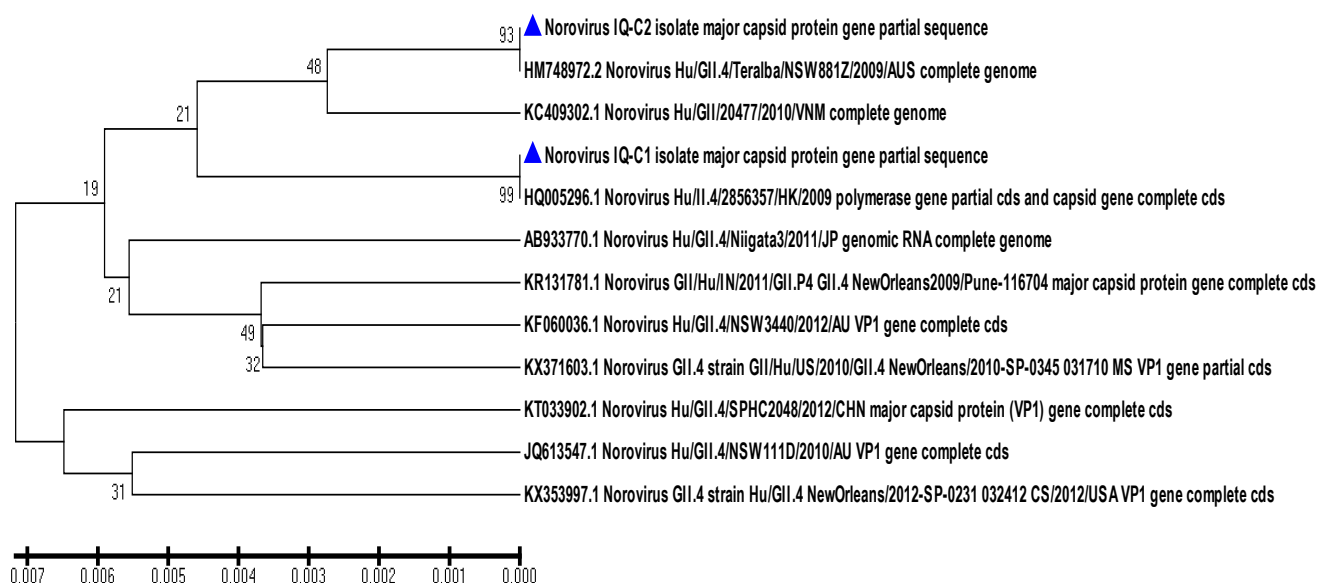


Figure (4-13): Phylogenetic Tree Analysis Based on VP6 Gene Partial Sequence That Used for Norovirus Confirmative Detection and Genetic Relationship Analysis . The Phylogenetic Tree Was Constructed Using Unweighted Pair Group Method with Arithmetic Mean (UPGMA Tree) in (MEGA 6.0 Version). The Local Norovirus Isolate (IQ-C1) Were Show Closed Related to NCBI-Blast Norovirus(HQ005296.1) Genotypegii.4 and the Local Norovirus Isolate (IQ-C2) Were Show Closed Related to NCBI-Blast Norovirus (HM748972.2) **Genotypeg.4** , Whereas, NCBI-Blast Norovirus Were Shown Less Different and Out of Tree.

Chapter Five

Discussion

5. Discussion

The current study was performed to evaluate the prevalence of rotavirus, norovirus and co-infections among the children with acute gastroenteritis, and their association with demographic and some parameters including age, sex, feeding type and clinical symptoms in children under five years of age.

5.1. Distribution of Rotavirus and Norovirus detected in stool specimens of diarrheal group.

Out of 100 samples, 35% are positive with rotavirus and 9% are positive with norovirus whereas 10% mixed infection with noro-rotavirus by using RT-qPCR analyses. The results in the present study are higher than those obtained by (Nasab *et al.*, 2016) who are indicated that among 170 samples, 49 (28.8%) and 15 (8.8%) are positive for rotavirus and norovirus infections, respectively, and 6 (3.5%) samples are positive for both rotavirus and norovirus. Many reports have established the importance of rotaviruses and noroviruses as the causes of outbreaks of gastroenteritis (Elliott, 2007). Whereas the results of current study are Matched with other studies by (Lanata *et al.*, 2013) who was indicated among 779 stool specimens tested for rotavirus, 263 were positive (33.8%), and norovirus positive specimens were 79 out of 318 (24.8%) specimens tested. Most of the clinical manifestations of gastroenteritis caused by these two viruses are quite similar with no significant difference. Several researchers which is consistent with this results such as reported by (Kargar *et al.*, 2012; Parashar *et al.*, 2009). The results are also matched with the results of (Shoja *et al.*, 2014) and (Moradi-Lakeh *et al.*, 2014), who reported 11.36% and 79% of rotavirus in two different regions of Iran. The co-infection of rotaviruses and norovirus have been reported by some investigators in different regions of the world. In a

study conducted by (Sai *et al.*, 2013) in China, the frequency of rotavirus and norovirus infections were 34.4% and 10.4%, respectively, and their co-infection was 1%.

Diarrhea remains the second leading cause of death due to infections among children under the age of five years worldwide (Lanata *et al.*, 2013). These results show that norovirus is a major viral pathogen, secondary to rotavirus, in children hospitalized with acute diarrhea in Iraq.

5.2. Sex Distribution of Rotavirus-Positive in Diarrheal Group of Children under 5 Years' Old

The prevalence of rotavirus in the present study among males is 31 (68.9%) are higher than those in females 14 (31.1%) within children under 5 years old with significant differences between them. This finding corresponds with other previous studies by (Nasab *et al.*, 2016). A higher rate of rotavirus infection is detected in males more than females. Also these results in line with another study by (Moyo *et al.*, 2014; Center, 2013) who was found male infected with rotavirus was (61.2%) higher than in female was (38.8%). the 421 Rotavirus cases seen at the hospital, 235/421 (56%) were males and 186/421 (44%) were females (RÄSÄNEN, 2016). The overall proportion of rotavirus infection was comparable between males and females. Also the results are matched with the results in Taiwan by (Yang *et al.*, 2010) who was show the mean of male infected was 56.1% was higher than those in female. More male children (53%) experienced diarrheal diseases compared to female children (47%) and are more susceptible to rotavirus infection. This suggests that boys become more frequently sick than girls (Seheri, 2013). The reason for the gender difference is not determined, but the of this

study results are consistent with the findings that had been reported previously by (Nguyen *et al.*, 2004; Kim *et al.*, 2005).

5.3. Sex Distribution of Norovirus-Positive in Diarrheal Group of Children under 5 Years' Old

Norovirus is considered as the second most common cause of viral gastroenteritis with a prevalence rate of 6% -19% globally (Nasab *et al.*, 2016). The percentage of Norovirus-positive in the present study among males is (57.9%) which is higher than those in females (42.1%) within diarrheal group of children under 5 years old, is the same as the results reported from Japan by (Ozawa *et al.*, 2007; Center, 2013).

In Tokyo, is found male infected with norovirus was 60.5% higher than in female was 39.5. Also these results resemble to another by (Moyo *et al.*, 2014) in which the infected male was 54.5% more than in female was 45.5%. Also the results match with other like (Yang *et al.*, 2010) who was observed the males were more infected with norovirus than females.

5.4. Age Distribution of Rotavirus-Positive in Diarrheal Group of Children under 5 Years Old.

The results of this present study reveal that are significant differences between the age groups and the percentage of rotavirus-positive patients with highest value is (64.4.9%) at age group of (1-2) years, and the lowest one is (0%) at age group of (4-5) months. This result is in line with the data obtained in Tehran by (Nasab *et al.*, 2016) which scored that the highest value of rotavirus positive in children was 46.9% between (1-2) years, whereas the lowest value was (6.1%) at age group of (3-5) years. However, there are other results indicate to the percentage of

rotavirus-positive patients with highest value is (35.5%) at age group of (1-2) years, and the lowest one is (4.0%) at age group of (0-1) years (Yang *et al.*, 2010). Most of the children (233/421, 55%) were between (0-10) years of age, and 149/421 (35%) were between (1-2) years of age (RÄSÄNEN, 2016).

In this study, (13/45) of rotaviral diarrheas are identified in the age group of less than 2 years(11-15) months. This finding is in agreement with previous studies done in Turkey which showed a 63% rate of rotavirus in diarrheic patients who were 2 year of age or less (Karadag *et al.*, 2005). Rotavirus is the leading cause of gastroenteritis in infants and younger child with the findings of most studies, consistent demonstrating that rotavirus mainly affects younger children aged less than 5 years and the proportion of rotavirus infections among younger children was higher than that in the older age groups.(Wu *et al.*, 2008).

5.5. Age Distribution of Norovirus-Positive in Diarrheal Group of Children under 5 Years Old.

Many studies show that norovirus plays an important etiological role in diarrhea in infants and young children (Ramani and Kang, 2009). The study find that norovirus-associated diarrhea occurred main in children aged 2 years or younger. The results of present study reveal that are significant differences between the age groups and the percentage of norovirus-positive patients with highest value is(73.7.3%) at both age group of (1-2) years, and lowest one is (0%) at age groups of (3-4) and (4-5) years which is in accordance with the studies reported from Iran (Iran pour *et al.*, 2013) and UK (Payne *et al.*, 2013).

These results match with other like (Yang *et al.*, 2010) who was appear 38.2% which was highest value within (1-2 years) age group and the lowest one was within (0-1) years. Also the results in corresponding with the results by (Nasab *et al.*, 2016) who found the highest rate of norovirus infection was observed in children under 1 years (40%). However, some studies demonstrated that norovirus also has an impact on children younger than 1 years, which is consistent with the finding of the current study. The general belief is that norovirus is a major pathogen leading to diarrheal outbreaks and causes symptomatic infections in older children and adults (Subekti *et al.*, 2002).

5.6. Distribution of Rotavirus and Norovirus Infections According to Feeding Types.

During the study of the relationship between the incidence of rotavirus and the pattern of nutrition shows the rate of rotavirus gastroenteritis is the highest in children who were used bottle feeding with 22 (48.9%) children, followed by breast feeding and mixed feeding with 16 (35.6%) and 7 (15.6%), respectively. This variation of the above results is statistically significant ($p < 0.01$). Another similar local study found a similar infection with a predominate Rotavirus positive infection in children with Bottle feeding (Ali *et al.*, 2010). On the other hand, other studies showed different results according to the feeding type (Nakawesi *et al.*, 2010). The present study showed that bottle feeding children tend to be more susceptible to face RV diarrhea, comparing with infants who are breast-fed and mix-feed. This is due to the ease of transmission of viruses in contaminated feeding bottles. another a local study carried out in Kurdistan, Iraq, (Rashid and Sharif, 2006) found that only 3 breast feeding in 30 children were RV-positive. The researchers proposed that breast feeding reduces the risk of rotavirus, and they notice that 10% of

breast feeding infants showed less severe signs while norovirus gastroenteritis was found to be the highest in children who were used bottle feeding with 10 (52.6%) child, followed by breast feeding and mixed feeding with 5 (26.3%) and 4 (21.1%), respectively. Evaluation of the feeding status with acute diarrhea revealed that noroviruses - positive cases are significantly higher among those being bottle fed at the time of acute gastroenteritis. This result suggests that breast feeding may be a protective factor against enteric virus infection by conferring passive immunity from the mothers to her infant(Asensi *et al.*, 2006). It has been reported that human breast milk contains several factors that may play a role in preventing infection and of these is a glycoprotein, lactoferrin, which is able to bind to and prevent virus replication, beside the natural immune defense mechanisms(Asensi *et al.*, 2006). On the other hand, the lower frequency of norovirus infection among those of breast fed may be explained with the synergy of these one type of nutrition in reducing the amount of norovirus disease. The infection with diarrhea is increased in infants who used bottle feeding may be because of the contamination of the bottle nipples. This result was similar to those previously described in other studies (Modarres *et al.*, 2006).

5.7. Distribution of Rotavirus in diarrheal group of children under 5 years according to the months of the year.

In this study, the occurrence of the Rotavirus is recorded throughout the five months and shown a greater occurrence in december month. Some studies also have clearly demonstrated a seasonal trend for rotavirus but the highest incidence is observed during november (Parashar *et al.*, 2003; Parashar *et al.*, 2006; Coffin *et al.*, 2006) In neighbor countries a previous study from Iran (Amini *et al.*, 2008) which reported a higher incidence in

spring, this finding in contrast with another study that found the highest incidence of rotavirus during winter in Turkey (Ozkana *et al.*, 2007) while a prevalence of rotavirus was found in northern Jordan during may (Meqdam *et al.*, 1997). In contrast to these studies, no significant seasonal variation was detected in Iraqi Kurdistan (Ahmed *et al.*, 2006) and in Saudi Arabia (Tayeb *et al.*, 2008). These differences may be due to environmental factors that may affect the survival rate of the rotavirus outside the body (temperature, humidity and rainfall) and to contaminated water or its relation with level of chlorination of water, as rotavirus is inactivated only by chlorine, rather than other disinfectants (Khuri-Bulos and Al Khatib 2006).

In this study witnessed a peaked incidence during the winter months with low prevalence during summer months. The peak incidence rates of rotavirus infections observed are 40%, 22.2% and 20% during December, January and November, respectively, among children less than 5 years of age . these results in corresponding with other in developed countries with temperate climatic conditions (Nakagomi *et al.*, 2005; Gleizes *et al.*, 2006).

5.8. Distribution of Norovirus in diarrheal group of children under 5 years according to the months of the year.

The peak incidence rates of norovirus infections observed are 42.1%, 31.6% and 21.1% during December, January and November, respectively whereas low incidence in February and March among children less than 5 years of age these results are mismatched with other data obtained by (Yang *et al.*, 2010) who was observed the incidence of norovirus

infection was slightly higher in the southern region compared with the northern and central regions.

In Taiwan high prevalence of norovirus infection was in February, March and April) and low infection was during November and December. However, the prevalent season of norovirus infection is highly variable in different regions as shown in some studies (Xu *et al.*, 2009). Some studies indicate that the prevalence of norovirus infection is higher during cooler months in temperate zones (Junquera *et al.*, 2009) Also these results are in corresponds with the results by (Nasab *et al.*, 2016) which showed the noroviruses were detected in spring, autumn and winter but not summer. There was also a considerable increasing trend in the number of cases in the autumn season, which confirms the previous studies (La Rosa *et al.*, 2008; Vinje, 2014). In moderate-climate countries, the norovirus season traditionally begins in winter and lasts until spring, between October and March in the northern hemisphere (Wollants *et al.*, 2015). About three-quarters of norovirus cases occur in these cool months. The explanation for the seasonality of norovirus AGE is not known, but such factors as rainfall, cool temperatures and the emergence of new variants have been proposed (Ahmed *et al.*, 2013).

5.9.Distribution of Patients with Rotavirus and Norovirus According to the Clinical Symptoms.

All of rotavirus positive children are suffered from diarrhea (100%), in addition to vomiting , fever and Abdominal pain with 38(84.4%), 27 (60%) and 6 (13.3%), respectively ($p < 0.05$). A sudden onset of symptoms typically manifests in children 1 to 2 days after infection with rotavirus. The clinical picture of rotavirus gastroenteritis is characterized by 4 to 7 days of , vomiting, and watery, non bloody

diarrhea. This combination can lead to rapid dehydration without appropriate intervention. Secondary infection with rotavirus is clinically milder or asymptomatic (Anderson and Weber, 2004). The present study results show that diarrhea is the predominate symptom among rotavirus infected children included in the study. These results are different with (Al-Jabiry, 2009). In Thi-Qar province an incidence of vomiting and diarrhea as rotavirus symptoms with 60% and 40%, respectively. On other hand, our finding is similar to that found in Iran recently (Karger *et al.*, 2012). While in Norovirus vomiting seems the symptom most commonly reported in association with Norovirus diarrhea, followed by fever and abdominal pain. These findings are similar to the results observed Which that diarrhea and vomiting are the most common in patients 94% (Thwiny and Hasony, 2013) Vomiting is more common in children with norovirus infection as similar to other studies (Mattner *et al.*, 2006) Delay in gastric emptying may be responsible for the vomiting associated with this agent (Hoshino *et al.*, 2004) Vomiting is a prominent clinical signal observed for norovirus and infections. Clinical reports describe vomiting as an important manifestation for norovirus infection (Rockx *et al.*, 2002). The results of this study contrast with those reported children infected with norovirus at a hospital in Rio de Janeiro where vomit observed in 33.3% of the cases (Victoria *et al.*, 2007).



Conclusions & Recommendations

Conclusions and Recommendations

Conclusions:

According to the previous results, the present study concludes the following:

1-This study shows that rotavirus is more associated with diarrhea in children and than Norovirus.

2-Genetic analysis of Rotavirus and Norovirus that infect children in Thi-Qar province shows that the local Rotavirus isolate (IQ-C1 and IQ-C2) are show closed related to NCBI-Blast Rotavirus A(JQ069617.1) (G9P8) for rotavirus and the local Norovirus isolate (IQ-C1 and IQ-C2) seems closed related to NCBI-Blast Norovirus(HQ005296.1) (GII.4) .

3-Age specific frequency in children patients with diarrhea caused by rotavirus and Norovirus is high in aged 11-15 months and the frequency of male children patients with diarrhea caused by rotavirus and Norovirus is higher than their female .

4-Lack of breast –feeding is significantly associated with an increased risk of diarrhea in children. Also it is found high infection with rotavirus and norovirus in bottle and mixed feeding children.

genotypeGII.4 , Whereas,NCBI-Blast Norovirus appears less different and out of tree.

Conclusions and Recommendations

Recommendations:

1-Genotyping and subserotyping studies are more necessary for detection of new rotavirus and norovirus strains which is more important to improve both rotavirus and norovirus treatment and vaccination.

2-Further studies are required to identify the infection with rotavirus and norovirus in young and old age human.

3-Study the frequency of other viral causes of childhood diarrhea.

4-Physicians need to make a request for rotavirus and norovirus identification for children with diarrhea who need to be hospitalized.

5-Training the staff for the prevention of transmission of this disease between children that hospitalized for any reason .

6-Large scale survey studies should be done to follow up the disease status in Thi-Qar province and another parts of Iraq.

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Appendices

Table (1): Homology sequence Identity (%)of local Rotavirus A isolates and NCBI-Blast Rotavirus A isolates using NCBI- BLAST alignment tool.

NCBI-Blast Rotavirus A isolate No.	NCBI-Genbank Accession number	Genotype	Country	Homology sequence Identity (%)	
				local Rotavirus A isolate (IQ-C1)	local Rotavirus A isolate (IQ-C2)
Human Rotavirus A No.1	JQ069617.1	G9P8	Canada	100%	100%
Human Rotavirus A No.2	EF472951.1	G9	India	99%	99%
Human Rotavirus A No.3	HQ702256.1	G9P8	USA	99%	99%
Human Rotavirus A No.4	JX027875.1	G1P8	Australia	99%	99%
Human Rotavirus A No.5	KX638603.1	I1	India	99%	99%
Human Rotavirus A No.6	JQ069537.1	G3P8	Canada	99%	99%
Human Rotavirus A No.7	JQ069597.1	G1P8	Canada	99%	99%
Human Rotavirus A No.8	KX778589.1	I1	China	99%	99%
Human Rotavirus A No.9	MF469414.1	G3P8	USA	99%	99%
Human Rotavirus A No.10	MF469445.1	GXP(X)	USA	99%	99%

Table (2): Homology sequence Identity (%)of local Norovirus isolates and NCBI-Blast Norovirus isolates using NCBI-BLAST alignment tool.

NCBI-Blast Norovirus isolate No.	NCBI-Genbank Accession number	Genotype	Country	Homology sequence Identity (%)	
				local Norovirus isolate (IQ-C1)	local Norovirus isolate (IQ-C2)
Norovirus No.1	HQ005296.1	GII.4	Hong Kong	100%	99%
NorovirusNo.2	HM748972.2	GII.4	Australia	99%	100%
NorovirusNo.3	JQ613547.1	GII.4	USA	99%	99%
NorovirusNo.4	KC409302.1	GII.4	India	99%	99%
NorovirusNo.5	KF060036.1	GII.4	Australia	99%	99%
NorovirusNo.6	AB933770.1	GII.4	Japan	99%	99%
NorovirusNo.7	KR131781.1	GII.4	India	99%	99%
NorovirusNo.8	KX353997.1	GII.4	USA	99%	99%
NorovirusNo.9	KX371603.1	GII.4	USA	99%	99%
NorovirusNo.10	KT033902.1	GII.4	China	99%	99%

Appendices

DNA Sequences	Translated Protein Sequences
Species/Abbrev	
1. Human rotavirus A IQ-C1 isolate (VP6) gene partial sequence	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
2. Human rotavirus A IQ-C2 isolate (VP6) gene partial sequence	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
3. MF469445.1 Rotavirus A strain RVA/Human-wt/USA/SSCRIV_00031/2009/G1	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
4. MF469414.1 Rotavirus A strain RVA/Human-wt/USA/SSCRIV_00009/2009/G1	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
5. KX778589.1 Rotavirus A isolate RVA/Human-wt/CHN/kml5094/G9P8 s	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
6. KX638603.1 Rotavirus A strain RV1001 VP6 (VP6) gene complete c	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
7. JX027875.1 Rotavirus A strain RVA/Human-wt/AUS/CK00088/2009/G1	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
8. JQ069617.1 Human rotavirus A strain RVA/Human-wt/CAN/RT073-09/A	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
9. JQ069597.1 Human rotavirus A strain RVA/Human-wt/CAN/RT188-07/A	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
10. JQ069537.1 Human rotavirus A strain RVA/Human-wt/CAN/RT002-07/A	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
11. HQ702256.1 Human rotavirus A strain human/Bethesda/DC8/2009/G1	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT
12. EF472951.1 Human rotavirus A strain ISO912 capsid protein VP6	ATCGAGTATTTTATTGATTTTCATTGATAATGTGTGATGGATGAAATGGCAAGGAGTCT

Figure(1): Multiple sequence alignment analysis of the partial capsid gene sequence for local Rotavirus A isolates(IQ-C1 and IQ-C2) with NCBI-Blast Rotavirus A capsidprotein (VP6) geneby using (MEGA 6.0, multiple alignment analysis tools). The multiple alignment analysis similarity (*)in capsid genenucleotide sequences.

Appendices

DNA Sequences		Translated Protein Sequences	
Species/Abbrv			
1. Norovirus IQ-C1 isolate major capsid protein gene partial sequence	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
2. Norovirus IQ-C2 isolate major capsid protein gene partial sequence	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
3. AB933770.1 Norovirus Hu/GII.4/Niigata3/2011/JP genomic RNA complete	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
4. HM748972.2 Norovirus Hu/GII.4/Teralba/NSW8812/2009/AUS complete geno	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
5. HQ005296.1 Norovirus Hu/II.4/2856357/HK/2009 polymerase gene partial	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
6. JQ613547.1 Norovirus Hu/GII.4/NSW111D/2010/AU VPI gene complete cds	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
7. KC409302.1 Norovirus Hu/GII/20477/2010/VNM complete genome	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
8. KF060036.1 Norovirus Hu/GII.4/NSW3440/2012/AU VPI gene complete cds	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
9. KR131781.1 Norovirus GII/Hu/IN/2011/GII.P4_GII.4_NewOrleans2009/Pune	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
10. KT033902.1 Norovirus Hu/GII.4/SPHC2048/2012/CHN major capsid protei	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
11. KX353997.1 Norovirus GII.4 strain Hu/GII.4_NewOrleans/2012-SP-0231	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		
12. KX371603.1 Norovirus GII.4 strain GII/Hu/US/2010/GII.4_NewOrleans/2	GATGGCGTGCCTAGGTACTACCCAACTGTCCTCCGTCAACATCTGCACCTTCAGAG		

Figure(2): Multiple sequence alignment analysis of the partial major capsid gene sequence for local Norovirus isolates(IQ-C1 and IQ-C2) with NCBI-Blast Norovirus major capsidprotein (VP6) geneby using (MEGA 6.0, multiple alignment analysis tools). The multiple alignment analysis similarity (*)in capsid genenucleotide sequences.

Questionnaire forma.

Questionnaire forma	
Date	
Age	
Sex	
Address	
Feeding type	
clinical Symptoms	

الخلاصة

هذه الدراسة صممت لتحديد اثنين من الفيروسات المعوية الأكثر أهمية (Rotavirus and Norovirus) في عينات البراز في الأطفال الذين تقل أعمارهم عن 5 سنوات. وزعت العينات تبعا للعمر, الجنس, نوع التغذية, الموسم, مكان المعيشة والعلامات السريرية.

المجموع الكلي لعينات البراز كان 100 عينة والتي جمعت من الأطفال والرضع تحت عمر 5 سنوات في محافظة ذي قار جنوب العراق خلال 5 أشهر من تشرين الثاني 2017 إلى آذار 2018. حيث تم جمع 60 عينة من مدينة الناصرية (مستشفى محمد الموسوي للأطفال ومستشفى بنت الهدى التعليمي والعيادات الخاصة), و40 عينة جمعت من الناصرية, سيد دخیل, الشطرة, سوق الشيوخ, لرفاعي, الغراف والبطحاء ومن 100 عينة وجدنا (63) من المرضى هم ذكور و(37) إناث حيث ان الإصابة الأكثر تكرارا كانت في الذكور مقارنة مع الإناث.

صنفت العينات إلى 5 مجموعات تبعا إلى أعمار الأطفال من 0-1 سنة, 1-2 سنة, 2-3 سنة, 3-4 سنة, 4-5 سنة. حيث ان العمر الأكثر عرضة للإصابة بهذين الفيروسين كان 1-2 سنة.

نسبة الأطفال الذين يعانون من الإسهال الناتج عن الإصابة ب Rotavirus كانت 35% بينما المشخصة كإصابة ب Norovirus كانت 9% ومن جهة أخرى الإصابات المختلطة كانت 10% من مجموع العينات الكلي.

أعلى معدل للإصابة ب Rotavirus كان في شهر كانون الاول حيث بلغت 18 حالة (40%) واقل معدل كان في شهر آذار 1 حالة (2.2%), بينما Norovirus كان في شهر اذار صفر حالة (0%).

تبعا للنتائج كانت نسبة الإصابة ب Rotavirus بالاعتماد على نوع التغذية (16) طفل (35.6%) تغذيتهم كانت طبيعية, (22) طفل (48.9%) كانت تغذيتهم صناعية و(7) أطفال (15.6%) كانت تغذيتهم مختلطة. بينما في الأطفال المصابين ب Norovirus وبالاعتماد على نوع التغذية اظهرت ان 5 أطفال (26.3%) تغذيتهم كانت طبيعية, (10) أطفال (52.6%) تغذيتهم كانت صناعية و(4) أطفال (21.1%) كانت تغذيتهم مختلطة.

العلامات السريرية للمرضى تضمنت إسهال, تقيؤ, حمى وألم في البطن, العلامات السريرية الأكثر شيوعا في حالة الإصابة ب Rotavirus كانت الإسهال بنسبة 100% مقارنة

مع الإعراض الأخرى ,بينما الإعراض الأكثر شيوعا في حالة الإصابة ب Norovirus كانت القي والإسهال بنسبة 100% مقارنة مع الإعراض الأخرى .

تم استخدام جهاز RT-qPCR لتحديد الفيروسين بالاعتماد على Vp6 gene للروتا فايروس و Capsid gene للنورو فيروس وأيضا استخدمت تقنية conventional RT.PCR على العينات التي كانت موجبة باستخدام تقنية RT.qPCR لمعرفة التتابع النيوكليوتيدي بطريقة DNA sequencing .

اظهرت نتائج تحليل الشجرة الوراثية لل Rotavirus للعزلات المحلية IQ-CI and IQC2 علاقة مقربة من (EF472951.1)(JQ069617.1) NCBI-Blast rotavirus التي تنتمي الى النمط الوراثي (G9P8) مع NCBI-Blast بنسبة تطابق 99-100% وخضعت إلى NCBI-Genbank وأعطت الرقم المضاف IQC1(MH463489) ,IQC2(MH463490)

بينما اظهرت نتائج تحليل الشجرة الوراثية لل Norovirus للعزلات المحلية (IQ-CI and IQC2) علاقة مقربة من NCBI- BlastNorovirus (HMQ005296.1)(HM748972.2) التي تنتمي الى النمط الوراثي GII.4 مع NCBI-Blast بنسبة تطابق 99-100% وخضعت لل NCBI-Genbank واعطت الرقم المضاف IQC1(MH490799) ,IQC2(MH490800)



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وزارة التعليم العالي والبحث العلمي
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عزل وتشخيص فيروس الروتا وفيروس النورو من الأطفال دون سن الخامسة من العمر

رسالة مقدمة إلى

مجلس كلية العلوم- جامعة ذي قار
وهي جزء من متطلبات نيل درجة الماجستير
في علوم الحياة- الأحياء المجهرية

من قبل الطالب

محمد نزال فنجان

بكالوريوس في علوم الحياة-2015

بإشراف

الأستاذ المساعد الدكتور

باسم عبد الحسين جاراالله

2018م

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