



ORIGINAL ARTICLE

Estimating the sensitivity and Specificity of Reverse transcriptase polymerase chain reaction (RT-PCR) with Rota virus latex agglutination test (Virogen Rotatest) in calves suffering from diarrhea in Karbala province

Hayder A. Muhammid¹, Ihab Ghazi Mahdi AL-Shemmari² and Mohmmmed Assad S. Alkabi²

¹ Veterinary Microbiology Department, Veterinary Medicine College, Karbala University, Iraq.

² Preventive and Internal Medicine Department, Veterinary Medicine College, Karbala University, Iraq.

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Corresponding author:

Hayder A. Muhammid
Email: drhayder93@gmail.com
Veterinary Medicine College
Karbala University
Iraq.

ABSTRACT

Objective: The aim of this study was to estimate the sensitivity and specificity of reverse transcriptase polymerase chain reaction (RT-PCR) in the detection of Rotaviral diarrhea in calves, and to compare with Rota virus latex agglutination test (Virogen Rotatest).

Methods: This study was conducted from November 2016 to April 2017 on 40 diarrheic fecal samples were taken from calves aged 1-30 days in un-organized dairy cow farm in Kerbala province, Iraq.

Results: Among forty fecal samples were collected from all diarrhoeal calves, 26 rotavirus in fecal samples were detected by virogen rota virus latex agglutination test. Result of chi square test showed that non-significant association between sex of calves and number of Rota virus detection by Rota virogen test kit ($P>0.05$), otherwise, results showed that 1-10 days possesses higher Rota virus observation (100%) as compared to the other age. The sensitivities of RT.PCR and Virogen Rota test were equal (92%) and (88.4%), respectively. While the specificity of virogen rota test (85.7%) was superior to that of RT.PCR (80%) with a total of 3 false positive results found on RT.PCR.

Conclusion: A commercially available latex agglutination test (Virogen Rotatest) and reverse transcriptase polymerase chain reaction (RT-PCR) protocol has been screened for bovine group A rotavirus infection, these results will help the readers for grasping advantages and limitations of two different diagnostic tools for screening diarrheal samples for Rota virus detection.

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INTRODUCTION

Rota viruses are major causes of calf scour that happened in the first weeks after birth¹. rotavirus is a double-stranded RNA virus which is recognized as a major cause of enteric disease in calves living in all areas of the world². Since rotaviruses are hard to cultivate directly from bovine fecal samples, generally immunoassay methods are suitable for identify rotavirus infections as direct detection of rotavirus antigens in

diarrheal samples³. Several causes associated with the calf scour which considered a clinical sings that characterized by diarrhea in calf, Noninfectious causes and infectious causes make the etiology of calf diarrhea is complex and often involving environmental, immunological and nutritional factors as well as number of infectious causes. Inadequate nutrition of the pregnant dam (colostrums) and inadequate environment for the

newborn calf considered the noninfectious causes, while viruses, bacteria, protozoa, yeasts and molds considered the infectious causes⁴. Cause severe destruction and damage to the epithelia which lined the intestine caused by these infectious agents, and these intestinal altered lead to many changes inside the body (dehydration, acidosis, and loss of electrolytes) as well as disturbance in digestion and absorption which may lead to death^{1,4}. The role of rotavirus has been established as a cause of diarrhea in calves and other mammals⁵. Epidemiological surveys serologically have also appear that antibodies against rotavirus are distributed among the calves population, and Anjan M. *et al*(2012)⁶ mentioned after using the PCR test for genotyping that Rotavirus and corona virus are causes a lot of economic damage to the farmers as well as suffering to the animal because they are the most common viral causes of neonatal calf diarrhea.as well as John F. (2009)⁷ mentioned that 37% of the diarrheic calves were due to rota virus, and Logan, C *et al* (2006)⁸ found that PCR test one of the excellent tests to detect Rota virus, In addition Yousif A *et al*(2001)⁹ show during their study on rotavirus infection that PCR is a rapid molecular methods for the routine screening of stool samples in hospital laboratories to provide rapid definitive diagnoses.

The goal of this study was to evaluate the specificity and sensitivity of two different diagnostic test reverse transcriptase polymerase chain reaction (RT-PCR) and latex agglutination test (Virogen Rotatest) in the detection of Rota viral diarrhea in calves.

MATERIALS AND METHDS

Samples and Animals: 40 Holstein calves suffering from diarrhoea between 1 – 30 days of age from different un-organized dairy farms in kerala province were used in this project. Samples were collected between November 2016 to April 2017. sex class were recorded; fresh diarrheal samples should be directly collected from diarrheic calves into a sterile container with rectal stimulation by avoiding environmental contamination (such as urine, soil)¹⁰. In rare cases when the rectum was empty, the sample volume was neglected. All samples collection were classified into two main categories, The first category, including one milliliter was prepared of freshly evacuated diarrhea pellets directly in order to get RNA isolation procedures, either way, the second category have included the use of latex agglutination test (Virogen Rotatest) through the preparation of 20% (vol/vol) suspension of liquid feces in 0.01 M PBS (pH7) and then centrifuged at 1,500 χ g, the supernatants were tested and then stored in sterile vials at - 80°C for further study⁹.

The first Category was involved nucleic acid extraction by Extract - Master™ Fecal DNA Extraction Kit (EPICENTRE ® Biotechnologies), to isolate rotavirus dsRNA from Galf fecal samples. The kit uses a detergent lysis process combined with an inhibitor removal resin was collected in a glass container and spin columns wrapped in foil, then all were autoclaved at 121°C for 10 min using a liquid cycle to avoid RNase

contamination, RT-PCR was carried out in a one tube format using MonsterScript™ Reverse Transcriptase (Epicentre, Madison, WI) depend on manufacture company to produce cDNA.

Primer synthesis

Primers were designed after comparison of the few available from the sequence of genome segment 6 inner capsid protein VP6 gene which is available in a gene bank have accession number (KX212868.1, KX655532.1, KJ752066.1 and KF636260.1) (<http://www.ncbi.nlm.nih.gov/>) and amplifying a fragment of 232 bp was designed with the use of Primer3 software (<http://frodo.wi.mit.edu/>). The sequences of primers were as follows: upstream primer, 5'- AAGAAATGGAATCGCACCAC-3'; downstream primer, 5'- TAGCCACATCGTACCCATCA-3'. Use of these primers in PCR would result in PCR product encompassing the partial length of inner capsid protein VP6 gene (232 bp).

Reverse transcriptase PCR (RT-PCR)

In collage of veterinary medicine , Kerala University, microbiological laboratory cDNA product were subjected to RT-PCR using a Three-step thermal cycling, including denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 68 °C for 2 min, was performed for 35 cycles. The amplified gene products were subjected to agarose gel electrophoresis using 1.5% agarose and then visualized by UV gel documentation system with a molecular weight marker (1.5 kb DNA Ladder).

Latex agglutination test (Virogen Rotatest)

The second category was included latex agglutination slide test (Virogen Rotatest) for rotavirus detection in bovine fecal samples by following the manufacturer's instructions. It is a rapid slide test in which latex particles are coated with antibodies specific for group A rotavirus antigens present in a fecal supernatant. These tests are read with the naked eye in 3 min.

Statistical Analysis

Data analysis were performed by chi square test using SPSS statistical software, the sensitivity and specificity of the results were also calculated by the following formula.

I : sensitivity : (true positive / true positive + false negative) \times 100 , II: specificity : (true negative / true negative +false positive) \times 100¹¹.

RESULTS

Two methods were used for detecting the Rota virus associated with calves diarrhea, latex agglutination test and reverse transcriptase polymerase chain reaction (RT-PCR). Table 1 explains sensitivity and specificity of these procedures; the results found that latex agglutination test was sensitive to detecting the agents of Rota virus Figure 1; its show sensitive by 88.4% while RT-PCR shows 92 % , otherwise, 12 out of 15 (80%) calves with negative results are truly negative and 3 (14.20%) calves test positive for a Rota virus disease which they do not have.

Table 1. Numbers of infected animals with Rota virus according to tests which used in this study

Test	RT-PCR		Total	
	Yes	No		
(Virogen Rotatest)	Yes	23 *True positive	3 False positive	26
	No	2 False negative	12 **True negative	14
Total	25	15	40	

*Sensitivity = [True Positive/(True Positive + False negative)] \times 100.
 **Specificity = [True negative/(True Negative + False positive)] \times 100.

In this study, 40 calves from both sex as male (n: 17) and female (n: 9) were analyzed by Latex agglutination test to detect Rota virus in diarrhea samples Table 2, The prevalence of anti-rotavirus antibodies in the diarrhea samples obtained from male and female were 77% and 50%, respectively, there were non- significant relationship between the virogen rotas test kit and sex of these animals. Table 3 shows the significant detection of anti-rotavirus antibodies in the group 1-10 day (100%).

Table 2. Latex agglutination test used according to the sex of Calves.

Gender	Virogen Rotatest					X ² &P-value
	+ve	%	-ve	%	No.	
Male	17	77	5	23	22	X ² = 3.237 P=0.0719 No Significant
Female	9	50	9	50	18	
Total					40	

Table 3. Latex agglutination test used according to the age of Calves.

Age range (months)	Virogen Rotatest					X ² &P-value
	+ve	%	-ve	%	No.	
1-10 days	11	100	0	0	11	X ² = 8.216 P= 0.0164 Significant
11-20 days	8	50	8	50	16	
21-30 days	7	54	6	46	13	
Total					40	

In PCR assay, Nucleic acids isolation with the Extract Master Kit yielded the expected 232 bp of RT-PCR amplicon, then isolates was observed in agarose gel electrophoresis Figure 2 PCR technique is a highly sensitive and susceptible rapid technique. The specific primers used in this technique were designed following identification, sequence determination and analysis.

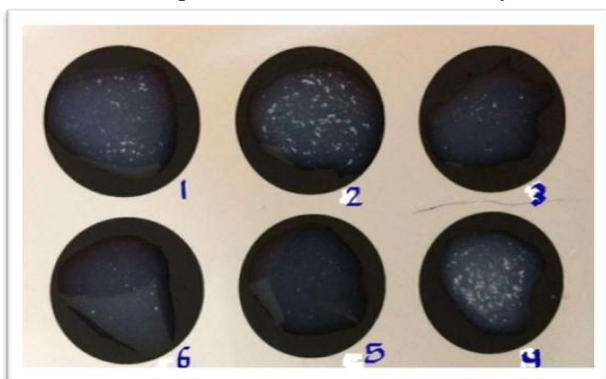


Figure 1. showed the positive results of infected calves by using latex agglutination test. Circle 1 represent control positive, Circle 5 represent control negative while circles 2,3,4,6, represent the positive cases.

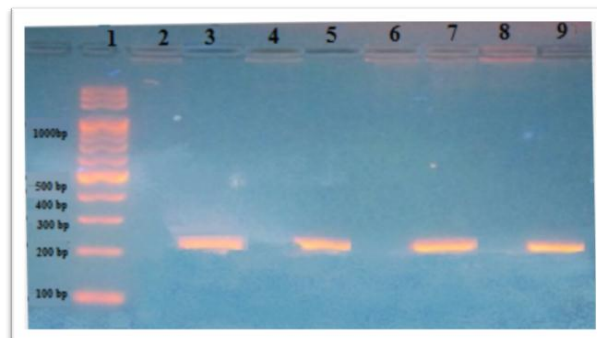


Figure 2. Agarose gel analysis of RT-PCR products The 232 bp amplicon of positive identifying of rotavirus have that appears in lanes 2, 3, 4 and 5 (nucleic acids isolated by ExtractMaster™) corresponds to inner capsid protein VP6 gene and Lane 1, 1500 bp ladder.

DISCUSSION

The distribution of the infection by rota during the winter season in Iraq and this agree with Millie R. C et al (2015)¹² when they noticed that the highest infection by rotavirus diarrhea in the winter months. Cold may cause increase stress on calves which led to the infection by causes of diarrhea¹³.

The good performance of the RT-PCR assay was reflected too in its ability to detect RNA of the Rota virus in 2 of 25 calves (8%) for whom latex agglutination test (Virogen Rotatest) was negative. Because of Rotavirus is a major factor associated with acute diarrhea in Human and animal species¹⁴, this study must be reveal the best and reliable methods to detect Rota virus in stool, the Al- Yousif, Y *et al*(2001)¹⁵ showed that Latex agglutination tests are easy to perform in a short time and it does not require skilled personnel and expensive equipment, with the reagents have long shelf lives. In other words, An assays with higher sensitivity are desirable to diagnose Rota virus in calves, especially when low levels of virus are shed in the feces, the polymerase chain reaction (PCR) has been adapted with high sensitivity or detection of RNA viruses by addition of a complementary DNA (cDNA) synthesis step, using reverse transcriptase, before cyclic amplification¹⁶. The result agree with Sanjay S. B. *et al*(2010)¹⁷ after collected 128 diarrhoeic faecal samples from cattle and buffalo calves during winter months from Pantnagar and Dehradun by using RT-PCR and he observed the overall prevalence of rotavirus was 10.15%.

However inhibitory substance in ruminant sample has adversely affected the specificity of RT-PCR. An obstacles in applying PCR methods to animal feces has been attributed to the difficulty of removing this inhibitor¹⁸ several curatives have been introduced to eliminate this trouble, including the uses of inhibitor removal resin in the Master™ Fecal DNA Extraction Kit (Epicentre, Madison, WI) which is used in the RNA purification against RNase contaminations¹⁹. The reason for consider the latex agglutination test (Virogen Rotatest) is gold standard due to fast, sensitive, simple, and specific diagnostic techniques for the detection of

viral agents **Figure 2** causing gastroenteritis in calves, while in the case of in handling samples a RT-PCR, the laboratories need to be very needs to experience, especially in the initial stages of RNA extraction and RT-PCR amplification, or maybe the choice of oligonucleotide primer is critical for accurate diagnosis in the clinic, It also takes a long time²⁰. on the other hand, the specificity of latex agglutination test was 85.7% was more than specificity of RT-PCR 80%, due to the molecular techniques depend on the amount of RNA in the fecal samples and how much is required to turn into cDNA product, otherwise, A variety of specificity reverse transcription polymerase chain reaction (RT-PCR) methods have been developed based on primers specific for several different rotavirus genes, because calves rotavirus Groups A, B, and C have distinct patterns of gene-segment distribution, There was a clear difference between the measurement of protein of VP7 and amplification of cDNA product, it would appear that The rotavirus serogroups are further divided into serotypes based on the outer capsid proteins, VP7 (G types) and VP4 (P types). At least 14 G and 12 P serotypes are recognized in group A rotaviruses²¹.

The results do not correspond to what Hasso, S. A. *et al* (1986)²² indicated by the use of agar gel precipitation test to check bovine antibodies of rotavirus in 562 of calves serum and rotavirus antigen of 347 calves feces, he found the significantly more females had rotavirus antigen in the feces and rotavirus antibodies in the serum than did male calves, but our results agree with Hahmed A. *et al* (2014)²³ when they found there is no significant differences between Male and female (37.5%, 40% respectively) were seen infected. In contrast to Dash, S.K. *et al* (2011)²⁴ which found that female diarrheic calves (12.76%) were found to be less infected than Male diarrheic calves to rotavirus infection (20.37%). Along with the antigen of the rotavirus has been detected by EISA. while Samuel J.(2013)²⁵ in their study on diarrheic calves after they divided them to different ages group were under 30 d old, and they found many of them infected with rota virus bovine and they found that they highest incidence of the infection by rota virus occurs in the 8-14 after birth .

Villarroel, A. (2009)²⁶ explained that calves can be infected with multiple infectious agents at the same time—for example, Rotavirus, Coronavirus, and Cryptosporidia in their first few weeks of life usually 1 to 3 weeks, one of the most important reasons to getting diarrhea in calves was hemolytic *E. coli* which highly associated with the diarrhea in the first weeks, the *E. coli* could adhere to enterocytes and were capable of producing a heat-stable and heat-labile enterotoxin all these factors corresponding to appear of rotavirus in the feces (a virus known to destroy enterocytes) these cases coincid with the initiation of diarrhea^{27,28}. On the other hands, The milk up taken by calves can provide a good condition for rotaviruses survival because great stability

of rotavirus in milk and its resistance to gastric pH changes in addition to intestinal epithelial cell²⁹. Several tests are routinely used in diagnostic laboratories for rotary virus in stool samples. These includes enzyme linked immunosorbent assay (ELISA), passive hemagglutination and electron microscopy³⁰, There are several studies to suggest use pair of primer to detect bovine rotaviruses in feces^{31,32}.

It is evident from present results and earlier related reports, that latex agglutination test provides rapid identification of Rota virus and can be used as rapid diagnostic method.

CONCLUSIONS

The result study showed that both technique (RT-PCR) and latex agglutination test are clearly a reliable and rapid methods for the detection of calf suffering from diarrhea, farther study concluded the RT-PCR of diarrhea with higher sensitivity and Latex agglutination test with higher specificity can be useful for Rota virus detection, In the other words, The results of this study should be compared with other studies because the concentration of Rota virus in feces of calves may vary by geographical position or management of work systems.

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