

Diagnostic Study on NSP5 of Human Rotavirus in Najaf Governorate

دراسة عن البروتين غير التركيبي ٥ لفيروس روتا الانسان في محافظة النجف

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الخلاصة

الهدف: يعتبر فايروس روتا الانسان من المسببات الشائعة لمرض الاسهال والوفيات بين الاطفال الصغار وحديثي الولادة، والذي يسبب حالات اسهال شديدة قد تؤدي احيانا الى الجفاف والموت.

المنهجية: تتناول هذه الدراسة مدى انتشار فيروس الروتا خلال فترة تمتد من شباط ولغاية كانون الاول ٢٠١٤. تم فحص ١٢٠ عينة خروج من الاطفال المرضى بعمر ٥ سنوات فأقل والذين تم جلبهم الى وحدة العناية الطبية في مستشفى الاطفال والولادة في محافظة النجف بواسطة فحص التلازن بطريقة اللاتكس المعتمد على الاضداد وحيدة النسيلة والخاصة بالبروتين الفيروسي VP4 و VP7 ومن ثم اجراء فحص الـ PCR.

النتائج: تم تحديد الإصابة الموجبة في ٨٠ عينة ونسبة ٦٦,٧ % من مجمل العينات المفحوصة بطريقة اللاتكس بالإضافة الى المحاولة الى الزرع الخلوي للفايروس، في حين اظهرت تقنية تفاعل البلمرة real-time PCR الخاص بتحديد البروتين غير التركيبي NSP5 وجود الإصابة في ٨٥ عينة ونسبة ٧٠,٨ % من مجموع الحالات. كما تم تشخيص الإصابة في الاطفال الاصحاء والذين تم تضمينهم كمجموعة سيطرة، ٥٠:١ (٢%) بطريقة اللاتكس مقابل ٥٠:٣ (٦%) تم تشخيصها بطريقة تفاعل بلمرة الجين المشفر عن البروتين غير التركيبي NSP5. وقد تم انتقاء ١٠ عينات من فايروس الروتا لإصابة خلايا الخط الزرع rhabdomyosarcoma cell line لتحديد فيما لو كانت هذه الخلايا ذات فعالية في اظهار الآثار المرضية لفايروس الروتا، حيث اظهرت ٨ عزلات فايروسية (٨٠%) الإصابة في هذه الخلايا.

الاستنتاج: نستنتج من هذه الدراسة ان تقنية تفاعل البلمرة real-time PCR تعتبر نوعية وحساسة في الكشف عن الجين المشفر عن البروتين غير التركيبي NSP5، وان خلايا الخط الزرع RD ناجحة في تنمية فايروس روتا الانسان.

التوصيات: ونوصي بأجراء الدراسات العلاجية المستقبلية باعتماد استنتاجات هذه الدراسة.

ABSTRACT

Aims: Human rotavirus is the common cause of diarrhea-related illness and death among infants and young children, can lead to severe and sometimes lethal dehydration .

Objective : This study deals with the prevalence of rotavirus during a period extending from March to December 2014 . A total of 120 samples of diarrheal samples from patients up to 5 years age, who were admitted for medically care unit in the hospital of pediatrics and maternity of Al-Najaf governorate were investigated by latex agglutination, based on specific monoclonal antibodies directed against VP4 and VP7 of human rotavirus then the detection by PCR technique and isolation of the virus on thssue culture.

Results : Rota virus was detected in 80 cases (66.7%) of the total cases investigated by latex agglutination test, while PCR positivity was recorded in 85 cases (70.8%). Rota virus infection was also detected in healthy children who were involved as control group; 1/50(2%) detected by Latex agglutination assay versus 3/50 (6%) detected by NSP5 gene PCR assay. From the total of ten isolates that selected to propagate via cell culture using rhabdomyosarcoma (RD) cells, confirmation of the detection was accomplished by 8 isolates (80 %).

Conclusions : We concluded that polymerase chain reaction is sensitive and specific technique for the detection of NSP5 coding gene and RD cell line is successful for the propagation of rotavirus.

Recommendations : we recommended to achieve future therapeutic studies depending on this study conclusions.

Key words: RD cell culture, rotavirus NSP5, real-time PCR.

INTRODUCTION

Rotavirus, a major causative agent of infantile diarrhea, it contains two independent outer shell protective antigens (VP4 and VP7) that elicit neutralizing antibodies and induce resistance to infection when used for immunization ^(1, 2). Viral shedding may continue days to weeks after symptomatic recovery, depending on immunocompetence in both humans and animal models ⁽³⁾. The virus contains 11 double-stranded RNA segments that code for six structural proteins, namely, VP1 to VP4, VP6, and VP7, and six nonstructural proteins, NSP1 to NSP6 ^(4,5). During

infection, viral mRNAs direct the synthesis of viral proteins and serve as templates for genomic double-stranded RNA. Since rotavirus is a cytoplasmic virus, the processes of viral RNA packaging, assortment, replication, and assembly of the double-layered particle are thought to occur in large discrete cytoplasmic electron-dense particulate structures called viroplasms⁽⁶⁾. Several rotavirus proteins (VP1, VP2, VP3, VP6, NSP2, NSP5, and NSP6) have been found in viroplasms during infection^(6,7). Rotavirus NSP5 is a non-structural phosphoprotein with putative autocatalytic kinase activity, and is present in infected cells as various isoforms having molecular masses of 26, 28 and 30–34 kDa, that encoded by genome segment 11 of rotavirus A and accumulates in the viroplasms⁽⁸⁾. Although the insolubility of NSP5 is not determined by hyperphosphorylation, NSP5 similarities to keratins and its presence in viroplasm-like structures suggest the potential for NSP5 to fulfill similar virus-specific cytoskeleton-like functions during infection⁽⁹⁾. The efficacy of specific rotavirus vaccine required enough data about diagnostic protocol and virulence factors in order to prepare a future strain specific vaccine against severe rotavirus gastroenteritis in infants in developing countries in Asia^(10,11). In our recent study, rotavirus isolates were investigated latex agglutination diagnostic assay, NSP5 gene real-time PCR assay and also involved investigation of RD cell line to propagate rotavirus particles with the confirmation of the detection by indirect fluorescent assay.

Objectives of the study:

Materials and Methods

Study subjects and case definition

One hundred and twenty infants and children suffering from acute diarrhea with ages of 1 month to 5 years admitted to the hospital of Maternity and Pediatrics in Al-Najaf governorate. Fifty healthy children without any apparent infections were also involved as a control group. Data about each patient involved age, gender, and being acute or chronic. Patients diagnosed as having an acute watery diarrhea if they had ≥ 3 liquid stools per day with duration period no more than 8 days⁽¹²⁾. Healthy persons (control group) were individuals without diarrhea.

Detection of rotavirus by latex agglutination (LA) test

Rotavirus latex test kit was used to perform agglutination test according to the recommendations of the manufacture company (plasmatic laboratory products, UK). It is a rapid qualitative test for direct detection of rotavirus particles in stool samples by agglutination.

RNA extraction.

Viral RNA was extracted from fecal materials and from a preparation of the tissue culture-adapted isolates using the NORGEN Viral RNA purification kit (NORGEN, Canada), according to the manufacturer's instructions. The concentration and the purity of the extracted total RNA were determined by measuring the absorbance ratio

at wavelength 260 nm over 280 nm using scandrop spectrophotometer (analyticajena-Germany) about 53-243.34 ng.

Detection of NSP5 gene by RT-PCR.

The extracted RNA was denatured at 95°C for 10 min, and reverse transcriptase PCR (RT-PCR) was carried out by using a ingene One Step RT-PCR kit (Ingene/UK). The RT-PCR was carried out with an initial reverse transcription step of 10 min at 55°C, followed by PCR activation at 95°C for 8 min, 50 cycles of amplification (10 s at 95°C, 60 s at 60°C) for NSP5 with a final extension of 7 min at 60°C.

Cell culture assay

Rhabdomyosarcoma (RD) cell line monolayer was prepared in 25 cm³ flask and lab-tek slides for the viral propagation and revealing of the specific viral cytopathic effects. Detection of virus was achieved by indirect immunofluorescent antibody assay. pH of growth and maintenance media were adjusted at 6.8-7.2. The cells was incubated at 37 °C and examined daily until complete monolayer was formed. Growth medium was supplemented with 10% fetal calf serum (FCS) and maintenance was supplemented with 2 % fetal calf serum, 100IU/ml penicillin and 100µg/ml streptomycin for cells culture. Viral particles were treated with 0.5µg of trypsin per ml and incubated for 30 min, cell monolayer was then inoculated with 0.5 ml volume of treated sample to each 25 cm³ cell culture flask. After virus adsorption at 37 °C for 1 hr. (with continuous rolling every 10 min), flasks were washed three times with maintenance medium then incubated at 37 °C and checked daily for virus growth by inverted microscope for detection of any cytopathic effect (CPE) ⁽¹³⁾.

RESULTS

LA and PCR results

Table 1 : The positivity of rotavirus detected by latex agglutination test and by RT-PCR technique in diarrheal samples as compared with control group

Cases examined	Latex test positivity	NSP5 gene PCR positivity
Diarrhea-related illness	80 / 120(66.7%)	85/ 120 (70.8%)
Healthy individual	1/ 50 (2%)	3 /50(6%)

P< 0.05

This table showed that The positivity of rotavirus latex agglutination among infants and young children was detected in 80 (66.7%) of a total samples, whereas 40 samples (33.3%) were negative. LA test was done for all samples obtained from the infants and young children in al Najaf governorate. The LA test was aimed to detect

specific monoclonal antibodies directed against VP4 and VP7 of human rotavirus. From 50 stool samples related to healthy infants and children; two cases (2%), and 3 cases (6%) were positive to latex and PCR respectively. In case of RT-PCR technique, the total positive results were 85 cases out of 120 examined samples (70.8%) Fig.-1.

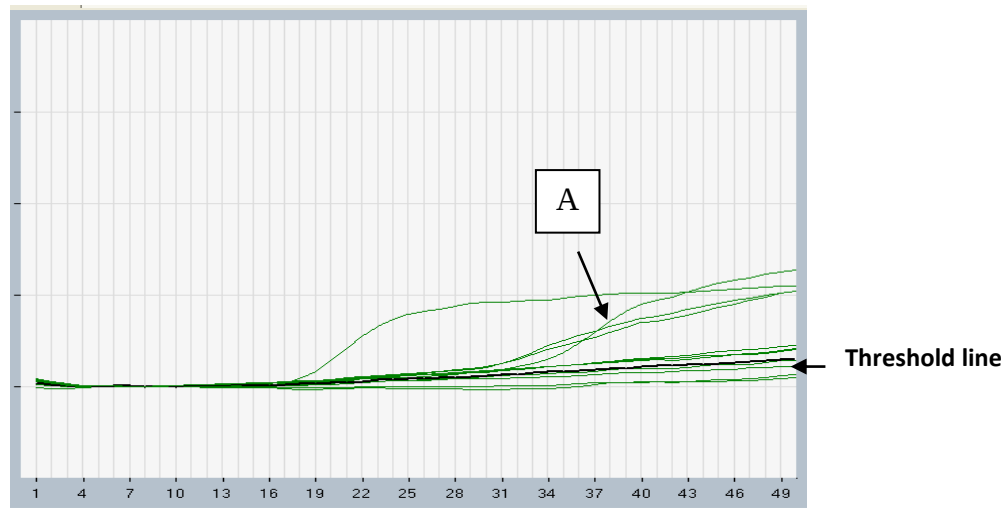


Figure -1: Fluorescence data (FAM) collection during 60°C extension for rotavirus, their curves higher the threshold line were positive results and the negative result the curves under threshold line in RT-PCR for rotavirus detection. (A: positive control while other line graphs were extracted genome applied to PCR).

Cell culture results

From the total of 10 RT-PCR positive isolates there were eight (80%) revealed positive viral results and give specific rotavirus intracytoplasmic inclusions as detected in cell culture propagation. The cellular localization of NSP5 during the infection cycle was examined by immunofluorescence. The cells were infected with rotavirus, fixed with paraformaldehyde at different time points postinfection, and then stained for NSP5-specific immunofluorescence. The NSP5 was visible in the cytoplasmic inclusions characteristic of rotavirus infection already 2 h after infection. As the infection proceeded, the viroplasmic inclusions increased in size and released proviral particles. However, NSP5 remained in the viroplasmic inclusions even late in infection, suggesting that the polypeptide forms part of a structure involved in Figure (3-D).

Detection of Rota virus in cell culture

The demonstration of specific rotavirus antigen in infected cells at each passage was accomplished by Indirect Fluorescent Antibody Technique (IFAT) on Lab-Tek slides, which had been carried out after 48 hours PI and from the first passage by

using RD cells. This character was increased with subsequent passages represented by the appearance of fluorescence cells with bright cytoplasm fluorescence which occupied most of the cytoplasm in severe extensive infection of passages with the peak of TCID₅₀ of 10⁵/0.1 ml in the sixth passage (Figure 2-B and Fig.3-C).as compared to confluent monolayer remained without change (Fig. 2-A)

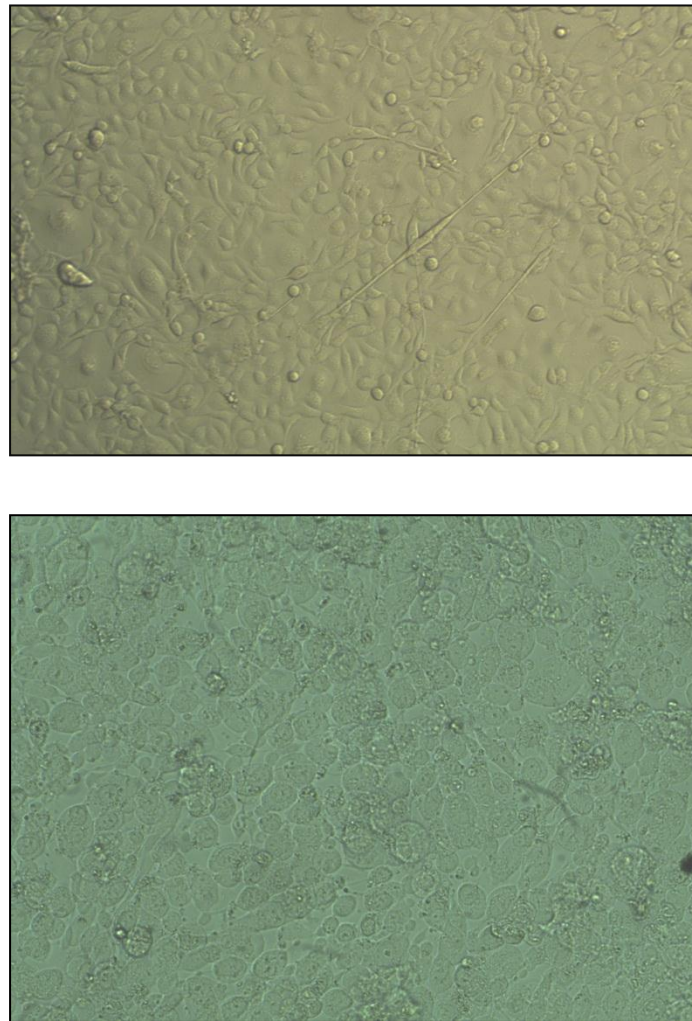


Fig. -2: Cytopathic effect of rotavirus infection on RD cells: A-Normal RD cells B- Infected RD cells after 48 hrs. PI at Sixth passage (100X magnification).

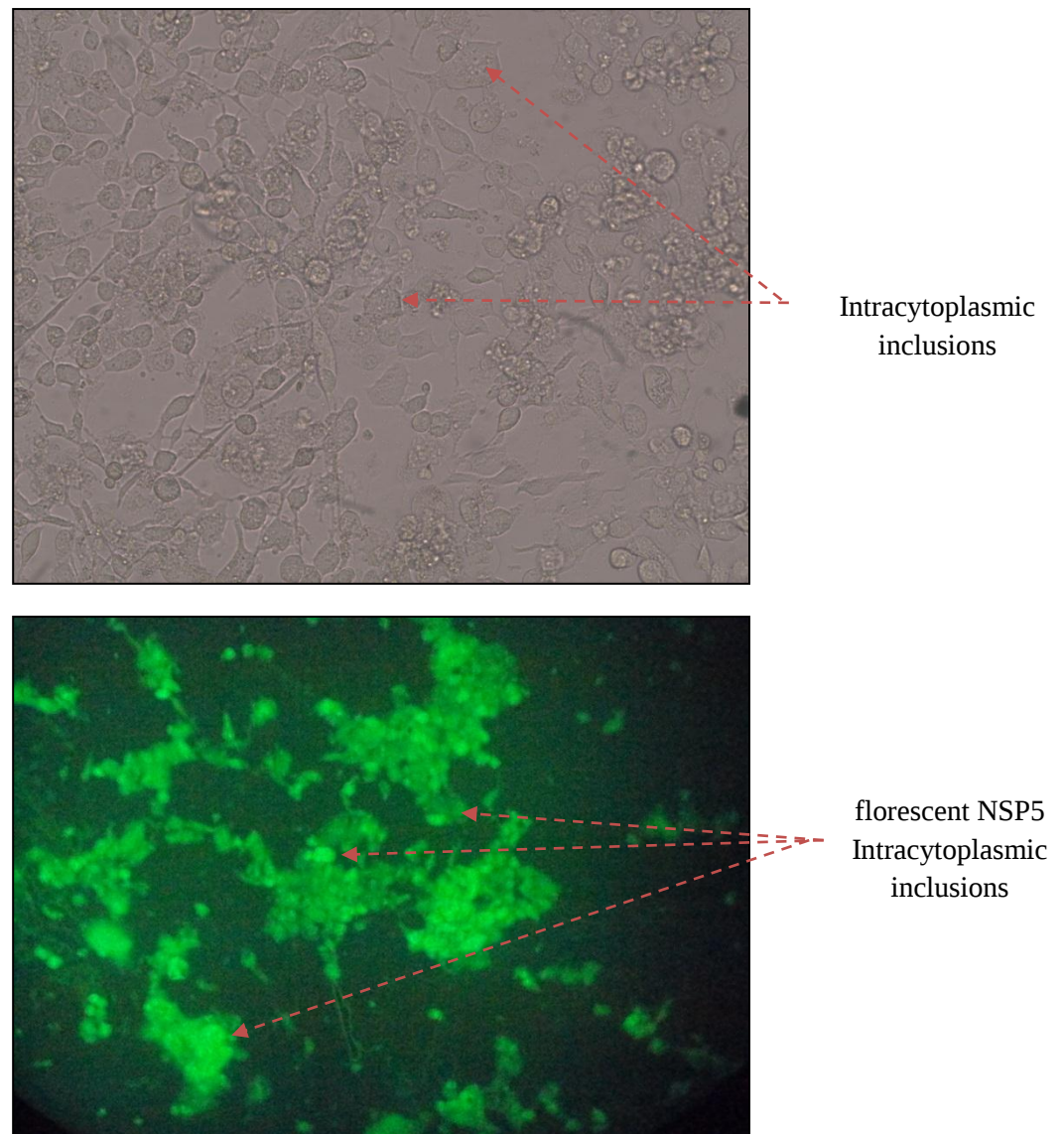


Figure (3): C- Infected RD cells after 72 hrs PI: D- rotavirus infection on RD cell line as detected by IFAT at Sixth passage (100X magnification).

DISCUSSION

NSP5 gene PCR positivity was detected in 85/ 120 (70.8%) and slightly more as compared to latex agglutination testing, 80 / 120(66.7%).The results were statistically not significant($P>0.05$). Real-time PCR detected all specimens identified as positive for rotavirus by latex agglutination or antigen detection, the design, development, and application of real-time PCR assays is rapid and specific ^(14,15) .

Some rotavirus infections are asymptomatic, that detected in 1/50 (2%) with latex and 3/50 (6%) with PCR which suggests that both viral and host factors can affect disease severity ⁽³⁾. The viral factors are the following. (i) some alleles of VP4 may be associated with asymptomatic disease ^(16,17). (ii) virus strains can be attenuated,

particularly by passage in cell culture. Attenuation generally results in a restricted ability to replicate and cause disease in the host ⁽¹⁸⁾. (iii) virus strains seem to be adapted for growth in particular host species ⁽¹⁷⁾.

Our results were came in agreement with Londrigan *et al.*,2000⁽¹⁸⁾ who mentioned that RD Cells can support rotavirus infection and propagation, and any replication of human rotaviruses on (RD, HepG2, Caco-2, COS-7 and MA104) expressed $\alpha 2\beta 1$. Previously, it has been shown that cellular integrins $\alpha 2\beta 1$, $\alpha 4\beta 1$ and $\alpha X\beta 2$ are involved in rotavirus cell entry. Londrigan *et al.*,2000⁽¹⁸⁾ mentioned that only RD cells expressed $\alpha 4\beta 1$. Nejmeddine *et al.*(2000)⁽¹⁹⁾ also used IF technique for the monitoring about the HRV-VP4 proteins, and mentioned that VP4 is located in the space between the periphery of the viroplasm and the outside of the endoplasmic reticulum in rotavirus-infected cells (MA-104) as a green fluorescent proteins. These fluorescent areas became obvious after 24 hrs. post inoculation. The indirect immunofluorescent antibody technique depended on the reaction of antigen and antibody conjugated to fluorescent isothiocyanate (FITC). The fluorescent particles were regarded as intracytoplasmic inclusions which contained viral particles. Our results indicate that the addition of trypsin (0.5% of maintenance media) is very helpful for the cultivation of rota virus on RD cells. This result was also came in agreement with Corthesy *et al.*,2006⁽²⁰⁾ who used the same protocol for viral particles trypsin pretreatment before infection. These results were also came in agreement with Arnold *et al.*,2009⁽²¹⁾,who mentioned that Cultivation of animal and human RVs in cells requires proteolytic activation of the viral attachment protein using trypsin.

The work presented here shows that the viroplasms gradually increased in size during infection and that NSP5 remained in these structures, suggesting that it forms part of a scaffold for the early steps of virion morphogenesis. NSP5 accumulates together with VP6 in viroplasms ⁽²²⁾. However, NSP5 alone cannot be the viroplasm organizer, since expression of the polypeptide in the absence of the other rotavirus gene products did not lead to formation of viroplasm-like inclusions. Instead, material reactive with NSP5 antibodies formed a diffuse staining in the cytoplasm, even when the posttranslational modification of the polypeptides apparently was the same as in infected cells. We concluded that polymerase chain reaction is sensitive and specific technique for the detection of NSP5 coding gene and RD cell line is successful for the propagation of rotavirus.

CONCLUSIONS :

We concluded that polymerase chain reaction is sensitive and specific technique for the detection of NSP5 coding gene and RD cell line is successful for the propagation of rotavirus.

RECOMMENDATIONS :

We recommended to achieve future therapeutic studies depending on this study conclusions.

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