

Detection of Human Torovirus Like Particles and Adenovirus Type F in Children Attending to Babylon Maternity and Children Hospital

Dr. Ziton Abdulrida Ighewish Al-Khafaji
College of Medicine University of Babylon, Hilla PO Box 473, Babylon, IRAQ
Email: torovirus@yahoo.com

Dr. Ghanim About Al-Mola
College of Science for Women University of Babylon, Hilla, Babylon, IRAQ
Email: almolaghanim@yahoo.com

Dr. Youins A. K. Al-Khafaji
College of Dentistry, University of Babylon, Hilla, Babylon, IRAQ

Abstract

Toroviruses are enteric viruses belonging to the Nidovirales order that infect different animal species and human. Torovirus-like particles (TVLPs) that are immunologically related to BRV have been reported as etiological agents of gastroenteritis in humans.

The lack of “in vitro” culture systems for toroviruses, except for the prototype Berne virus or BEV, isolated originally from an infected horse, has hampered their study and the development of diagnostic assays. This study describes a real time RT-PCR method to detect human torovirus-like particles (TVLPs) RNA in clinical stool samples using primers corresponding to the gene coding for the nucleocapsid protein which are conserved in all (TVLPs) strains known to date. During this study, the *CT* value measured during real-time PCR analysis was used as an indication of the viral load found in the stool sample. The assay was evaluated with 72 stool samples from children attending the Babylon maternity and children hospital. Fifty two out of 72 (72.2%) children were shedding virus at the time of sample collection, indicating a high incidence of TVLPs infection in Babylon Province. This is the first study attempted for estimating the presence of TVLPs in Iraq. The real time RT-PCR assay described in this study provides a rapid, highly sensitive, specific and reliable detection and quantization method enabling future TVLPs epidemiological studies. In addition to that the study included the development of real-time PCR assays for the detection of group F *Adenovirus* in 250 stool samples of pediatric subjects exhibiting symptoms of diarrhea and/ or vomiting which were examined. PCR results of 10 positive Adenovirus group F diarrheic stool samples were confirmed by electron microscopy examination which gave clear positive Adenovirus appearance. Till now there was no successful virus culture growth for isolation of diarrhegenic type 40 and 41 grow in routine cell culture. The result of this study by real time reverse transcription – PCR assay reflected in 72.2 % and 58 % torovirus and adenovirus group F respectively. The genotyping results of adenoviruses (genotype 40 and 41) highlight the significance of rapid molecular methods for the routine screening of stool samples in diagnostic laboratories to provide rapid and efficient methods.

Keywords: Human Torovirus, Adenovirus, RT-PCR, Electron Microscopy.

Introduction

Gastrointestinal infections are the third most common nosocomial infection in children, accounting for up to 20% of nosocomial infections in pediatric facilities (Gubbay, *et al.*, 2012). In developed countries, rotavirus, astrovirus and adenovirus are the predominant pathogens found in most studies (Shastri, *et al.*, 1998).

Adenoviruses are classified in the genus *Mastadenovirus*, which contains 7 known HAdV species (HAdV-A to HAdV-G) (Seanne, *et al.*, 2012).

Adenoviruses are non-enveloped, ds DNA viruses with 52 recognized serotypes. The serotypes 40 and 41 enteric adenoviruses are associated with gastroenteritis in pediatrics. Investigations on gastroenteritis prevalence illustrated that the incidence of enteric adenovirus infection is nearly 3 times greater in developing countries than developed ones (Akan, *et al.*, 2009, Mohammad M., *et al.*, 2013).

A decade later, torovirus was the most commonly identified cause of viral gastroenteritis (Jamieson, *et al.*, 1998). However, the etiologic agent can remain undiagnosed in over half the cases of suspected infectious gastroenteritis despite advances in diagnostic technology (Kotloff, *et al.*, 1989).

These enveloped RNA viruses measure 100–140 nm at their largest diameter and contain a tightly coiled tubular nucleocapsid that generally assumes a donut or torus shape in the virion (Weiss, *et al.*, 1983, Duckmanton, 1999). According to the International Committee on Taxonomy of Viruses (ICTV) there are four

main torovirus species: equine torovirus (EToV), bovine torovirus (BToV), porcine torovirus (PToV) and human torovirus like particles (TVLPs). Although previous study indicate that toroviruses are dispersed widely over the world and are highly prevalent in animals, there is little detailed information about them. However, BToV can be obtained in large quantities from calves infected experimentally which has allowed the development of different diagnostic tests, including ELISA (Brown, *et al.*, 1987), Southern blot (- Koopmans, *et al.*, 1991) and RT-PCR methods (Duckmanton, *et al.*, 1998, Hoet, *et al.*, 2003). Hence, most of the epidemiological information about toroviruses is related to BToV which has been detected in United States (Duckmanton, *et al.*, 1998), several European countries (Reuter, *et al.*, 2007), South Africa (Vorster and Gerdes, 1993), New-Zeland (Koopmans and Horzinek, 1995), Japan (Ito, *et al.*, 2007), and South Korea (Park, *et al.*, 2007). On the other hand, very little is known on the incidence of Human TVLPs.

Diagnosis is traditionally by cell culture or antigen detection Methods. diarrheagenic types 40 and 41 will not grow in routine cell culture. Direct antigen detection from clinical specimens may be used for diagnosis of acute infections (Mandel, *et al.*, 2009).

Real-time PCR offers a rapid and sensitive alternative to culture, antigen detection, and serology (Stroparo, *et al.* 2010).

Due to the limitations of conventional diagnostic methods for HAdV and torovirus, the aim of this study was directed to develop and validate a real-time PCR assay for torovirus and group F HAdV from diarrheic stool sample.

Aims of study

The present study aimed to detection of Human Torovirus like particles and Adenovirus type F in children under five years by RT-PCR assay.

Materials and methods

Specimen collection

A total of 322 stool samples were collected from diarrheic children under five years exhibiting symptoms of diarrhea and/or vomiting received at the Microbiology laboratory, Babylon Maternity and Children Hospital from June 2012 to April 2013, The patients' specifications, such as age, sex, and clinical symptoms, including the number of defecation in 24 hours, nausea, fever, respiratory, and feed symptoms, were recorded through a questionnaire.

The samples were obtained only once from each patient and divided into aliquots and stored at - 80°C until analyzed using molecular methods. The frozen sample were transported to public veterinary company in Al - Najif Governorate and Veterinary Medicine College in Al- Qadissiya University.

AccuPower® RocketScript™ RT PreMIX Kit

A restriction manual of this kit were used to synthesized c DNA at a temperature of 42- 72 °C.

Nucleic acid extraction from stool specimens

Fecal samples were thawed immediately before RNA and DNA isolation. All specimens were processed and extracted in a dedicated laminar flow hood.

Total RNA were extracted using *AccuZol™* as protocol to RNA Isolation (Bioneer company) for Torovirus and the DNA extraction for adenovirus by using the *AccuPrep®* stool DNA extraction kit (Melzak, *et al.*, 1996). as the following procedure:

Reverse transcription and real time PCR amplification

Reverse transcription (RT) reaction was performed using Rocket script reverse transcriptase (Bioneer. co. kr.) Briefly, 10 µl of viral RNA purified from stool samples were mixed with 1µl of oligonucleotide plus 9 µl of DEPC for each sample and mixing by exispin device (3000 rpm, vortex 10, cycle 20), than put in the BIO-RAD device (thermal cyler). The reaction mix was incubated for 1 min at 25 °c, followed by 45 min at 50 °c and finally 5 min at 95 °c to inactivate reverse transcriptase. The cDNA preparations were aliquoted, and conserved at - 20 °c until use.

Real time PCR amplification

Real time PCR was performed using SYBR Green detection and melting temperature (Tm) analysis. Power PCR SYBR Green master mix was purchased from Applied Biosystems (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA) for adenovirus amplification and *AccuPower®* GreenStar qPCR PreMix (Bioneer, co, kor.) for the amplification of torovirus.

Electron microscopy exam:

EM and. Stool specimens were kept in tightly closed plastic transport containers at 4°C. Negative-contrast EM was performed by applying a 20% stool suspension in 1% ammonium acetate to a 400-mesh grid precoated with polyvinyl formal and carbon. Excess fluid was removed with filter paper, and the grid was stained for 1 min with a drop of 2% phosphotungstic acid (pH 7.0). The dried grids were placed under a UV source for 5 min for inactivation of virus and were examined in an electron microscope (Philips CM10 EM) at a magnification of x 60, 000.

Results

Among the 322 cases, a virus was identified in 186 torovirus, 52, rotavirus 75; and adenoviruses 41 figure (1). This study was designed to determine whether there was an association between torovirus, adenovirus excretion and gastroenteritis, a retrospective analysis was performed of symptomatic patients whose stool specimens were submitted for bacteriologic testing as a part of the routine investigation outside the study protocol. This analysis revealed no bacterial pathogens in stool specimens from all specimens suspected with torovirus and adenovirus group F for whom bacteriologic testing was done.

The infection was most prevalent during the autumn and winter season (twenty and eleven cases respectively). An analysis of the type of milk (mother's milk vs dry milk) the children were fed showed that most cases belonged to children fed with dry milk (25 cases), (10 cases) mixed fed and only 6 cases belonged to a child fed with mother's milk in adenovirus infection (table 1) while in torovirus infection most of them are mixed feeding.

The concentrations of adenovirus detected in this study ranged between 10^1 genome copies/ μL and 10^6 genome copies/ μL . Of the 250 samples collected over a 1-year period, 30 samples were positive for adenovirus giving a detection rate of 9.3%.

Figure (2) Showed the amplification plot in adenovirus group F.

Figure (3) Showed the standard curve.

The highest concentration of adenovirus ranging between 10^3 genome copies/ μL and 10^6 genome copies/ μL were recorded in samples taken from children infected in rural area.

Result of Human Torovirus in stool sample detected by real time PCR assay

To evaluate the real time RT-PCR method for detection of human torovirus in stool samples. Seventy-two samples collected from diarrheic children were analyzed, fifty-two were positive with real time RT-PCR test as determined by their Ct values ($Ct < 32$), figure 4 and the analysis of melting temperature as in figure 5.

Electron microscopy examination

PCR results of 10 positive Adenovirus group F diarrheic stool samples were confirmed by electron microscopy examination which gave clear positive Adenovirus appearance.

Virus appearance in negative stain

Adenovirus seen in negative stain Phosphotungstic acid (PTA) is a naked virus, icosahedral. The result of electron microscopy examination reflected a large number of adenovirus particles were detected by a negative staining technique. (Figure 6).

Discussion

Gastroenteritis is one of the commonest diseases in children especially in developing countries (Girard, *et al.*, 2006). More than 20 types of viruses cause this illness (Girard, *et al.*, 2006). The medically most important enteric viruses are, group A rotaviruses, caliciviruses, adenoviruses and astroviruses (Jakab, *et al.*, 2005). Rotavirus and adenovirus are important causes of viral diarrhea in many countries and have led to noticeable deaths in children in the past decade (Bernstein, 2009).

HAdV is also a common cause of gastrointestinal disease, and detection of HAdV DNA in stool may precede viremia (Lion, *et al.* 2010). This study describes the development of molecular assays for detection of two agents responsible for viral gastroenteritis from a single stool sample, namely group F adenovirus and human torovirus. A novel method was developed for the prevention of false negatives due to the high levels of PCR inhibitors present in stool samples.

In many different areas of clinical microbiology, molecular diagnostics have been introduced in recent years. The main reasons for introduction of molecular diagnostics are the short time to results and the possibility to detect unculturable or difficult-to-culture viruses. Also in the field of viral gastroenteritis, more molecular diagnostic assays have been published for detection of causative viruses (Logan, *et al.*, 2007, Pang, *et al.* 2004, van Doornum, *et al.*, 2007).

Because human adenoviruses (HAdVs) are double-stranded DNA viruses, they have remarkable stability with regards to several physical conditions such as pH, temperature, and moisture. In addition, their resistance to commercially available disinfectants or wastewater treatments contributes significantly to their persistence in the environment (Maier, *et al.*, 2010).

During this study, the CT (Cq) value measured during RT-PCR analysis was used as an indication of the viral load found in the stool sample. Although RT-PCR allows for the determination of a more exact viral load (in copies or genome equivalents). Melting temperature analysis is the detection phase of the assay and is performed at the end of the analysis after amplification has occurred.

This study provides strong evidence for a causative role for torovirus in gastroenteritis and describes the development of a real time RT-PCR method designed to enable the detection of HTovVLPs in stool samples.

This is the first diagnostic tool for torovirus research based on real time RT-PCR. Molecular methods previously described to detect HTovVLPs are based either on conventional RT-PCR (Matiz, *et al.*, 2002), or electron microscopy analysis (Duckmanton, 1999).

Real time PCR technology using SYBR green as fluorescent dye reduces both the timing of the test, and the risk of sample contamination by lessening sample handling.

Furthermore this approach facilitates the analysis of a large number of samples in a short time. Due to these advantages, real time PCR has been used extensively to develop robust virus diagnostic assays. This approach has been very useful for epidemiological studies involving the analysis of large number of samples (Oka, *et al.*, 2006, Kim, *et al.*, 2007, Meleg, *et al.*, 2008).

In this study the real time RT-PCR is based on the amplification of a highly conserved region of the HToVLPs Np gene. This method has proven to be sensitive and specific for HToVLPs, but it can also be applied to detect other types of toroviruses like BToV. The detection of BToV could be expected since it was described previously that the most recent BToV strains identified in Europe (Smits, *et al.*, 2003). The real time RT-PCR method was evaluated using stool samples from infected infant and children from Hilla province. Fifty two of the total 72 samples analyzed in this study were shown to be HToV-positive. As expected, the nucleotide sequences of all the RT-PCR amplified fragments were shown to be related to torovirus Np gene sequences, thus confirming the specificity of the method.

This study considered as the first formal evidence of the presence of HToVLPs in Iraq. In addition, the data obtained indicate a high incidence of HToVLPs infection in the analyzed stool samples from infected children. The results obtained are in agreement with those of a previous serological and molecular study (Koopmans, 1997, Duckmanton, 1999). Therefore, extensive studies involving more samples from more Geographical areas will be required to establish the prevalence of HToVLPs in Iraq.

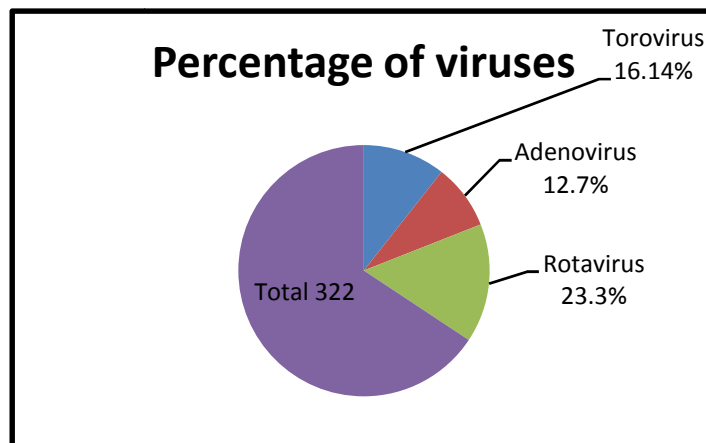
Conclusions

This study report the design, development, and application of real-time PCR assay for the rapid, specific, and highly sensitive detection of group F Adenovirus and Human Torovirus as causative agent in gastrointestinal infection.

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Figure(1) Percentage of viruses

Table (1) . Show type of feeding and type of diarrhea in infected children .

Type of feeding	No. of cases	Type of diarrhea
Mother milk (Brest feeding)	6	Watery
Mixed feeding	10	Watery
Dry milk (Artificial dried milk)	25	Watery

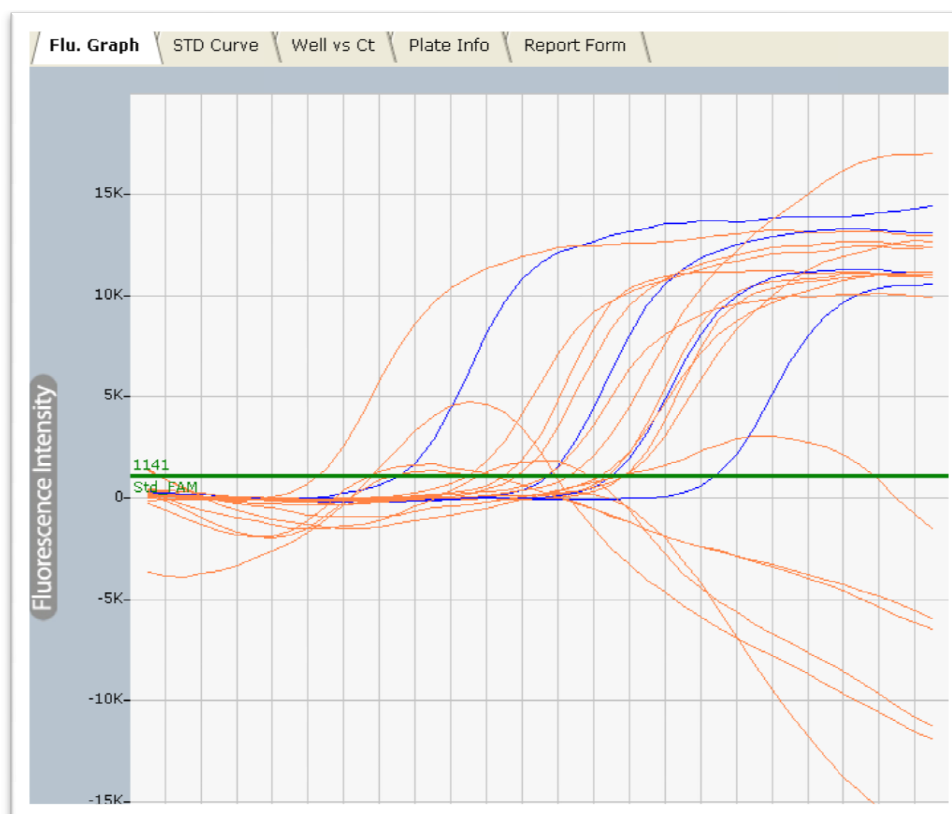


Figure (2) Showed the amplification plot in adenovirus group F by real –time PCR assay .

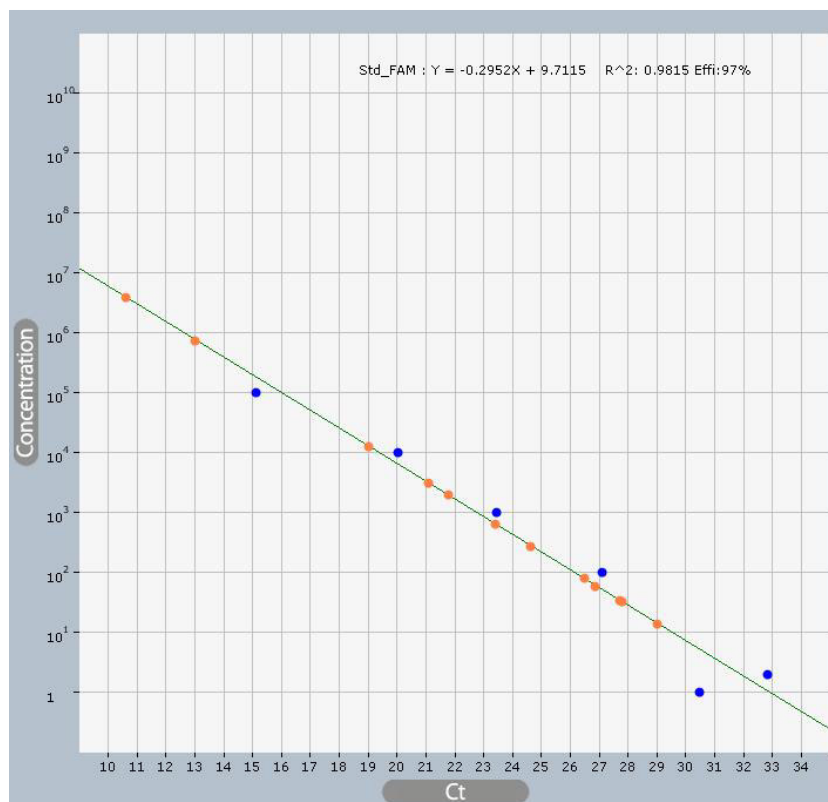


Figure (3) : (Distribution of positive (red circle) and negative (blue circle) samples on the stander curve). for adenovirus types F and G by Real time PCR assay.

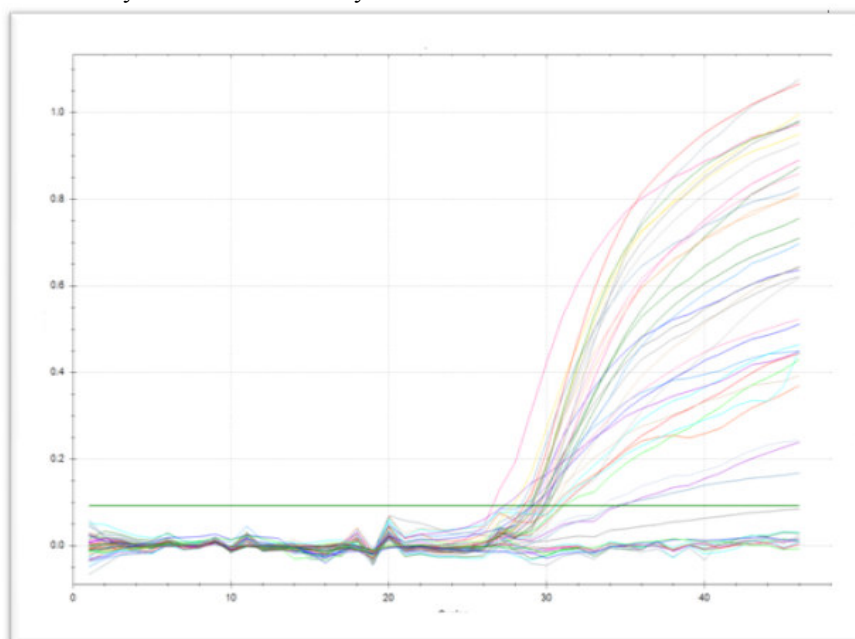


Figure (4) Amplification plot showing the Rn on the y axis (where Rn is the fluorescence intensity of the dye) against the cycle number on the x axis.

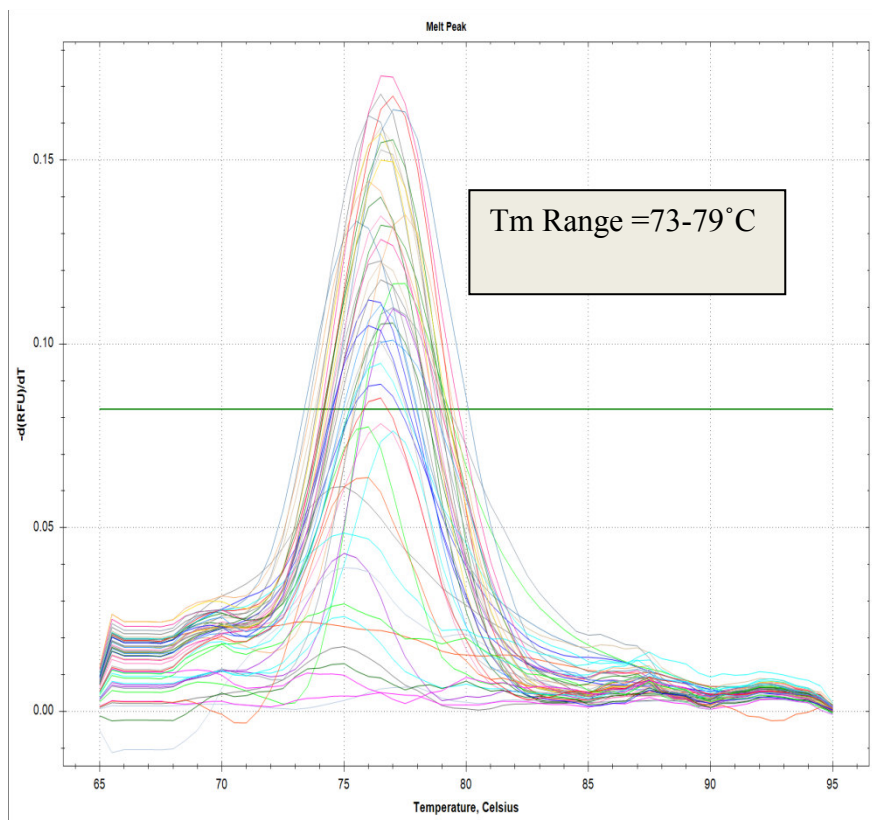


Figure (5) : Melt peak analysis of the results real- time PCR assay . Most of the HToVLPs will melt at 75°C , but the melting point can range from 73°C to 79 °C .

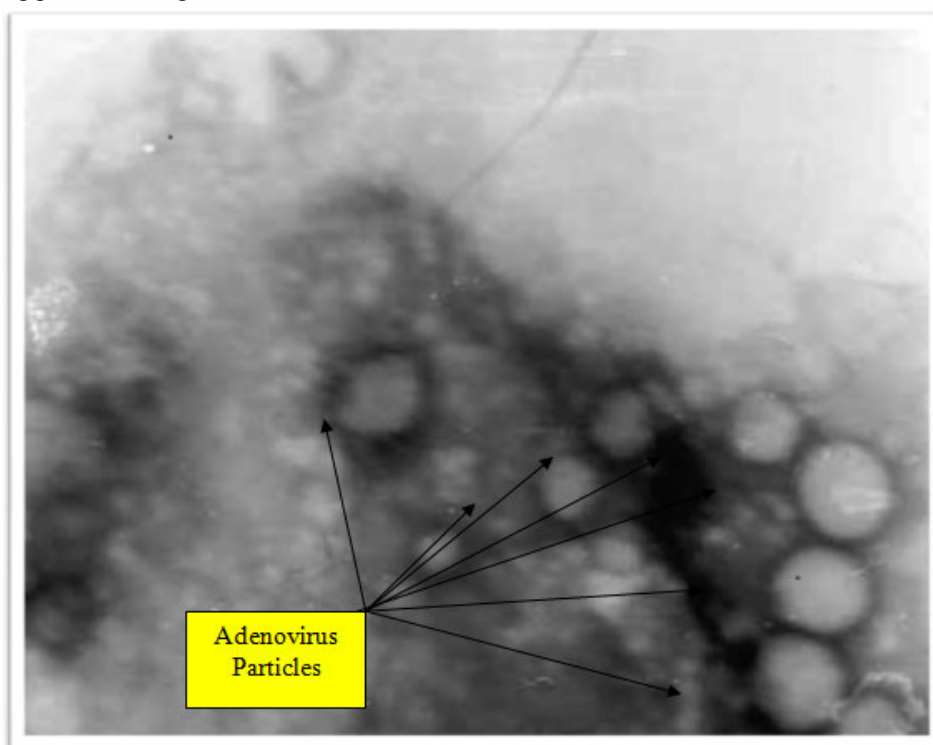


Figure (6) : Transmission electron micrograph image of stool sample from 12 - month- old child (male) with diarrhea , showing viral particles characteristic of enteric adenovirus (Adv.)Magnification 100 , 000 . Negative stain of a large naked icosahedral adenovirus .

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