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Comparative Study: Conventional and Real-Time PCR of enterovirus 70 and 71 as causative agents of eye infections in Thi Qar province

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Abstract.

Enteroviruses EV70 and EV71 are important viral pathogens; EV70 is primarily associated with acute hemorrhagic conjunctivitis, while EV71 is linked to hand-foot-and-mouth disease with occasional ocular involvement. This study aimed to evaluate the performance of conventional PCR and real-time PCR in detecting these viruses from conjunctival swabs in Thi-Qar Province. A total of 120 conjunctival swabs were collected from patients suspected of viral eye infections, and viral RNA was extracted and tested using both conventional PCR and real-time PCR. All samples were negative by conventional PCR, whereas real-time PCR detected infections mainly in children aged 1–10 years, with EV70 being the predominant virus. These findings suggest a potential association of EV70 and EV71 with pediatric eye infections and emphasize the importance of sensitive molecular diagnostics for early and accurate detection

Keywords: Enterovirus 70،Enterovirus 71 ،Viral conjunctivitis ،PCR ،Real-time PCR ،Eye infections

الملخص

تعد الفيروسات **EV70** و**EV71** من العوامل الممرضة المهمة، حيث يرتبط **EV70** بالتهاب الملتحمة النزفي الحاد، بينما يُعرف **EV71** بمرض اليد والقدم والفم مع تقارير محدودة عن إصاباته العينية. تهدف هذه الدراسة إلى تقييم كفاءة الـ **PCR** التقليدي والـ **Real-time PCR** في الكشف عن الفيروسين من مسحات الملتحمة في محافظة ذي قار. جُمعت 120 مسحة ملتحمة من مرضى يُشتبه إصابتهم بعدوى فيروسية، واستُخلص الـ **RNA** الفيروسي ثم فُحصت العينات باستخدام كل من **PCR** التقليدي و**Real-time PCR**. أظهرت جميع العينات نتائج سلبية بالـ **PCR** التقليدي، بينما كشف **Real-time PCR** عن إصابات خاصة لدى الأطفال بعمر 1–10 سنوات، مع سيادة فيروس **EV70**. تشير النتائج إلى ارتباط محتمل للفيروسين **EV70** و**EV71** بالتهابات العين لدى الأطفال، وتؤكد أهمية الاعتماد على تقنيات التشخيص الجزيئي الحساسة للكشف المبكر والدقيق.

الكلمات المفتاحية : الفيروس المعوي 70،الفيروس المعوي 71،التهاب الملتحمة الفيروسي، تفاعل البوليمراز المتسلسل (PCR)**، تفاعل البوليمراز المتسلسل بالزمن الحقيقي** (Real-time PCR)

Introduction

Viral conjunctivitis is the most common and highly contagious disease with a self-limiting course characterized by acute follicular conjunctival reaction and preauricular lymphadenopathy, though the signs and symptoms at presentation are variable [1,2]. The prevalence varies according to the viral causative agent, the patient's age, and the season of the year [3,4]. Up to 80% of all cases of infectious conjunctivitis, both in the general population and adults, are caused by viruses [5,6]. With over 70 serotypes, enteroviruses are a varied collection of viruses;[8] EV70 and EV71 are two of the most clinically important [9]. Since its initial isolation in 1969 during a conjunctivitis outbreak in the United States,[10] EV70 has been associated with

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several outbreaks globally, especially in Asia [11].

However, EV71 has been linked to severe neurological conditions like encephalitis and meningitis and is well-documented for its relationship to hand, foot, and mouth disease (HFMD), especially in children [12]. In contrast to other enteroviruses, human enterovirus 70 (EV70) causes acute hemorrhagic conjunctivitis by reproducing in the eye's conjunctiva and corneal epithelium cells [13] Human enterovirus 70 (EV70), unlike other enteroviruses, replicates in conjunctiva and corneal epithelium cells of the eye and leads to acute hemorrhagic conjunctivitis [14]. The most common symptoms are swelling, congestion, watering, and pain in the eyes, as well as subconjunctival and bulbar oculi hemorrhage. Enterovirus [15,16].

Material and Methods

The study was conducted on 120 patients diagnosed with conjunctival infections. The participants included 70 males and 50 females, ranging from 10 to 80 years, from November 20, 2024, to May 20, 2025. The clinical history of each case and complete information was taken directly from the patient. All information was arranged in a clear and informative manner, such as patient name, age, etc.

All 120 swab samples were collected from Al-Shatrah General Hospital (Outpatient Department), Al-Habboubi Teaching Hospital in Nasiriyah, and private clinics in Al-Shatrah. Inclusion criteria for patients encompassed a range of symptoms from mild to moderate and severe, as evaluated by specialized physicians. Patients diagnosed with conjunctivitis exhibited symptoms such as excessive discharge, tearing, redness, high temperature, and swelling. Samples were collected using special viral swabs that contain specific nutrients essential for viral preservation, in addition to TransZol Up reagent (TransGen Biotech Co., Ltd., Beijing, China), which aids in maintaining the integrity of the virus for

Molecular Identification

RNA Extraction Kit the viral RNA was extracted using Magic Pure® Up Viral DNA/RNA Kit (Cat. No. EC341), supplied by TransGen Biotech Co., Ltd., China. for RNA Extraction from Swabs Samples

This kit is designed for rapid lysis of aquatic and livestock samples, releasing viral DNA/RNA, followed by purification of viral. DNA/RNA using silica-based magnetic beads. It is suitable for extracting viral DNA/RNA from ≤200 µl whole blood, plasma, serum, oral/nasal fluids, urine, tissues, feces, feed, and environmental samples. The resulting product is highly pure and suitable for experiments such as PCR, RT-PCR, qPCR, and qRT-PCR.

Kit Contents

The kit enables efficient lysis of biological and environmental samples, with subsequent binding and isolation of viral DNA/RNA using silica-coated magnetic beads [5].

Reverse Transcription PCR (RT-PCR)

One-step RT-PCR is a streamlined method that combines reverse transcription and PCR

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amplification into a single reaction, eliminating the need for separate cDNA synthesis and PCR steps. This is achieved using a specially formulated one-step master mix, which contains all the necessary reagents for both reverse transcription and PCR amplification in a single tube.

Reverse Transcription PCR (RT-PCR) Using One-Step Master Mix

The PCR master mix reaction was prepared following the manufacturer's instructions provided by TransGen Biotech Inc., China.

Description

One-Step RT-PCR combines the first-strand cDNA synthesis with PCR in the same tube to simplify the reaction setup and reduce the possibility of contamination. Only gene-specific primers can be used for One-Step RT-PCR. TransScript II RT and Trans TaqR HiFi DNA Polymerase are used in the kit.

Measuring RNA with NanoDrop

Measuring the amount and purity of purified RNA is crucial for determining the amount of each sample to use in downstream applications, such as reverse transcription or RT-PCR. NanoDrop Spectrophotometers (NDS), such as the one below, are very convenient instruments for assessing RNA quantity and quality. This is how to use the NDS to measure RNA quantity, followed by a few points on interpreting the 260/280 and 260/230 ratios, important indicators of RNA quality.

Discussion and Results

Results of conventional PCR

The PCR test results showed that all samples were negative, and neither Enterovirus 70 nor Enterovirus 71 was detected, as presented in Table 6.

A.COX gene

The agarose gel electrophoresis image represents the PCR amplification results targeting the COX gene (specific for Coxsackievirus A16), where the expected amplicon size was approximately 550 bp. Despite multiple optimization attempts—including adjusting the RNA template concentrations, modifying annealing temperatures, and employing various cycling conditions—no visible bands corresponding to the expected size were observed across all 64 samples. The absence of PCR products suggests that no detectable levels of Coxsackievirus A16 RNA were present in the tested samples. Several factors could explain the lack of amplification. Primarily, it may be attributed to a very low or absent viral load within the clinical samples, falling below the detection limit of the conventional PCR technique. Additionally, RNA degradation, possibly due to improper sample storage or repeated freeze-thaw cycles, may have compromised the integrity of the template, leading to amplification failure. Another potential cause is inefficient reverse transcription during cDNA synthesis, particularly if the initial RNA quality was suboptimal.

Furthermore, inhibitors co-purified during RNA extraction could have affected the PCR efficiency, preventing proper amplification. Collectively, these findings emphasize that either the viral concentration in the samples was extremely low or absent, or technical factors related to RNA quality and reaction inhibition contributed to the negative results. To overcome these limitations, it may be necessary to employ more sensitive techniques, such as real-time PCR (qPCR) with specific fluorescent probes, which can detect even very low levels of viral RNA and provide quantitative results.

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Enterovirus 71 gene

The provided gel electrophoresis image represents the PCR amplification attempt of the VP1 gene specific to Enterovirus 71, where an expected product size of approximately 264 base pairs was anticipated. Despite multiple optimization strategies, including adjustments in RNA template concentrations, annealing temperatures, and PCR cycling conditions, the gel shows no distinct, specific bands corresponding to the expected VP1 amplicon size in any of the samples. This indicates a failure to detect Enterovirus 71 in the tested samples under the applied conditions. Several possible reasons can explain the absence of detectable bands. Firstly, a very low viral RNA load in the clinical samples could have resulted in insufficient template molecules for successful amplification, especially considering that Enterovirus 71 may exhibit low copy numbers in certain stages of infection or sample types. Secondly, RNA degradation during extraction or handling might have compromised the integrity of the viral RNA, making it unsuitable for reverse transcription and amplification. Another possibility is the presence of PCR inhibitors co-extracted with RNA, which can interfere with enzyme activity and amplification efficiency.

Furthermore, suboptimal changes in temperature may hamper efficient amplification due to secondary structures in the target region, primer design, or primer-dimer formation. Lastly, it could be that the samples lacked Enterovirus 71 RNA, indicating either the absence of infection or viral clearance at the time of the collection, given that amplification products were absent even after optimizations. When combined, these results emphasize how crucial it is to use internal positive controls, high-quality RNA, and sensitive methods of detection in viral diagnostic research in order to preserve accuracy and rule out errors in technology. More sensitive methods, which can identify even extremely low quantities of viral RNA and produce quantitative data, would be necessary to get around the constraints.

Results RT-PCR

Results of patient infections diagnosed with viral conjunctivitis caused by enteroviruses. The analysis revealed in Table 3 that 19 patients (15.8%) tested positive for Enterovirus 70 (EV70), whereas 17 patients (14.2%) were infected with Enterovirus 71 (EV71). A statistically significant difference was observed in the distribution of enterovirus types (EV70 and EV71) among the patient group.

It is noted in Table 4. The results showed that Enterovirus 70 causes viral eye infection at a higher rate in males (52.6%) compared to females (47.4%). There are statistically non-significant differences between males and females in relation to enteroviral infection 71

It is noted in Table 5. The results showed that Enterovirus 71 causes viral eye infection at a higher rate in males (70.6%) compared to females 29.4%. There are statistically significant differences between males and females in relation to enteroviral infection 71.

Molecular detection using Real-Time PCR for Enterovirus 70 indicated that the highest incidence of viral conjunctivitis was observed in the 1- to 10-year-old age group, accounting for 47.4% of the cases. This was followed by the age group of >10 to 20 years, with an infection rate of 26.3%. Older age groups showed significantly lower infection rates: 10.5% in individuals aged 60-70 years, and 5.3% in those aged 70-80 years. Notably, no infections were

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recorded among individuals aged 30 to 60 years, indicating a potential decline or absence of infection within these adult age groups. A statistically significant difference was observed in the age groups in enterovirus 70 in Table 7.

Molecular detection using Real-Time PCR for Enterovirus 71 in Table 8 indicated that the highest incidence of viral conjunctivitis was observed in the 1- to 10-year-old age group, accounting for 47.1% of cases. This was followed by the age group of >10 to 20 years with an infection rate of 23.5%. Older age groups showed significantly lower infection rates (5.9%) in individuals aged 30.3–40.3, 50.5–60.5, and 60.6–70.6 years. Notably, no infections were recorded among individuals aged 40.4–50.4, 70.7–80.7 years, indicating a potential decline or absence of infection within these adult age groups.

The enterovirus symptoms observed in all patients suffering from enterovirus 70 and 71 in eye diseases include fever, redness, pain, secretion, and swelling. As in Table 9, fever was the most common clinical sign associated with all viral infections, followed by secretion, which was the most frequent among sick children, and the other clinical symptoms fluctuated between appearance and disappearance. The statistical evaluation of clinical symptoms among patients infected with Enterovirus 70 and Enterovirus 71 revealed significant associations between specific symptoms and virus type. A highly significant difference was observed in the presence of pain, secretions, swelling, fever, and tearing, indicating that these symptoms were more prevalent among virus-positive patients. However, redness did not show a statistically significant association. As can be seen from Table 10, the rate of viral infections was high during April, followed by an increase in the infection rate in November, March, and then the infections gradually decreased during January, December, and February. The statistical analysis revealed a statistically significant difference.

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Thi-Qar province can be divided into an urban area that includes Al-Nasiriyah region, which has the highest infection rates among all studied renter viruses, 70, as shown in Table 12. Rural areas, which included four areas from which samples were collected to study the spread of enteroviruses, had the highest number of infections recorded within AL-Nasiriyah, followed by Al-Shatrah. In contrast, the lowest number of infections appeared within the regions AL-Nasar and AL-Garraf. The statistical analysis revealed a statistically significant difference.

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Discussion

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Regardless of age group or geographical origin, Enterovirus 70 and Enterovirus 71 were not detected by the usual PCR method in any of the samples that were collected (Thi Qar Province, Nasiriyah). This is most likely due to the low viral load in the samples, which may be below traditional PCR's detection threshold due to its relatively low sensitivity.

Additionally, enteroviruses are classified as positive-sense single-stranded RNA (+ssRNA) viruses, making them particularly prone to degradation during sample handling and RNA extraction [18], thus further compromising detection efficiency. In contrast, real-time reverse transcription PCR (RT-PCR) demonstrated superior sensitivity and successfully detected the viral RNA, highlighting its diagnostic advantage in identifying low-titer infections and confirming its reliability for enteroviral surveillance in clinical samples [19].

The results of RT-PCR showed that the highest incidence of Enterovirus 71 infection (14.2%) was in the age group of 1 to 10 years, with 8 (47.1%) positive cases recorded. The lowest incidence of this virus was in the age group of 30 to 50 years (0%,5.9%), with zero cases reported in the 40–50 age range, and five (26.3%) cases in the 10–20 age group. As for Enterovirus infection 70(15.8%), the lowest number of infections was observed in the 40–50 age group (0%). The results showed that the highest incidence of Enterovirus 70 infection was among the age group of 1 to 10 years, with 9(47.4%) positive cases detected in the examined samples.

Enterovirus was the second most common etiological agent (31.81%) in this study, consistent with previous findings (33.3%) [11]. EV-70 was detected in 77% of enterovirus-positive cases. AHC outbreaks, commonly caused by EV-70, are widespread in developing countries and may affect over half the population in endemic areas [20].

In 1994, one of the most significant outbreaks of enterovirus 70 (EV70) was documented, with a total of 7,509 reported cases, primarily affecting individuals aged 11 to 15 years [13]. The study was conducted in Japan, specifically in cases from Okinawa Prefecture (62% of the total).[21]

A study conducted at a tertiary medical center in Turkey investigated viral causes of conjunctivitis [22,23]. Enterovirus 70 and Enterovirus 71 were each isolated from conjunctival samples at a rate of 6.4%. Additionally, 33.3% of Enterovirus 71 isolates and 16.7% of Coxsackievirus A16 isolates were found in conjunctival samples.[24,36]

Between 1998 and 2007, a total of 1,571 severe cases of hand, foot, and mouth disease (HFMD/HA) were reported in Taiwan [25]. The average age of patients was 2.2 years, with a male-to-female ratio of 1.5:1[26]. Most severe cases occurred in children under 4 years old, with 75% under 2 years. Viral isolation was conducted in several reference laboratories [27,35]. During this period, Coxsackievirus A16 and EV71 were the most common serotypes, accounting for 24% and 17% of identified cases, respectively, followed by Coxsackievirus B3, Echovirus 6, Echovirus 4, Coxsackievirus A10, and Coxsackievirus A6 at lower rates [28,29,30].

In this study, Enterovirus 70 and 71 were isolated from the eyes of individuals across a wide range of ages [31,32]. Notably, the findings revealed infection rates in the 1 to 15-year age group that closely resemble those reported in previous studies [33]. This substantial similarity reinforces the reliability of the current results and suggests a

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potential age-related vulnerability to ocular infections caused by these enteroviruses. Such consistency across studies may point to shared environmental, immunological[34] or behavioral factors that contribute to higher infection rates among children and adolescents [38,39,40].

Conclusion

Enterovirus 70 and 71 are important pediatric viruses associated with ocular infections and severe systemic complications. Molecular techniques facilitate early detection, enabling timely clinical management. Continuous epidemiological surveillance is critical to reducing the risk of diseases and enhancing public health efforts.

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Table 1: Primer sets used in this study with their reference

Primer name	Sequences 5'-'3	TM	PCR product	Source of primer
Human enterovirus 71	Forward primer: GARAGYTCTATAGGRGAYAG Reverse primer: AGAGGGAGRTCTATCTCYCC	48.2°C	264bp	[6]
Coxsackievirus A16	F: AGGGTAATGGARTGTGGTGAYT R: TGTGTGTTGAACCATCACTC	57°C	550bp	[6]

Table 2: Distribution of enterovirus Detected in swab specimens of patient Group by RT_PCR

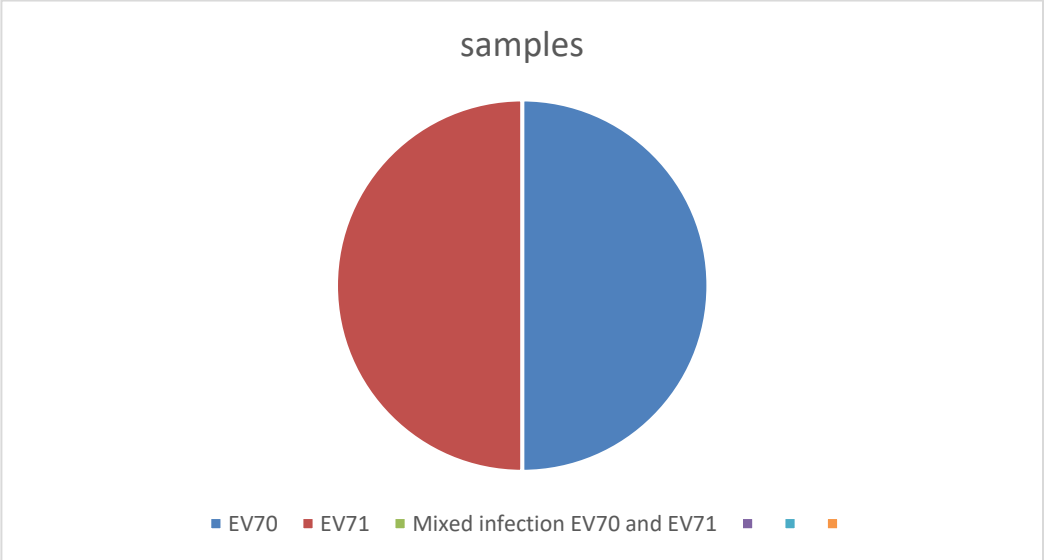

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Table 3: Distribution of Enterovirus70 infection according to sex by RT_PCR in patients' group

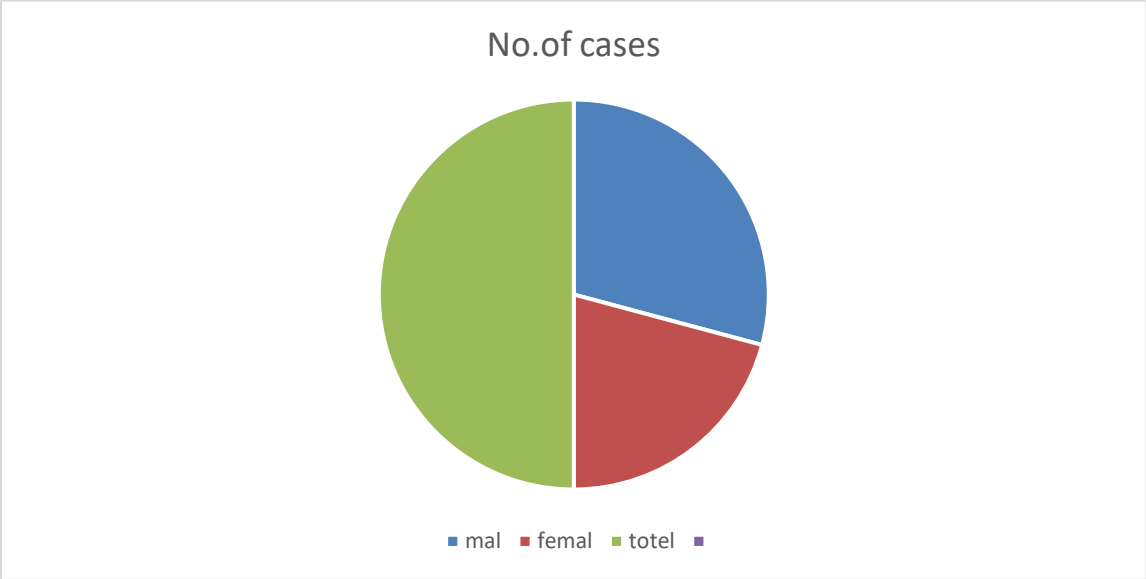

Table 4: Distribution of Enterovirus71 infection according to sex by RT_PCR in patients' group

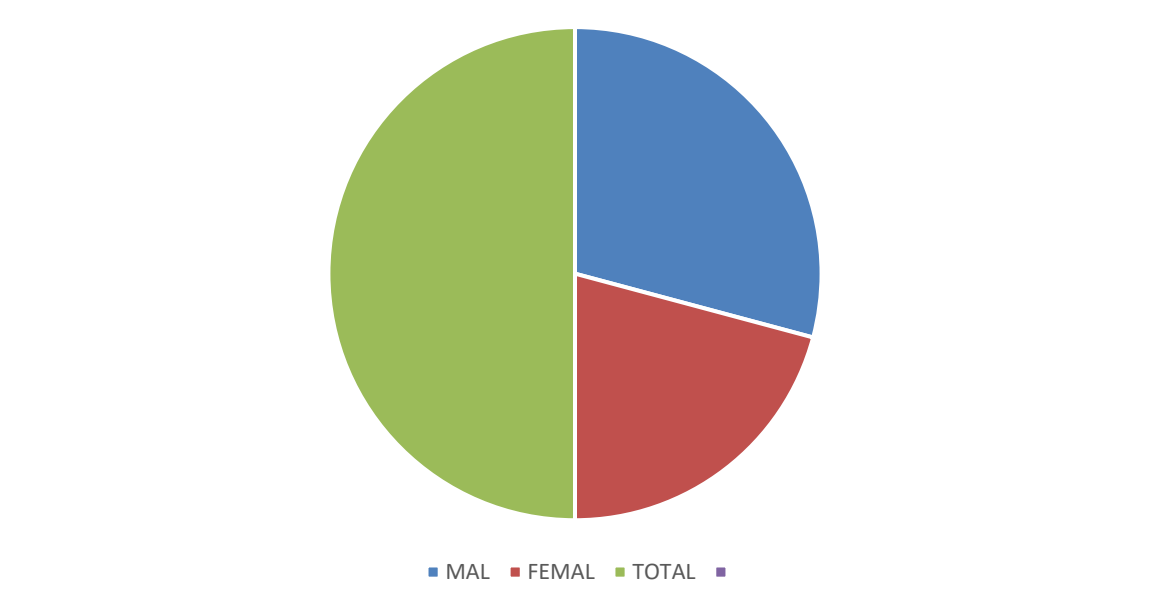

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Table 5: Age Distribution of Enterovirus 70,71 PCR

Age groups (Years)	No. of cases	EV. of +ve cases	%of +Ve cases
1-10	33	0	0%
10.1-20.1	18	0	0%
20.2-30.2	16	0	0%
30.3-40.3	22	0	0%
40.4-50.4	17	0	0%
50.5-60.5	7	0	0%
60.6-70.6	4	0	0%
70.7-80.7	31	0	0%
Total	120	0	0%
Chi-Square: χ^2 (P-value)	--	--	0.00 NS --
Cal: χ^2 =0.00 Tab: χ^2=14.067 DF= 7 p-value: --			
NS: Non-Significant.			

Table 6: Age Distribution of Enterovirus 70 by RT-PCR

Age groups (Years)	No. of cases	EV. of +ve cases	%of +Ve cases
1-10	33	9	47.4%
10.1-20.1	18	5	26.3%
20.2-30.2	16	2	10.5%
30.3-40.3	22	0	0%
40.4-50.4	17	0	0%
50.5-60.5	7	0	0%
60.6-70.6	4	2	10.5%
70.7-80.7	31	1	5.3%
Total	120	19	100%
Chi-Square: χ^2 (P-value)	--	--	14.0257 ** (0.0001)
Cal: χ^2 =14.0257 ** Tab: χ^2=0.057 DF= 7 p-value:0.0001			
* (P≤0.01).			

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Table 7: Age Distribution of Enterovirus 71 by RT _ PCR

Age groups (Years)	EV. of +ve cases	%of +Ve cases
0_10.1	12	70%
10.1_20.1	5	29.4%
20.1_30.1	0	0%
30.1_40.1	0	0%
40.1_50.1	0	0%
50.1_60.1	0	0%
60.1_70.1	0	0%
70.1_80.1	0	0%
Total	17	99.4%
Chi-square: (P-value)	---	10.803 ** (0.0001)
Cal: χ^2 =10.803**	**Tab: χ^2=0.057**	**DF= 7** **p-value:0.0001**
**** (P≤0.01).**		

Table 8: Distribution of patients with Enterovirus 70 and 71 according to the Clinical Symptoms

Type of viruses	En. Of cases	Redness	Pain	Secretion	Swelling	Fever	Tears	P-value
Enterovirus Cox 71	17 positives negative	25% 35%	50% 20%	75% 25%	25% 15%	75% 50%	25% 50%	0.0001 **<0.01
P-value		0.084 NS	0.0071 **	0.0001 **	0.049 *	0.0047 **	0.0081 **	--
Enterovius70	19 positives negative	25% 35%	50% 20%	75% 25%	25% 15%	75% 50%	25% 50%	0.0001 **
P-value		0.084 NS	0.0071 **	0.0001 **	0.049 *	0.0047 **	0.0081 **	--
* (P≤0.05), ** (P≤0.01).								

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Table 9: Distribution of Enterovirus 70 According to the Months of the Year in the 'patients' group

Month		Patient group	
	No. of cases	Ev. Of +ve cases	%of +ve cases
November	24	5	26%
December	12	1	5.3%
January	10	2	10.5%
February	15	1	5.3%
March	27	4	21.1%
April	32	6	31.6%
Total	120	19	100%
Chi-Square: χ^2 (P-value)	--	--	8.902 ** (0.0067)
Cal: χ^2 =8.902 ** Tab: χ^2=0.05.5 DF= 5 p-value:0.0067 * (P≤0.01).			

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Table 10: Distribution of Enterovirus 71 According to the Months of the Year in the 'patients' group

Month		Patient group	
	No. of cases	Ev. Of +ve cases	%of +ve cases
November	24	3	17.4%
December	12	2	11.8%
January	10	1	5.9%
February	15	1	5.9%
March	27	2	11.8%
April	32	8	47.1%
Total	120	17	100%
Chi-Square: χ^2 (P-value)	--	--	10.784 ** (0.0052)
Cal: χ^2 =10.784 ** Tab: χ^2=3.84 DF= 5 p-value:0.0054 * (P≤0.01).			

Table 11: Distribution of Enterovirus 70 According to the different Cities in Thi-Qar province

City		EV group	
	NO. Of cases	Ev. Of +ve cases	%of +ve cases
AL-Nasiriyah	50	8	42.1%
AL-Shatrah	45	7	36.8%
AL-Garraf	15	2	10.5%
AL-Nasar	10	2	10.5%
Total	120	19	100%
Chi-Square: χ^2 (P-value)	--	--	9.446 ** (0.0064)
Cal: χ^2 =9.446** Tab: χ^2=0.05.3=7.815 DF= 3 p- value0.0064 * (P≤0.01).			

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Table 12: Distribution of Enterovirus 71 According to the different Cities in Thi-Qar province

City		EV group	
	NO. Of cases	Ev. Of +ve cases	%of +ve cases
AL-Nasiriyah	50	7	41.2%
AL-Shatrah	45	5	29.4%
AL-Garraf	15	3	17.6%
AL-Nasar	10	2	11.8%
Total	120	17	100%
Chi-Square: χ^2 (P-value)	--	--	9.935 ** (0.0042)
Cal: χ^2 =9.935 ** Tab: χ^2=0.05.3=7.815 DF= 3 p-value:0.0042 * (P≤0.01).			

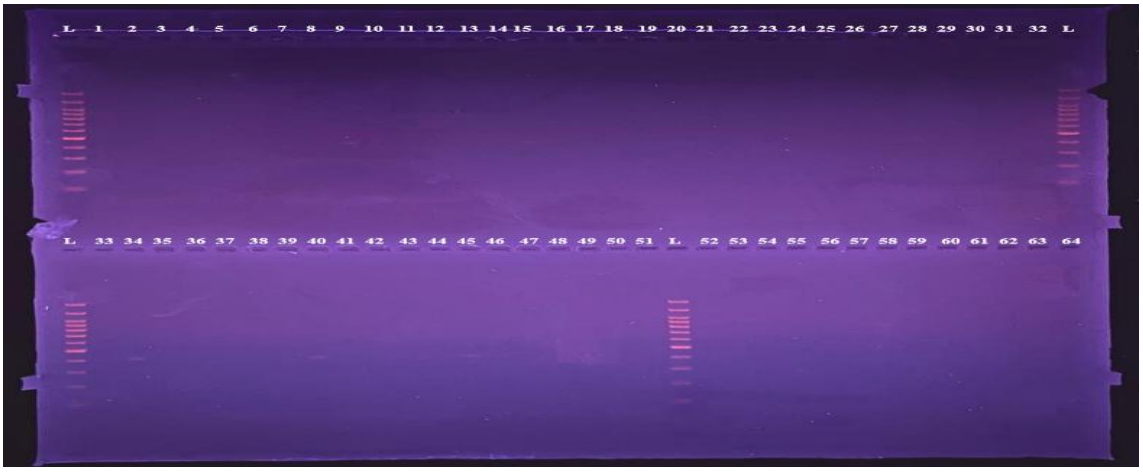

Figure 1: Agarose gel electrophoresis image showing the RT-PCR amplification results targeting the COX gene (expected product size ~550 bp) for Coxsackievirus A16 detection. No specific bands were observed across all 64 samples, indicating negative amplification despite optimization attempts involving RNA concentration adjustments and thermal cycling modifications.

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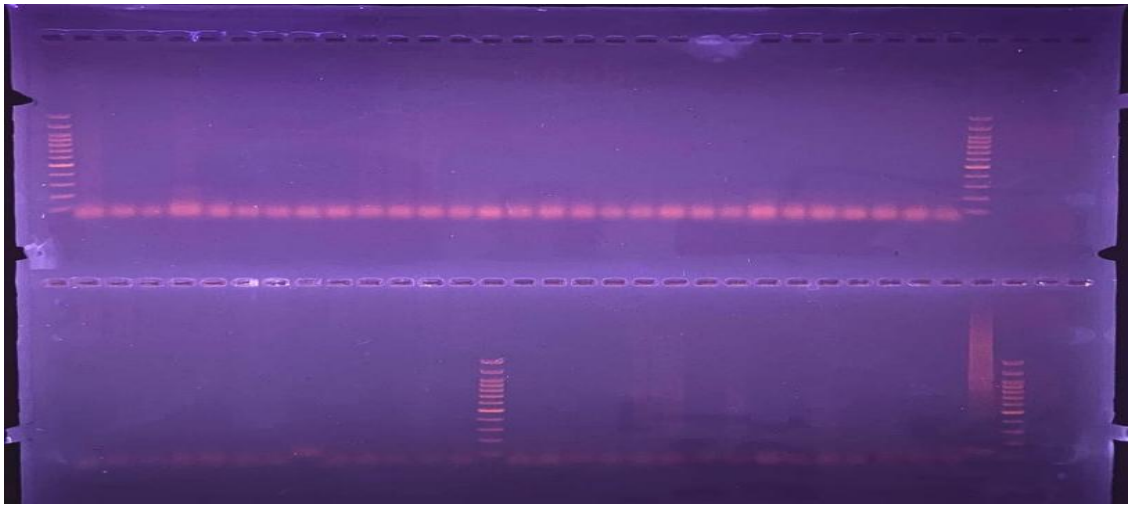

Figure (2): Gel electrophoresis image of RT-PCR amplification targeting the VP1 gene of Enterovirus 71. The expected amplicon size was 264 bp; however, no specific bands were observed across all samples despite multiple optimization attempts, suggesting either a low viral RNA concentration, RNA degradation, the presence of PCR inhibitors, or the actual absence of the virus.