

The effects of extraction methods on the positivity rate of rotavirus infection

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Received 5 /4 /2005 : accepted 22 /5 /2005

Abstract

The laboratory diagnosis of human rotavirus (HRV) based on many different methods. The latex agglutination test was the only method used in several hospitals in Iraq to detect HRV in stool samples. The frequency of positivity rate was proportional to the concentration of virions in the sample. The aim of this work was to study the efficiency of different extraction methods as filtration, sonication, ultracentrifugation and Freon-ether) on the results of Latex test also on the virus yield in tissue culture. The effectiveness was determined semi-quantitatively by LAT. All methods give statistically significant differences at $p < 0.001$ except the low speed centrifugation gave differences at $p < 0.005$. The results reveal that there are clear effects on positivity rate between the use of available methods and the sonication, ultracentrifugation and Freon -ether methods. The results of tissue culture revealed that the cytopathic effects were visible when the virus preparation processed by the mentioned methods cause CPE after 12,24 and 48 hrs respectively in comparison with the available methods. The recommended methods for detection of RV particles from clinical specimens were sonication, ultracentrifugation and freon-ether.

تأثير طرق الاستخلاص على معدل ايجابية الإصابة بفيروس الروتا غانم عبود جابر المولى

المستخلص

الفحص المختبري للبحث عن فايروس الروتا يتم على اساس اختيار عدة طرق و يعتبر اختبار تلازن اللاتكس من الفحوصات الشائعة في معظم مختبرات المستشفيات في العراق للتحري عن فايروس الروتا في النماذج المرضية وبما ان نسبة الحالات الموجبة تتناسب مع تركيز جزيئات الفايروس في النموذج . لذا كان هدف هذه الدراسة تقييم كفاءة طرق الاستخلاص المختلفة (الترشيح، الموجات فوق الصوتية، الطرد المركزي العالي و طريقة الفريون- أثير) وتأثير ذلك على نتائج فحص تلازن اللاتكس وبالمقارنة مع الطرق المتبعة. وقد بينت النتائج ان هنالك قيمة احصائية معنوية بمقدار $p < 0.001$ لكافة الطرق عدا طريقة الطرد المركزي المنخفض ذا قيمة معنوية $p < 0.005$. كذلك بينت الدراسة ان هنالك تأثيرا واضحا عند استخدام طرق الاستخلاص اعلا مع النتائج الموجبة كما اوضحت الدراسة عند استخدام المزارع النسيجية ان حقن المستحضرات الفايروسية بالطرق الواردة اعطت تأثيرات معلة (CPE) بعد 12,24,48 ساعة بالمقارنة مع الطرق الاعتيادية. توصى الدراسة باستخدام الطرق الواردة لغرض تنقية فايروس الروتا للحصول على نتائج جيدة من العينات السريرية.

Keywords: Extraction methods, Positivity rate, Rotavirus, Infection

Introduction

Rotavirus is known to be a major cause of acute diarrheal disease in the young of numerous species including human. Rotavirus particles multiply in the cytoplasm of enterocytes and damage their transport mechanism (1). Damaged cells may slough into the lumen of the intestine and release large quantities of virus, which appear in the stool (up to 10¹⁰ particles per gram of feces) (2, 3) There is a special need for a sensitive and rapid methods for the identification of the virus in the feces. The laboratory diagnosis of human rotavirus (HRV) based on many different methods. In addition to electron microscope (EM), enzyme immunoassay, immunofluorescence tests, Latex agglutination test and more recently, several sensitive and specific techniques have been developed, which include dot-immunobinding assay, radio-immunoassay (RIA), and polymerase reaction test (PCR) (4). Since the Latex agglutination test was the only method used in several hospitals in Iraq to detect HRV in stool sample, therefore only this technique will be discussed here. The evaluation of a Latex test for HRV detection revealed that the frequency of positive test was appeared to be proportional to the concentration of virions in the stool, as the sensitivity was decreased in stool specimens contained low RV level. (5,6) The Latex test required greater numbers of rotavirus particle for detection in comparison with other methods (7,8). Therefore the aim of this work was to study the efficiency of different extraction methods on the results of Latex test to improve the test results.

Materials and Methods

In all experiments 10% of fecal specimens were processed by four

separate methods before testing by Latex agglutination test (LAT): blending centrifugation method, sonication method, filtration method, blending Freon-ether method. For the first method, the specimens was suspended in buffer and well mixed by stirring followed by centrifugation at 800 g for 10 min. In the second method the samples were sonicated for 45 second at 5000 MHZ with ultrasonic homogenizer (Cole Parmer Inst.Co.USA), the third method centrifugation was made at 4000rpm for 30 min to get rid of fat, solid substance and other contaminants then the supernatant fluid was filtered through a membrane sterile filters with pore size 0.45mm, 0.2mm. The fourth method consist of several steps, the blended material was mixed with an equal volume of Freon 12 (1,1,2-trichloro-1,1,2-trifluoro ethan) and blended an additional 2min at 4C. The aqueous phase was vigorously mixed with ether to decontaminate the samples, and dissolved Freon was removed with the separated ether phase.

The primary isolates for the virus was adapted on a secondary Fetal Bovine Kidney cell cultures which were obtained for this experiments from AL-Kindy Company For Vaccines and Sera (Baghdad-Iraq) cells monolayer were prepared with Eagle's minimum essential medium (MEM) containing 10% fetal calf serum. The processed fecal specimens were mixed with equal amount of trypsin at concentration of 20 mg/ml and the mixture was inoculated onto confluent monolayer (0.2ml/flask) after incubation for 30 min at 37C, MEM with antibiotic was added. The cell culture were then incubated at 37 C until the cytopathic effect (CPE) was essentially completed.

Results

The primary rotavirus isolate examined by the current study were extracted by six different methods (table 1). The amount of agglutination of RV antigen and the reaction was scored as weak positive (WP), moderate positive (MP) and Strong positive (SP). The Fisher statistic test was used for analysis. Each method was compared with stander method (given by manufacturer) as follows: using low speed centrifugation method 23.8%WP, 36.3%MP and 40%SP at $p < 0.05$. In filtration method the percentages were 33.8%WP, 28.8%MP and 37.5%SP: $p < 0.001$. The results of ultrasonication method were 26.3% WP, 13.8%MP, and 52.5%SP. The ultracentrifugation was 33.8%WP, 13.8%MP, and 52.5% SP: $P < 0.001$. The Freon-Ether method was 27.5%WP, 21.3% MP and 51.3% SP: $p < 0.001$. The methods were compared statistically with each others given significant differences.

The Filtration method was compare with each of Ultrasonication, Ultracentrifugation and Freon-Ether method gave $p < 0.001$, $p < 0.001$ and $p < 0.001$ respectively. Similar differences were obtained by the comparison of Low speed centrifugation method with each of ultrasonication, ultracentrifugation and Freon -ether method. The statistical comparison was significant between ultrasonication method with each of ultracentrifugation and Freon -ether method at $p < 0.01$ and $p < 0.05$ respectively and the last statistical comparison between ultracentrifugation and Freon-ether was significant with $p < 0.01$.

The primary rotavirus isolate from the specimens examined in the current study was extracted by the six procedures mentioned above were inoculated onto washed cell culture.

The virus preparation processed by the Sonication method caused CPE after 12hr while cell destruction by sample treated by stander procedure was less obvious. The sample extracted by ultracentrifugation, Freon-ether and filtration procedures give CPE better than samples extracted by Low-speed centrifugation and stander procedure (Fig 1, a, b, c, Fig 2, a, b).

Discussion

Previous studies carried out in many developing countries indicated that RV is an important pathogen responsible for 7%-65% of diarrhea in patients less than 5 years old.(2,9) RV may persist in the stool for 2to3 weeks but in the majority of cases the virus was not detectable although large number of virus particles were shed in the stool following multiplication in epithelial cells of the small intestine .The shedding may persist for 10 days or more after the illness, but maximal virus shedding occurs in 2to5 days after the onset as reported by many authors(10,11,12).The purification is defined as freeing virions from associated cell debris or even from viral protein synthesized in excess in infected cells. IN this study the determination of the effectiveness of the purification methods was determined semi-quantitatively by LAT and scored as weak, moderate and strong positive.

All methods statistically gave significant differences at $p < 0.001$ except the low speed centrifugation gave significant differences at $p < 0.05$ with stander method .This is because the two methods depended upon the use of low speed centrifugation in comparison with stander methods. The Filtration method was less effective in comparison with stander at $p < 0.001$. The other three methods i.e. Sonication, Ultracentrifugation and Freon-Ether gave significant

differences. The results revealed that there is a clear effects on the positivity rate between the use of available laboratory methods and the other three methods (Sonication, Ultra-centrifugation and Freon- ether). The

basic facts underline the purification of viruses that all viruses contain substantial quantities of protein and hence is more or less susceptible to protein fractionating. The second that the size and densities of viruses are not

Table 1: Relation between different purification methods with amount of rotavirus antigen in stool specimens by using latex agglutination test.

Purification methods	Weak positive		Moderate Positive		Strong Positive		p-value
	No.	%	No.	%	No.	%	
1- Standard	15	18.8	32	40	33	41.3	P<0.05
2- Low speed centrifugation	19	23.8	29	36.3	32	40.0	P<0.05
3-Filtration	27	33.8	23	28.8	30	37.5	P<0.001
4-Sonication	21	26.3	17	21.3	42	52.5	P<0.001
5-Ultra centrifugation	27	33.8	11	13.8	42	52.5	P<0.001
6-Freon-ether	22	27.5	17	21.3	41	51.3	P<0.001

P- Value for the comparison of the methods with standard

-Significant difference between filtration or low speed centrifugation and each of sonication, ultra centrifugation and Freon-ether at $p < 0.01$, $p < 0.001$ and $p < 0.01$.

-Significant difference among sonication, ultra centrifugation and Freon-ether at $p < 0.05$ or $p < 0.01$.

-Significant difference between ultra centrifugation and Freon-ether at $p < 0.01$

readily sedimented in low gravitational fields. The next its well known that coupling of centrifugation with other techniques potency the purification. It can be concluded that the use of higher centrifugation speed with combination of two methods enhance the effectiveness of the purification. Richard *et al* (6) mentioned that the ultrasound enhance the results of LAT and offers a rapid economical alternative to diagnostic methods. The blending centrifugation procedure required fewer steps but several of undiluted samples obtained by this procedure had significant bacterial contamination. The Freon-ether treatment can cause large reduce in the infectivity of RV. It is well known that the human RV has been difficult to cultivate isolate from the clinical

specimens need several passage. The production of infectious viruses defined by rapid evolution of CPE was significantly enhanced by high concentration of trypsin (20mg/ml) in the growth medium (13). The enhancement of RV infectivity by proteolytic enzyme treatment was demonstrated by number of investigator (14,15) These studies show that proteolytic effect is apparently not due to dispersal of viral aggregates, direct effect of trypsin on the cells, or the digestion of viral inhibitors. The mechanism of adaptation is not known either inhibitors were present in the fecal material which limit RV growth in continuous cell lines or the progeny viruses in primary cells are some how different from virus particles in the

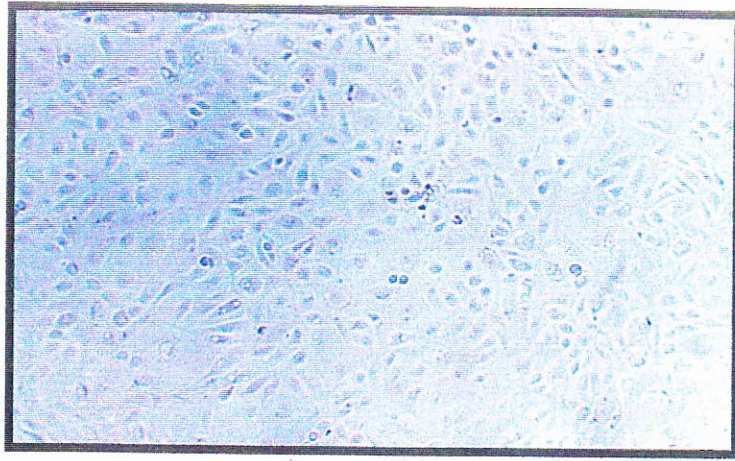
stool preparations or due to the extraction methods of virus particle from the clinical specimens. In the current study the results revealed that the virus preparation processed by sonication method caused CPE after 12 hrs in comparison with the sample treated by stander procedure. Also the sample extracted by ultracentrifugation, Freon-ether and filtration procedures gives CPE better than others. The infection with RV results in that columnar cells being replaced by more cubical and squamous cells of the villi become shorter and the monolayer cells infiltration in the lamina propria (16,17) While the cytopathic effects are not striking or even visible except when fairly large doses of virus are inoculated into cultures. Moribund RV-infected cell don't round up but become partially detached from the glass (18, 19) The CPE are more pronounced in secondary tissue culture than in the primary tissue culture. In conclusion the recommended methods were Sonication, Ultracentrifugation and Freon-Ether methods for the detection of RV particles from the clinical specimens.

Refernces

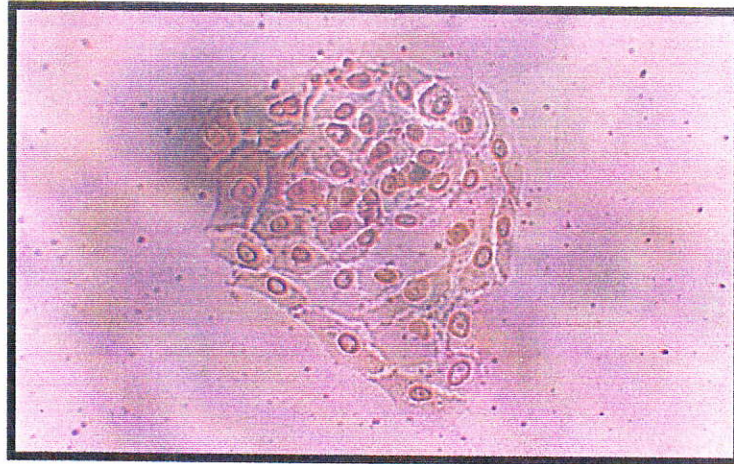
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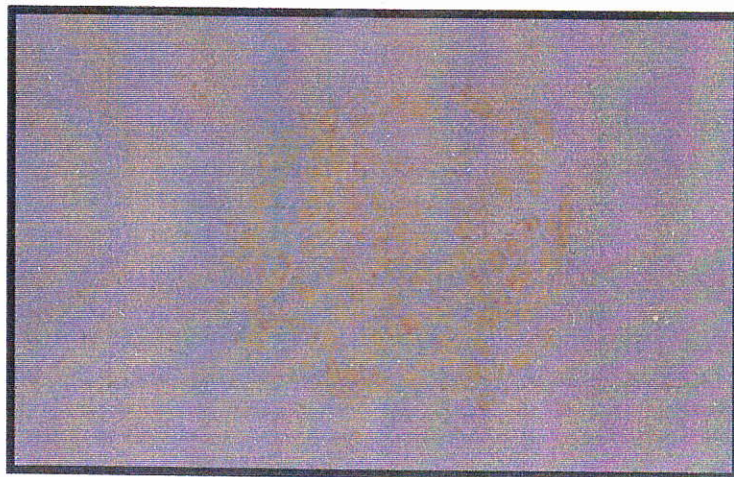
(A)



(B)

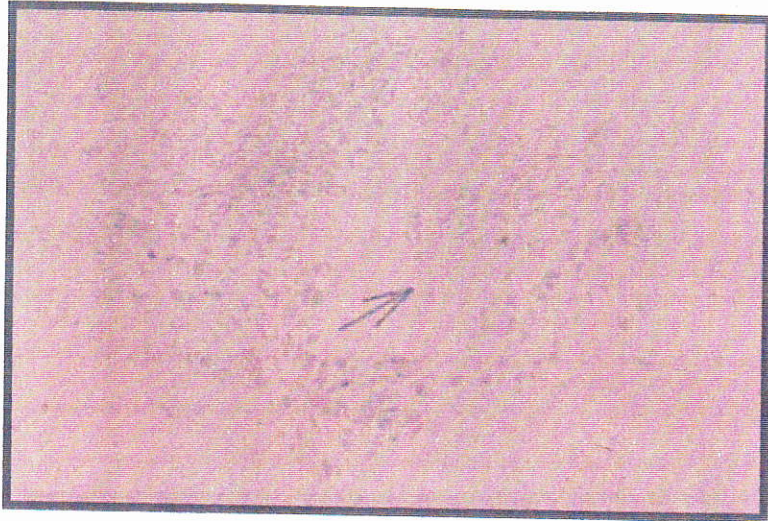


(C)

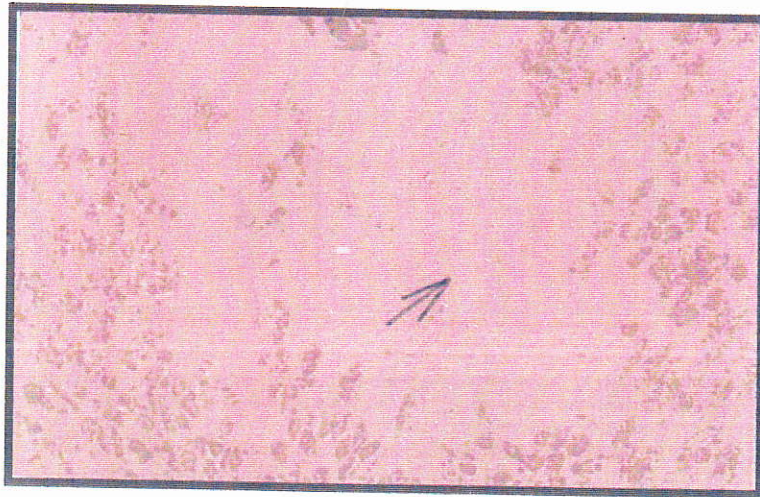


**Fig.1. A. Normal Bovine Kidney cell culture.
B. The infected cells after 12hrs of virus inoculation.
C. The infected cells after 24hrs.**

(A)



(B)



**Fig 2. A. The infected cells after 36hrs.
B. The infected cells after 24hrs.**