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Immunological And Molecular Study among *Rotavirus* Infection children In Babylon Province.

A Thesis

Submitted to the Council of the College of Medicine, University of Babylon, in Partial Fulfillment of The Requirements for the Degree of Master In science of Medical Microbiology

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بِسْمِ اللهِ الرَحمَنِ الرَحِيمْ

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Dedication

This thesis work is dedicated to my parents, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

This work is also dedicated to my husband, who has been a constant source of support and encouragement during the challenges of graduate school and life. I am truly thankful for having you in my life.

To my children who have been affected in every way possible by this quest.

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To my husband who encouraged me to pursue my dreams and finish my dissertation.

Nagham Ali

2022

Summary

Rotavirus gastroenteritis is a leading cause of severe diarrhea in pediatric aged less than five years of age worldwide, with higher mortality in lower-income countries. This study aimed to provide baseline information on the epidemiology of *Rotavirus* and host responses in children in Babylon Province . A prospective hospital-based case-control study was undertaken at Babylon Maternity and pediatric teaching Hospital- Al Noor pediatric Hospital from September 2021 to February 2022.

Children under the age of five years who were admitted to the hospital with acute gastroenteritis were enrolled in the study. Demographic and clinical data were gathered. Stool samples were analysed for *Rotavirus* antigen and faecal Immunoglobulin IgA.

Serum samples were collected to measure IgA and Interleukin (IL) 6 levels. One hundred and fifty children up to five years of age (100 with *Rotavirus* diarrhea and 50 healthy controls) were enrolled.

Most of the children with *Rotavirus* gastroenteritis were under the age of two years, predominantly between six to eleven months of age. Breastfeeding rates were lower in the *Rotavirus* group than the control group ($p < 0.001$). There was no association between *Rotavirus* diarrhea and residential location (rural versus urban) ($p > 0.05$).

Serum and faecal IgA levels, and serum IL-6 levels were all higher in the children with *Rotavirus* than the control group ($p < 0.01$ for each).

RT-PCR was performed for direct detection Human *Rotavirus* based on specific amplification of the *NSP4* gene.

The DNA sequencing method was carried out to identification genetic variation (substation Mutations) analysis in non-structural protein *NSP4* gene of local Human *Rotavirus* A isolates (IQH.1 - IQH.5) and NCBI-Blast related Human *Rotavirus* A isolates.

Summary……..……

The phylogenetic tree genetic relationship analysis was showed that local Human *Rotavirus* A isolates (IQH.1- IQH.4) were showed closed related to NCBI-BLAST Human *Rotavirus* A strain RV1326 , the local Human *Rotavirus* A isolates (IQH.5) were showed closed related to NCBI-BLAST Human *Rotavirus* A strain G1P at total genetic changes (0.0080-0.0020%).The homology sequence identity between local Human *Rotavirus* A isolates (IQH1 - IQH.4) and NCBI BLAST related Human *Rotavirus* A strain RV1326 were showed genetic homology sequence identity ranged from (99.37-99.84%). Local Human *Rotavirus* A isolates (IQH.5) and NCBI BLAST related Human *Rotavirus* A strain G1P were showed genetic homology sequence identity ranged from (99.52%).

The genetic variation (substitution Mutations) analysis in non-structural protein (*NSP4*) gene between local Human *Rotavirus* A isolates and NCBI-Blast related Human *Rotavirus* A isolate were find (1-4) substitution mutations at total genetic variation percentage ranged (0.16-0.63%).

Finally, the local Human *Rotavirus* A isolates (IQH.1 - IQH.5) were submitted into NCBI Genbank and identified by accession numbers .

BankIt2566061 Seq1 ON087846
BankIt2566061 Seq2 ON087847
BankIt2566061 Seq3 ON087848
BankIt2566061 Seq4 ON087849
BankIt2566061 Seq5 ON087850

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List of Abbreviations

Abbreviation	Meaning
A(7)	**Interferon antagonist**
ADRV	**Adult Diarrhoea *Rotavirus***
AGE	**Acute Gastroenteritis**
APCs	**Antigen-presenting cells**
C(2)	**Core-shell protein**
cDNA	**Complementary DNA**
Cryo-Em	**Cryo-electron microscope**
DEPC	**Diethylpyrocarbonate**
DLP	**Double Layered Protein**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxynucleoside triphosphate**
dsRNA	**double-stranded RNA**
DTT	**Dithiothereitol**
E(10)	**Enterotoxin**
EDIM	**Epidemic Diarrhea of Infant Mice**
ELISA	**Enzyme-Linked Immunosorbent Assay**
EIAs	**Enzyme Immunoassay**
G	**Glycoprotein**
G(6)	**Glycoprotein**
H(11)	**Phosphoprotein**
HRP	**Horseradish peroxidase**
HRV	**Human *Rotavirus***
I(5)	**Intermediate capsid shell**
IAHA	**Immune Adherence Hemagglutination Assay**
ICT	**Immuno Chromatography Test**

ICTV	**International committee on taxonomy of virus**
Ig	**Immunoglobulin**
IRF	**Interferon regulatory factor**
$M^{(3)}$	**Methyltransferase**
MatAbs	**Maternal antibodies**
MDG	**Millennium Development Goal region.**
$N^{(8)}$	**NTPase**
Nm	**Nanometer**
NSP	**Non-structural proteins**
OD	**Optical Density**
ORS	**Oral Rehydration Solution**
ORV	**Oral *Rotavirus* Vaccine**
P	**Protease sensitive**
$P^{(4)}$	**Protease-sensitive**
PAGE	**Polyacrylamide Gel Electrophoresis**
PCR	**Polymerase Chain Reaction**
$R^{(1)}$	**RNA-dependent RNA polymerase**
RdRp	**RNA-dependent-RNA-polymerase**
RPM	**Revolutions Per Minute**
RT-PCR	**Reverse transcription-polymerase chain reaction**
RV	***Rotavirus***
SES	**socio-economic settings**
$T^{(9)}$	**Translation enhancer**
TLP	**Triple layered protein**
TLR	**Toll-like receptors**
TMB	**Tetramethylbenzidine**
WHO	**World Health Organization**

Chapter One

Introduction and Literature Review

1.1 Introduction

Rotavirus infection is the most common cause of acute gastroenteritis globally in children under five years of age and is responsible for approximately 5% of all child deaths yearly (Muendo, *et al*., 2018).

Human serotypes group A *Rotavirus* (RV) is the major etiologic agent of viral gastroenteritis and is responsible for 29 to 45% of hospitalizations worldwide(Ali, *et al*., 2022).

The outer capsid layer of *Rotavirus* consists of two structural proteins and they were *VP4* and *VP7*. A traditional dual classification scheme based on the genetic variation of two outer proteins, *VP7* (glycosylated, G-type) and *VP4* (protease-sensitive, Ptype), exists. At present 27 G genotypes and 35 P genotypes have been discriminated so far (Santiso-Bellón and colleagues, 2020).

In addition, an alternate classification strategy based on *NSP4* gene nucleotide sequence variation was presented. Recently, a total of 27 *NSP4* genotypes have been identified and designated as E (enterotoxin) genotypes (E1–E27) (Khalkhali, *et al*., 2021). *NSP4* is a 175 amino acid multifunctional three pass trans-membrane glycoprotein (aa). *NSP4* is required for virus pathogenesis and morphogenesis (Hyser *et al*., 2010).

Gastrointestinal symptoms in contrast to gastroenteritis caused by bacterial pathogens, *Rotavirus* infections cause non-bloody diarrhoea that lasts for a relatively short duration and is associated with a limited inflammatory response. Indeed, inflammatory markers such as serum C-reactive protein and fecal calprotectin are almost unaltered in children with *Rotavirus* infection. Thus, *Rotavirus*-induced diarrhea is considered non-inflammatory and has two proposed mechanisms: osmotic diarrhea due to malabsorption (secondary to enterocyte damage or death, or to decreased epithelial absorptive function) and secretory diarrhoea due to

the effects of *NSP4* and activation of the enteric nervous system (ENS) (Crawford *et al.*, 2017).

*Rotavirus*es also affect several animal species, but interspecies spread is rare with a few documented cases of bovine *Rotavirus* strains infecting a child. (Ali *et al*., 2022).

IgA is one of the major immune effector products present in the gastrointestinal tract yet its importance in protection against gastrointestinal viral infections has been difficult to prove. In part this has been due to a lack of small and large animal models in which pathogenesis of and immunity to gastrointestinal viral infections is similar to that in humans (Blutt and Conner, 2013).

Cytokines, proteins of low-molecular-weight, are the main components of the immune system, which contribute to signal transduction between cells and regulate the immune responses (Delirezh *et al*., 2016). Meanwhile, pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factors alpha (TNF-α), play a major role in this regard. These cytokines are mainly produced by mononuclear phagocytes. The other type of cytokine, known as anti-inflammatory cytokine owing to its function, is produced by the immune cells to regulate the secretion of pro-inflammatory cytokines and control the associated tissue damage. The IL-6 is one of the anti-inflammatory cytokines released by many activated immune cells, controlling the inflammatory pathways in several diseases. Therefore, this cytokine can be considered an appropriate indicator to monitor the activity of the immune system. (Beheshtipour and Raeeszadeh, 2020).

Aim of the study:

To identify the *Rotavirus* disease immunology, epidemiology, and molecular aspects of circulating strains among children under the age of five in Babylon province.

Objectives and Study Plan:

In the current study, the Objectives can be mentioned to include a variety of approaches, with these approaches including:

1- Determine the prevalence and age distribution of *Rotavirus* gastroenteritis in children under the age of five in Babylon province.

2- Immunochromatographic test (ICT) for detecting *Rotavirus* antigens in feces.

3- Immunological assays employing ELISA to detect immunoglobulin's (IgA) in feces and serum samples, as well as interleukin 6 in children's serum samples.

4- RT-PCR-based molecular approaches for detecting *Rotavirus NSP4* in feces and sequences for it.

1.2 Literature Review

1.2.1 Discovery and Historical Events

Acute gastroenteritis (AGE) is a major cause of hospitalization for children less than five years of age and *Rotavirus* is a leading cause of AGE requiring hospitalization and resultant mortality in this age population worldwide (Huang *et al*.,2015 ; Mohammed, 2021). From 1986 to 2000, *Rotavirus* infections were responsible for up to half a million deaths with at least 2 million children being hospitalized every year (Saha *et al*., 2020).

Rotavirus was first identified as an enteric pathogen by Ruth Bishop and colleagues in 1973, when identified new 'virus-like' particles, from the epithelial cells of duodenal mucosa of children suffering from non-bacterial gastroenteritis and admitted to the Royal Children's Hospital Melbourne (Australia). Through electron microscopic examination, it was revealed that these ''virus-like" particles were distinct from the parvovirus group or Norwalk-like agent but had a similar resemblance to orbiviruses. Owing to its 'wheel-like' morphology, the causative agent was subsequently named '*Rotavirus*' (Latin word ''rota", meaning wheel) under the family Reoviridae (Bishop, 1973). The *Rotavirus* vaccine has been recommended by the World Health Organization (WHO) since 2009 and had been introduced by 108 countries by 2019 (Schollin Ask *et al*., 2021).

1.2.2 General characteristics

1.2.2.1 *Rotavirus* Structure and Genome Organization

Rotavirus is a type of Reoviridae virus that has double-stranded RNA (Morelli, *et al.*, 2015). The virus has an 11-segment genome, ranging in size from 664 to 3,302 nucleotides, encoding six structural viral proteins (VP)and six nonstructural proteins (NSP) (Faleye *et al*., 2020).

Rotavirus (RV) capsids are multi-layered icosahedral capsids that are not enveloped (Rodríguez, Javier, and Daniel , 2019). The viral capsid is formed by three concentric layers: a central core, an inner protein layer, and an outer protein layer. The outer protein layer is composed of *VP4* and *VP7*, the two major antigens of the virus, and the middle layer is composed of *VP6* molecules arranged as trimers. The central core is composed mainly of *VP2* and contains the gene segments and enzyme complexes responsible for the processes of RNA transcription and replication (Rahman *et al*., 2007).Figure (1-1).

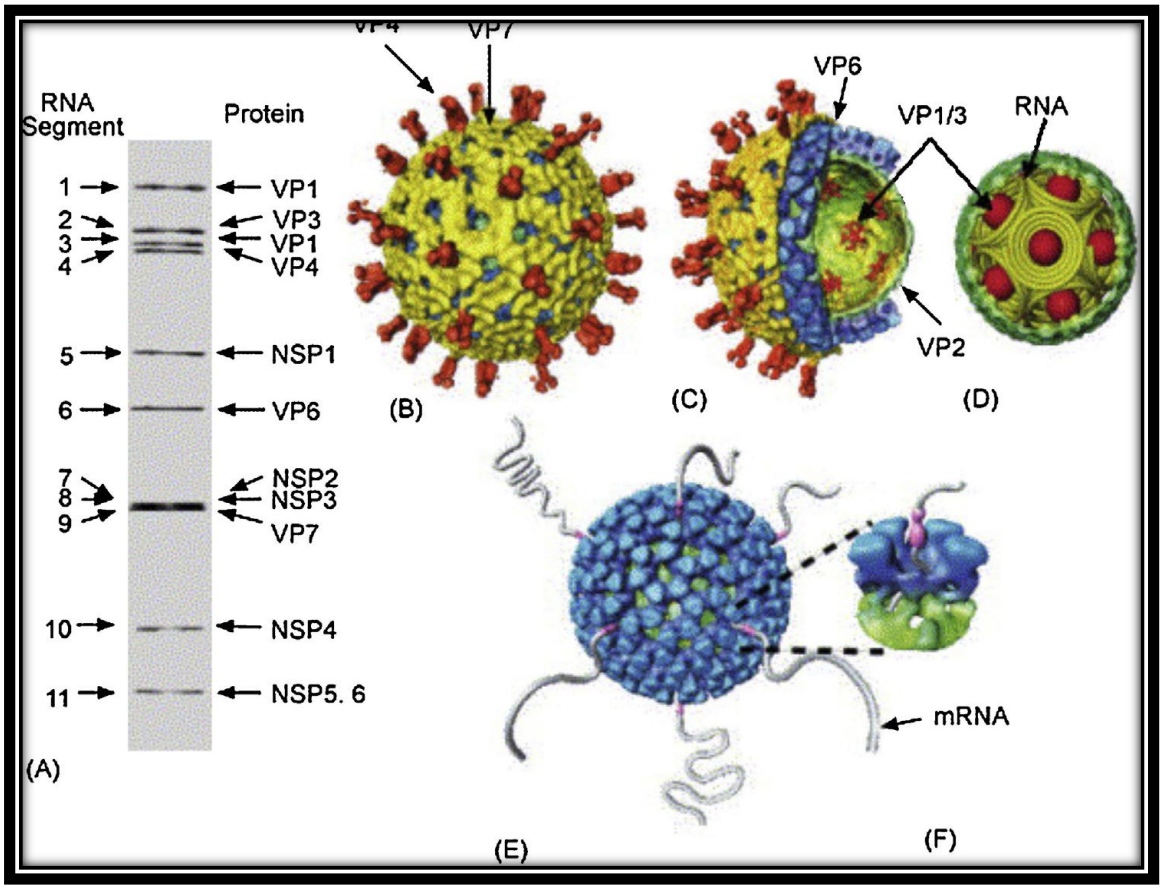

Figure (1-1): Aspects of *Rotavirus* structure. (A) PAGE gel showing 11 dsRNA segments comprising the *Rotavirus* (RVA) genome. The gene segments are numbered on the left and the proteins they encode are indicated on the right. (B) Cryo-EM reconstruction of the *Rotavirus* triple-layered particle. The spike protein *VP4* is colored in orange and the outermost *VP7* layer in yellow. (C) A cutaway view of the

Rotavirus TLP showing the inner *VP6* (blue) and *VP2* (green) layers and the transcriptional enzymes (in red) anchored to the inside of the *VP2* layer at the fivefold axes. (D) Schematic depiction of genome organization in *Rotavirus*. The genome segments are represented as inverted conical spirals surrounding the transcription enzymes (shown as red balls) inside the *VP2* layer in green. (E and F) Model from Cryo-EM reconstruction of transcribing DLPs. The endogenous transcription results in the simultaneous release of the transcribed mRNAs from channels located at the fivefold vertices of the icosahedral DLP.From (Jayaram *et al*.,2004).

1.3 Classification of *Rotavirus*

1.3.1 *Rotavirus* Groups

According to the serological reactivity and genetic variability of the middle layer protein *VP6*, at least 10 different groups, also termed species, have been differentiated (termed RVA-RVJ) (Matthijnssens *et al*., 2012; Mihálov-Kovács *et al*., 2015; Bányai *et al*., 2017).

The high genomic and antigenic diversity of RVs has been explored further. Following the classification of all RNA segments of RVAs into genotypes (Matthijnssens *et al*., 2008), recent accumulation of sequence data of non-species A RVs has led to similar pan-genomic genotyping schemes for RVs of species B (Marthaler *et al*.,2014; Shepherd *et al*., 2018), species C (Suzuki and Hasebe, 2017), and species H (Suzuki and Inoue, 2018) (Table 1-1). For the other RV species there are only a few publications which are insufficient to propose whole genome genotyping classification (Deol *et al*., 2017; Chen *et al*., 2017; Banyai *et al*., 2017).

RV isolates obtained from common shrews have been proposed as members of novel species K and L (Johne *et al*., 2019). Bats were found to be the hosts of a large diversity of RVAs which have completely host-specific genotypes, provide evidence for bat-to-human transmission, or are reassortants between bat and human RVAs (Simsek *et al*., 2021; Komoto *et al*., 2021).

Table (1-1) Whole genome classification of *Rotavirus*es of different species

Species				**Number of genotypes per genome segment**							
	R (1)	**C (2)**	**M (3)**	**P (4)**	**I (5)**	**G (6)**	**A (7)**	**N (8)**	**T (9)**	**E (10)**	**H (11)**
	VP1	***VP2***	***VP3***	***VP4***	***VP6***	***VP7***	***NSP1***	***NSP2***	***NSP3***	***NSP4***	***NSP5***
A[a]	**22**	**20**	**20**	**51**	**26**	**36**	**31**	**22**	**22**	**27**	**22**
B[b]	**5**	**5**	**5**	**5**	**13**	**26**	**8**	**10**	**6**	**4**	**7**
C[c]	**4**	**6**	**6**	**21**	**13**	**18**	**9**	**8**	**6**	**5**	**4**
H[d]	**3**	**4**	**7**	**6**	**6**	**10**	**6**	**2**	**4**	**6**	**3**

(Matthijnssens *et al*, 2008):

b-(Shepherd *et al*., 2018 ; Marthaler *et al*., 2014).

c-(Suzuki and Hasebe, 2017).

d-(Suzuki and Inoue, 2018).

1.3.2 *Rotavirus* Serotypes

Based on the genetic and antigenic similarity of *VP4* and *VP7*, Group A *Rotavirus*es (RVA) can be further divided into P or G groups. *VP4* (P protein for 'protease-sensitive' due to its trypsin mediated cleavage required for virus adsorption into cells) determines the P serotypes. *VP7* (G protein for 'glycoprotein' forming the matrix of the capsid) defines G serotypes (Falkenhagen *et al*., 2020). For G types, serotypes determined by neutralization assay) and genotypes (determined by RT-PCR). Thus, the serotype of prototype human *Rotavirus* strain Wa is described as G1P.

To date, at least 27 G types and 37 P types have been found in humans and animals (Mhango and Mandolo, 2020).

1.4 *Rotavirus* Replication

Rotavirus infects enterocytes in the small intestine, where the virus exhibits tropism towards mature enterocytes and only infects the middle and top portions of the villi. *Rotavirus* also infects sensory enterochromaffin (EC) cell, but these cells are few, less than 1% of the intestinal epithelium. Histopathological analysis has demonstrated vacuolization of the enterocytes and shortening and blunting of villi in the *Rotavirus*-infected intestine (Hellysaz *et al*., 2021).

The TLP is the infectious form of the virus that attaches to and enters into host cells. However, during the cell entry process, the outer *VP4–VP7* layer of the TLP is shed, depositing a double-layered particle (DLP) into the cell cytoplasm. *VP1* polymerases within the DLP synthesize single-stranded, positive-sense RNAs (+RNAs), which acquire a 5' cap structure by the activities of *VP3*. These +RNAs serve as mRNA templates for protein synthesis, and they are also selectively assorted and packaged into an early assembly intermediate where they serve as templates for genome replication by *VP1*(Long and McDonald, 2017).

The attachment of the virus to the host cell is initiated by *VP4*, which attaches to molecules, called glycans, on the surface of the cell (Rodríguez and Luque, 2019). The virus enters cells by receptor-mediated and forms a vesicle known as an endosome. Proteins in the third layer (*VP7* and the *VP4* spike) disrupt the membrane of the endosome, creating a difference in the calcium concentration. This causes the breakdown of *VP7* trimers into single protein subunits, leaving the *VP2* and *VP6* protein coats around the viral dsRNA, forming a double-layered particle (DLP)(Baker and Prasad, 2010).

The eleven dsRNA strands remain within the protection of the two protein shells and the viral RNA-dependent RNA polymerase creates

mRNA transcripts of the double-stranded viral genome. By remaining in the core, the viral RNA evades innate host immune responses including RNA interference that are triggered by the presence of double-stranded RNA(Arnold, 2016).

During the infection, *Rotavirus*es produce mRNA for both protein biosynthesis and gene replication. Most of the *Rotavirus* proteins accumulate in viroplasm, where the RNA is replicated and the DLPs are assembled. In the viroplasm, the positive sense viral RNAs that are used as templates for the synthesis of viral genomic dsRNA are protected from siRNA-induced RNase degradation (Silvestri, Taraporewala, and Patton, 2004; Kaelber and Jiang, 2020). Viroplasm is formed around the cell nucleus as early as two hours after virus infection, and consists of viral factories thought to be made by two viral nonstructural proteins: *NSP5* and *NSP2*. Inhibition of *NSP5* by RNA interference in vitro results in a sharp decrease in *Rotavirus* replication. The DLPs migrate to the endoplasmic reticulum where they obtain their third, outer layer (formed by *VP7* and *VP4*). The progeny viruses are released from the cell by lysis(Ruiz *et al*., 2009; Buttafuoco *et al*.,2020).

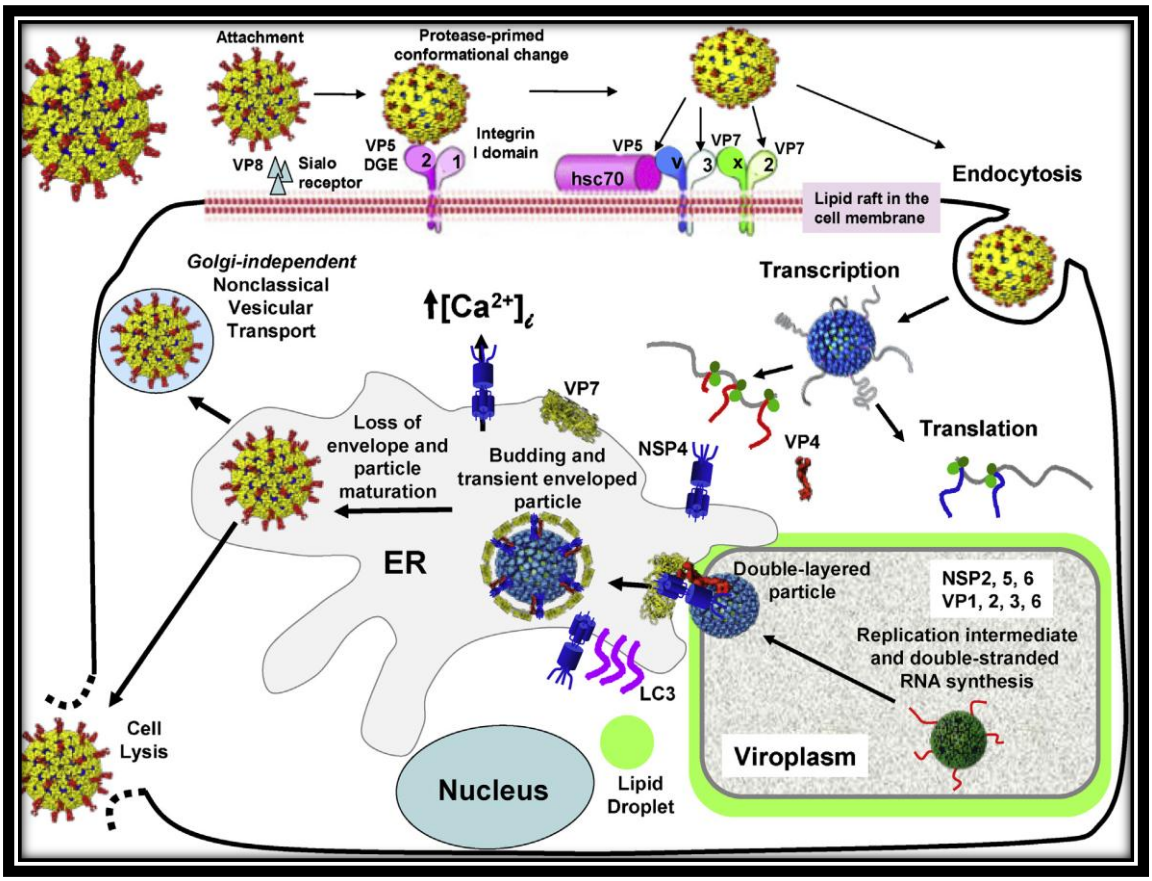

Figure (1-2). The *Rotavirus* replication cycle.

The *Rotavirus* triple layered particles (TLPs) first attach to sialo-glycans (or histo-blood group antigens) on the host cell surface, followedby interactions with other cellular receptors, including integrins and Hsc70. Virus is then internalized by receptor-mediated endocytosis. Removal of the outer layer, triggeredby the low calcium of the endosome, results in the release of transcriptionally active double-layered particles (DLPs) into the cytoplasm. The DLPs start rounds of mRNAtranscription, and these mRNAs are used to translate viral proteins. Once enough viral proteins are made, the RNA genome is replicated and packaged into newly made DLPsin specialized structures called viroplasms, which interact with lipid droplets. The newly made DLPs bind to *NSP4*, which serves as an endoplasmic reticulum (ER) receptor, and bud into the ER. *NSP4* also acts as a viroporin to release Ca2+from intracellular stores. Transiently enveloped particles are seen in the ER. The transient membranes are removed as the outer capsid proteins *VP4* and *VP7* assemble, resulting in the maturation of the TLPs. The progeny virions are released through cell lysis. In polarized epithelialcells, particles are released by a non-classical vesicular transport mechanism (Estes and Greenberg, 2013).

1.5 Mechanisms/pathophysiology

When humans ingest food and water contaminated with the virus, protein *VP4* on the external surface of the *Rotavirus* is fractionated by the enzymes pancreatin, trypsin, or elastase into two smaller proteins (*VP5* and *VP8*). Subsequently, the virus adheres to and infects enterocytes, destroying enzymes like maltase, sucrose, and lactase. This destruction prevents disaccharides from breaking and being absorbed, thereby increasing the osmolarity of the intestinal lumen and raising the influx of liquid (Liu *et al*., 2016).

Furthermore, intestinal bacteria may act on unabsorbed sugar, which results in elimination of feces with acid pH and may accentuate the diarrhea of osmotic nature(Ball *et al*., 1996). The viral enterotoxin (protein *NSP4*, which resembles the cholera toxin) also decreases the activity of Na/K ATPase, which underlies the intestinal absorption of sodium coupled to glucose, reducing the absorption of sodium and water. Activation of the enteric nervous system by infection can also induce diarrhea, to culminate in secretion of intestinal fluid, electrolyte liquid, and thus diarrhea (Lundgren, 2000).

Rotavirus infections cause non-bloody watery diarrhea, vomiting of short duration, and fever, and are associated with a limited inflammatory response. *Rotavirus* infects the mature enterocytes in the mid and upper part of the villi of the small intestine. *Rotavirus*-induced diarrhea is considered osmotic and non-inflammatory with diarrhea most likely due to extracellular accumulation of solutes and mal-absorption as a result of enterocyte damage or death; or decreased epithelial absorptive function. Another proposed mechanism is secretory diarrhea due to the effects of the viral enterotoxin, *NSP4*, on activation of the enteric nervous system. *Rotavirus* can exert effects on the central nervous system through nerve

gut-brain communication, via the release of mediators, such as the *Rotavirus* enterotoxin *NSP4*, which stimulates neighboring enterochromaffin cells in the intestine to release serotonin and activate both enteric neurons and vagal afferents to the brain (Hellysaz and Hagbom, 2021). There is evidence that the serotonin (5-HT3) receptor is involved in *Rotavirus*-induced diarrhea by promoting intestinal motility (Istrate *et al*., 2014) but not increased permeability. There is also evidence of extra-intestinal *Rotavirus* infection with antigenemia, and viremia commonly detected in hospitalized children within 10 days after onset of symptoms (Zweigart *et al*., 2021).

1.6 Signs and Symptoms

The major symptoms of *Rotavirus* infection of the small intestine are diarrhea and vomiting, which can cause rapid dehydration in young children and even lead to organ failure and death (Hellysaz *et al*., 2021).

In infants <1 month of age, *Rotavirus* infections are caused by distinct strains and are often asymptomatic or mild, presumably because of protection conferred by maternal antibodies that are transferred through the placenta or through breast. The first *Rotavirus* infections in infants >3 months of age are likely symptomatic and accordingly the incidence of *Rotavirus* disease peaks between 4 and 23 months of age(Crawford *et al*., 2017). In children, the manifestation of *Rotavirus* disease ranges from no symptoms to mild, watery diarrhea of short duration and to severe diarrhea with vomiting and fever that can result in rapid dehydration with shock, electrolyte imbalance and death. As previously mentioned, reinfections are common, although the severity of disease usually decreases with each repeat infection. However, *Rotavirus* can cause limited disease in older children and adults, especially parents and caretakers of children with *Rotavirus* diarrhea, immune-compromised

individuals, travelers and elderly individuals. These illnesses are generally mild to moderate in severity (Carvalho *et al*., 2019).

1.7 Routes of Transmission

Rotavirus is shed in large quantities in stools during episodes of *Rotavirus*-associated diarrhea. The virus is transmitted predominantly through the fecal-oral route, mainly by close person-to-person contact (Estes *et al*., 2013), About 10 particles of the virus are excreted per gram of feces, and the maximum viral excretion occurs on the third and fourth days since the onset of the first symptoms. During the acute phase of the disease, viral elimination may begin two days before the onset of diarrhea and may persist for 10 days after the onset of the symptoms. If we consider immunodeficient individuals, the virus can be detected for over 30 days after infection.Only 10 to 100 viral particles are necessary to infect humans, increasing the potential risk of infection and making epidemics common. In addition because the *Rotavirus* can survive for weeks or even months on non-disinfected surfaces, it is also an important cause of nosocomial diarrhea (Gray, 2011; Dennehy, 2012). Other sources of the virus such as airway secretions, objects, surfaces, toys, water, and food have been pointed out in most emissions from pre-schools and schools.

1.8 Epidemiology

1.8.1 Global *Rotavirus* Infection in Human

Rotavirus illness kills 527,000 children under the age of five each year, according to the World Health Organization (WHO). *Rotavirus* kills 82 percent of children in the world's poorest countries (AL-Shuwaikh *et al*., 2015). Globally, it is estimated that RV infection causes approximately 25 million medical examinations, 2 million hospital admissions and 215,000

deaths every year(Gualano *et al* .,2018). In Italy, over 250,000 cases of RVGE occur every year, resulting in approximately 125,000 pediatric medical visits, 52,000 emergency room visits, 14,500 hospital admissions and 5 to 11 deaths(Conforti, 2016). Studies from western Europe found that 50% of cases of gastroenteritis in children younger than 5 years of age who were treated in emergency departments were caused by *Rotavirus* and that the infection resulted in 230 deaths per year (Troeger, 2018).

1.8.2 Local *Rotavirus* Infection in Human

Three studies conducted in Baghdad such as (Musa *et al*., 2019) who found 21.4% (21 out of 98) among children with acute gastroenteritis in two hospitals which are Children's Protection Teaching Hospital and Al-Alawiya Children's Hospital from October 2018 to the end of January 2019 using multiplex RT-PCR (Al-Janabi, 2020).

(Abd-Al Fattah *et al*., 2020) who reported 32.6% of *Rotavirus* group A among 150 children with diarrhea admitted to Maternity and Children Hospital in Ramadi city at Al-Anbar governorate using rapid test (AL-Sadawi *et al*.,2017) found that viral infections were mainly identified in infants in Al-Najaf Province, Iraq (12%).

In Diyala community (Nasser, 2021) found All the investigated strains in this study belong to G1P[8] genotype, with no noticeable assortment events between human and animal *Rotavirus* strains in Diyala province. Several Iraqi studies conducted in the North of Iraq, such as (Jaff *et al*., 2016). Who detected 22% (22 out of 100) of children with gastroenteritis fewer than five years. (Badry *et al*., 2014) in Kurdistan Region, Iraq, found that 13.21% of viruses were isolated from children suffering from diarrhea.

Numerous Iraqi studies conducted in the South of Iraq, such as (Jarullah and Mohammed,2019) revealed that 45% of 100 infants and

children under five years of age in Thi-Qar Province south of Iraq for five Months (From November 2017 to March 2018) using RT-qPCR.

(Habash and Habeeb,2018) find a routine *Rotavirus* stool test review of children with serious diarrheal episodes to avoid un-rational antibiotic prescribing and best practices in the integrated treatment of childhood disease in Basrah Province.

1.9 Morbidity and Mortality

The vast majority of *Rotavirus*-associated deaths occurred in children in resource-limited countries, particularly in sub-Saharan Africa and in the Indian subcontinent, deaths due to *Rotavirus* were rarely reported in resource-rich countries. Possible reasons for low mortality could be related to better hygiene and access to timely healthcare resources, however, the morbidity burden is still significant in these settings. Regardless of economic level, *Rotavirus* accounted for an estimated 1,537,000 global hospital admissions due to diarrheal disease among children fewer than 5 years of age in 2016 suggesting that it is a significant global public health burden both in terms of mortality and morbidity (Saha *et al*., 2021). In 2017, 47% of an estimated 5.4 million deaths in children aged <5 years occurred within 1 month of birth, with more than three-quarters (77%) occurring in sub-Saharan Africa and South Asia (United Nations Children's Fund, 2018).

1.10 Diagnosis of *Rotavirus*

variety of conventional and molecular methods are used for direct detection of human RV in clinical specimens.

1.10.1 Electron Microscope

Electron microscopy is highly specific for detection of *Rotavirus* and is as sensitive as some EIAs. Electron microscopy has become an important method for identifying noncultivatable viral agents in stool samples from

patients with acute nonbacterial gastroenteritis. However, the method is too labor intensive for routine detection of *Rotavirus* in large numbers of stool specimens. In addition, EM requires an expensive instrument and highly trained personnel and cannot distinguish between *Rotavirus*es of different groups (Steele *et al*., 2004; Alkhafaji, 2015).

1.10.2 Cell culture isolation

Cultivation of *Rotavirus* from clinical fecal specimens is achieved through the use of several primary cell types and continuous cell lines. *Rotavirus*es can be isolated in African green monkey kidney cells and continuous cell lines, including Rhesus monkey kidney (MA104) cells. The virion can be purified from RV-infected cell lysate using cesium chloride gradients. Purified virus is analyzed by sodium dodecyl sulfate – PAGE to verify *Rotavirus* recovery. Plaque assay can be formed to determine the viral titer in plaque-forming units per milliliter of virus based on the cytopathic effect caused by *Rotavirus* in cultured cells .Diagnosis of *Rotavirus* in stool samples using cell culture method is not performed routinely because it is time consuming, labor intensive, and prone to contamination (Esona and Gautam, 2015).

1.10.3 Serological Methods

1.10.3.1 Immunochromatographic tests

Immunochromatographic assays also called lateral flow tests or strip tests, can be used for qualitative detection of *Rotavirus* antigens in patient fecal samples. Immunochromatographic assays are based on the principle of sandwich immunochromatography where antibodies against RVA-specific *VP6* protein are used to detect *Rotavirus* antigen in stool samples. In immunochromatography strips, the *Rotavirus* antigen present in the

stool sample reacts with the antihuman antibody in the membrane strip first and then is captured by the recombinant antibody, appearance of a colored(Esona and Gautam, 2015; Akhafaji, 2015).

The sensitivity of rapid antigen detection is generally higher than that of conventional methods (e.g., EM and IAHA) although lower than that of molecular methods (Marie-Cardine *et al.,* 2002). However, these assays are particularly useful in the pediatric setting; the sensitivity is higher in this population because children often shed gastrointestinal viruses at higher titers and for longer time periods than do adults.

1.10.3.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme immunoassay has been proven to be very sensitive and specific for the detection of group A and C *Rotavirus*es in fecal specimens, especially if monoclonal antibodies are used (Ellen *et al*., 2007).

Enzyme-linked Immunosorbent Assay and latex agglutination are the stool tests used most frequently for *Rotavirus* because they are easy to perform, provide rapid results, and are more sensitive than many of the other tests. Such tests, which detect the abundant *VP6* protein present in the middle layer of the triple layer capsid, have sensitivities of 70% to 98% and specificities of 71% to 100%. ELISA is preferred and most common method used for screening for *Rotavirus* in children. Several rapid methods as latex agglutination have been evaluated and compared to ELISA showing a wide range of sensitivity and specificity (Yadav *et al*., 2019).

1.10.4 Molecular Techniques

1.10.4.1 Conventional molecular methods

PCR has become the method of choice in the epidemiologic survey of gastrointestinal viruses. Currently, PCR is widely employed as a tool for

the routine diagnosis of Astrovirus, Norovirus, Sapovirus, *Rotavirus*, adenovirus, and Torovirus infections. These PCR assays are highly sensitive, specific, and easy to perform. The most reliable marker for the diagnosis of virus infection is the presence of viral nucleic acid in stool specimens. Therefore, the specimen of choice is stool samples from patients with diarrhea. To facilitate the molecular analysis, the amplification of the viral genome and sequencing of the amplification products should be performed, and virus genotypes can be identified based on their sequence analysis (Sidoti *et al*., 2015). Therefore, PCR assays and nucleic acid sequence analysis are widely used for the detection and genotype identification of viruses causing gastroenteritis. Gradually, these techniques have replaced the traditional immunological tests and have become the gold standard for diagnosis of gastrointestinal viruses for almost two decades. Nested PCR assays were also developed to increase both sensitivity and specificity (Izzo *et al*., 2012).

At last, multiplex RT PCRs have been widely described. In particular, multiplex RT -PCRs for the detection of groups A, B, and C *Rotavirus*es and identification of G and P genotypes of group A *Rotavirus*es have been developed gastroenteritis, multiplex molecular assays have the potential to consolidate laboratory workflow reducing the time to result, improving diagnostic accuracy and allowing to simultaneously detecting different pathogens (Binnicker, 2015)

1.10.4.2 Real -time PCR

Real-time PCR technology provides results more quickly than conventional PCR assays and shows improved sensitivity and specificity. Although reagent and instrument costs are higher for real-time PCR technology compared to conventional molecular methods, real-time PCR requires less hands-on time per specimen than traditional PCR,

particularly nested PCR, which is labor intensive(Barsoum *et al*., 2020). Automation of the extraction process and the use of real-time PCR further reduce the hands-on time in the clinical laboratory. Moreover, real-time PCR technology offers advantages over conventional PCR by providing lower risk of false-positive results due to amplicon contamination and quantification of viral load. Real -time PCR assays that detect the most common gastrointestinal viruses in large numbers of stool specimens have been developed (Sidoti *et al*., 2015).

1.11 Prevention and Control of *Rotavirus* Infection

Prevention of infection remains the cornerstone of effective management of the rotaviral disease. The provision of clean water, maintaining hygienic practices like hand washing, and promoting exclusive breastfeeding for 6 months is beneficial to reduce the risk of *Rotavirus* infections (Saha *et al*., 2021).

1.11.1 *Rotavirus*-specific antivirals

There is a scarcity of approved antiviral drugs efficacious against enteropathogenic viruses (De Clercq and Li, 2016). This means that management of disease currently focuses on supportive therapy aiming at the treatment of dehydration (Parashar *et al*., 2013). The importance of antiviral immunity in controlling disease is highlighted by the observation that the symptomology is more severe in children with more immature immune systems (Crawford *et al*., 2017; Bányai *et al*., 2018), with disease being the worst in the youngest children, where as severe dehydration, electrolyte disturbances and emesis are not uncommon following *Rotavirus* infection (Leung *et al*., 2005; Parashar *et al*., 2013; Hartman *et al*., 2019).

1.11.2 Probiotics

An accidental finding led to the discovery of protection from *Rotavirus* disease in mice by segmental filamentous bacteria (SFB) which induce increased intestinal epithelial proliferation, are active in both, immunocompetent and immunocompromised mice and thus independent of immune responses, and potentially interfere with the replication of other viruses (Shi *et al*., 2019). This raises the possibility of using specific bacteria for treatment and/or prevention of RV-induced disease. Bifidobacteria and Lactobacilli have shown promise in preventing diarrhoea in pre-clinical models (Vlasova *et al*., 2019; Azagra-Boronat *et al*., 2020).

Some lactic acid bacteria (LAB) strains are able to impact on human and animal health by modulating the mucosal and systemic immune systems. Those immunoregulatory probiotic LAB, known as immunobiotics, provide protection against viral infections by modulating innate and adaptive antiviral immunity. Thus, several reports have shown that immunobiotic LAB shorten the duration of diarrhea, reduce the number of episodes , diminish RVs shedding, normalize gut permeability, and increase the production of RVs-specific antibodies (Villena *et al*., 2016).

1.11.3 Fluid and Electrolyte Management

The treatment of viral gastroenteritis is based on symptomatic support . The most important goal of treatment is to maintain hydration status and effectively counter fluid and electrolyte losses. Fluid therapy is a fundamental part of treatment. Intravenous fluids may be administered to those individuals who appear dehydrated or to those unable to tolerate oral fluids. Antiemetic medications such as ondansetron or

metoclopramide may be used to assist with controlling nausea and vomiting symptoms. Patients demonstrating severe dehydration or intractable vomiting may require hospital admission for continued intravenous fluids and careful monitoring of electrolyte status (Stuempfig and Seroy, 2021). Electrolyte abnormalities may be addressed on an individual level, although often these are caused by an overall fluid volume depletion which, when corrected, will also cause electrolytes to normalize. Both saline and lactated Ringer's solutions appear to be effective for the treatment of dehydration due to viral gastroenteritis.

For oral rehydration, some studies have shown that commercially available oral rehydration solutions containing electrolytes are superior to sports drinks and other forms of oral rehydration. However, a recent study using children with mild dehydration demonstrated no differences between children receiving oral rehydration solutions versus ad lib oral intake (Freedman *et al.*, 2016).

1.12 Physiochemical Properties

*Rotavirus*es are quite resistant to freezing and ultrasound but they are susceptible to boiling, action of acids and alkalis. They live in faeces for the period up to 7 months; they can be kept in tap water at the temperature of 40°C for2 months, on the vegetables at t = 4°C for30 days(Piku *et al*., 2017).

1.13 Immune Response to *Rotavirus*

Authors who studied the immunological changes in the body of sick person noted that at the early stage of disease the protection takes place at the expense of secretory IgA (sIgA). In several days, IgM are included, and during *Rotavirus* discharge from organism the antibodies to IgG occur providing the easier disease course in case of reinfection. The inhibition of cellular component, in particular T-helpers and destruction of

phagocytosis are observed. During the acute phase, the increase of serum interferon is noted, on the 7th-8th day their decrease is noted, however, the function of interferon production of leukocytes is still reduced (Piku *et al*., 2017).

MatAbs are transferred to the infant via 2 distinct routes: (1) placental transfer of immunoglobulin G (IgG) into infant circulation, and (2) breast milk transfer of primarily IgA into the infant gastrointestinal tract (Palmeira *et al*., 2012). Most studies investigating the role of matAb interference focus on placentally transferred IgG (Niewiesk, 2014). However, evidence from both population-level observational and animal modeling studies suggests that breast milk–derived matAb also interferes with RV vaccine efficacy (Appaiahgari *et al*., 2014; Yang *et al*., 2019).

VP6 is the most immunogenic RV protein. *VP6* does not induce neutralizing antibodies (Abs), although some *VP6*-specific polymeric immunoglobulins A (IgA) are protective in vivo,probably via transcytosis through epithelial cells (Burns, 1996 ; Schwartz-Cornil *et al*., 2002). *VP7* is known as the major antigen inducing neutralizing Abs (Kapikian, Hoshino, and Chanock, 2001). This Abs can passively protect experimental animals from RV-induced diarrhea. In humans, RV-induced Abs probably play an important role in the resolution of viral infection and against reinfections, as suggested by studies with adult volunteers, naturally RV-infected children, and infants from candidate vaccine clinical trials (Jiang, Gentsch and Glass, 2002; Holloway and Coulson, 2013). The B-lymphocyte population, which provides the specific anti-RV Abs, appears to be involved in other aspects of the host response, especially in the early phase of infection. Actually, intestinal infection with RV induces a rapid and massive T-lymphocyte-independent expansion of B cells that results in early anti-RV IgM production (Blutt *et al*., 2002). Furthermore, naive B lymphocytes were shownto be the

antigen-presenting cells responsible for intestinal IgA production after subcutaneous RV injection in mice. Because RV does not infect B cells, naive B lymphocytes probably take up RV via pinocytosis or receptor-mediated endocytosis.

A central goal of vaccine research and the study of natural history of viral infections is to identify vaccine induced or naturally induced immune responses that predict protection from infection or disease. Among the predictors of protection from *Rotavirus* infection and disease the most recognized are serum *Rotavirus* IgA (>1:800) and *VP7* specific IgA (>1:200), followed by serum *Rotavirus* IgG (>1:6400) and *VP7* specific IgG (>1:800), respectively. Fecal IgA, antibody secreting cell (ASC), *Rotavirus* CD4+, *Rotavirus* specific or neutralizing antibodies that recognize *NSP4*, *VP6*, *VP4*, *VP7*, and also epitope-specific neutralizing antibodies to *VP7* have been also studied (Clarke and Desselberger, 2015). Sterilizing immunity could not be induced by natural *Rotavirus* infection or vaccination due to antigenic drift and shift caused by the error-prone viral RNA polymerase, and also due to other evolutionary characteristics of *Rotavirus* like gene reassortment, gene recombination and interspecies transmission (Angel *et al*., 2014).

1.13.1 Innate responses

Rotavirus infection triggers an innate immune response of the intestinal epithelial cells, activating proinflammatory signalling pathways with the release of type I and type III interferons (INFs) and other cytokines . These responses are critical to reduce *Rotavirus* replication and to build up protective immunity in later stages of the infection (Gandhi *et al*., 2017).

An increasing number of studies have reported the effect of biotherapeutic substances on antiviral immune responses and the underlying cytokine mediated pro- and anti-inflammatory responses for

the inhibition of *Rotavirus* replication.

The intestinal epitheiial cell IEC senses viral dsRNA through pattern recognition receptors (PRRs), such as TLR3, retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5), and cellular signaling cascades are activated to react to viral infection .One of the major innate responses against dsRNA viruses relies on the activation of those PRRs, which leads to the production of cytokines and chemokines by IECs and immune cells. Thus, RVs dsRNA triggers the production of IL-8, IP-10, IL-6, TNF-α, and IL-15 in IECs via the TLR3-, RIG-I-, and MDA5-activated pathways inducing recruitment and activation of macrophages and NK cells and stimulating adaptive B- and T-cell immune responses. As a result of PRRs activation, interferons (IFNs) and IFN-regulated gene products are also produced and they play a key role in establishing an antiviral state for virus clearance and restriction of spread (Villena *et al.*, 2016).

1.13.2 The Role of Interleukin 6 during Viral Infections

IL-6 is a pleotropic cytokine produced in response to tissue damage and infections (Tanaka *et al.*, 2014). Multiple cell types including fibroblasts, keratinocytes, mesangial cells, vascular endothelial cells, mast cells, macrophages, dendritic cells, and T and B cells are associated with the production of this cytokine (Mauer *et al*., 2015).

Accordingly, the biological consequences of IL-6 production have been associated with both pro- and anti-inflammatory effects (Scheller *et al*., 2011), highlighting IL-6's pivotal role in the activation and regulation of the immune response. Biological activities affected by production of IL-6 include: control of the differentiation of monocytes into macrophages by regulating the expression of macrophage colony-stimulating factor, increasing B-cell IgG production by regulating the expression of IL-21

(Yang *et al*., 2016), negative regulation of dendritic cell maturation by activation of the STAT3 signaling pathway, as well as the promotion of the Th2 response by inhibiting Th1 polarization. Two different mechanisms have been described to promote the inhibition of Th1 polarization by IL-6: (1) IL-6 stimulates CD4 T cells to secrete IL-4 and direct the response to Th2, and (2) IL-6 affects the secretion of IFNγ by CD4 T cells, an essential interferon to promote Th1 polarization. A similar effect is produced in Th1 cells, where inhibition of IFNγ secretion in these cells affects CD8 T cell activation (Green *et al.*, 2013).

Moreover, in combination with the transforming growing factor beta, IL-6 induces the differentiation of naïve CD4 into Th17 cells, which are important for the defense against pathogens at mucosal sites. Also, IL-6 synergic interactions with IL-7 and IL-15 induce the differentiation and cytolytic capacity of CD8 T cells. Importantly, IL-6 is a potent pyrogenic cytokine, and has an essential role organizing lymphocyte trafficking to lymphoid organs during febrile events (Evans *et al*., 2015).

In addition to its roles modulating the host immune response, IL-6 has been implicated in the progression of several viruses infectious. IL-6 is considered one of the most important cytokines during an infection, along with interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α) (Velazquez-Salinas *et al.*, 2019).

1.13.3 Specific responses

*Rotavirus*es elicit both B and T cell immune responses. Antibodies to the *Rotavirus VP4* and *VP7* proteins neutralise viral infectivity in vitro and in vivo (Ward, 2009). Specific antibodies of the classes IgM, IgA and IgG are produced, which have been shown to protect against *Rotavirus* infection by the passive transfer of the antibodies in other animals (Vega *et al*., 2012). Maternal trans-placental IgG might play a

role in the protection neonates from *Rotavirus* infections, but on the other hand might reduce vaccine efficacy (Mwila *et al*., 2017).

Historic studies in a gnotobiotic pig model of *Rotavirus* infections have shown that protective immunity against diarrhea depends on the production of IgA and IgG antibody-secreting cells and memory B cell responses at the site of viral replication in the ileum, IgA memory B-cells decline substantially in about 3 months after infection (Patton, 2012). Studies of natural *Rotavirus* infections in children showed a similar positive association between IgA and IgG antibodies and protective immunity against *Rotavirus* infection and diarrhea, but after adjusting for age, the effect was only partial (Lewnard *et al*., 2017). *NSP4*-specific antibodies may be important in protecting against clinical symptoms of *Rotavirus* infection .In children with *Rotavirus* gastroenteritis and in animal models; *NSP4* induces humoral immune responses with modest IgG seroconversion rates (54–70%) and IgA being barely or not detected at all. The titers of *NSP4* specific IgG antibody have been found to be transient, increasing with age, peaking between 12–23 months of age and dropping to minimal levels afterwards. A significant proportion of children who did not develop diarrhea associated with *Rotavirus* infection had antibodies to *NSP4* in acute-phase serum. There is evidence that humans can circumvent the extensive serotype diversity of circulating *Rotavirus* strains by generating frequent heterotypic neutralizing antibody responses to *VP7*, *VP8*, and most often, to *VP5* after natural infection (Zweigart *et al*., 2021).

1.13.4 IgA in the gastrointestinal tract

Immunoglobulin A (IgA) is present in all mammals and birds. It is found in large amounts in the mucosal secretions of gastrointestinal tract and in other secretions, including saliva and breast milk. However, IgA is

also present in serum at lower concentration (about 2–3 mg per mL). In humans, daily IgA production is higher than any other immunoglobulin isotype (up to ~60 mg per kg of body weight) (Pietrzak *et al*.,2020).

Monomeric IgA is present in serum, whereas in mucosal secretions is found secretory IgA (SIgA). It is diferent from the structure of IgA present in the serum because SIgA generally occurs in a polymeric form stabilized by joining chain (J chain), in particular in dimeric or tetrameric setup.Additionally, SIgA contains a secretory component (SC) derived from polymeric Ig receptor (pIgR) utilized for transcytosis through epithelial cells during secretion. In humans, there are two subclasses of IgA: IgA1 and IgA2. In serum subclass IgA1 dominates, whereas in mucosal secretions the proportion between IgA1 and IgA2 depends on the site of production, e.g., up to: 60% IgA1 in saliva 90% IgA1 in nasal and 60% IgA2 in intestinal secretions. In the human colostrum approximately 48% of immunoglobulins correspond to IgA2 and 40% to the IgA1 subclass (Sánchez-Salguero *et al*., 2019) that confers an adaptation to protect against potentially harmful pathogens, and which is also a way to regulate the colonization of the microbiota in newborns.

1.14 Rotavirus Vaccine

There are currently four approved vaccines available. Two of these vaccines (Rotarix and RotaTeq) have been used extensively globally over the past years. Rotarix is a live-attenuated vaccine derived from a human G1P[8] *Rotavirus* isolate, while RotaTeq is a live-attenuated, pentavalent vaccine consisting of five human bovine reassortants (human G1, G2, G3, or G4) in a bovine background as well as human P[8] in a bovine backgroun]. The human parent G4 strain was originally isolated from a patient in France, but all other strains originate from the United States (Falkenhagen *et al*., 2020).

South Africa was the first African country to introduce the monovalent RVA vaccine, Rotarix®, into its Expanded Program on Immunization (EPI) in September 2009 (World Health Organization 2009) In the first year, after the vaccine was introduced, RVA infections indicated by laboratory confirmed results and hospitalizations were reduced significantly by approximately 58% (Mwangi *et al*., 2020).

As the post-2015 MDGs take hold, continued surveillance of the leading pathogens associated with acute childhood diarrhea remains a priority for infectious disease specialists (Liu *et al.,* 2015). Moreover, prioritizing implementation of licensed interventions like *Rotavirus* vaccines and institutingfollow-up studies to monitor the impact of such interventions are increasing lyurgent to help document benefits that can guide new interventions to address emerging needs. Increasing evidence for the efficacy and effectiveness of *Rotavirus* vaccination against morbidity and mortality among children under five from all MDG regions has continued to be reported (Lamberti *et al.,* 2016).

1.14.1 Reasons for decreased effectiveness of *Rotavirus* vaccines in countries of low socio-economic conditions

RV vaccination does not completely protect young children against infection, but it reduces the severity of RVGE. RV vaccines are highly effective in preventing severe gastroenteritis in young children during the first five years of their life, particularly in developed countries (Arnold, 2018). The SES of a country seems to influence RV vaccine effectiveness. Vaccination was predicted to prevent 93, 86, and 51% of severe RVGE in high, middle, and low SES, respectively.

Analysis of the data for the Asia region found median vaccine effectiveness of 94% in low child mortality countries, 64% in medium child mortality countries, and 49% in high child mortality countries

(Lestari, 2020). While there is substantial evidence that RVA vaccination is effective in different socio-economic settings (Bányai *et al*., 2018); (Pindyck *et al*., 2018), it has recently been concluded that RV vaccine efficacy is lower and wanes more rapidly in regions of high-mortality than in those with low-mortality, but is beneficial even in areas of high-mortality (Clark *et al*., 2019 ; Lee, 2021).

In low- and middle - income countries the degree of protection achieved is often 20–30% lower for a variety of reasons (Desselberger, 2017; Parker *et al*., 2018).

Risk factors associated with impaired vaccine efficacy were identified by (Parker *et al*., 2018) by comparing vaccinated infants in Malawi, India and the UK. Maternal antibody levels and microbiota diversity were shown to interfere with vaccine efficacy (Parker *et al*., 2021). These and additional factors understood to be negatively affecting vaccine efficacy are listed in table (1-2) (Vlasova *et al*.,2019). In addition, vaccination of immunocompromised infants with live attenuated RVA strains (all licensed RVA vaccines are live attenuated) can have adverse clinical effects (Chiu *et al*., 2019).

It was found that the instantaneous efficacy and duration of RV vaccines were inversely correlated with the degree of RV-associated mortality in countries of different socioeconomic standard. Various interventions to improve oral RV vaccine performance in low-income countries have been tried, but have so far not been overtly successful, alternative ways to improve oral vaccine efficacy are urgently needed (Church *et al*., 2019; Lee, 2021).

Table (1-2): Host conditions affecting the health of children in low-income countries and of relevance for *Rotavirus* vaccine efficacy.

	a. via breastmilk
	a. zinc deficiency
	b. avitaminoses (vitamin A, vitamin D)
	b. transplacentally
1	Co-infections of the gut
2	Environmental enteropathy
3	Genetic factors
4	Infant immunological immaturity
5	Intestinal microbiome
6	Malnutrition
7	Transfer of maternal antibodies

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Patients

2.1.1 Study Design

This study is a case control study to observe frequency of *Rotavirus* infections in individuals and to estimate IL6 and IgA in serum and stool samples respectively, in addition to molecular study.

2.1.2 Study population

This current study conducted in Babylon province-Iraq during the period of September 2021 to February 2022. A total of 150 children under five years of age (100 children suffering from *Rotavirus* diarrhea and 50 children healthy as control) were enrolled. Bloody gastroenteritis cases were excluded from the study and acidic stool sample included. The data of consideration in the present study were age, sex, address and type of feeding. Fecal and serum samples were collected from children in the neonatal period, and a baseline assessment of the domestic environment was conducted by interview.

2.1.3 Sample collection

2.1.3.1 Stool Samples

All stool samples were transported in ice to the laboratory and were processed immediately for the detection of *Rotavirus*. A microscopic examination was performed for each stool sample looking for any abnormality as well as parasites. Stool samples were also sent for *Rotavirus* utilizing (Rapid test) Stool specimens should be placed in sterile screw-cup containers, properly labeled. Samples can be stored temporarily at 4–8°C for up to one month. Ice packs can be used to keep

samples cool. Freeze-thaw cycle should be avoided where possible. If prolonged storage is necessary, store at -70°C, as evidence suggests that ability to characterize *Rotavirus*es declines during storage for years at -20°C.

2.1.3.2 Blood samples

Three to five (ml) of venous blood were collected aseptically from 150 children included in this study. Blood samples were left to clot at room temperature or at 25° C for 30 minutes to allow the clot to form, followed by separation of serum from the clot. Serum was separated by centrifugation at 3000 RPM for 5 minutes. The collected separated sera samples were divided into small aliquots (200 µl) numerated and kept at (-20° C) deep freeze (Tarabily and Moustafa, 2019).

All these samples were collected from children under five years of age to perform the following serological and molecular tests:-

1-Immuno-chromatographic test (ICT) for detection of *Rotavirus* antigens from stool.

2-Immunological tests for detection of immunoglobulin (IgA and IL6) from stool and serum sample respectively by ELISA test.

3-Molecular techniques for detection of *Rotavirus NSP4* in stool by RT-PCR.

4- DNA sequencing for *Rotavirus NSP4*.

2.1.4 Ethical approval

All subjects involved in this work are informed and the agreement was obtained verbally from each one before the collection of samples.

This study was approved by the committee on publication ethics at college of medicine, University of Babylon, Iraq, under the reference No **BMS/0231/016**.

2.2 Materials

2.2.1 Equipment and Instruments

Table (2-1): The equipment and instruments that used in this study with their companies and countries of origin:

No.	Equipment & instrument	Company
1	Cool box	Chine
2	Disposable plastic tubes10 ml	Bio-Hit – Finland
3	Disposable sterile syringes 5 ml	Korea/ Seoul
4	Disposable tip	Bio-Hit/Finland
5	Electrophoresis	Bioneer/ Korea
6	ELISA unit	Biotech/USA
7	Eppendorf tubes	Bioneer/ korea
8	Exispin vortex centrifuge	Bioneer/ korea
9	Gel tubes	Afcovac/ Jordan
10	Gloves	
11	High Speed Cold centrifuge	LabNet /USA
12	Micropipettes 5-50, 0.5-10, 100-1000µl	CYAN/ Belgium
13	Nanodrop	THERMO/ USA
14	PCR T100 Thermal cycler	BioRad /USA
15	Refrigerator	Concord /Lebanon
16	U.V transilluminator	Wised/Korea
17	Vortex	CYAN/ Belgium
18	Water Bath	Plymouth/USA

2.2.2 Kits

Table (2-2): The kits used in this study with their companies and countries of origin:

No.	Kit	Company	Country
1	Human IgA(Immunoglobulin A)ELISA Kit	Elabscience	China
2	Human Interleukin 6 ELISA Kit	Bioassay Technology Laboratory's	China
3	One-Step RT-PCR Premix Kit for cDNA synthesis and PCR	iNtRON	Korea
4	Rapid test kit	CerTest Rota+Adeno+Astro	Spain
5	Trizol 100ml		
6	Viral RAN extraction AccuZol™ RNA extraction kit	Bioneer	Korea
7	OptiScript™ RT System		
	RT-PCR buffer (10×)		
	DNTPs		
	*i-StarTaq*TM DNA polymerase		
	Stabilizingbuffer		

2.2.3 Primers

The RT-PCR primers for detection Human *Rotavirus* A based capsid protein *NSP4* gene were designed according to (Teimoori *et al*., 2018) These provided by (Scientific Researcher. Co. Ltd, Iraq) as following table(2-3).

Table(2-3) PCR primers

Primer	**Sequence 5'-3'**		**Amplicon**
Human *Rotavirus* A *NSP4* gene	F	TTAAAAGTTCTGTTCCGAGAGAGCG	744bp
	R	GTCACAYTAAGACCRTTCCTTCCAT	

2.2.4 Chemicals

Table (2-4): The chemicals with their companies and countries of origin used in this study:

No.	**Chemical**	**Company and Origin**
1	10x TBE buffer	iNtRON (Korea)
2	Absolute ethanol	BDH (England)
3	Agarose	iNtRON (Korea)
4	Chloroform	BDH (England)
5	DEPC water	Bioneer/ Korea
6	DNA Marker ladder (100bp)	iNtRON (Korea)
7	Ethidium Bromide 10mg/ml	BioBasic (Canada)
8	Isopropanol	BDH (England)
9	Nuclease free water	BioLabs/ UK

2.3 Method

The main methods included in current study are shown in the figure (2-1).

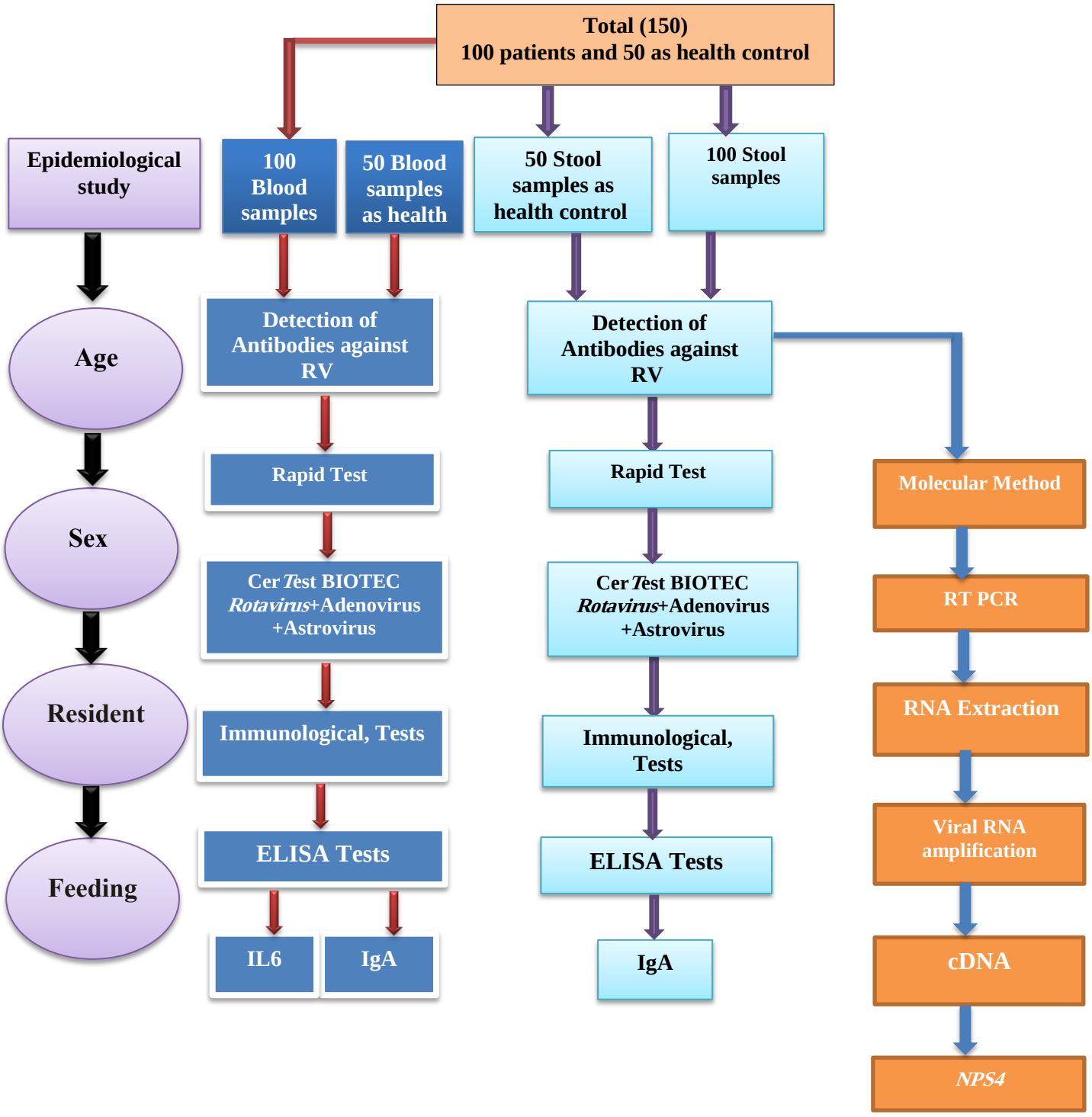

Figure (2-1):study design

2.3.1 Immunological Tests:

2.3.1.1 Rapid test (*Rotavirus*- Adenovirus-Astrovirus)(CerTest)

Rotavirus - Adeno - Astro antigen Certest is an in vitro qualitative immuno-chromatographic assay for the rapid detection of *Rotavirus*, adenovirus and astrovirus antigens in human stool specimens. The test results are intended to aid in the diagnosis of *Rotavirus*, adenovirus and astrovirus infections and to monitor the effectiveness of therapeutic treatment.

Principle of the assay:

Rotavirus-Adeno-Astro Certest is a sandwich solid phase immunochromatographic assay.

To perform the test, an aliquot of diluted stool sample is added to the sample well of test cassette. The sample flows through a pad containing antibodies against *Rotavirus* and adenovirus coupled to red-coloured colloidal gold. If the sample contains *Rotavirus* or adenovirus antigens, the antigens will bind to antibody coated on the colloidal gold particles to form antigen-antibody-gold complexes. Theses complexes move on nitrocellulose membrane by capillary action toward the test line region on which *Rotavirus* and adenovirus specific antibodies are immobilized separately. As the complexes reach the test line, they will bind to the antibody corresponding to the virus on the membrane to form a line, A red control line will always appear in the result window to indicate that the test has been correctly performed and the test device functions properly. If virus is not present or lower than the detection limit of the test, only the control line will be visible. If control line does not develop, the test is invalid.

Test procedure:

Stool samples and all reagents including test device were put at room temperature (20-30 °C) before use.

1- Bring all materials and specimens to room temperature (8-30 °C).

2- Remove the test card from the sealed foil pouch.

3-Hold the sample bottle upright with the tip pointed away from the test performer, snap off the tip.

4-Hold the bottle in vertical position over the sample well of the test card, deliver 3 drops (120-150 µl) of diluted stool sample to the sample well.

5-Read the result after 10 minutes. A strong positive sample may show result earlier.

2.3.1.2 Human IgA ELISA Kit

Human immunoglobuline (IgA) ELISA was used in this study for quantitative determination IgA in patient and healthy control serum samples and done according to company instruction (Elabscience Inc.-china) as following:

Table (2-5) A. ELISA components:

No	Reagent	quantity
1	Biotinylated Detection Ab Concentrate	1vial 120µL
2	Biotinylated Detection Ab Diluent	1vial 14mL
3	ELISA Micro Plate	8 wells ×12 strips
4	HRP Conjugate Concentrated	1vial 120µL
5	HRP Conjugate Diluent	1vial 14mL
6	Reagent for Substrate	1vial 10mL
7	Reference Standard & Sample Diluent	1vial 20mL
8	Standard of Reference	2 vials
9	Stop Solution	1vial 10mL
10	Wash Buffer Concentrate (25 x)	1vial 30mL

B. Assay procedure:

1-100µL of Standard, Blank, or Sample was added per micro ELISA plate well. After that solutions mixed gently and cover the plate with sealer, and then incubated for 90 minutes at 37℃.

2-The liquid of each well were removed, and immediately 100µL Biotinylated Detection Ab working solution was added to each well and covered with the plate sealer and then incubated for 1 hour at 37°C.

3- All plate wells were aspirated and washed, and repeated the process three times. The wash done by filling each well with Wash Buffer (approximately 350µL) using a squirt bottle.

4- 100µL of HRP Conjugate working solution was added to each well and covered with the plate sealer. And then incubated for 30 minutes at 37°C.

5- The wash process was repeated for five times as conducted in step 3.

6- 90µL of substrate Solution was added to each well and covered with a new Plate sealer, then incubated for about 15 minutes at 37°C.

7-50µLof Stop Solution was added to each well. Then, the colour turns to yellow immediately.

8- for determine the optical density (OD value) of each well at once, used a micro-plate reader set at 450 nm.

C. Calculation of results:

The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was plotted by the mean OD value for each standard on the X-axis against the concentration on the Y-axis and draw a best fit curve through the points on the graph.

2.3.1.3 Human Interleukin 6 ELISA Kit

Human Interleukin 6 (IL-6) ELISA was used in this study for quantitative determination of IL-6 from in patient and healthy control serum samples and done according to company instruction (BT-LAB) as following:

A. ELISA components:

No	Reagent	quantity
1	Biotinylated Human IL-6 Antibody	1ml x1
2	Plate Sealer	2 pics
3	Pre-coated ELISA Plate	12 * 8 well strips x1
4	Standard Diluent	3ml x1
5	Standard Solution (640ng/L)	0.5ml x1
6	Stop Solution	6ml x1
7	Streptavidin-HRP	6ml x1
8	Substrate Solution A	6ml x1
9	Substrate Solution B	6ml x1
10	User Instruction	1
11	Wash Buffer Concentrate (25x)	20ml x1
12	Zipper bag	1 pics

B. Assay procedure:

1- All reagents, standard solutions and samples were prepared according to kit instruction and bring at room temperature before use.

2- A 50µl standard serial dilution was added into to standard well. **Without** added antibody to standard well because the standard solution contains biotinylated antibody.

3- A 40µl sample was added to sample wells and then add 10µl anti-IL-6

antibody to sample wells, then 50µl streptavidin-HRP added in to sample wells and standard wells (Not blank control well).

4- The ELISA mixed well and covered the plate with a sealer. Then incubated at 60 minutes at 37°C.
5- The sealer was removed and wash the plate 5 times with wash buffer. wells socked
6- with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash.
7- A 50µl substrate solution A was added to each well and then 50µl substrate solution B added to each well.
8- The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.
9- A 50µl Stop Solution was added to each well, then the blue color will change into yellow immediately.
10- The optical density (OD value) was determined using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

C. Calculation of results:

The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was plotted by the mean OD value for each standard on the X-axis against the concentration on the Y-axis and draw a best fit curve through the points on the graph.

2.3.2 Molecular Tests

2.3.2.1 Viral RNA Extraction

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

1- A 250 µl stool suspension specimen was transferred by pipette into sterile and clean 1.5ml eppendorf tube, then 1ml Accuzol reagent an mixed by vortex.
2- Chloroform (200 µl) was added to each eppendorf tube and mixed 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, 4°C, for 15 minutes.
5- Supernatant was transferred to a new Eppendorf tube, and 500µl isopropanol was added.
6- The mixture Mixed by inverting the tube 4-5 times and incubated at 4°C for 10 minutes.
7- The mixture was centrifuged at 12,000 rpm, 4°C, 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, 4°C 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 (50µl) was added to RNA pellet tubes and mixed by vortex to dissolved the RNA pellet.
12- The extracted RNA sample was kept at -80 deep freezers.

2.3.2.2 Estimation of extracted RNA from stool samples

The extracted RNA were estimated by using Nanodrop spectrophotometer that used to measurement the RNA concentration and purity at absorbance 260/280 nm at ratio (1.8-2.0) 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.

2.3.2.3 cDNA synthesis

The RNA samples were used in cDNA synthesis step by using **M- MLV Reverse Transcriptase kit** and done according to company instructions as following tables:

Table (2- 6): M-MLV Reverse Transcriptase kit (**Step 1**).

RT master mix	Volume
Total RNA 100 ng/ul	8µl
Random Hexamer primer	1µl
DEPC water	1µl
Total	**10µl**

Then RNA and primer was denatured for 10 min at 65° C, after that immediately cool on ice.

Table (2-7): M-MLV Reverse Transcriptase kit (**Step 2**).

RT master mix	Volume
100mM DTT	2 µl
5X M-MLV RTase reaction buffer	4 µl
dNTP	2 µl
M-MLV RTase (200 µl)	1 µl
RNase inhibitor	1 µl
Step 1 RT master mix	10 µl
Total	**20 µl**

Then the tubes were placed in vortex and briefly spinning down. The RNA converted into cDNA in thermocycler under the following thermocycler conditions:

Table (2-8): Thermocycler conditions

Step	Temperature	Time
cDNA synthesis (RT step)	42 °C	1 hour
Heat inactivation	95 °C	5 minutes

2.3.2.4 Reverse Transcription PCR (RT-PCR)

RT-PCR was performed for direct detection Human *Rotavirus* based on specific amplification of *NSP4* gene. The RT-PCR was done according to (Teimoori *et al*., 2018).as following steps:

1- **RT-PCR master mix preparation:**

RT-PCR master mix was prepared by using **(RT-PCR Premix Kit**) and done according to the company instructions in two step the following **tables(2-9):**

RT-PCR master mix components:

PCR master mix	Volume
cDNA template 5-100ng	5µL
DEPC water	5 µL
Human *Rotavirus* *NSP4* forward primer (10pmol)	1µL
Human *Rotavirus* *NSP4* reverse primer (10pmol)	1µL
RT-PCR premix	8µL
Total	**20µL**

These RT-PCR reaction mixes were placed in sterile 0.2ml PCR tubes and mixed and centrifuged for 3000rpm for 3 minutes by Exispin centrifuge, after that transferred into PCR thermocycler.

2. PCR thermo cycler conditions:

PCR thermo cycler conditions was set by using Optimase protocol writer and according to primer annealing temperature as in the following **tables(2-10):**

Step	Condition	Cycle
Annealing	50 °C 30 sec.	35
Denaturation	95 °C 30 sec.	
Extension	72 °C 1min.	
Final extension	72 °C 5 min.	1
Hold	4 °C	Forever
Pre-Denaturation	95 °C 5 min.	1

2.3.2.5 RT PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis following steps:

1- A 1.5% 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 solidify for 15 minutes at room temperature, then the comb was removed gently from the tray.
4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer.

5- A 10µl of RT PCR product were loaded in to each comb well and 5µl of (100bp Ladder) in first well.
6- Then electric current was performed at 90 volt and 80 AM for 1.5hour.
7- RT-PCR products were visualized by using UV ttransilluminator.

2.3.2.6 DNA sequencing method

The RT-PCR products of positive *Rotavirus NSP4* protein gene were sent to Macrogen Company in Korea for performed the DNA sequencing by (AB DNA sequencing system). The DNA sequencing analysis was conducted by using phylogenetic tree UPGMA method (MEGA 6.0 version), Multiple alignment analysis based CrlustalW alignment analysis, and NCBI-BLAST for homology sequence identity.

2.3.2.7 Statistical analysis

Statistical analysis was carried out using SPSS version 16. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means ± SD). Pearson's chi square (X^2) and Fisher-exact tests were used to find the association between categorical variables. A *p*-value of ≤ 0.05 was considered as significant (Daniel and Cross, 2018).

Chapter Three

Results and Discussion

3. Results and Discussion

3.1 Population Study

3.1.1 The frequency distribution of children with *Rotavirus* and control group according to sex and comparison of mean age between study groups

Comparison of mean age and the frequency distribution of children with *Rotavirus* and control group according to sex is shown in table (3-1). There was no significant difference in the frequency distribution of individuals according to sex between control group and patients group (p = 0.289). But the proportion of males with *Rotavirus* diarrhea was more than that of females, 63(63.0 %) versus 37 (37.0 %) making the male to female ratio (1.7:1). There was also no significant difference in mean age between control group and patients group (p = 0.310).

Table (3-1): Comparison of mean age and the frequency distribution of children with *Rotavirus* and control group according to sex

Characteristic	**Control group *n* = 50**	***Rotavirus* *n* = 100**	***P***
Sex			
Male, *n* (%)	27 (54.0 %)	63 (63.0 %)	0.289 C NS
Female, *n* (%)	23 (46.0 %)	37 (37.0 %)	
Age (Months)			
Mean ±SD	9.32 ±5.15	8.33 ±5.83	0.310 I NS
Range	2- 36	1- 48	

n: number of cases; **C**: chi-square test; **I**: independent samples *t*-test; **NS**: not significant

In the present study, the proportion of male children with *Rotavirus* was 63 (63.0 %) and that of female patients was 37 (37.0 %), thus, it appears that *Rotavirus* is more common in males than in females and the male to female ratio was 1.7: 1.

In the study of Hussein and Hassan, (2000) the rate of males was 57.8 and that of females was 42.2 %. In the study of (Ojobor *et al*., 2020), there was no significant difference in the *Rotavirus* infection between male and female children but the preponderance of the infection was more in males. *Rotavirus* levels are higher but negligible infection. It has been claimed that males have higher levels of testosterone than females (Aminu *et al.*, 2008), Sex-related factors could affect the immune response to rotaviral infection. The *Rotavirus* entry into the cell stimulates the innate immune response by recognition of pathogen-associated molecular patterns by host Toll-like receptors and type I interferon (IFN) expression, which is mediated by macrophages and cytokines(Di Fiore *et al* .,2015). Estrogen promotes innate immune system pathways, including production of type I IFN, possibly inhibiting virus entry, which may be related to the ability of estradiol to increase the expression of IFN induced antiviral genes (Tasker *et al.*, 2014). The genetic factors play a role in regulating the immune modulation of immune-related genes, Toll-like receptors such as TLR7 and TLR8 are important for *Rotavirus* recognition and are encoded on the X chromosome, leading to gene dosage effects that may be relevant for immune response (Xu, *et al.*, 2009). In very young children, hormonal and genetic component differences may contribute to sexual dimorphism. Mini puberty has been suggested as a critical window of programming with lifelong implication. In infant boys, the postnatal rise in luteinizing hormone and the follicle-stimulating hormone is associated with an increase in testosterone. In girls, the endogenous production of estrogen increases after birth, and estradiol levels fluctuate, decreasing gradually toward the second year of life. Thus, the excess of rotaviral infections in childhood in males may be related to an interaction between hormonal and chromosomal differences (Renau, *et al.*, 2020). Higher but insignificant *Rotavirus* infection in

males than females has been reported (Aminu *et al*., 2008), while others have reported significantly higher infections in males than females (Ndze *et al*., 2012; Tagbo *et al*., 2019). The reasons for the difference in detection rate between males and females are however, not known (Ojobor *et al*., 2020). In the present study, we chose the control group to have comparable age range, therefore, there was no significant difference in mean age, in addition, comparable proportions of boy s and girls were also considered. Indeed, statistical matching with respect to age and sex is mandatory to avoid bias in the results in such cases control study. The mean age of children with *Rotavirus* diarrhea was 8.33 ±5.83 months. According to (Hussein and Hassan, 2000), the most common age affected was between 6 to 12 months and this finding supports our finding. In addition, similar results was obtained by (Deus *et al*., 2018) who found that the highest prevalence of *Rotavirus* infection was observed in children between 6 and 11 months old. This age is more affected because of reduced effect of immunoglobulins taken from mothers because of lack of breast feeding and depending on artificial feeding (Chen *et al*., 2017).

3.1.2 The frequency distribution of children with *Rotavirus* and control group according to type of feeding

The frequency distribution of children with *Rotavirus* diarrhea and control group according to type of feeding is shown in table (3-2). The most important observation was that breast feeding was more frequently encountered in control group and less seen in patients group, 24 % versus 4% respectively, in addition mixed feeding and bottle feeding in combination was more frequently in children with *Rotavirus* diarrhea in comparison with control group (40 % + 56 % = 96 % versus 56 % + 20 % = 76 % respectively) making the difference in exclusive breast feeding between study group and control group significant ($p < 0.001$).

Table (3-2): The frequency distribution of children with *Rotavirus* and control group according to type of feeding

Characteristic	**Control group** ***n* = 50**	***Rotavirus*** ***n* = 100**	***P***
Type of feeding			
Breast feeding, *n* (%)	12 (24.0 %)	4 (4.0 %)	< 0.001 C **
Bottle feeding, *n* (%)	28 (56.0 %)	40 (40.0 %)	
Mixed feeding, *n* (%)	10 (20.0 %)	56 (56.0 %)	

n: number of cases; **C**: chi-square test; ******: significant at $p \leq 0.01$

In the Iraqi study by Habash and Habeeb, (2018), bottle feeding with or without complementary diet had been reported in higher frequency of studied patients (59.2%) with positive *Rotavirus* stool test and this rate is close to the result in current study. In one previous study, artificial feeding was reported in the majority of *Rotavirus* diarrhea (86.8%) (Wobudeya *et al*., 2011) and this finding supports our finding that the majority of children with diarrhea have bottle feeding.

The benefits of breastfeeding on infant and child morbidity and mortality are well documented, with observational studies dating back to the 1960s. breastfeeding demonstrates a dose–response relationship of protection against diarrheal disease morbidity and mortality in infancy, exclusive breastfeeding, defined as feeding only human milk with no other liquids or foods, is known to offer maximum protection against diarrhea to infants younger than 6 months of age, whereas partial breastfeeding offers intermediate protection compared with no breastfeeding (Turin and Ochoa, 2014).

It's worth noting that most infants are better protected from enterovirus infection thanks to antibodies acquired from their mothers, as breastfeeding reduces the risk of infection with *Rotavirus*, astrovirus, and

adenovirus, as well as the inability of infants to infect themselves with the virus through contaminated hands, which explains why children under the age of one year are less likely to be infected. Because the body or intestine can get rid of a high rate of the virus through feces, defecation is 5-7 times per day for children who are dependent on breastfeeding. The infection will be mild or without symptoms, but the possibility of infection in older age groups is more due to passive, diminished immunity (Saravanan *et al*., 2004).

3.1.3 The frequency distribution of children with *Rotavirus* and control group according to residence

The frequency distribution of children with *Rotavirus* and control group according to residence is shown in table (3-3). There was no significant association between *Rotavirus* and residence (rural versus urban) ($p = 0.561$).

Table (3-3): The frequency distribution of children with *Rotavirus* and control group according to residence

Characteristic	**Control group *n* = 50**	***Rotavirus* *n* = 100**	***P***
Residence			
Urban, *n* (%)	24 (48.0 %)	43 (43.0 %)	0.561 C NS
Rural, *n* (%)	26 (52.0 %)	57 (57.0 %)	

n: number of cases; **C**: chi-square test; **NS**: not significant

In the current study, the finding did not report a significant association between *Rotavirus* and residence. On contrary to our observation, (Basharat *et al*., 2021) reported significantly higher rate of *Rotavirus* diarrhea in children from urban residence in comparison with children from rural residence and they attributed such significant variation to population density and increased transmission may be to blame for the disparity in prevalence rates between the two regions. Similarly, (Deus *et*

al.,2018) reported higher rate of viral diarrhea in urban residency.

On the other hand, results obtained by (Page *et al*., 2014) in which rural areas were affected more than urban areas. In one of the previous studies, rural areas were more than urban, which contradicts with the results of our study (Hussein and Hassan, 2000).Variation in the spread of *Rotavirus* infection may be due to the social behaviors of the population, such as personal hygiene or environmental changes that may be associated with the growth of *Rotavirus* pathogens, especially contaminated water or less vaccination.

Therefore, it appears that the prevalence of *Rotavirus* diarrhea with respect to rural and or urban residence is determined factors other than the residence itself such as social and cultural habits and availability of and accessibility to vaccine and health facilities.

3.1.4 The results of rapid test and RT-PCR in patients with *Rotavirus* and in control group

The results of rapid test and RT-PCR in patients with *Rotavirus* and in control group are shown in table (3-4). Out of all patients detected by rapid test, 80 % showed positive results following RT-PCR and this suggested reduced specificity of rapid antigen test in comparison with RT-PCR, and this suggestion was further confirmed by table (3-5).

Table (3-4): The results of rapid test and RT-PCR in patients with *Rotavirus* and in control group:

Characteristic	**Control group *n* = 50**	***Rotavirus* *n* = 100**	***P***
Rapid Test *Rotavirus*			
Positive, *n* (%)	0 (0.0 %)	100 (100.0 %)	< 0.001 C **
Negative, *n* (%)	50 (100.0 %)	0 (0.0 %)	
RT-PCR-*Rotavirus*			
Positive, *n* (%)	0 (0.0 %)	80 (80.0 %)	0.001 C **
Negative, *n* (%)	50 (100.0 %)	20 (20.0 %)	

n: number of cases; **C**: chi-square test; ******: significant at $p \leq 0.01$

Table (3-5): Sensitivity test

		RT-PCR (gold standard test)	
		Positive *n* = 80	Negative *n* = 70
Rapid antigen test	Positive	80	20
	Negative	0	50
Sensitivity %	100.0		
Specificity %	71.4		
PPV %	80.0		
NPV %	100.0		
Accuracy %	86.7		

n: number of cases; **PPV**: positive predictive value; **NPV**: negative predictive value

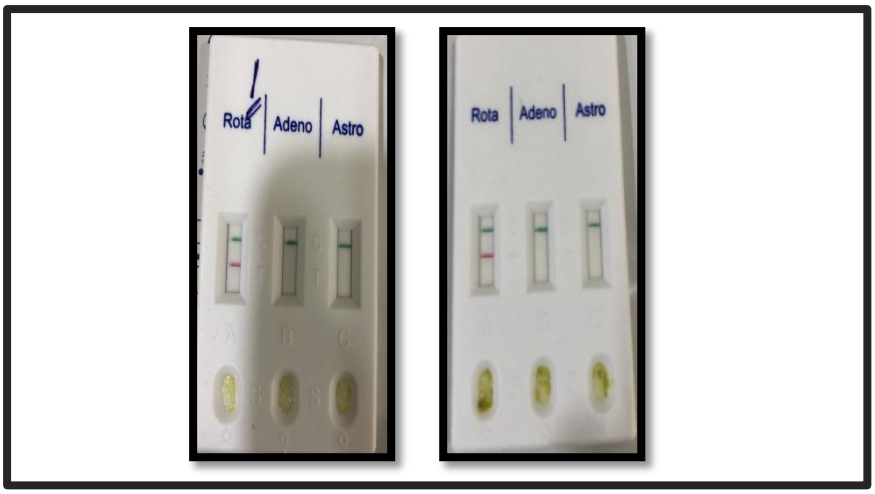

Figure (3-1): The result of Human *Rotavirus* Detected by Imunochromotography test (ICT) for stool**.**

Figure(3-2): Agarose gel electrophoresis image that showed the RT-PCR product analysis of *NSP4* gene in Human *Rotavirus* A from stool children diarrhea samples. Where M: marker (1500-100bp) and Lane (1-20) showed some positive samples for *NSP4* gene in Human *Rotavirus* A at (744bp) RT-PCR product.

In the current study, there was discrepancy in the results of rapid test and RT-PCR in such a way that 20 % of cases which were diagnosed as positive by rapid test were proved to be negative using RT-PCR. *Rotavirus* is a common cause of gastroenteritis in children younger than 3 years worldwide. Rapid Antigen Testing (RAT) is a quick and easy tool to detect virus antigen in stool samples and is more specific than sensitive (higher specificity and lesser sensitivity). Reverse transcription-polymerase chain reaction (RT-PCR) and PCR are more sensitive and specific than RAT. Sensitive and specific tools are required for true diagnosis (Barsoum, 2020).

In the current study rapid antigen test has a sensitivity level of 100 %, but the specificity level was 71.4 %. Thus, it appears that the rapid test is specific but less sensitive when compared to results of RT-PCR. In one previous study, Of 71 samples that were positive for RV by RT-PCR, 69, 68 and 63 were also recognized by RAT kits, indicating 97.2, 95.8 and 88.7% sensitivity for RAT kits, with only one false positive result in one of the three RAT kits (specificity up to 100%) (Khamrin *et al*., 2011).

When comparing the sensitivity of rapid antigen test for group A *Rotavirus* detection, it is clearly demonstrated that among various immunochromatography kits, sensitivity and specificity for group A *Rotavirus* infection were a bit different. In addition, it was observed that several *Rotavirus* genotypes G1, G3 and G9 were reacted with these kits. Therefore, genotype variations of *Rotavirus* may not be a problem for false negative results (Khamrin *et al*., 2011). RT-PCR assay was found to be specific to *Rotavirus* and broadly reactive to *Rotavirus* genogroups1-4, 9, 10 and 12 (Jothikumar *et al*., 2009). Specificity testing did not identify any cross-reactivity of the assay with a panel of 36 non- *Rotavirus* enteric virus specimens (Jothikumar *et al*., 2009). Highly sensitive and specific methods such as one-step RT-PCR are still required for true diagnosis of viral gastroenteritis following clinical suspicion of gastroenteritis and gastroenteritis associated complications and for *Rotavirus* vaccine efficacy trials (Barsoum, 2020).

3.1.5 Comparison of serum levels of IgA and IL-6 and stool level of IgA between patient with *Rotavirus* and control group

Comparison of serum levels of IgA and IL-6 and stool level of IgA between patient with *Rotavirus* and control group is shown in table (3-5). Serum IgA, stool IgA and serum IL-6 were all significantly higher in patients group in comparison with control group (p <0.01).

Table (3-6): Comparison of serum levels of IgA and IL-6 and stool level of IgA between patient with *Rotavirus* and control group

Characteristic	Control group *n* = 50	*Rotavirus* *n* = 100	*P*
IgA serum			
Median (IQR)	90.31 (1.36)	96.18 (2.70)	< 0.001 M **
Range	72.61 -95.24	79.62 -99.56	
IgA stool			
Median (IQR)	78.88 (23.29)	96.36 (30.52)	0.001 M **
Range	24.65 -106.76	6.83 -97.85	
IL-6 serum			
Median (IQR)	75.31 (51.78)	188.43 (221.46)	< 0.001 M **
Range	15.65 -144.66	35.78 -474.46	

n: number of cases; **M**: Mann Whitney U test; ******: significant at $p \leq 0.01$

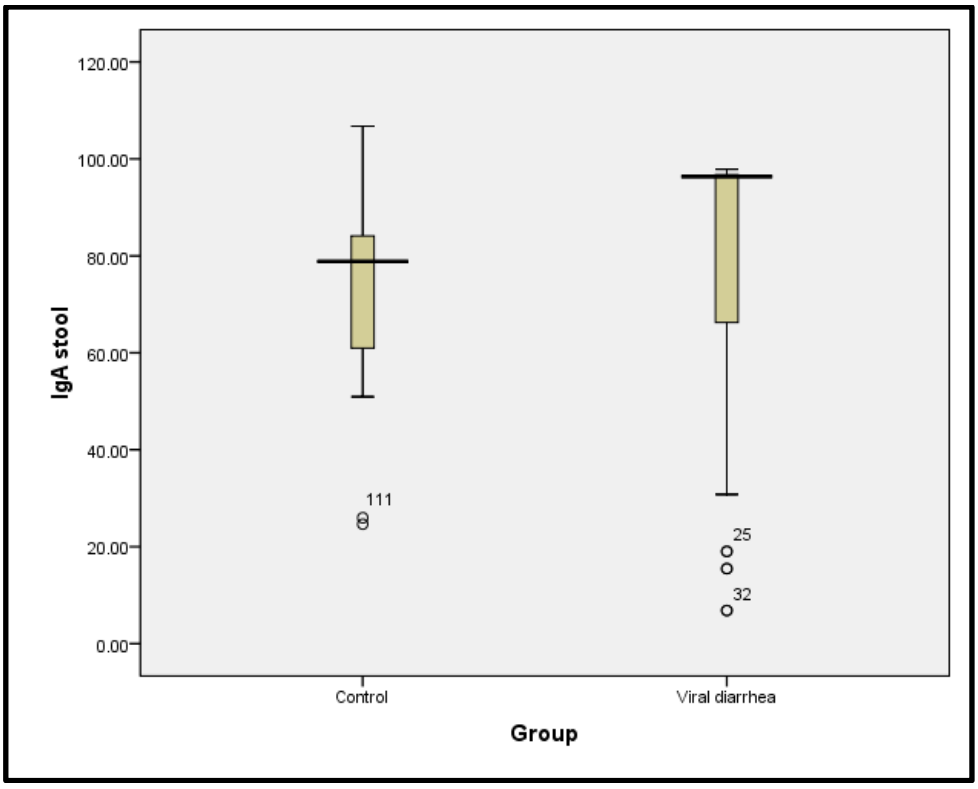

Figure (3-3): Box plot showing comparison of stool IgA level between patients with *Rotavirus* and control group

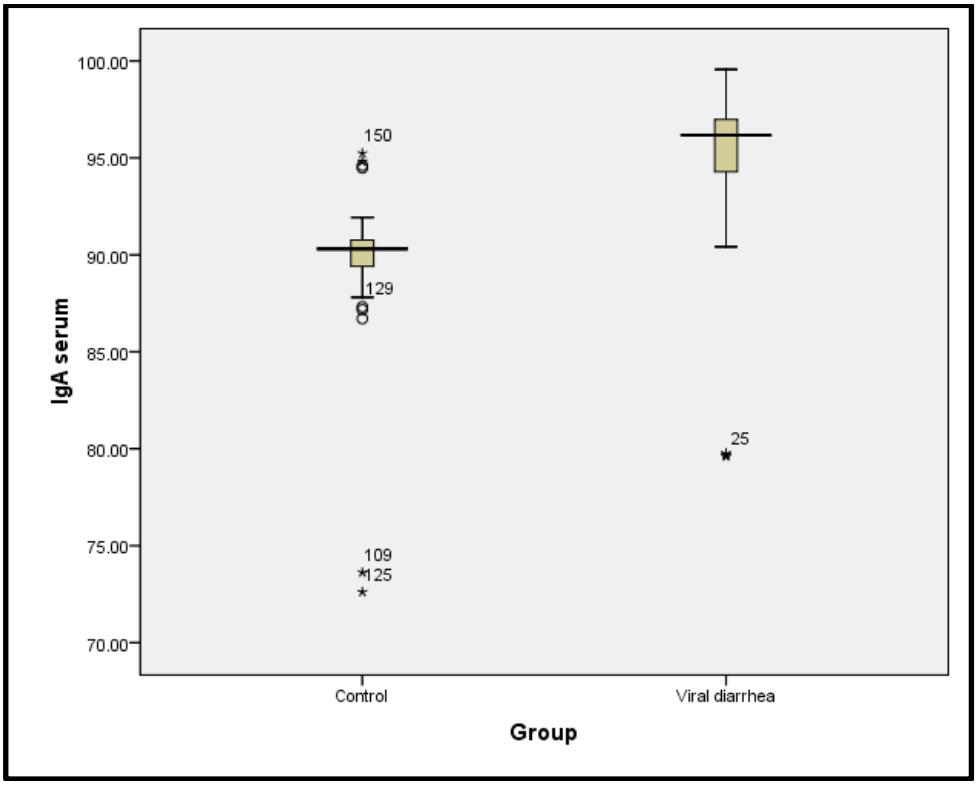

Figure (3-4): Box plot showing comparison of serum IgA level between patients with *Rotavirus* and control group

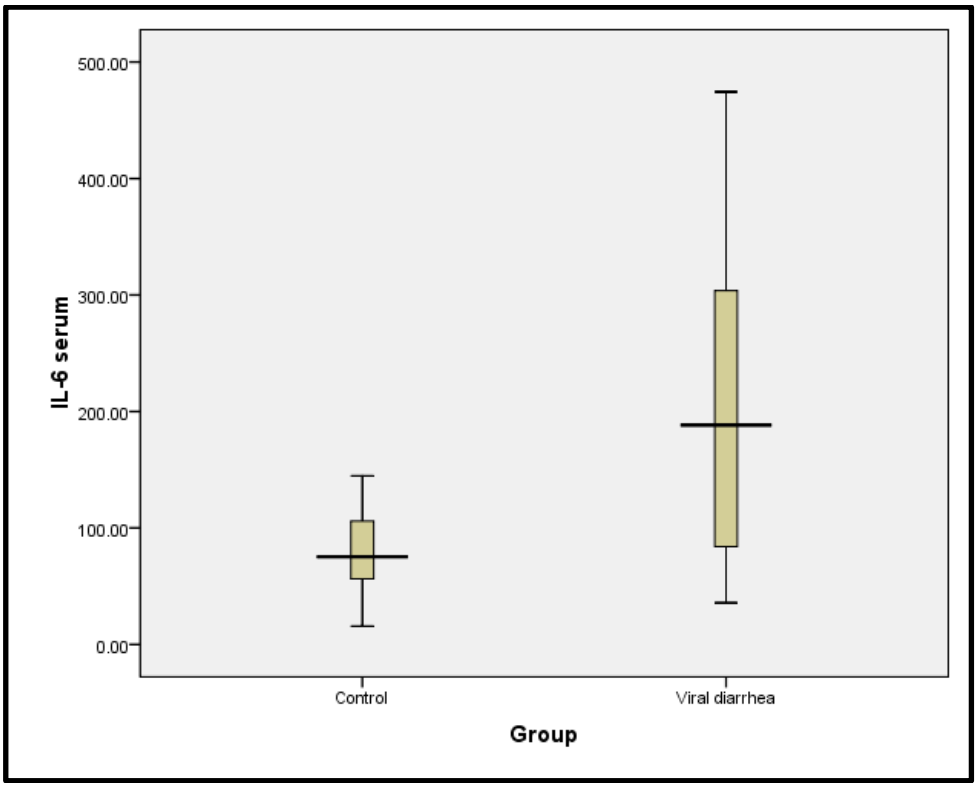

Figure (3-5): Box plot showing comparison of serum IL-6 level between patients with *Rotavirus* and control group.

3.1.6 Correlation study

Correlations among immune markers are shown in table (3-7). Serum IgA was significantly and positively correlated to stool IgA. Serum IL-6 was not correlated to serum IgA or stool IgA level.

Table (3-7): Correlations among immune markers

	IgA stool		**IL-6 serum**	
	R	***P***	***R***	***P***
IgA serum	0.261	0.009 **	0.027	0.791
IgA stool			-0.012	0.909

**: significant at $p \leq 0.01$

In the current study, serum and stool IgA and serum IL-6 were higher in *Rotavirus* diarrhea group in comparison with control group. Significant positive correlation was also found between serum and stool IgA indicating that changes in serum IgA are accompanied by parallel changes in stool IgA. However, no significant correlations were found between serum or stool IgA and serum IL-6.

Previous reports have highlighted the role of measuring serum IgA concentration as a marker of post-vaccine protection against severe *Rotavirus* gastroenteritis (Velázquez *et al*., 2000; Cheuvart *et al*., 2014) and in one of previous systemic reviews it has been concluded that "a significant correlation between IgA titers and *Rotavirus* vaccine efficacy and hypothesize that a critical level of IgA antibody titer is associated with a sufficient level of sustained protection after *Rotavirus* vaccination" (Patel *et al*., 2013).

On the other hand we found that higher levels of serum IgA are associated with infection when compared to healthy control subjects. One of previous reports have shown that previous infection with *Rotavirus*

offer higher and protective levels of serum IgA in comparison with immunized children indicating that natural infection provides better immunity than vaccination (González *et al*., 2005) and this finding may explain partly the higher level of serum IgA in infected children in current study in comparison with non-infected children.

In addition, measuring fecal IgA levels in previous reports have been linked to seroconversion post-vaccination however, weak correlation has been found between stool IgA level and seroconversion (Paul *et al*., 2016 ; Bishop *et al.*, 1996), observed that among young infants, levels of fecal antibodies fluctuated widely during the first few weeks of life while breast-feeding was being established, possibly due to the amount of breast milk ingested and intervals between evacuation of feces. Another study speculated that the fluctuations in anti-*Rotavirus* fecal antibody levels reflect the fluctuating production in the small intestine, as a response to a recurrent asymptomatic infection or to a persisting infection (Matson *et al*., 1993).

Previous reports have shown valuable diagnostic performance of serum IL-6 between *Rotavirus* and norovirus infection in children (Chen *et al*., 2012).

In current study we observe that serum IL-6 in children with *Rotavirus* infection was higher than that reported in control children. Therefore, measuring serum IL-6 in children with *Rotavirus* diarrhea may provide an efficient tool in discriminating virus diarrhea from other causes of diarrhea.

3.1.7 Clinical features

The frequency distribution of children with *Rotavirus* infection according to clinical features was as following: 55 % of patients were complaining of fever. Vomiting was reported in 33 % of patients. Diarrhea was reported in all patients. Dehydration was reported in 30 % of patients and it ranged from mild to severe degree.

According to (Yu *et al*., 2015), besides diarrhea, vomiting was the most common clinical features with 32.8% of cases. Other common symptoms include fever (22.4%), and dehydration (3.6%). Therefore, the clinical features found in our study are comparable to this study. In general, in children, the clinical features of *Rotavirus* disease ranges from no symptoms to mild, watery diarrhoea of short duration and to severe diarrhoea with vomiting and fever that can result in rapid dehydration with shock, electrolyte imbalance and death (Crawford *et al*., 2017). In addition to diarrhoea, *Rotavirus* infection can induce vomiting, malaise and fever. Indeed, vomiting is a hallmark of *Rotavirus* disease (Hagbom *et al*., 2011), contributes to dehydration and can hamper the effectiveness of therapeutic interventions, such as oral rehydration therapy (Leung and Robson, 2007). The cause of vomiting and diarrhea in case of Rota virus is the stimulation of secretion of serotonin (Crawford *et al*., 2017).

Rotavirus infection induces fever. Although the exact mechanism of how *Rotavirus* infection induces fever, significantly increased levels of IL-6 and TNF have been reported in the serum of children with fever following *Rotavirus* gastroenteritis compared with levels in children who did not have gastroenteritis (Crawford *et al*., 2017).

3.1.8 DNA Sequence

The DNA sequencing method was carried out to identification genetic variation (substation Mutations) analysis in non-structural protein (*NSP4*) gene of local Human *Rotavirus* A isolates (IQH.1 - IQH.5) and NCBI-Blast related Human *Rotavirus* A isolates.

The phylogenetic tree genetic relationship analysis was showed that local Human *Rotavirus* A isolates (IQH.1 - IQH.4) were showed closed related to NCBI-BLAST Human *Rotavirus* A strain RV1326 , the local Human *Rotavirus* A isolates (IQH.5) were showed closed related to NCBI-BLAST Human *Rotavirus* A strain G1P at total genetic changes (0.0080-0.0020%), as showed in figure (3-7).

The homology sequence identity between local Human *Rotavirus* A isolates (IQH.1 - IQH.4) and NCBI BLAST related Human *Rotavirus* A strain RV1326 were showed genetic homology sequence identity ranged from (99.37-99.84%). local Human *Rotavirus* A isolates (IQH.5) and NCBI BLAST related Human *Rotavirus* A strain G1P were showed genetic homology sequence identity ranged from (99.52%).

The genetic variation (substitution Mutations) analysis in non-structural protein (*NSP4*) gene between local Human *Rotavirus* A isolates and NCBI-Blast related Human *Rotavirus* A isolate were find (1-4) substitution mutations at total genetic variation percentage ranged (0.16-0.63%), As showed in table (3-8).

Finally, the local Human *Rotavirus* A isolates (IQH.1 - IQH.5) were submitted into NCBI Genbank and identified by accession numbers .

BankIt2566061 Seq1 ON087846
BankIt2566061 Seq2 ON087847
BankIt2566061 Seq3 ON087848
BankIt2566061 Seq4 ON087849
BankIt2566061 Seq5 ON087850

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KX638732.1      ACCCTGGAATTGCGTATTTTCCATATATTGCATCTGTTCTAACGGTTTTATTCACATTAC
IQH.3           ACCCTGGAATGGCGTATTTTCCATATATTGCATCTGTTCTAACGGTTTTATTCACATTAC
IQH.2           ACCCTGGAATGGCGTATTTTCCATATATTGCATCTGTTCTAACGGTTTTATTCACATTAC
IQH.4           ACCCTGGAATGGCGTATTTTCCATATATTGCATCTGTTCTAACGGTTTTATTCACATTAC
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GQ465021.1      ACCCTGGAATGGCGTATTTTCCATATATTGCATCTGTTCTAACAGTTCTGTTCACATTAC
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IQH.5           ATAAGGCTTCAATTCCAACAATGAAAATAGCATTAAAAACGTCAAAGTGTTCATATAAAG
KX638732.1      ATAAAGCTTCAATTCCAACAATGAAAATAGCATTAAAAACGTCAAAATGTTCATATAAAG
IQH.3           ATAAAGCTTCAATTCCAACAATGAAAATAGCATTAAAAACGTCAAAATGTTCATATAAAG
IQH.2           ATAAAGCTTCAATTCCAACAATGAAAATAGCATTAAAAACGTCAAAATGTTCATATAAAG
IQH.4           ATAAAGCTTCAATTCCAACAATGAAAATAGCATTAAAAACGTCAAAATGTTCATATAAAG
IQH.1           ATAAAGCTTCAATTCCAACAATGAAAATAGCATTAAAAACGTCAAAATGTTCATATAAAG
KU048729.1      ATAAAGCTTCAATTCCAACAATGAAAATAGCATTAAAAACGTCAAAATGTTCATACAAAG
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IQH.3           TGATCAAGTATTGTATAGTCACAATTATTAATACTCTTTTAAAATTGGCAGGGTATAAAG
IQH.2           TGATCAAGTATTGTATAGTCACAATTATTAATACTCTTTTAAAATTGGCAGGGTATAAAG
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IQH.2           AATTTAATCAGAAGAATATAAAAACGCTAGATGAATGGGAGAACGGAAAAAATCCATATG
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Figure (3-6): Multiple sequence alignment analysis of non-structural protein (*NSP4*) gene in local Human *Rotavirus* A isolates and NCBI-Genbank Human *Rotavirus* A related isolates. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in non-structural protein (*NSP4*) gene between isolates.

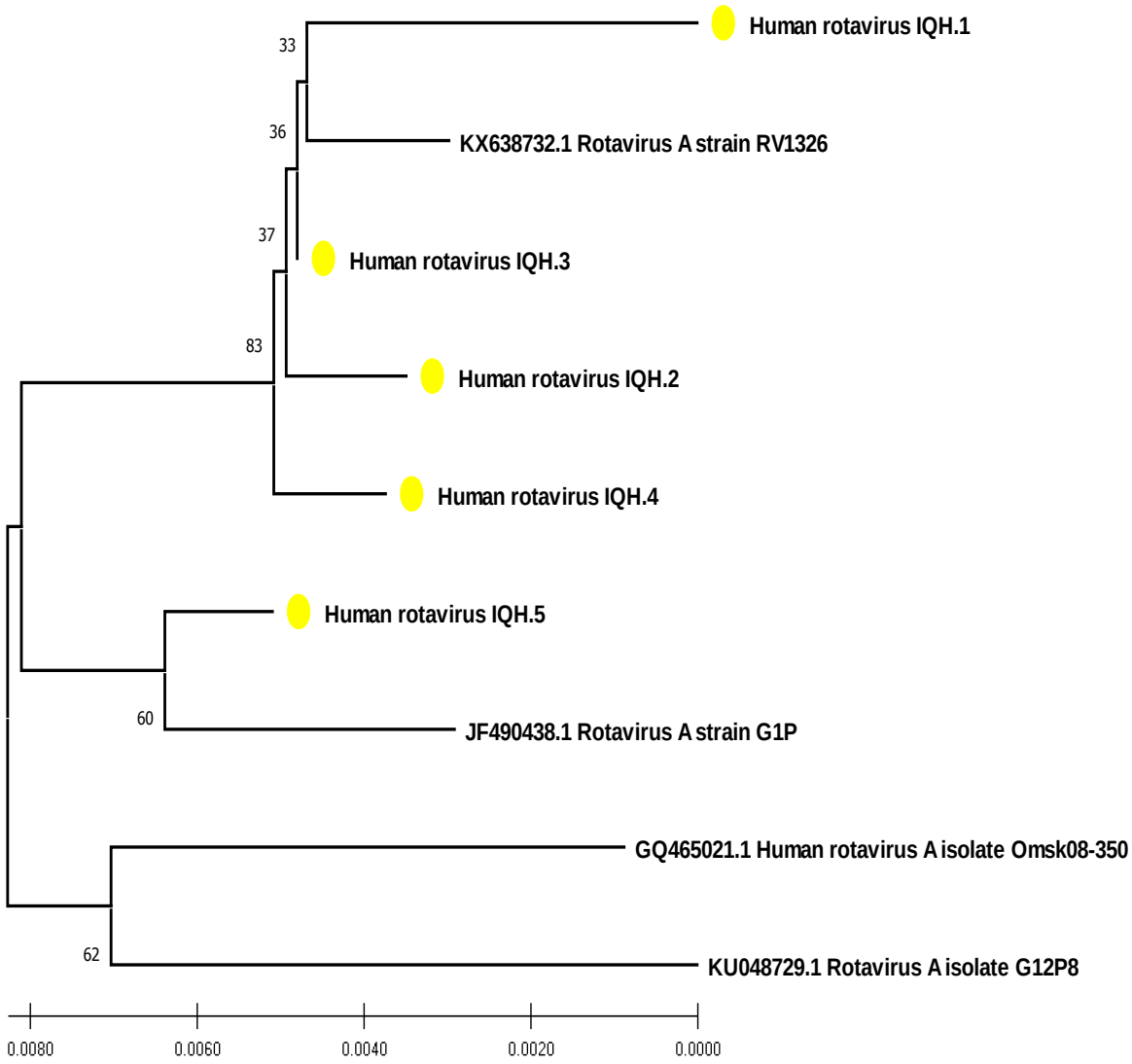

Figure (3-7): Phylogenetic tree analysis based non-structural protein (*NSP4*) gene partial sequence in local Human *Rotavirus* A isolates that used for genetic relationship analysis. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local Human *Rotavirus* A isolates (IQH.1 - IQH.4) were showed closed related to NCBI-BLAST Human *Rotavirus* A strain RV1326 , the local Human *Rotavirus* A isolates (IQH.5) were showed closed related to NCBI-BLAST Human *Rotavirus* A strain G1P at total genetic changes (0.0080-0.0020%).

Table (3-8): The NCBI-BLAST genetic variation analysis between local Human *Rotavirus* A isolates and NCBI-BLAST closed related Human *Rotavirus* A isolate:

***Rotavirus* A isolates**	**Accession number**	**Homology sequence identity (%)**			
		Number Mutations	**Type of Mutation**	**Mutation %**	**Identity (%)**
IQN.No.1	ON087846	4	T/G, G/A, A/G, T/C	0.63%	99.37%
IQN.No.2	ON087847	4	T/G, T/C	0.32%	99.68%
IQN.No.3	ON087848	1	T/G	0.16%	99.84%
IQN.No.4	ON087849	2	T/G, T/C	0.32%	99.68%
IQN.No.5	ON087850	3	A/G, A/G, T/C	0.48%	99.52%

The current study was aimed to identification genetic variation (substation Mutations) analysis in non-structural protein (*NSP4*) gene in local Human *Rotavirus* A isolates (IQH1-IQH.5) based DNA sequencing method. The phylogenetic tree genetic relationship analysis results were showed closed related to NCBI-BLAST Human *Rotavirus* A strain RV1326 at total genetic changes (0.0080-0.0020%). The homology sequence identity between local Human *Rotavirus* A isolates (IQH1 - IQH.5) and NCBI BLAST related Human *Rotavirus* A strain RV1326 were showed genetic homology sequence identity ranged from (99.37-99.84%). The DNA sequence analysis study of non-structural protein (*NSP4*) gene *Rotavirus* A was mention in previously study by many researchers, because this gene is most important and multifunctional protein for *Rotavirus* A infection (Ball *et al*,. 2005). This gene

responsible to encoding a protein is important for *Rotavirus* morphogenesis, pathogenesis and efficiently stimulates immune response and can be targeted for the development of an effective vaccine (Hyser *et al* ., 2010 ; Trask , McDonald and Patton 2012). The *NSP4* is a multifunctional three-pass transmembrane glycoprotein with 175 amino acids. *NSP4* is essential for virus morphogenesis and pathogenesis (Kavanagh *et al*., 2010). Non-structural protein 4 (*NSP4*) is a critical protein for *Rotavirus* replication and assembly. This protein has multiple domains and motifs that predispose its function and activity. *NSP4* has a sequence divergence in human and animal RVs. Recently, 14 genotypes (E1-E14) of *NSP4* have been identified, and E1 and E2 have been shown to be the most common genotypes in human (Teimoori *et al*., 2018). The current study was found (1-4) substitution mutations at total genetic variation ranged (0.16-0.63%) (substitution Mutations) in non-structural protein (*NSP4*) gene between local Human *Rotavirus* A isolates and NCBI-Blast related Human *Rotavirus* A submitted isolates. These genetic diversity may be used to genetic differentiation many genotypes of Human *Rotavirus* A isolates. This suggestion was consistent with a study by Khalkhali in Iran who revealed that genetic diversity and variation analysis of viroporin, which is encoded by the *Rotavirus NSP4* segment (Khalkhali *et al* ., 2021).

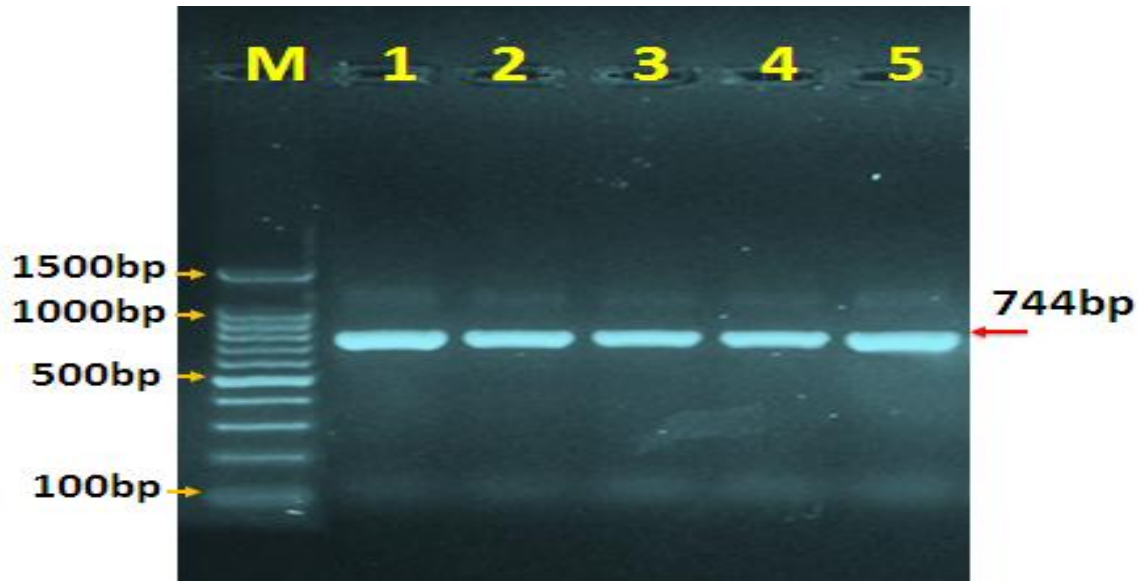

Figure (3-8): Agarose gel electrophoresis image that showed the RT-PCR product analysis of *NSP4* gene in Human *Rotavirus* A from stool children diarrhea samples. Where M: marker (1500-100bp) and Lane (1-5) showed some positive samples for *NSP4* gene in Human *Rotavirus* A at (744bp) RT-PCR product**.**

Conclusions

And

Recommendations

Conclusions:

1- *Rotavirus* diarrhea is more common in the male than in female .

2- The breastfeeding was more frequently encountered in the control group and less seen in the patient's group and the difference in breastfeeding between study group was significant ($p < 0.001$).

3- Serum IgA, stool IgA, and serum IL-6 were all significantly higher in the patient's group in comparison with the control group ($p <0.01$).

4- Out of all patients detected by rapid test, 80 % showed positive results following RT-PCR and this suggested reduced specificity of rapid antigen test in comparison with RT-PCR.

5- Significant positive correlation was also found between serum and stool IgA indicating that changes in serum IgA are accompanied by parallel changes in stool IgA. However, no significant correlations were found between serum or stool IgA and serum IL-6.

6- The DNA sequencing method was carried out to identification genetic variation (substation Mutations) analysis in non-structural protein (*NSP4*) gene of local Human *Rotavirus* A isolates (IQH1 - IQH.5) and NCBI-Blast related Human *Rotavirus* A isolates.

Recommendations:

1- *Rotavirus* vaccination is recommended for all infants because it significantly decreases the incidence and morbidity associated with *Rotavirus* infection.

2- Determine the prevalence and titer of serum antibodies against several *Rotavirus VP8* proteins from different P genotypes in children.

3- Study the incidence and genetic diversity of *Rotavirus* (RV) infection among children under 5 years of age in a community in Babylon province.

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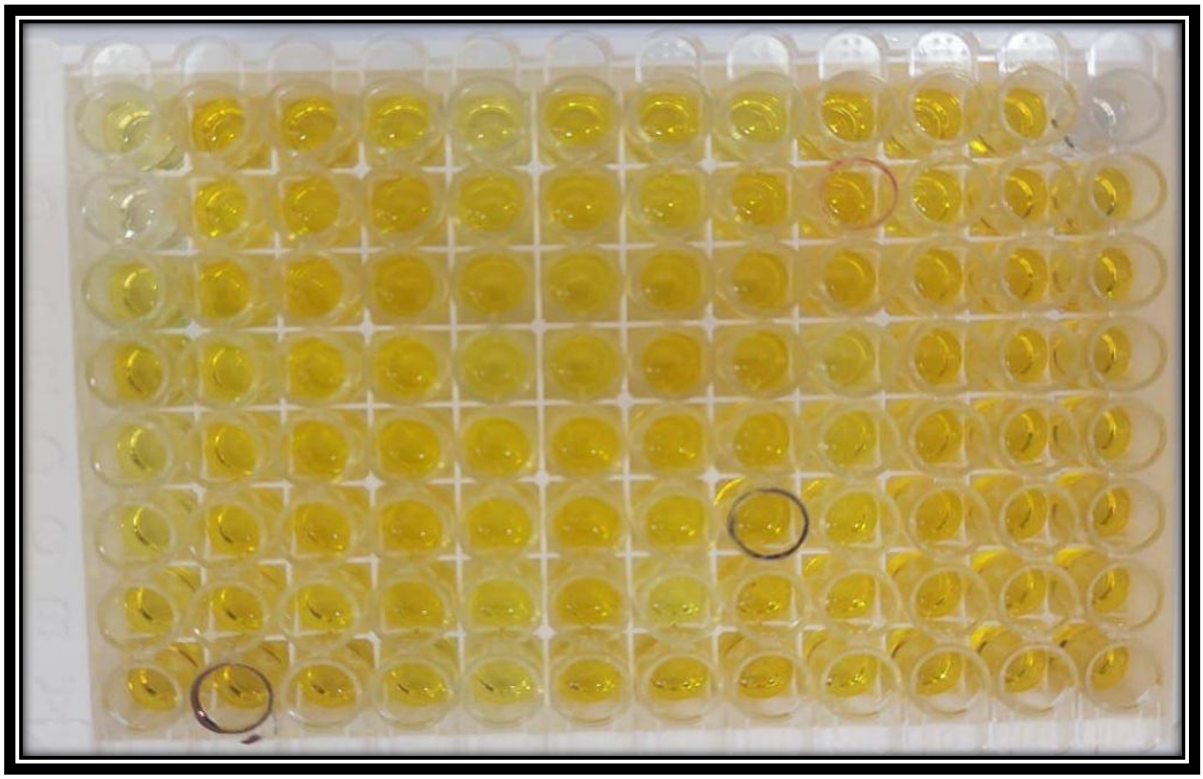
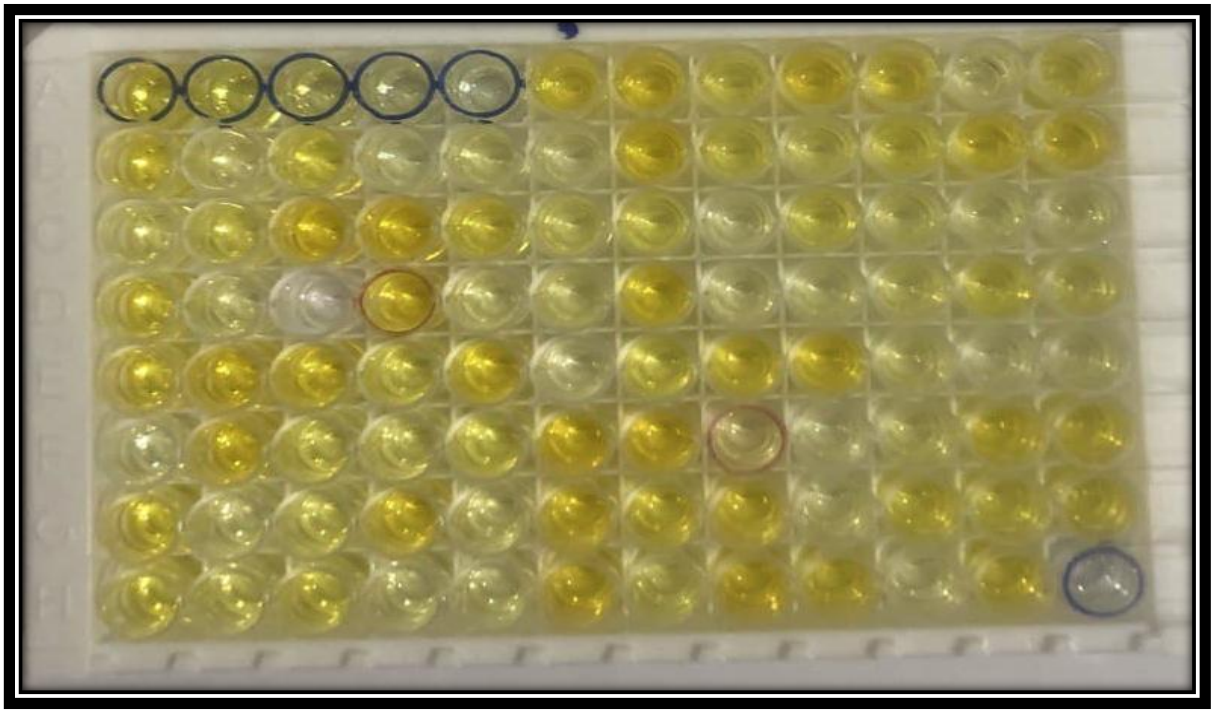
Appendices

Appendices……..……...



Appendix- 1: Detection of IgA in serum samples by ELISA.



Appendix- 2: Detection of IL6 in serum samples by ELISA.

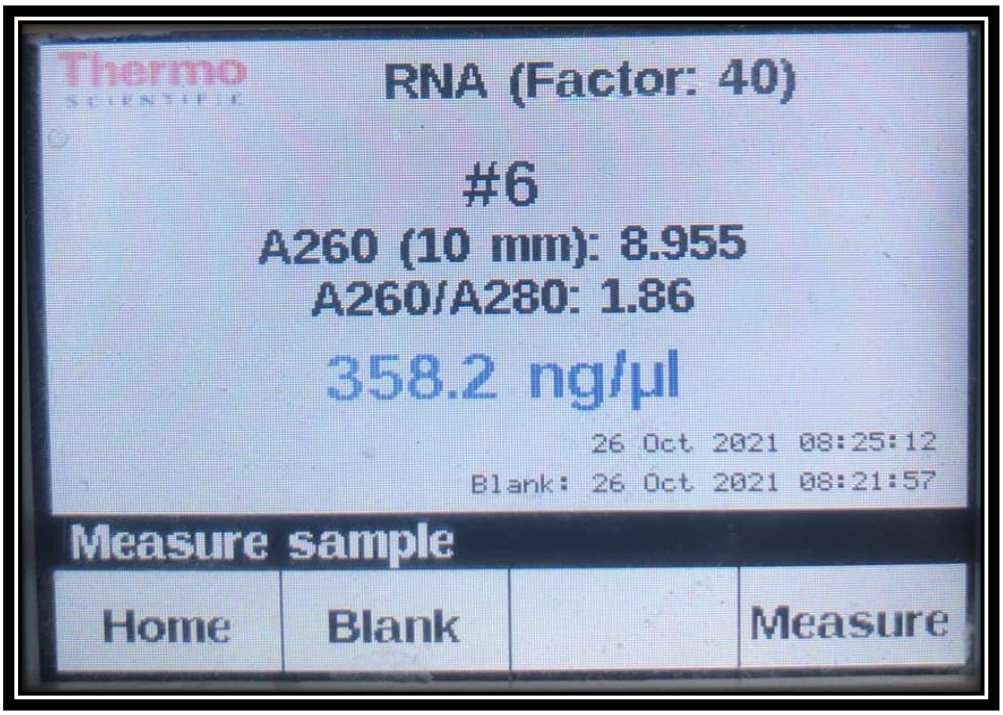
	01	02	03	04	05	06
A	SMP01 2.920	SMP02 2.920	SMP03 2.920	SMP04 2.778	SMP05 1.193	SMP06 2.920
B	SMP13 2.035	SMP14 2.930	SMP15 2.519	SMP16 2.930	SMP17 1.684	SMP18 2.930
C	SMP25 1.226	SMP26 2.915	SMP27 2.915	SMP28 2.915	SMP29 2.647	SMP30 2.915
D	SMP37 1.066	SMP38 2.581	SMP39 2.925	SMP40 2.925	SMP41 2.700	SMP42 2.925
E	SMP49 2.209	SMP50 1.865	SMP51 2.896	SMP52 2.907	SMP53 1.454	SMP54 2.020
F	SMP61 1.141	SMP62 2.916	SMP63 2.916	SMP64 2.916	SMP65 2.242	SMP66 2.032
G	SMP73 0.450	SMP74 2.424	SMP75 2.944	SMP76 2.944	SMP77 1.885	SMP78 2.505
H	SMP85 0.692	SMP86 2.931	SMP87 2.931	SMP88 2.128	SMP89 0.922	SMP90 2.149

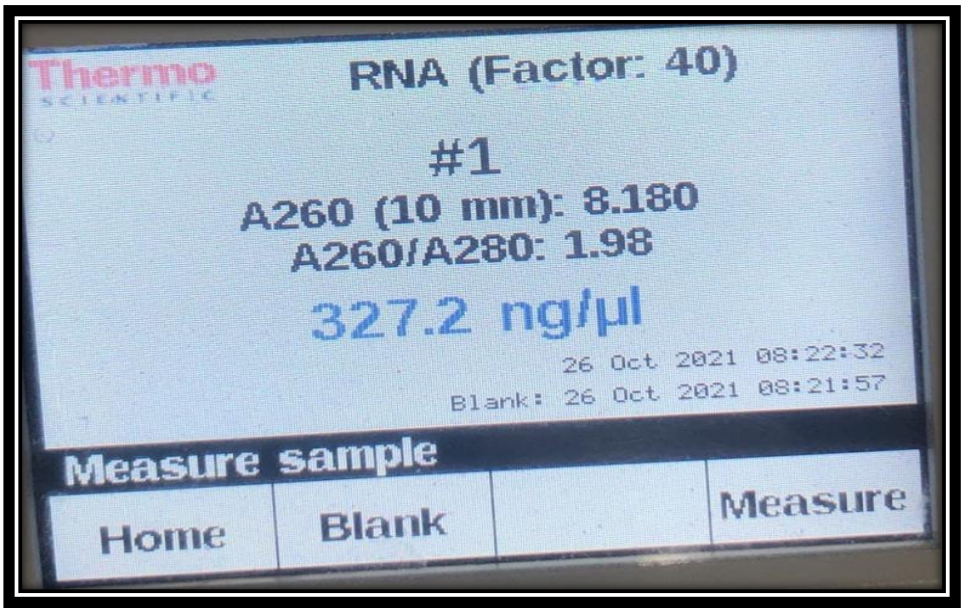
Next Exit

	07	08	09	10	11	12
A	SMP07 2.920	SMP08 2.920	SMP09 2.586	SMP10 2.920	SMP11 2.920	SMP12 2.920
B	SMP19 1.114	SMP20 2.930	SMP21 2.731	SMP22 2.930	SMP23 2.930	SMP24 2.930
C	SMP31 2.915	SMP32 2.915	SMP33 2.497	SMP34 2.915	SMP35 2.915	SMP36 2.915
D	SMP43 2.925	SMP44 2.925	SMP45 1.902	SMP46 2.925	SMP47 2.925	SMP48 2.925
E	SMP55 2.907	SMP56 2.636	SMP57 1.347	SMP58 2.907	SMP59 2.907	SMP60 2.907
F	SMP67 2.646	SMP68 2.651	SMP69 2.916	SMP70 2.916	SMP71 2.916	SMP72 2.916
G	SMP79 1.799	SMP80 2.633	SMP81 2.944	SMP82 2.539	SMP83 2.944	SMP84 2.542
H	SMP91 2.332	SMP92 1.318	SMP93 2.931	SMP94 2.931	SMP95 2.931	SMP96 0.315

Next Exit

Appendix- 3: Detection of IgA in serum samples by ELISA reader under absorbance 450nm.





Appendix- 4: Measurement the concentration and purity *Rotavirus* in stool sample by Nanodrop.

Appendices……..……...

Dehydration	Diarrhea	Vomiting	Fever	IL6 Serum	IgA Stool	IgA Serum	RTPCR	RAT	Residence	Type Of Feeding	Gender	Age	Group
1	1	1	1	311.50	96.77	97.85	1	1	1	1	1	10.00	2
	1			353.69	97.22	97.26	1	1	1	3	2	11.00	2
	1			371.40	96.54	96.77	0	1	1	2	2	7.00	2
1	1	1	1	256.45	81.51	97.22	1	1	1	2	1	1.00	2
	1			45.72	49.28	96.54	1	1	2	2	1	7.00	2
	1			180.31	96.59	96.99	0	1	2	1	1	12.00	2
1	1	1	1	271.49	74.44	96.18	1	1	2	2	1	10.00	2
	1			56.15	97.26	96.59	1	1	1	3	1	12.00	2
	1			190.25	96.77	79.62	0	1	1	1	1	7.00	2
	1	1	1	35.78	78.72	97.26	1	1	2	1	2	15.00	2
1	1			58.81	96.54	96.77	0	1	1	3	2	11.00	2
	1		1	40.38	96.99	97.22	0	1	1	2	1	7.00	2
1	1	1		419.17	95.69	96.54	0	1	2	3	1	48.00	2

	1		**1**	**419.17**	**96.59**	**96.99**	**1**	**1**	**1**	**2**	**2**	**12.00**	**2**
	1			**75.79**	**97.85**	**96.18**	**0**	**1**	**2**	**1**	**1**	**7.00**	**2**
1	**1**	**1**		**136.17**	**97.26**	**96.59**	**0**	**1**	**1**	**2**	**2**	**7.00**	**2**
	1		**1**	**262.27**	**90.38**	**97.85**	**1**	**1**	**1**	**1**	**2**	**1.00**	**2**
1	**1**		**1**	**302.04**	**97.22**	**97.26**	**1**	**1**	**2**	**3**	**1**	**12.00**	**2**
	1	**1**	**1**	**126.71**	**96.54**	**96.77**	**1**	**1**	**1**	**3**	**1**	**12.00**	**2**
	1		**1**	**173.52**	**96.99**	**97.22**	**0**	**1**	**1**	**1**	**2**	**21.00**	**2**
	1		**1**	**474.46**	**96.18**	**96.54**	**0**	**1**	**1**	**2**	**1**	**11.00**	**2**
1	**1**	**1**	**1**	**441.72**	**96.59**	**96.99**	**1**	**1**	**2**	**2**	**1**	**7.00**	**2**
	1			**204.80**	**97.85**	**96.18**	**1**	**1**	**2**	**2**	**1**	**1.00**	**2**
	1			**204.80**	**61.12**	**96.59**	**1**	**1**	**1**	**3**	**2**	**7.00**	**2**
1	**1**	**1**	**1**	**307.38**	**19.03**	**79.75**	**0**	**1**	**2**	**2**	**2**	**7.00**	**2**
1	**1**	**1**		**85.97**	**41.13**	**95.15**	**0**	**1**	**2**	**2**	**1**	**4.00**	**2**
1	**1**	**1**		**350.06**	**84.48**	**94.56**	**0**	**1**	**1**	**3**	**2**	**12.00**	**2**

	1	**1**	**1**	**71.42**	**86.86**	**94.07**	**0**	**1**	**2**	**1**	**1**	**7.00**	**2**
	1	**1**		**93.25**	**30.77**	**94.52**	**0**	**1**	**2**	**2**	**1**	**2.00**	**2**
1	**1**	**1**		**331.38**	**66.25**	**98.34**	**0**	**1**	**1**	**3**	**2**	**2.00**	**2**
1	**1**	**1**	**1**	**85.49**	**50.18**	**94.29**	**0**	**1**	**1**	**2**	**1**	**6.00**	**2**
	1			**152.42**	**6.83**	**93.48**	**0**	**1**	**2**	**1**	**1**	**8.00**	**2**
1	**1**	**1**		**120.41**	**96.77**	**93.89**	**0**	**1**	**2**	**2**	**1**	**11.00**	**2**
	1		**1**	**280.94**	**97.22**	**90.42**	**0**	**1**	**2**	**3**	**1**	**5.00**	**2**
	1			**395.40**	**96.54**	**94.56**	**1**	**1**	**2**	**2**	**1**	**10.00**	**2**
1	**1**	**1**	**1**	**309.56**	**96.99**	**94.07**	**0**	**1**	**2**	**3**	**1**	**9.00**	**2**
	1		**1**	**134.47**	**56.25**	**94.52**	**0**	**1**	**2**	**2**	**1**	**4.00**	**2**
	1		**1**	**295.49**	**56.79**	**98.34**	**1**	**1**	**1**	**2**	**1**	**6.00**	**2**
1	**1**	**1**	**1**	**43.05**	**78.09**	**94.29**	**0**	**1**	**1**	**2**	**2**	**7.00**	**2**
	1			**131.32**	**62.06**	**93.48**	**0**	**1**	**2**	**3**	**2**	**7.00**	**2**
1	**1**	**1**		**228.56**	**96.77**	**93.89**	**0**	**1**	**2**	**3**	**1**	**5.00**	**2**

	1		**1**	**308.10**	**15.47**	**95.15**	**1**	**1**	**2**	**2**	**2**	**10.00**	**2**
	1	**1**		**64.15**	**96.54**	**94.56**	**0**	**1**	**1**	**2**	**1**	**6.00**	**2**
	1			**39.90**	**96.99**	**94.07**	**1**	**1**	**1**	**2**	**1**	**5.00**	**2**
1	**1**	**1**	**1**	**59.54**	**96.18**	**94.52**	**1**	**1**	**1**	**2**	**1**	**11.00**	**2**
	1			**64.63**	**84.43**	**93.84**	**1**	**1**	**2**	**2**	**2**	**6.00**	**2**
1	**1**	**1**	**1**	**399.77**	**46.30**	**94.29**	**0**	**1**	**1**	**1**	**1**	**10.00**	**2**
1	**1**	**1**		**119.68**	**70.30**	**93.48**	**0**	**1**	**1**	**3**	**1**	**10.00**	**2**
1	**1**	**1**	**1**	**97.86**	**96.77**	**98.39**	**0**	**1**	**2**	**2**	**1**	**6.00**	**2**
	1	**1**		**126.96**	**97.22**	**99.56**	**0**	**1**	**2**	**1**	**1**	**16.00**	**2**
	1		**1**	**355.88**	**96.77**	**97.85**	**0**	**1**	**1**	**2**	**2**	**12.00**	**2**
	1		**1**	**332.84**	**97.22**	**97.26**	**0**	**1**	**2**	**2**	**1**	**12.00**	**2**
1	**1**	**1**	**1**	**58.33**	**96.54**	**96.77**	**0**	**1**	**1**	**2**	**1**	**10.00**	**2**
	1		**1**	**44.75**	**81.51**	**97.22**	**0**	**1**	**1**	**2**	**1**	**7.00**	**2**
	1		**1**	**69.97**	**49.28**	**96.54**	**0**	**1**	**2**	**1**	**2**	**18.00**	**2**

	1		**1**	**277.55**	**96.59**	**96.99**	**0**	**1**	**1**	**1**	**2**	**11.00**	**2**
	1			**254.27**	**74.44**	**96.18**	**0**	**1**	**2**	**1**	**1**	**3.00**	**2**
	1		**1**	**305.44**	**97.26**	**96.59**	**0**	**1**	**1**	**1**	**1**	**5.00**	**2**
1	**1**	**1**		**88.40**	**96.77**	**79.62**	**0**	**1**	**1**	**2**	**2**	**12.00**	**2**
	1		**1**	**128.41**	**78.72**	**97.26**	**0**	**1**	**2**	**3**	**1**	**4.00**	**2**
	1			**343.75**	**96.54**	**96.77**	**0**	**1**	**2**	**1**	**2**	**5.00**	**2**
	1			**80.88**	**96.99**	**97.22**	**0**	**1**	**1**	**2**	**2**	**5.00**	**2**
	1		**1**	**358.54**	**95.69**	**96.54**	**0**	**1**	**2**	**2**	**1**	**22.00**	**2**
1	**1**	**1**		**205.04**	**96.59**	**96.99**	**0**	**1**	**2**	**2**	**1**	**12.00**	**2**
	1		**1**	**254.27**	**97.85**	**96.18**	**0**	**1**	**2**	**2**	**2**	**4.00**	**2**
	1			**64.15**	**97.26**	**96.59**	**0**	**1**	**1**	**2**	**1**	**10.00**	**2**
	1			**203.83**	**90.38**	**97.85**	**0**	**1**	**2**	**2**	**1**	**4.00**	**2**
	1		**1**	**175.94**	**97.22**	**97.26**	**0**	**1**	**2**	**2**	**1**	**3.00**	**2**
	1			**256.21**	**96.54**	**96.77**	**0**	**1**	**1**	**2**	**2**	**24.00**	**2**

	1		**1**	**223.71**	**96.99**	**97.22**	**0**	**1**	**1**	**1**	**1**	**11.00**	**2**
	1			**153.39**	**96.18**	**96.54**	**0**	**1**	**1**	**2**	**2**	**6.00**	**2**
	1		**1**	**186.61**	**96.59**	**96.99**	**0**	**1**	**2**	**1**	**2**	**11.00**	**2**
	1			**80.88**	**97.85**	**96.18**	**0**	**1**	**1**	**2**	**2**	**9.00**	**2**
	1		**1**	**82.34**	**61.12**	**96.59**	**0**	**1**	**2**	**2**	**1**	**10.00**	**2**
	1			**427.90**	**19.03**	**79.75**	**0**	**1**	**2**	**1**	**1**	**5.00**	**2**
	1		**1**	**119.44**	**41.13**	**95.15**	**0**	**1**	**2**	**2**	**2**	**7.00**	**2**
	1			**384.49**	**84.48**	**94.56**	**0**	**1**	**2**	**3**	**1**	**6.00**	**2**
1	**1**	**1**	**1**	**294.04**	**86.86**	**94.07**	**0**	**1**	**1**	**2**	**2**	**5.00**	**2**
	1			**58.33**	**30.77**	**94.52**	**0**	**1**	**2**	**2**	**2**	**5.00**	**2**
1	**1**	**1**	**1**	**302.04**	**66.25**	**98.34**	**0**	**1**	**2**	**2**	**1**	**6.00**	**2**
	1			**128.41**	**50.18**	**94.29**	**0**	**1**	**2**	**3**	**1**	**10.00**	**2**
	1		**1**	**343.75**	**6.83**	**93.48**	**0**	**1**	**2**	**1**	**2**	**6.00**	**2**
	1			**80.88**	**96.77**	**93.89**	**0**	**1**	**2**	**2**	**1**	**1.00**	**2**

1	**1**	**1**	**1**	**358.54**	**97.22**	**90.42**	**0**	**1**	**2**	**2**	**1**	**5.00**	**2**
	1		**1**	**205.04**	**96.54**	**94.56**	**0**	**1**	**2**	**1**	**1**	**7.00**	**2**
1	**1**	**1**	**1**	**254.27**	**96.99**	**94.07**	**0**	**1**	**1**	**2**	**1**	**5.00**	**2**
1	**1**			**64.15**	**56.25**	**94.52**	**0**	**1**	**2**	**2**	**2**	**6.00**	**2**
	1		**1**	**203.83**	**56.79**	**98.34**	**0**	**1**	**1**	**2**	**1**	**7.00**	**2**
	1			**175.94**	**78.09**	**94.29**	**0**	**1**	**2**	**2**	**1**	**5.00**	**2**
1	**1**	**1**	**1**	**256.21**	**62.06**	**93.48**	**0**	**1**	**2**	**1**	**1**	**4.00**	**2**
	1		**1**	**223.71**	**96.77**	**93.89**	**0**	**1**	**2**	**2**	**2**	**7.00**	**2**
	1		**1**	**153.39**	**15.47**	**95.15**	**0**	**1**	**1**	**1**	**1**	**2.00**	**2**
	1			**186.61**	**96.54**	**94.56**	**0**	**1**	**1**	**2**	**1**	**10.00**	**2**
	1		**1**	**80.88**	**96.99**	**94.07**	**0**	**1**	**2**	**2**	**2**	**8.00**	**2**
	1			**82.34**	**96.18**	**94.52**	**0**	**1**	**1**	**2**	**1**	**7.00**	**2**
	1		**1**	**427.90**	**84.43**	**93.84**	**0**	**1**	**2**	**1**	**1**	**9.00**	**2**
	1		**1**	**119.44**	**46.30**	**94.29**	**0**	**1**	**2**	**3**	**2**	**6.00**	**2**

	1		**1**	**384.49**	**70.30**	**93.48**	**0**	**1**	**2**	**2**	**2**	**9.00**	**2**
	1		**1**	**294.04**	**96.77**	**98.39**	**0**	**1**	**2**	**3**	**1**	**3.00**	**2**
	1		**1**	**58.33**	**97.22**	**99.56**	**0**	**1**	**2**	**3**	**2**	**4.00**	**2**
				137.14	**96.54**	**91.33**	**0**	**0**	**1**	**2**	**2**	**7.00**	**1**
				117.74	**96.99**	**91.11**	**0**	**0**	**2**	**1**	**1**	**7.00**	**1**
				103.68	**83.98**	**90.37**	**0**	**0**	**2**	**2**	**2**	**7.00**	**1**
				136.66	**84.66**	**91.91**	**0**	**0**	**2**	**2**	**1**	**4.00**	**1**
				57.36	**83.85**	**90.81**	**0**	**0**	**1**	**3**	**2**	**5.00**	**1**
				144.66	**24.65**	**90.34**	**0**	**0**	**2**	**1**	**1**	**7.00**	**1**
				80.64	**81.73**	**90.32**	**0**	**0**	**2**	**2**	**1**	**2.00**	**1**
				59.78	**88.26**	**90.93**	**0**	**0**	**1**	**3**	**2**	**2.00**	**1**
				64.15	**77.73**	**73.62**	**0**	**0**	**1**	**2**	**1**	**6.00**	**1**
				58.09	**50.94**	**91.13**	**0**	**0**	**2**	**3**	**1**	**5.00**	**1**
				64.15	**25.96**	**90.91**	**0**	**0**	**1**	**2**	**2**	**7.00**	**1**

				85.49	**96.59**	**90.43**	**0**	**0**	**2**	**3**	**2**	**7.00**	**1**
				112.89	**61.84**	**89.71**	**0**	**0**	**1**	**3**	**1**	**12.00**	**1**
				93.49	**61.25**	**89.17**	**0**	**0**	**1**	**3**	**2**	**12.00**	**1**
				79.43	**60.75**	**89.87**	**0**	**0**	**1**	**3**	**2**	**10.00**	**1**
				112.41	**61.21**	**89.23**	**0**	**0**	**2**	**3**	**2**	**36.00**	**1**
				33.11	**60.53**	**90.76**	**0**	**0**	**1**	**3**	**1**	**12.00**	**1**
				120.41	**60.98**	**90.31**	**0**	**0**	**1**	**3**	**2**	**9.00**	**1**
				56.39	**78.18**	**89.61**	**0**	**0**	**1**	**3**	**1**	**24.00**	**1**
				35.53	**78.58**	**90.71**	**0**	**0**	**2**	**2**	**1**	**10.00**	**1**
				39.90	**84.12**	**89.41**	**0**	**0**	**2**	**2**	**2**	**9.00**	**1**
				33.84	**79.26**	**89.21**	**0**	**0**	**2**	**3**	**2**	**10.00**	**1**
				39.90	**78.76**	**89.42**	**0**	**0**	**1**	**3**	**1**	**12.00**	**1**
				61.24	**79.21**	**89.12**	**0**	**0**	**2**	**2**	**2**	**12.00**	**1**
				112.89	**78.54**	**72.61**	**0**	**0**	**2**	**3**	**1**	**6.00**	**1**

				69.24	**78.99**	**88.24**	**0**	**0**	**1**	**3**	**1**	**6.00**	**1**
				79.43	**78.18**	**87.81**	**0**	**0**	**1**	**2**	**1**	**7.00**	**1**
				112.41	**83.08**	**87.17**	**0**	**0**	**2**	**2**	**1**	**10.00**	**1**
				105.86	**79.84**	**87.32**	**0**	**0**	**1**	**3**	**2**	**6.00**	**1**
				120.41	**79.26**	**94.61**	**0**	**0**	**2**	**3**	**1**	**6.00**	**1**
				56.39	**78.76**	**90.31**	**0**	**0**	**1**	**3**	**2**	**11.00**	**1**
				35.53	**83.71**	**89.81**	**0**	**0**	**1**	**3**	**2**	**8.00**	**1**
				88.40	**83.04**	**89.41**	**0**	**0**	**2**	**3**	**1**	**6.00**	**1**
				33.84	**78.99**	**86.71**	**0**	**0**	**1**	**2**	**1**	**10.00**	**1**
				136.90	**96.18**	**90.23**	**0**	**0**	**1**	**2**	**1**	**9.00**	**1**
				61.24	**78.58**	**90.39**	**0**	**0**	**1**	**2**	**1**	**12.00**	**1**
				105.37	**106.76**	**90.46**	**0**	**0**	**2**	**2**	**2**	**12.00**	**1**
				85.97	**86.14**	**94.82**	**0**	**0**	**2**	**3**	**2**	**8.00**	**1**
				71.91	**58.55**	**90.73**	**0**	**0**	**2**	**3**	**1**	**10.00**	**1**

				104.89	**89.57**	**89.71**	**0**	**0**	**2**	**2**	**1**	**11.00**	**1**
				74.09	**58.50**	**89.81**	**0**	**0**	**1**	**3**	**1**	**8.00**	**1**
				112.89	**93.84**	**91.93**	**0**	**0**	**2**	**3**	**2**	**12.00**	**1**
				73.12	**84.48**	**90.37**	**0**	**0**	**2**	**2**	**1**	**12.00**	**1**
				76.52	**84.88**	**90.41**	**0**	**0**	**1**	**3**	**1**	**11.00**	**1**
				32.38	**54.41**	**90.45**	**0**	**0**	**1**	**2**	**2**	**8.00**	**1**
				26.32	**58.55**	**89.61**	**0**	**0**	**2**	**3**	**2**	**10.00**	**1**
				32.38	**58.05**	**90.24**	**0**	**0**	**1**	**3**	**2**	**7.00**	**1**
				53.72	**58.50**	**89.76**	**0**	**0**	**2**	**2**	**2**	**9.00**	**1**
				84.03	**62.33**	**94.48**	**0**	**0**	**2**	**2**	**1**	**10.00**	**1**
				15.65	**58.28**	**95.24**	**0**	**0**	**2**	**3**	**1**	**7.00**	**1**

Appendix- 5: Data raw

الخلاصة

يُعد التهاب المعدة والأمعاء الناتج عن الفيروس العجلي سبباً رئيسياً للإسهال الحاد لدى الأطفال الذين تقل أعمارهم عن خمس سنوات في جميع أنحاء العالم ، مع ارتفاع معدل الوفيات في البلدان منخفضة الدخل. هدفت هذه الدراسة إلى توفير معلومات أساسية عن وبائيات الفيروس العجلي واستجابات المضيف لدى الأطفال في محافظة بابل. أُجريت دراسة مستقبلية قائمة على الحالات المستندة إلى المستشفى في مستشفى بابل للولادة والأطفال - مستشفى النور للأطفال من سبتمبر 2021 إلى فبراير 2022 ، تم تسجيل الأطفال دون سن الخامسة الذين تم إدخالهم إلى المستشفى مصابين بالتهاب المعدة والأمعاء الحاد في الدراسة ، تم جمع البيانات الديموغرافية والسريرية وتحليل عينات البراز لمستضد الفيروس العجلي والغلوبيولين المناعي في البراز(IgA)، تم جمع عينات المصل لقياس مستويات IgA و Interleukin IL 6.
تم تسجيل مائة وخمسين طفلاً حتى سن الخامسة (100 مصاب بالإسهال الناجم عن الفيروس العجلي و 50 من الاصحاء)، كان ثلاثة وستون (63٪) من الأطفال المصابين بالإسهال الناجم عن الفيروس العجلي من الذكور ، كان معظم الأطفال المصابين بالتهاب المعدة والأمعاء الناجم عن الفيروس العجلي أقل من عامين ويتراوح عمرهم غالباً بين ستة إلى أحد عشر شهراً، كانت معدلات الرضاعة الطبيعية أقل في مجموعة الفيروس العجلي من مجموعة التحكم (P <0.001).

لم يكن هناك ارتباط بين الإسهال الناجم عن الفيروس العجلي والموقع السكني (الريف مقابل الحضر) (P> 0.05).

كانت مستويات IgA في المصل والبراز ومستويات IL-6 في الدم أعلى في الأطفال المصابين بالفيروس العجلي مقارنة بمجموعة التحكم (p <0.01 لكل منهما).

تم تنفيذ طريقة تسلسل الحمض النووي لتحديد تحليل التباين الجيني (طفرات الفرعية) في جين البروتين غير الهيكلي (*NSP4*) لعزلات الفيروس العجلي البشري المحلي (IQH1 - IQH.5) و NCBI-Blast المتعلقة بفيروس الروتا البشري A.

أظهر تحليل العلاقة الوراثية لشجرة النشوء والتطور أن العزلات المحلية للفيروس العجلي البشري (IQH.1 - IQH.4) A قد تم إغلاقها فيما يتعلق بسلالة الفيروس العجلي البشري NCBI-BLAST A RV1326 وأظهرت عزلات فيروس الروتا البشري المحلي (IQH.5) مغلق مرتبط بسلالة الفيروس العجلي البشري NCBI-BLAST A G1P عند إجمالي التغيرات الجينية (0.0020-0.0080٪).

تم عرض هوية تسلسل التماثل بين العزلات المحلية للفيروس العجلي البشري A (IQH1-IQH.4) وسلالة NCBI BLAST المتعلقة بالفيروس العجلي البشري A RV1326 تراوحت هوية تسلسل التماثل الجيني من (99.37-99.84٪) ، أظهرت العزلات المحلية للفيروس العجلي البشري A (IQH.5) و NCBI BLAST ذات الصلة بالفيروس العجلي البشري A سلالة G1P هوية تسلسل تماثل جيني تراوحت من (99.52٪).

تم العثور على تحليل التباين الجيني (طفرات الاستبدال) في جين البروتين غير الهيكلي (*NSP4*) بين عزلات الفيروس العجلي البشري المحلي وعزلة NCBI-Blast المتعلقة بالفيروس العجلي البشري A (1-4) طفرات استبدالية في نسبة الاختلاف الجيني الإجمالية التي تراوحت (0.16 -0.63٪).

واخيراً تم تقديم العزلات المحلية للفيروس العجلي البشري نوع A -IQH.1) IQH.4) الى NCBI Genbank وتم تحديدها من خلال ارقام الانضمام :

BankIt2566061 Seq1 ON087846

BankIt2566061 Seq2 ON087847

BankIt2566061 Seq3 ON087848

BankIt2566061 Seq4 ON087849

BankIt2566061 Seq5 ON087850

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية الطب / فرع الأحياء المجهرية

الدراسة المناعية والجزيئية للفيروس الدوار بين الاطفال المصابين في محافظة بابل

رسالة مقدمة إلى مجلس كلية الطب / جامعة بابل وهي جزء من متطلبات نيل

درجة الماجستير في العلوم/الأحياء المجهرية الطبية

تقدم بها:
نغم علي كاظم جاسم
بكالوريوس احياء مجهرية / كلية العلوم / جامعة بابل 2006

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