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Investigation of Astrovirus and IFN-γ Polymorphism in Iraqi Childhood Patients with Gastroenteritis

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Abstract

Background: Human astroviruses are considered acute gastroenteritis agents (AGE) and are largely reported in children worldwide. Astrovirus causes endemic childhood diarrhea; worldwide, it is responsible for 3-9% of diarrheal illness. Transmission is primarily person to person via the fecal-oral route and also via contaminated food and water.

Objectives: To determine the rate of human *Astrovirus* infections genome and the role of IFN-γ in limitation the severity and prevalence of virus among children and infants with gastroenteritis by assess it *Astrovirus* genome by quantitative Real Time PCR and also IFN-γ by conventional polymerase chain reaction among study population.

Patients and methods: Case control study was carried out on 150 children and infant aged between (6-120 months) with gastroenteritis who attended the middle Euphrates hospitals for Maternity and Children as well as private laboratories, during the period from February 2021 till September 2019. Stool, blood, body fluids samples were collected from each participant and stored as frozen at -70 °C to RNA extraction *Astrovirus* genome and IFN-γ genome by using qReal Time PCR and Conventional PCR test.

Results: The mean of age in patients with gastroenteritis was (43.56) while the control group (40.6) , also we found the infection in male more than female in percentage 58% for male and 42% for female. The rate of human *Astrovirus* infection according to the quantitative real time PCR was 18.1% (14 out of 77) while the negative result was 81.9 % (63 out of 77). The results of conventional polymerase chain reaction for IFN-γ for detection the relation with the limitation of virus severity demonstrated that only 21.3 % (32 out of 150) was positive for IFN-γ while the negative result was 78.7 % (118 out of 150).

Conclusion: : The genome of the human *Astrovirus* genome appears to play a major role in gastroenteritis among infants and children, and IFN-γ has an important role in limitation the severity of gastroenteritis among infected individuals.

Keywords: Interferon gamma (FN-γ); Gastroenteritis ; Real time PCR ; *Astrovirus*

Introduction

Astroviruses are small (28–30 nm), non-enveloped viruses belonging to the family Astroviridae. The viral genome comprises a single-stranded RNA of positive sense [1]. Astroviruses was identified in 1975 by electron microscopy (EM) in children suffering from diarrhea, it is belong to the family Astroviridae, which made up of single-stranded positive RNA viruses [2]. .Since then, enteric infections in humans caused by astrovirus have been reported worldwide mainly in infants and young children. Several studies suggest that astroviruses are the second most common cause of gastroenteritis in children after rotavirus infection [3].

The transmission of HAstV is through fecal-oral route. The replication of the virus in infected children takes place in the intestinal epithelium [4]. AstVs are transmitted through either direct interaction with feces or the consumption of contaminated food or water [5]. Both avian and mammalian species that congregate in large groups or live in highly dense populations provide highly permissive environments for AstV transmission. Avian migrations provide the opportunity for AstVs from different geographical regions to be introduced into new areas and populations of hosts [6].

Acute gastroenteritis (AGE) is a common illness of humans globally. It adversely affects the public health, especially the very young, the elderly, the malnourished, and those with an impaired immune system [7,8]. Gastroenteritis of viral origin is a major public health concern among children 5 years of age . It is estimated that one billion hospital admissions and about 1.6–2.5 million deaths occur annually among children under the age of 5 years in tropical Africa due to acute gastroenteritis. Human astroviruses (HAstVs) and other viruses such as rotavirus and norovirus are known pathogens responsible for gastroenteritis among children 5 years old worldwide [9-13].

Interferons (IFNs) are pleiotropic cytokines with antiviral, antitumor and immunomodulatory properties, being central coordinators of the immune response [14]. The term "interferons" comes from the description of molecules protecting cells by "interfering" with viral infection [15] . IFN-γ pleiotropic functions are mediated by cell-specific expression of hundreds of IFN-γ-regulated genes that encompass inflammatory signaling molecules, apoptosis and cell cycle regulators, and transcriptional activators [16]. Autocrine IFN-γ produced by APCs can act locally and contrib-ute to sustain self and neighbor cell activation, crucial for early control of pathogen spreading, while T lymphocytes are the major paracrine source of IFN-γ in adaptive immunity. Under physiological conditions, the constitutive expression of type I and II IFNs is tightly controlled, remaining localized to tissues, with-out systemic effects [17] .

Experiments

i.. Group of study

Our current study included 150 samples of infants and children with gastroenteritis and a non-infected group as a control group (n = 50). The infected people were confirmed according to the symptoms and after being diagnosed by the Pediatrician, then it was detected by diagnostic methods and their results were positive. As for the control group, they are healthy (uninfected)

ii. Experimental design

This study was makes through period from February 2021 to September 2021. About 150 specimens from (infants and children) in the age 6–120 months were taken from teaching hospitals as well as many private laboratories in Middle Euphrates provinces /Iraq .

iii. Sample types and collection

Stool swabs as well as blood from each study group of infants and children patients suffering from gastroenteritis should be enrolled, that classify into One hundred – fifty stool swabs as well as blood specimens from infants and children patients suffering from gastroenteritis, and Fifty stool swabs and blood specimens of apparently healthy persons as control group.

The samples collection included Endometrium and\or cervical swabs ; fetal fluids swab were in 3 ml liquid viral transport media tube (UTM),each specimen was aliquot into three cryotube containing 1000 µl of the sample which stored at (-20ºc) until genome extraction. After that, required part of specimens were taken and centrifuged at 10000 g/min for 5 minutes, discarded the supernatant except 100 µl of the solution was left to be used in re-suspension of the pellet for RNA/DNA extraction.

iv. Methods

1. Extraction the viral nucleic acid from gastroenteritis patients specimens

- ***Principle***

By using specific viral DNA/RNA extraction kit (Intron/Korea); the viral genomic was extracted ,purifying and migrated using agarose gel from the blood, stool and fetal fluid as a first step to amplify the target *Astrovirus* RNA.

2.Detection of Astrovirus (NoV) by Real Time Polymerase Chain Reaction (RT-PCR).

- ***Principle***

Real-time PCR (qPCR) is based on two major processes: Firstly, isolation of viral genome (DNA\ RNA) from specimens, and Secondly, Real time amplification for each sample . In real-time PCR, the accumulating

amplified product can be detected at each cycle with fluorescent dyes. This increasing signal allows to achieve sensitive detection and quantification of pathogens.

- ***Procedure***

GoTaq 1-Step RT-qPCR System(a,b) – (A6020/ Promega \ USA) combines GoScript™ Reverse Transcriptase and go Taq-qPCR master mix in a sigle step real time amplification reaction. the system which is optimized for RT-qPCR contains a propriety fluorescent DNA-binding dye, BRYT Green Dye.The system enables detection of RNA expression levels using a one-step RT-qPCR method, combining Go Script™ Reverse Transcriptase and GoTaq- qPCR Master Mix in a single step real-time amplification reaction.

The *Astrovirus* genome that was targeted by real time PCR involve two primers , the *Astrovirus* primer (IDT/USA) with nucleotide sequence ,Forward primer (5"-3") CAACTCAGGAAACAGGGTGT and Reverse primer with sequence (5"-3") TCAGATGCATTGTCTTGGT.

3.Detection of IFN-γ Genes Polymorphism By Polymerase Chain Reaction (PCR)

- ***Procedure***

Reactions were placed in a thermal cycler (Biometra-Germany) that had been preheated to 94°C and beforehand set up to the desired cyclic conditions. The target regions of *IFN-γ* genome , by using specific *IFN-γ* primer (IDT/USA) with product size (441bp) and nucleotide sequence ,Forward primer (5"-3") GGAGGATCCCTCCTGGGG and Reverse primer with sequence (5"-3") CATACACCCGTTCCTGTCCC.

The PCR amplification procedure was make according to the conditions , Initial denaturation / $95C^0$ /5 min , Denaturation / 95C0 / 30 sec, Annealing/58 C0 /30 sec, Extension 72 C0 /30 sec, Final extension/ 72 C0 /10min, and Number of cycles /40

Statistical analysis

All data were analyzed using one-way ANOVA, and means were compared using the Duncan test. The significance levels were $P<0.01$ & $P<0.05$ [18].

Results

I.Distribution of Patients with Gastroenteritis (GE) and Apparently Healthy Control (AHC) Groups According to Their Age.

Table 4-1 shows the mean age groups of the study population , where the mean age of the patients with GE was (43.56±8.31 months) was more than the mean age of the AHC (40.6± 10.96 months). there are non-significant statistical differences (p=0.42) between ARTI and AHC.

Table 1 : Distribution of Patients with GE and AHC according to Their Age.

Study groups	No.	Mean of age (Months)	S. D	S. E	Range		(P-value)
					Minimum	Maximum	
GE	150	43.56	8.31	2.304	6 months	120 months	**P=0.42 N S (P>0.05)**
AHC	50	40.6	10.96	4.59	8months	120 months	
Total	200						

II. Distribution of Patients with GE and AHC According to Their Gender.

Gender distribution is represented in Figure -2. Where the Fifty-eight percent (58%) (116 out of 200 cases) of the study population were male, while female represented 42% (84 out of 200 cases)

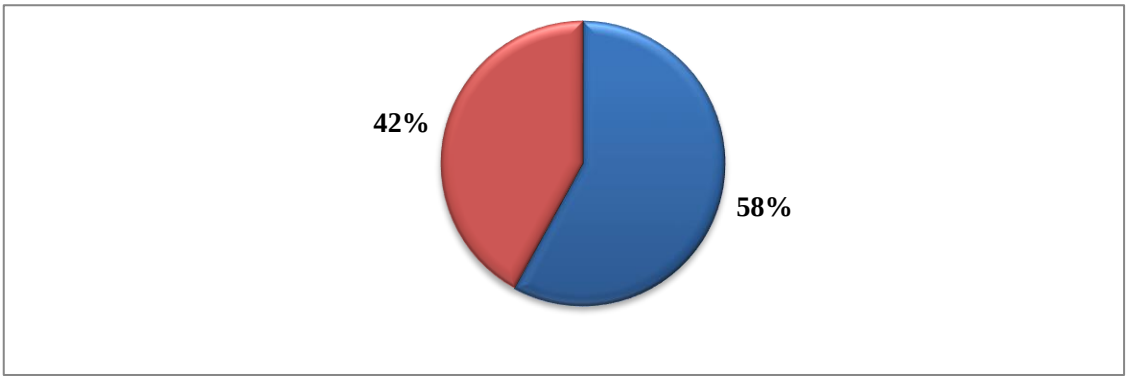

Figure 1: Gender Distribution of the Study Population.

III. Extraction of viral nucleic acid genome

Out of 150 stool swabs specimens involved in this study 51.3% (77 out of 150 cases) were found to have a viral infection more than 48.7%(73 out of 150 cases) patients who did not show have a viral genome as shown in Figures (2). While, no viral nucleic acid was detected among all the examined apparently healthy specimens (50) as control group . There were statistically significant differences (p = 0.03) between patients with the viral genome and those without the viral genome Table (2).

Table 2: Percentage of Viral Genome Extraction of Patients with GE and AHC Groups.

Viral Genome		Study Groups AHC No. (50)	Study Groups GE No. (150)	Chi-Square (P-value)
Positive	**N**	0	77	
	%	0%	51.3 %	**P=0.03**
Negative	**N**	50	73	**S.**
	%	100%	48.7%	**(P<0.05)**
Total	**N**	50	150	
	%	100%	100%	

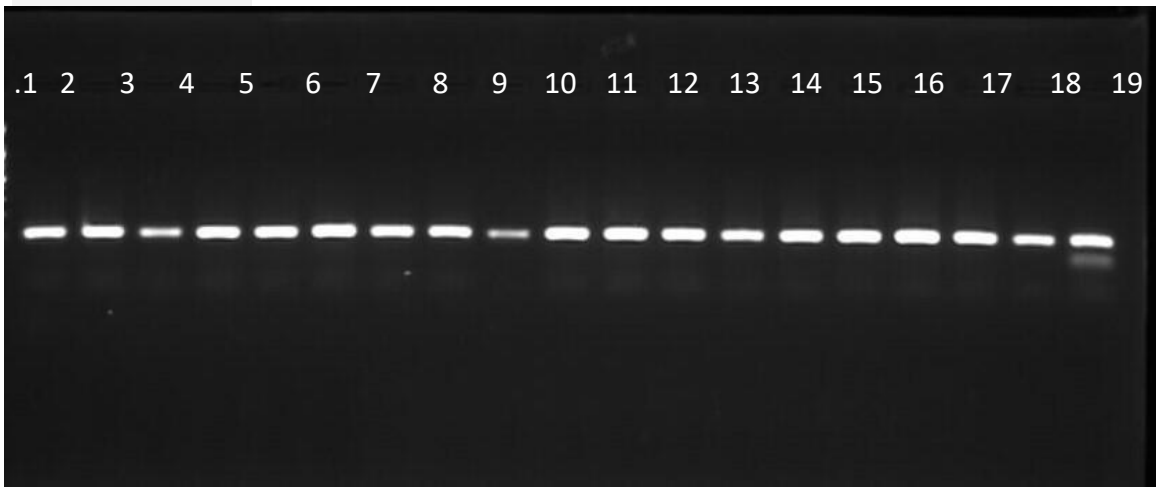

Figure 2: Extraction of Viral Genome from Patients with GE ,1 % Agarose Gel Electrophoresis , TBE 1X ,at Voltage 75 Volt for 45 min, Lanes (1-19) were Positive.

IV. Detection and Quantatiative of Astrovirus Genotypes By qRT.PCR

The total positive result of *Astrovirus* genome according to qRT-PCR shows 37.6% (29 out of 77 cases) as positive while less than 62.4% (48 out of 77 cases) as negative, as shown in Table -3 as well as Figures 3 (A & B) . Statistically significant differences (p = 0.03) among patients group.

Table 3: Percentage of AsV Positive Signals in Patients with GE by Using qRT.PCR Technique.

AsV Genome	No.	%	P value
Positive	**14**	18.1	**P=0.04**
Negative	63	81.9	**Sign**
Total	77	100	**>0.05**

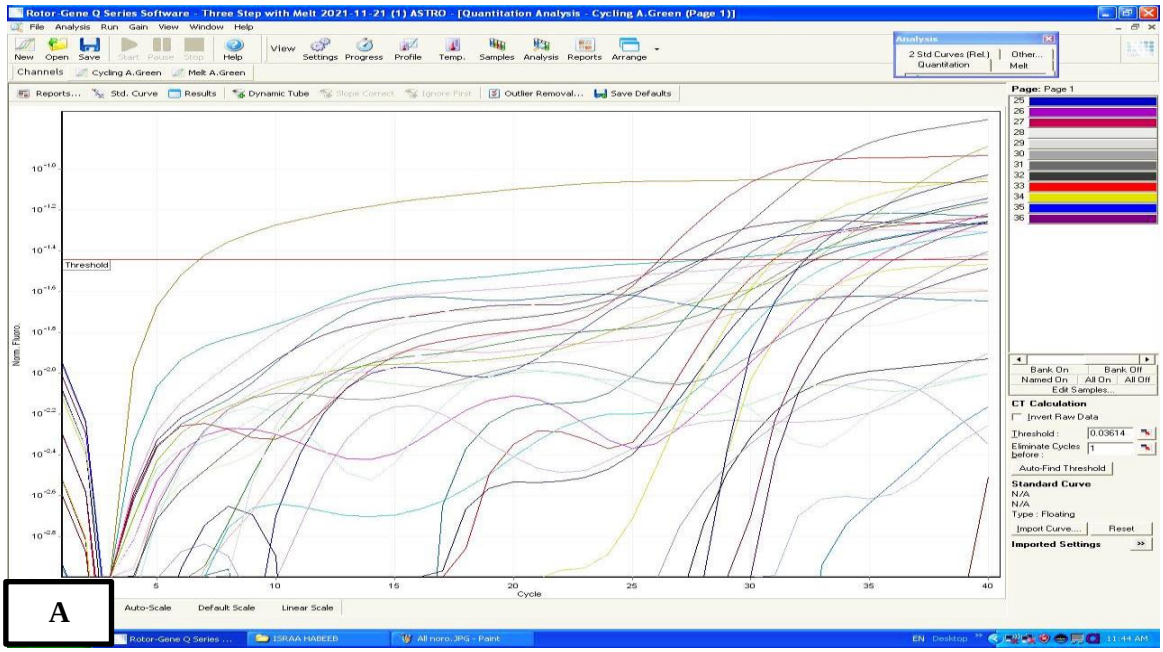

Figure 3: Detection the *Astrovirus* genome by real time quantitative PCR

A- Cycle threshold of *Astrovirus* genome that showing in many colored lines after 45 cycle in real time PCR

B- Quantitative & melting curve for *Astrovirus* genome .

4.5. Detection the IFN-γ Gene in patients with gastrointestinal

Figure 4 shows the IFN-γ gene using specific primer, where the revealed that the presence a single band (408 bp) of the target sequence of IFN-γ gene in agarose gel .

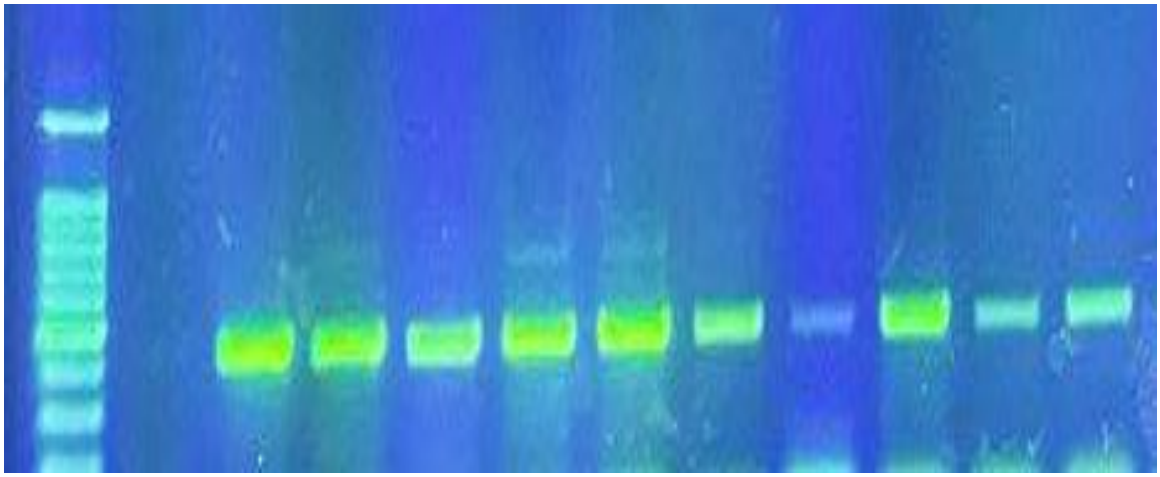

Figure 4 : PCR amplification with specific primers for IFN-γ gene on 1% agarose gel electrophoresis, TBE 1×, at voltage 75 for 60 min

Table 4 show the percentage of a single band (441 bp) of the target sequence of IFN-γ gene. The positive result, according to PCR amplification of a single band (441 bp) of IFN-γ gene in women patients with GE and AHC were 21.3% (32 of 150 cases) and 8% (4 of 50 cases), respectively . While, the negative results were in patients with GE and AHC were 78.7% (118 of 150 cases) and 92% (46 of 50 cases), respectively .

Table 4: Percentage of IFN-γ signals in patients with GE and AHC groups by PCR technique.

IFN-γ gene band	GE No.(%)	AHC No.(%)
Positive	32 (21.3%)	5 (8%)
Negative	118 (78.7%)	45 (92%)
Total	150 (100%)	100%)

The results showed that DNA polymorphism distribution were DNA polymorphism distributions according to GA ; CT ; **AT** and AG genotypes of **IFN-γ** polymorphism were respectively 53.1% (17 out of 32 cases) ; 21.9% (7 out of 32 cases); 15.6% (5 out of 45 cases) and 9.3% (3 out of 32 cases) in the GE patient group . While, polymorphism distributions according to GA and AG genotypes of **IFN-γ** polymorphism were 60%(3 out of 5 cases) and 40% (2 out of 5 cases) ; respectively in the control group. In addition , was found just transversion mutation in GE patients , while in control group just transition mutation in **IFN-γ** gene **Table (5)**. The frequency of transversion mutation more than the transition mutation (A\G).

Table 5: Comparison between patient with GE and HC on percentages of IFN-γ expressed gene polymorphism.

Polymorphism of IFN-γ gene	Type of Mutation	Study group		OR [Patients]	OR [Control]	*P value*	95% C.I for OR [Patients]	
		HC NO.(50)	GE NO.(150)				*lower*	*Upper*
G\A	**Transtion**	60%	53.1%	0.8	1.7	**0.001**	0.83	0.99
C\T	**Transversion**	0.0%	21.9%	0.7	1.4	**0.008**	0.77	0.97
A\T	**Transversion**	0.0%	15.6%	0.6	1.3	**0.006**	0.80	0.96
A\G	**Transtion**	40%	9.3%	0.8	1.1	**0.003**	0.88	0.95

Discussion

Gastroenteritis is one of the most common disease around the world. However, other than rotavirus, norovirus and enteric adenovirus, knowledge about the impact of infections caused by HAstV is still much needed. In an effort to understand better the role of HAstV as a cause of Gastroenteritis at infants and children , a survey on HAstV was carried out Hospitals in the Middle Euphrates, Iraq.

Gastroenteritis is one of the most common disease around the world. However, other than rotavirus, norovirus and enteric adenovirus, knowledge about the impact of infections caused by HAstV is still much needed. In an effort to understand better the role of HAstV as a cause of Gastroenteritis, a comprehensive survey on HAstV was carried out in Guangzhou, China. Whereas, the quantitative Real time PCR technique was used to detect the *Astrovirus* as a one of the major cause of intestinal diseases, especially gastroenteritis, and this is consistent with what was mentioned by the researchers Taco-Masias *et al* [19] and Lu *et al* [20] Muhammad and his group, who used the same technique and proved its effectiveness in diagnosing both HAstV and SaV and other associated intestinal pathogens. with AGE. The detection rate of HAstV in people with AGE in age stratum (3-36 months) was 9.1% in the present study, which is near to that previously reported in shanghai (5.22%), and Germany (5.0%), but was lower than the mean incidence worldwide of 11.0% [21,22]. other studies have reported a higher rate of infection with HAstV in viral gastroenteritis, ranging between 77 and 80% [23].

Our slightly lower positivity can be explained by differences in the sample size, geographic location, and detection method. where rotavirus was the most frequently detected enteropathogens responsible for coinfection in many studies [24]. so It can be suggested that coinfection with HAstV is not rare. Our data also confirmed HAstV was one of the as the enteropathogens responsible for AGE . However, quantitative real time PCR can pick up free nucleic acid and asymptomatic shedding of HAstV or other enteropathogens in patients, these finding consistent with Luo *et al* [25] . Astrovirus was most commonly detected in the 3-36 month age group (9.1%), which is close to what was previously mentioned, i.e. most infections with HAstV, SaV, Rotovirus, norovirus, and intestinal adenovirus occurred predominantly in infants and children. <5 years of age [26].

In the current study, the incidence of infection in males was higher than females, reaching 58% for males, while it was 42% for females, as shown in figure 1, and this agrees with a previous study showed that the infection rate higher in males (11.4%) than females (8.6%) [13], In addition, other studies have indicated that the incidence of infection is higher in both males and females it can be this information is supported by other studies [27] and may serve to guide possible future vaccination policy to target children who they are no more

than 5 years old. More infections were observed with HAstV among children 7-12 months and older between age groups [28] and this is consistent with what was mentioned in our previous results.

Moreover, the age distribution of children showed a great close attachment to shedding HAstVs, with a predominance among children aged 3-36 months and above in this study (p = 0.00). The reason for this may be connected with their growing stage and an immature immune system .This is an adventurous stage when most children progress from crawling to walking, which is usually characterized by falling and standing as they practice. They also have a common habit of putting dirt into their mouth without the knowledge of their mother. Furthermore, when children are together, the probability of spreading infection is high. Infants and toddlers usually wipe off saliva from their mouth with their hands and use the same hands to wipe or rub their noses and eyes, transferring the same hands to their toys and touching other children. The virus is then transmitted from child to child via bodily contact, toys, and other fomites [27,29]. Detection may be lower in older children be due to protective immunity. If children, especially in this area, are exposed at a younger age, they may have protective immunity and, therefore, the infection rate can be lower It is observed in older children [30].

In this study, we demonstrate that HAstV-1 induces IFN-γ at 21.3% (32 out of 150 cases) while in apparently healthy group shows at 8% (5 out of 50 cases) this increasing in the percentage of IFN-γ in patients with *Astrovirus* in contrast to the control group It means that the virus induce the IFN-γ production that lead to the limitation from infection these findings was consistent with the researcher Guix *et al* [31] who demonstrated that the production of IFN, which limits astrovirus replication in vitro and is important for the clearance of astrovirus in vivo .

In the current finding , we diagnosed IFN-γ and its role in reducing the severity of infection with the astro virus, and the importance of choosing this type of interferon was for its important role in reducing viral infection, as it is specialized for viruses, and this is consistent with what was mentioned by previous studies that mentioned type II IFNs (IFN-γ) are responsible for regulating and activating the immune response induced in virtually all cell types upon recognition of viral components, especially nucleic acids, by cytoplasmic and endosomal receptors, and type II interferon is induced by cytokines such as IL-12, and its expression is restricted to immune cells such as T cells and NK cells [32,33]. Other studies have indicated that the importance Clinical study of the relationship between interferon-gamma and astrovirus infection, extending extensively ranging from asymptomatic infections to fatal encephalitis, so the study of this type of interferon plays a major role in knowing the body's ability to end viral infection and reduce its symptoms that may affect the health of infected children, as they are at the beginning of the formation of the immune system and its functional and physiological completion [34].

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