

## Isolation and detection of rotavirus by Enzyme linked Immune assay in fecal specimens of buffalo calves

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### Summary

A total of 50 fecal samples were collected from buffalo calves ages between 3 days to 4 months, in middle area of Iraq (Baghdad, Salahaldden, Babylon, Diyala, and Wasit) between July 2016 to June 2017. All samples were examined by Immuno-chromatographic rapid tests for detection of buffalo rotavirus, twenty samples were positive. Enzyme-Linked Immunosorbent Assay. test was also used and revealed only 8 positive samples, the later samples were used for viral isolation on fetal bovine kidney cell culture for detection the cytopathogenic effects of the virus. The cytopathogenic effects of virus were induced rounded cells and gaps; as a result of rupture of these infected cells by rotavirus. Moreover Enzyme-Linked Immunosorbent Assay test used again for detection of rotavirus cytopathogenic effect in fetal bovine kidney cell culture for more confirmation. The TCID<sub>50</sub> was estimated for rotavirus on fetal bovine kidney cells 104.5 virus/0.05 ml.

**Keywords:** ELISA, buffalo calves, rotavirus.

### Introduction

Rotaviruses (RV) are the most common cause of neonatal diarrhea in calves (1). The virus is present in most cattle herds and typically causes diarrhea in calves up to 3 years. Clinical disease in calves older than one month is rare. However, periodic asymptomatic re-infection and shedding occurs in older cows and calves.(2). Rotavirus particles are non-enveloped, are triple-layered, measure around 75 nm in diameter by electron microscopic examination (3-5). There are eight types of this infection defined as A, B, C, D, E, F, G and H. The young buffalo and cattle calves essentially contaminated by species A, B and C. The surface of virus composed of two proteins VP4 and Vp7, which are essential in serotyping assurance(5). The most commonly used test for diagnosis of rotavirus infections are latex agglutination test, enzyme linked immunosorbent assays (ELISA), polyacrylamid gel electrophoresis, immune chromatographic methods, in addition the more selective molecular method Polymerase chain reaction (PCR) (6 -7).

The aim of this study was to detect of rotavirus buffalo calves by Rapid

Chromotography test and confirm that by using ELISA and tissue culture.

### Materials and Methods

After obtaining an official approval from the ethical committee of the college. A total of 50 fecal specimens of diarrheic buffalo calves were randomly collected from 5 Iraqi governorates (Baghdad, Salahaldden, Babylon, Diyala, and Wasit) from July 2016 to June 2017. The feces were collected directly into sterile disposable plastic containers, stored in a cool box and transported to the laboratory. Thereafter, each specimen was divided into several parts in tubes according to number of tests. Finally, all specimens were stored at -20oC until the next assay day. All samples were diagnosed by immune chromatographic test according to manufacturer procedure (ABON Biopharm Co., Ltd., Hangzhou, China) and confirmed by indirect ELISA test (Cusabio Biotech Co., Ltd, China) which contain 96 wells, according to manufacturer procedure, the wells of samples were compared with controls to calculate the values of rotavirus antigen according to the following equation:-Cut-off value = the average value of

OD<sub>negative</sub>+ 0.10. The sample was considered as positive when OD<sub>sample</sub> is more or equal to cut-off value while it was considered as negative when OD<sub>sample</sub> is less than cut-off value.

Preparation of fetal bovine kidney (FBK) cell culture: FBK tissue culture were used for cultivate rotavirus from fetal aged 3-4 months were obtained at aseptically conditions, treated with 0.25% of trypsin solution for 20 minutes in a trypsinizing flask and repeated 4 times to obtain maximum cells, suspended in growth medium contain 5%-10% fetal calf serum Gibco/USA, 0.1g/l streptomycin, 100000 I.U/l penicillin and 1% fungi zone (250 ug/ml), then cells were grown in 25 ml Tissue Culture Falcon at 37oC, after 5 days of incubation, the cells were then infected with the virus (0.5ml) and incubated for 30 minute at 37oC for virus adsorption, until obtaining a good cytopathogenic effect (CPE) of the virus after 24hrs, in other flask the cells were remained free of infection and kept as negative control, cells were examined daily for virus growth by inverted microscope ; also compared with control flasks was done (8).

The Maintenance Media (MM) was prepared by mixing (MEM) 97 ml (GIBCO-USA), Bovine serum 2 ml, antibiotic 1 ml, fungizone 0.5 ml and 7% NaHCO<sub>2</sub> 2.5-3.5 ml (8).

Virus titration: 0.1 ml from tenfold virus dilution ranging from 10<sup>-1</sup> to 10<sup>-8</sup> was used for the cells infection in each well of the 96 wells tissue culture plate. TCDI 50 value was calculated according to the method of (9).

$$\text{Proportional Distance} = \frac{\text{dil above } 50\% - 50\%}{\text{dil above } 50\% - \text{dil below } 50\%}$$

### Results and Discussion

Detection of (RV) in buffalo calves by chromatography and indirect ELISA test: The Chromatographic Immunoassay kits gave two color line for positive fecal samples, when tested 50 fecal samples by Chromatographic Immunoassay according to age of calves in the five governorates (10 for each one). That revealed 20 positive samples. The highest positive rate was recorded in Wasit governorates (80%) and the lowest positive rate was recorded in Dyala 10% samples. In the current study all the 20 positive samples from chromatographic immunoassay were tested then by ELISA method to reveal the

presence of rotavirus antigen which gave us 10 positive (50%). The highest positive result was recorded in Wasit (5) and the lowest were in Babylon(0 %), these positive samples shown in the (Table ,1).

**Table, 1: Results of ELISA test for detection rotavirus in 20 fecal samples according to age of tested calves revealed that 50% positive.**

Time/week city	AGE										Total
	1	2	3	4	5	6	7	8	12	14	
<b>Dyala</b>											
Positive	1	0	0	0	0	0	0	0	0	0	1
Negative	0	0	0	0	0	0	0	0	0	0	0
Total	1	0	0	0	0	0	0	0	0	0	1
<b>Salah alden</b>											
Positive	1	0	0	0	0	0	0	0	0	0	1
Negative	1	0	0	0	0	0	0	0	0	0	1
Total	2	0	0	0	0	0	0	0	0	0	2
<b>Babylon</b>											
Positive	0	0	0	0	0	0	0	0	0	0	0
Negative	1	1	0	0	0	0	0	0	0	0	2
Total	1	1	0	0	0	0	0	0	0	0	2
<b>Wasit</b>											
Positive	2	1	1	1	0	0	0	0	0	0	5
Negative	1	1	0	1	0	0	0	0	0	0	3
Total	3	2	1	2	0	0	0	0	0	0	8
<b>Baghdad</b>											
Positive	1	1	0	0	0	1	0	0	0	0	3
Negative	0	0	1	1	2	0	0	0	0	0	4
Total	1	1	1	1	2	1	0	0	0	0	7

In this study immuno-chromatographic rapid tests (FASTest® Strips) used as a screen test for detection rotavirus, 20 positive out of 50 samples, then confirmed these positive samples by using indirect ELISA test which revealed 10 positive samples. These result indicated that ELISA is more sensitive but the immuno-chromatographic rapid test is faster, simple and cheaper cost .

A previous study by using rapid test on calve fecal samples showed in Babylon, Karbala ,Najaf, AL-Qadisyia revealed that infection rate were 60%, 20% , 40% and 33% respectively (10). Chromatographic Immunoassay and ELISA are the simple and good standard methods for detection of rotavirus. These methods were declared low cost equipment and simple experience, which is available in many laboratories (11), which

help us to detect rotavirus of buffalo in Iraq. Another study showed that rotavirus were detected in diarrheic calves from 5 Iraqi Governorates showed 53 (42%), using ELISA (12) which were near to our results. But Al-Gburi results were higher (13) detected calf RV in 73.3% of calves in Al-Sawara city (Al-Nasser Station) using ELISA test.

Titration of isolated virus: Tissue culture plate was incubated at 37°C and TCID<sub>50</sub> was calculated at 5 days post infection. The result of virus titration, was calculated according to equation below (Table,2). The proportional distance (PD) was 0.5 and the titration of virus was 104.5 virus /0.05 ml.

Un-infection (free of virus) wells were also kept for each dilution(Fig.1). TCID<sub>50</sub> value was calculated according to (9), the titration of rotavirus was 104.5 virus /0.05 ml

$$\text{Proportional Distance (PD)} = \frac{\text{dil above } 50\% - 50\%}{\text{dil above } 50\% - \text{dil below } 50\%} = \frac{60 - 50}{60 - 40} = 0.5$$

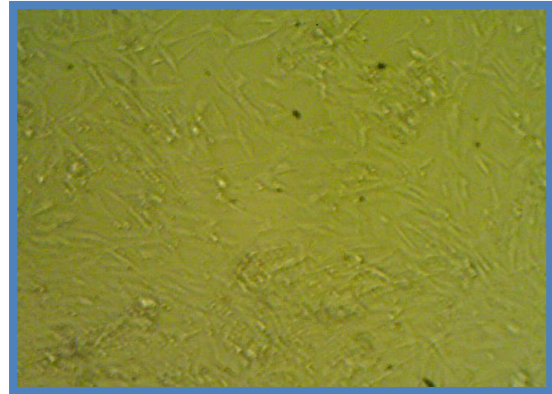
The titration of the virus =  $1 \times 10^{4+PD}$  Value  
 =  $1 \times 10^{4.5}$  virus/0.05ml

Table, 2: Titration of the rotavirus (TCID<sub>50</sub>)

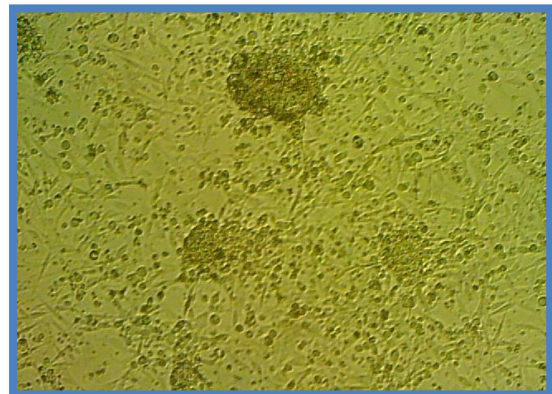
Virus Dilution	Infected wells	No infected wells	No. of wells that show positive CPE	No. of wells that show negative CPE	Percentage of positive results
10 <sup>-1</sup>	10	0	9	1	90%
10 <sup>-2</sup>	10	0	8	2	80%
10 <sup>-3</sup>	9	1	7	3	70%
10 <sup>-4</sup>	6	4	6	4	60%
10 <sup>-5</sup>	4	6	4	6	40%
10 <sup>-6</sup>	3	7	3	7	30%
Total			29	21	

Results of isolated virus on tissue culture: The cytopathic effect of isolated rotavirus on third passage of FBK cell culture, showed in (Fig. 2). The accumulated virus in calf kidney

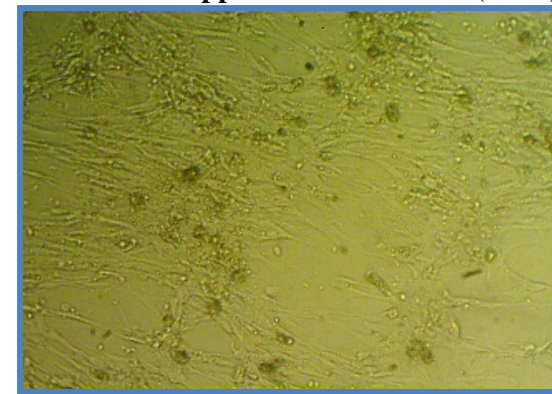
cell culture causing CPE after 3rd passage of RV, the cells appeared rounded at second day post infection, large syncytia formation after 24hrs, and at 72 hrs post infection the cells showed cytoplasmic vacuolation, degeneration and detachment of cells from the surface, also showing sloughing the infected cells from the surface after 72 hrs. (Fig. 3).



Figure, 1: Normal uninfected calf kidney cell culture (control) (200X)



Figure, 2: Calf kidney cell culture infected with RV after 24 hrs appeared rounded cell (200X)



Figure, 3: Calf kidney cell culture infected with RV after 72hrs (cytoplasmic vacuolation and detachment of cells from surface) (200X)

Viral culture is a laboratory test in which samples are placed with a cell type that the virus being tested for is able to infect. If the cells show changes known as cytopathic effects, then the culture is positive (14). The bovine rotavirus was inoculated in one type of cell culture to define the growth and characteristics of this virus on these cells (15 and 16). The ELISA positive samples in this study were cultivating on calf kidney cells that revealed (CPE) of rotavirus on these cells (fetal bovine kidney FBK) that agreed with Bartlett (15) then ELISA test was used again for these FBK cell culture only to confirm its rotavirus CPE. The virus has CPE on calf kidney cell culture. The characteristics of FBK cell culture are retain normal set of chromosomes and morphology, serially transferred (20-50) passage, while rotavirus in primary fetal bovine kidney cells was successfully carried through 37 serial passages was achieved by previous studies (17 - 19), this study showed, The CPE of isolated rotavirus appeared on third passage of FBK cell culture

A viral plaque is formed when a virus infects a cell within the fixed cell monolayer. Plaque formation can take 3-14 days depending on virus analyzed (20). This study finding stated that TCID<sub>50</sub> was 104.5 virus /0.05 ml. While in other rotavirus cultivating study was 107 to 108 (21). In contrast, these differences in the cytopathic effect of bovine rotavirus may depend on the strain and serogroup of rotavirus, adaptation and propagation of bovine rotavirus, cells used for cultivation, temperature-sensitive mutants of bovine rotavirus and effect of proteolytic enzymes (22).

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## عزل وتشخيص فايروس الروتا من عينات براز عجول الجاموس باستخدام اختبار الفحص

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#### الخلاصة

تم جمع 50 عينة براز من عجول الجاموس المشكوك بإصابتها بأسهال الفيروس البقري من محافظات وسط العراق بين شهر تموز 2016 إلى شهر حزيران 2017 وكانت أعمارهم بين 3 أيام إلى 4 أشهر. استخدمت الاختبارات السريعة المناعية الكروماتوغرافية (الأشرطة المناعية الخاصة للكشف السريع عن مستضد فيروس الروتا ) للكشف عن الفيروس البقري بالعجول أسفرت عن 20 نتيجة إيجابية. تم اختبار العينات الايجابية بواسطة اختبار الفحص المناعي المرتبط بلانزيم (الاليزا) فوجدنا 8 نتيجة إيجابية. تم استخدامها لتأكيد هذه الإصابات بزراعتها في الأنسجة باستخدام خلايا كلى الجنين البقري. ظهرت التأثيرات المرضية للفيروس على الخلايا عبارة عن استدارة الخلايا وظهور فجوات ناتجة عن تمزيق الفيروس لهذه الخلايا و باستخدام اختبار الفحص المناعي المرتبط بلانزيم (الاليزا) لفحص خلايا كلى الجنين البقري فقط وللمرة الثانية تم التأكد من إن المسبب لهذه التأثيرات المرضية هو فايروس الروتا. كانت المعيارية الأفضل للجرعة المصيبة لنصف الخلايا المحقونة TCID50 المستخدمة للفيروس على خلايا كلى الجنين البقري هي  $10^{-4.5}$  / 0.05 ml.

الكلمات المفتاحية: اليزا ، عجول الجاموس ، روتا فيروس