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University of Al-Qadisiya
College of Veterinary Medicine

Molecular Investigation of Bovine Rotavirus in Diarrheic Calves by Using RT-PCR

A Research Project
Submitted to the council of Department of the medicine College of Veterinary Medicine/ University of Al-Qadissiyia in Partial Fulfillment of the Requirements for the Degree of Bachelor in Veterinary Medicine

By

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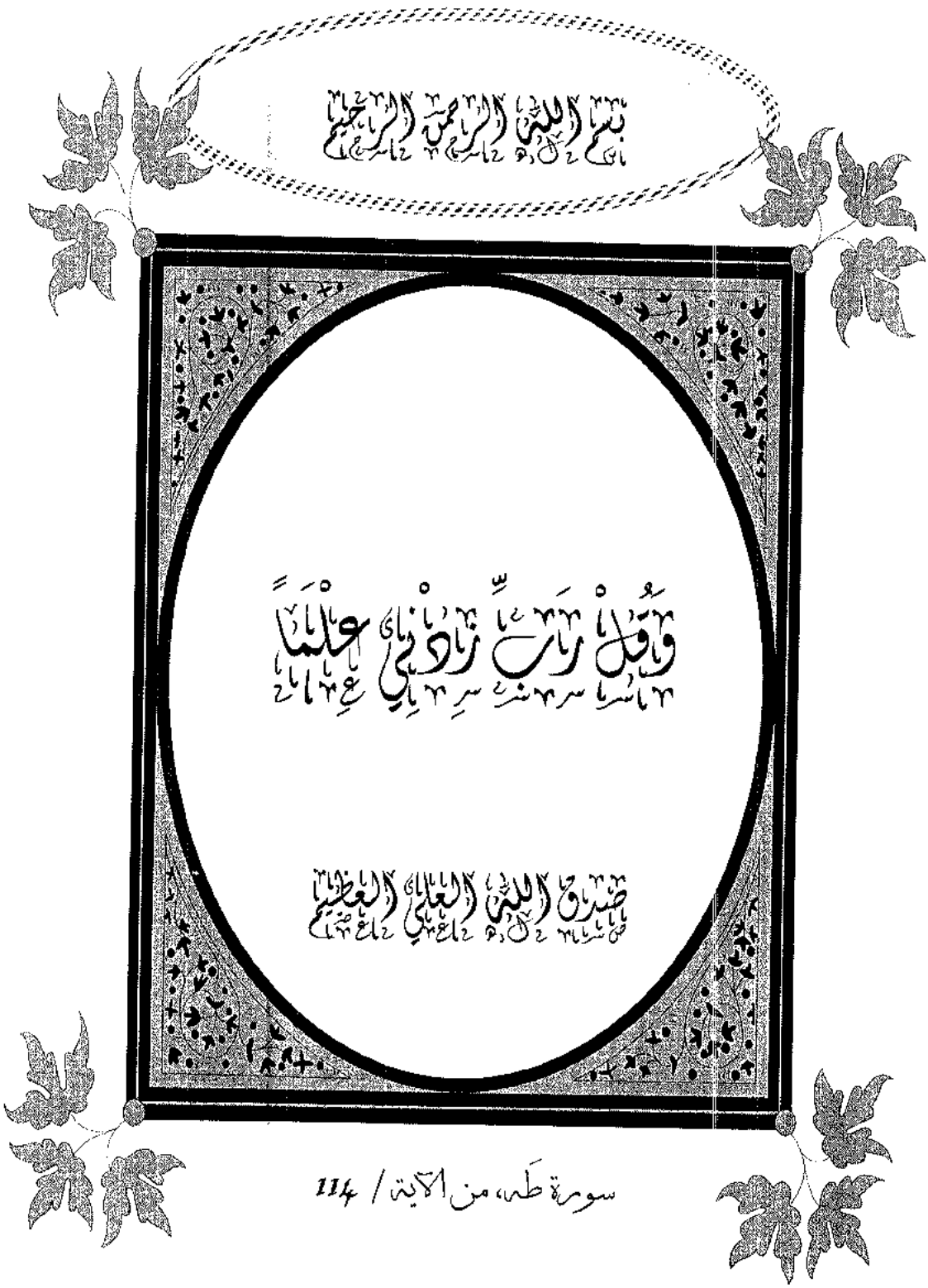

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Certificate of supervisor

I certify that ***Sama Hosean Ibraheem*** has completed the fulfillment of her graduation project entitled ***Molecular Investigation of Bovine Rotavirus in Diarrheic Calves by Using RT-PCR*** for the year 2015/2016 under my construction.

Lecturer

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April 2016

Certificate of Instructor

We certify that ***sama hosean ibraheem*** has completed the fulfillment of her graduation project entitled ***Molecular Investigation of Bovine Rotavirus in Diarrheic Calves by Using RT-PCR*** for the year 2015/2016 under our construction.

Instructor

Dr. Muthanna Hadi Hussain

2 . 4 . 2016

Head of Department of Internal and Preventive Medicine

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2 . *April 2016*

الأهداء

الى ...أبي وأمي ..وكل من وقف ورائي وقدم الدعم المادي والمعنوي وأنا اصعد سلالم درجات العلم والمعرفه... أهدي مجهودي الصغير .....

سما

شكروتقدير

أشعلت روحك بألآفاق مصباحا ورحت تزرع بألآوطان ارواحا
وأضحيت توقد بألآبدان مفتخرا عزيمة تغمر ألآكوان أصباحـــا

بعد مضـي خمسة أعوام من الحياة الدراسيـة أقف وكلي رهبة وأجلال في محراب الذين زرعوا في عقولنا وقلوبنا من العلم والمعرفة...وأتقدم بخالص الشكر والتقدير لأساتذتي الكرام الذين كانوا مشاعل من النور أضاءوا لي دروب العلم فـــي مراحـــل الدراسة والحياة وأخص بالذكر أستاذي المربـــي الفاضل الدكتور خليفة علي منصور الذي تكـــرم بأشرافه على بحثي المتواضع .....

سما

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Summery

Bovine Rotavirus Groups A and B consider is the most common viral cause of diarrhea in neonatal calves. but group A is high incidence and clinically important . group A Rotavirus is classified as G and P genotypes or serotypes according to the genetic or antigenic characteristics presented by the proteins VP7 and VP4, both located in the virus outer capsid.

In this study we used more sensitive techniques (reverse transcription polymerase chain reaction) and by using specific primers VP7 to Detection the infection by bovine Rota virus group A in diarrheic fecal samples which taken from diarrheic calves in age (1 -30)day .The fecal samples were collected from diarrheic calves in age under one month from different area in AL-Diwaniya province in winter season ,All samples were collected in Disposable container and storage until the laboratory diagnosis were done .

The result showed out of 10 samples only four sample were positive to infection by Rota virus. and we conclusion the Rota virus is the common viral causes of diarrhea in neonatal calves and the RT-PCR assay is more sensitive for detection small amount of DNA of Rota virus.

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Chapter one

Introduction

Diarrhea is one of the most causes of calf loss in beef calves and are the major cause of loss in dairy heifers born alive. Mortality from diarrhea, in dairy calves diarrhea and other digestive diseases account for about 5% of total mortality (1).

Viral diarrhea of calf consider is the one of the most widespread diseases, is a complex syndrome with a complex etiology and pathogenesis, causing important economic losses due to morbidity and mortality, treatment costs, and reduced growth rates in affected calves(2) .

The etiology of diarrhea is involves infectious agents (viruses, bacteria, and protozoa) and also noninfectious factors such as herd management, host nutritional, and immunological condition which affect the outcome of the disease (3).

Rotavirus and Coronavirus are two main causes of severe diarrhea in human infants and many animal species worldwide. Calves up to 3 months old can be affected by these viruses. The importance of these viruses in cattle industry is that it may cause substantial economic loss, treatment costs, and reduced growth rates in beef and dairy calves. Mixed infections caused by rotavirus and coronavirus can lead to severe form of diarrhea(4).

Since diarrhea in calf and other animals is a great problem which involves economic losses, several diagnostic methods are used to detect enteropathogenic agents. Diagnosis is done through collecting feces of animals suffering from diarrhea by a rectal swab or collecting intestinal contents (5).

(6) observed that numerous etiological agents may be implicated in this clinical sings , group A rotavirus is one of the major causes of acute gastroenteritis in infants and in many animal species .

(7) mentioned that group A rotaviruses, as the member of *Reoviridae* family, have clearly been established as causing significant viral diarrheal disease in infants and in the young of various mammalian and avian species .

Group A bovine rotavirus (BRV) is considered the major cause of severe diarrhea in calves worldwide

Group A rotavirus is classified as G and P genotypes or serotypes according to the genetic or antigenic characteristics presented by the proteins VP7 and VP4, both located in the virus outer capsid(8).

There are different methods to detect BCoV, but a high degree of sensitivity is required, especially in subclinically infected calves and chronic shedders of BCoV in faeces (9).

The RT-PCR assay is useful to detect small quantities of nucleic acid and is widely used for the diagnosis of infectious disease (10)

The objective of this study were designed with two aims. The first is to use RT-PCR methods for detection of bovine coronavirus and mixed infection with rota virus . The second was to determine the prevalence of bovine coronavirus in calves diarrhoea in four Iraqi governorates .

Chapter two

Review

2:Review

2:1:History of the virus

Rotaviruses are a group of viruses that have been associated with diarrhoea in the young of most mam - mammalian species and some avian species .' ' Rotaviruses were first isolated and characterized from mice in the early 1960s. Toward the end of the 1960s a similar virus was isolated from scouring newborn calves'; the infection was transmitted by inoculating calves with filtrates of diarrhea faeces. Virus particles 65 ,nm in diameter, were found in large numbers in the faeces of the infected animals. This work has been confirmed by a number of other groups and some isolates have been adapted to grow in tissue culture . Rotaviruses were discovered in the 1960s in animals. The virus was first described in humans when it was found by electron microscopy in duodenal biopsies from children with acute gastroenteritis (**15**).

2:2:Classification

Rotaviruses are 70-nm icosahedral viruses that belong to the family *Reoviridae*. Seven rotavirus serogroups (serogroups A to G) are described. Most human pathogens belong to groups A, B, and C. Group A rotaviruses are the most important from a public health standpoint(16)

2.3:Structure of the virus

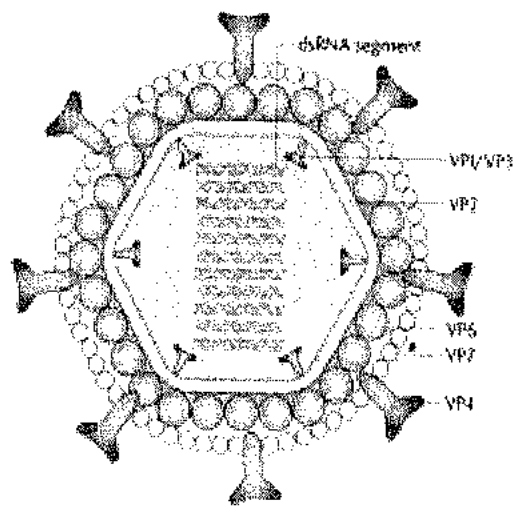
The virus is composed of three protein shells, an outer capsid, an inner capsid, and an internal core, that surround the 11 segments of double-stranded RNA (Fig.1). For the most part, each gene segment codes for a single protein. When mixed infection with more than one rotavirus strain occurs, the gene segments from the parental viruses may reassort independently, producing reassortants of mixed parentage, a source of viral diversity.43

Four major structural and nonstructural proteins are of interest in vaccine development: VP6, NSP4, VP7, and VP4. VP6, the most abundant viral structural protein, is found in the inner capsid (17). VP6 bears group-specific antigenic determinants. NSP4 is a nonstructural protein and has been shown to be an enterotoxin (16).

VP7 and VP4 are structural proteins found in the outer capsid. These two proteins define the serotype of the virus and are considered to be critical for vaccine development because they are targets for neutralizing antibodies that may provide both serotype-specific and, in some instances, cross-reactive

protection (18). The VP7 protein is glycosylated, and serotypes determined by this protein are termed G serotypes. Fourteen G serotypes have been identified.

VP4 is a protease-cleaved protein, and serotypes determined by this protein are termed P serotypes. P types have been difficult to characterize by traditional methods of virus neutralization; therefore, molecular methods have been used to define a genotype based on sequence analysis. These genotypes correlate well with known serotypes, so the genotypes are tentatively designated in brackets (e.g., P1A(19). Strains are generally designated by their G serotype specificities (e.g., serotypes G1 to G4 and G9).



Schematic representation of a rotavirus virion. The virus is composed of three protein shells, an outer capsid, an inner capsid, and an internal core, that surround the 11 segments of double-stranded RNA. The outer capsid proteins VP4 and VP7 are neutralization antigens and define the P and G serotypes, respectively. VP6, the inner capsid structural protein, is the subgroup antigen. (Reprinted from reference 1 by permission from Macmillan Publishers.)

2.4:Epidemiology and Transmission

(15)examined 134 samples for the detection of the virus associated with neonatal calf diarrhea .The presence of (rota virus and coronavirus) has been demonstrated by using the electron microscope and the florescent antibody

techniques ,they described that rota virus and coronavirus was found in 58 cases (54%) whilst the coronaviruses and the rotavirus were found singly in 34 cases (53%) and 45 cases (44%) respectively.

(16) mentioned that bovine coronavirus is ubiquitous in the cattle population and persists in adults as subclinical infections. However, under stressful conditions adult cattle can shed coronaviruses in faeces .

(17) mentioned that diarrhea is recognized as the main clinical feature of BCOV infection . BCOV also causes respiratory tract infections in calves. Results of comparative studies indicated that coronaviruses isolated from either the intestinal or respiratory tract of calves, replicated in both the intestinal and upper respiratory tracts of gnotobiotic or colostrum-deprived calves .

(18 (1987) proved that the rate of virus excretion on studying 132 cows and heifers with no previous BCoV vaccination history, they found the rate of virus excretion has been reported to increase by50% to 60% during the winter months, by 65% at parturition, and by 71% 2 weeks postpartum.

(19) observed that viral neonatal calf diarrhea (NCD) is a common disease affecting the newborn calf worldwide, threatening the cattle production along with significant morbidity and mortality and inducing severe economic losses. There are numerous infectious causes for NCD, bovine coronavirus (BCoV) and group A bovine rotavirus (BRV) are proved to be two major viral pathogens.

2.5:laboratory diagnosis

2.5.1: Electron microscope

2.5 .2:Direct fluorescent antibody (DFA)

2.5.3: Lateral flow immunoassays (LFT)

2.5.4: Hemagglutination (HA) test

2.5.5: Protein A-gold immunoelectron microscopy (PAG-IEM)

2.5.6:Haemadsorption elution assay (HEHA)

2.5.7:Antigen-capture ELISA(enzyme-linked immunosorbent assay)

2.5.8:RT-PCR(reverse transcriptase polymer chain reaction)

(20) Investigated the in vivo cross-protection between BRCV, CD and WD strains of BCOV and the occurrence of reinfections detected by RT-PCR and

nested PCR and compared their sensitivity with that of an antigen-capture ELISA previously developed by laboratory, they found that, the 1-step RT-PCR and nested PCR assays were 50 and 5000 times, respectively more sensitive than the antigen-capture ELISA to detect BRCV in nasal swab suspensions. In addition, the 1-step RT-PCR and nested PCR assays were highly sensitive to detect BCOV in nasal swab and fecal specimens. Therefore, these assays should be useful to diagnose BCOV infections in calves and adult cows.

2.6:Treatment

(21) mentioned that treatment should include correction of fluid loss and dehydration, electrolyte imbalance, acidosis, hypoglycemia, and hypothermia. This correction is achieved primarily through administration of oral electrolytes or intravenous polyionic isotonic crystalloid fluid therapy, and provision of a warm and dry environment. Selection of the type of fluids, the amount to provide, and rate and route of administration is based on the age and weight of the animal(s), severity and duration of clinical signs, level of metabolic acidosis, and whether the affected calf has a suckle reflex.

(22)showed that the treatment of viral diarrheas in newborn farm animals is essentially the same as described for acute undifferentiated specific therapy for viral diarrhea, but antimicrobial agents may be used both orally and parentally for the possible occurrence of secondary enteric and systemic bacterial infections .The withholding of milk for 24-48 is beneficial, but often not possible or practical with nursing beef calves or litters of pigs.

Chapter three

Material and methods

3.Material and Methods

3.1:Kits

Table (1): The kits used for extraction of viral genomic (RNA) and amplification by RT-PCR with their companies and countries of origin:

Kit	No.	Company	Country
AccuZol™ Total RNA Extraction Kit	**2**	**Bioneer**	**Korea**
Trizol 100ml			
AccuPower® RocktScript RT-PCR PreMix	**3**	**Bioneer**	**Korea**
- Rocket Script Reverse Transcriptase (200 u)			
- 5× Reaction Buffer (1×)			
- DTT (0.25 mM)			
- dNTP (250 µM each)			
- RNase Inhibitor (1 u)			

3.2:Primers

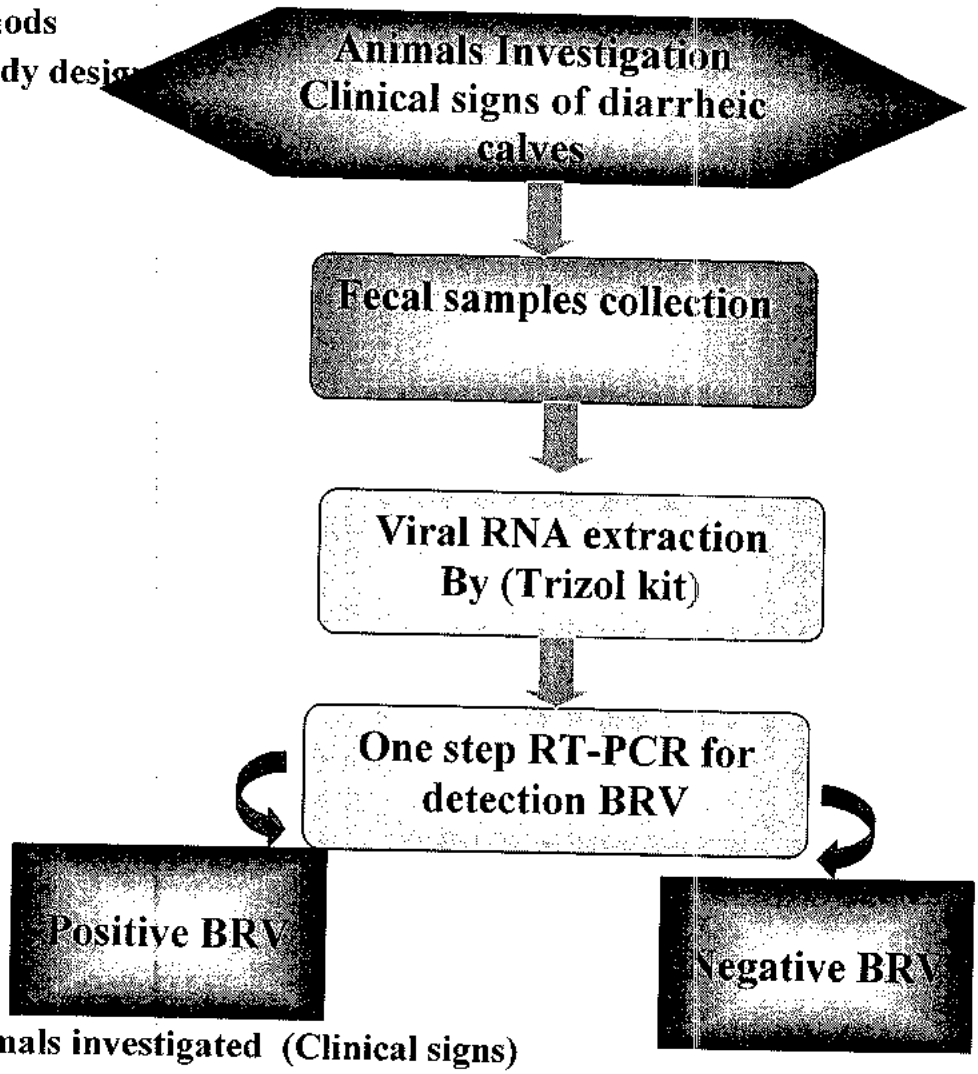
The oligonucleotide primers for bovine rotavirus were designed in this study using the published sequence of VP7 gene found NCBI-Gene Bank and Primer 3 design online. The primers provided by (Bioneer, Korea) company.

Table (2): The Primers and their sequences and RT-PCR product size.

Primer		Sequence	Product size
BRV (VP7gene)	**F**	GTATGGTATTGAATATACCAC	**344bp**
	R	GATCCTGTIGGCCATCC	

3.2:Methods

3.2.1:Study desig



3.2.2:Animals investigated (Clinical signs)

A total of 10 faecal sample were collected from diarrheic neonatal calves ,The occurrence of diarrhea is mostly in cold months all calves had clinical signs of Diarrhoea including dehydration ,all Animal investigated for other condition , reduced appetite or suckle reflex, progressive depression and weaknesses were are typical , a rectal temperatures, color and consistency of feces from each calf were observed . Feces were scored firm, pasty; semi mucoid;, liquid and, profuse diarrhea. The color yellowish watery , yellowish-white watery, greenish-brown sticky, pale yellowish, yellowish-white mucoid.

3.2.3:Fecal samples collection

10 faecal sample were collected from diarrheic and calves . their age was 1-24 day from both sex.For collection of faeces , rectal stimulation was made for the calves then the faeces were collected directly into disposable closed plastic containers ,all sample were collected transported under cold condition to the laboratory where the required test were done or storage at -20 C^{o}.

3.2.4:Viral RNA extraction

Fecal suspensions were prepared in DEPC-treated ultra-pure water to a 1:4 final dilution, clarified at 5,000xg/15min at 4°C and the supernatant submitted to RNA extraction with TRIzol.

The genomic RNA of Bovine Rota virus were extracted by using Trizol RNA extraction Kit (Bioneer, Korea) and done according to kit instructions as following steps:

3.2.5:One step RT-PCR for Detection Bovine Rota Virus

One step RT-PCR for Detection Bovine Rota Virus was performed using AccuPower® RT PCR PreMix reagent kit (Bioneer, Korea) and done according to method optimized in this study as following:

3.2.6:Preparation of RT PCR Master Mix (Rota virus)

RT PCR for detection Bovine Rota virus by using VP7 gene primers were prepared according to kit instructions as following table(3):

RT PCR master mix		Volume
Viral RNA template		5 µL
Primers	F	2 µL
	R	2 µL
DEPC water		11 µL
Total		20 µL

All these components of RT PCR master mix reaction were added into *AccuPower* RT PCR PreMix tube that contain PreMix pellet of all other components of one step RT PCR such as (Revese transcription enzyme for cDNA synthesis, Taq DNA polymerase, dNTPs, MgCl2, KCl, and Green loading dye). Then mixed by vortex for resuspension of PreMix pellet.

3.2.7:RT PCR Thermocycler Conditions (Rota virus)

RT PCR Thermocycler conditions for VP7 primer were done by using Optimase PCR protocol writer online in Simple PCR Thermocycler (TECH-Belgium) as following table(4):

Step	Temperature	Time	Number of cycles
cDNA synthesis	50	60 min	1
Predenaturation	95	5 min	1
denaturation	94	30 sec	35
annealing	55	30 sec	
Extension	72	30 sec	
Final extension	72	7 min	1
Hold	4	4	

3.2.8:RT PCR Product Analysis(Rota virus)

The final RT PCR products were subjected to gel electrophoresis as following steps:

1- A 1.5 gm agarose gel was prepared in 1X TBE buffer to get final 100ml of 1.5% concentration of agarose gel. Then dissolved at 100°C in hot starrier for 15minutes until boiling. After that, left to cool at 50°C.

2- 3uL of Ethidium Bromide were added to agarose gel solution and mixed well, then the tray adjusted and the comb placed at proper position, then the agarose poured in tray and left until solidified, then the comb has been removed.

3- 6uL of PCR product per each sample was loaded in agarose well as well as 6uL of 100bp ladder loaded in one well. Then the agarose gel filled by 1X TBE buffer and electrophoresis cover closed.

4- The electrophoresis device was run at 100 volts and 80 for 1 hour. The samples that showed as positive bands for VP7gene of Bovine Rota Virus visible at 344bp in the PCR product on UV light.

Chapter four

Result and discussion

4 :Result and Discussion

4.1:Clinical signs of bovine Rota Virus

Rotaviral syndrome Diarrhea is considered as one of the most important diseases, because mortality, cost of treatment, and extracted of growth rate would be great economic loss to the cattle industry **(11)**.The effects of diarrhea on the health and efficiency of calves cured from clinical courses of the disease may cause more economic loss (12).

In the present study most calves showed different clinical signs , including diarrhea with or without fleck of blood .The feces were pale yellow, mucoid, a yellow to blood-stained mucous, profuse watery diarrhea, and other clinical signs were severe dysentery, dehydration, loss of weight ,depression and these signs were similar to the study of(13).

10 samples were tested by One step RT-PCR only four samples were shown to be positive for rotavirus. Figure (1).

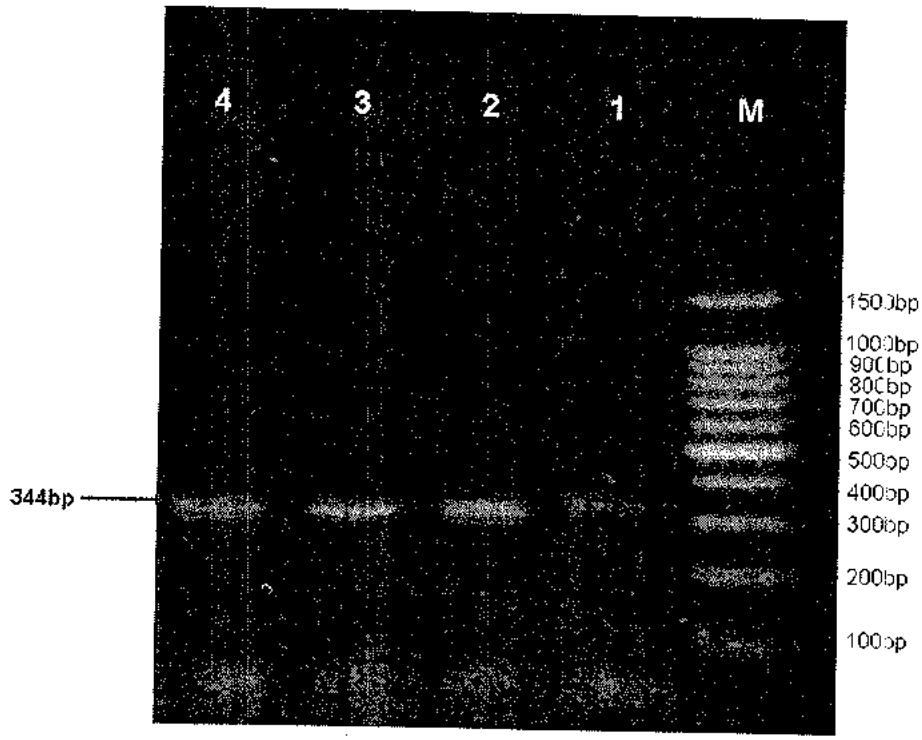


Figure (3): Ethidium bromide- stained Agarose gel of PCR amplified product from extracted RNA bovine rota virus DNA amplified with primer VP7 . The electrophoresis was performed at 80 Ampere for 1 hour product.

Lane marker (M), DNA molecular size marker (100 – 1500 bp ladder).

Lane (1 –4) showing the positive results of Bovine rota Virus primer at specific RT-PCR product size (344 bp)

In the present study by using One step TR-PCR , the mixed infection of coronavirus in diarrheic samples was 6.57% and bovine rotavirus was 40% out of 10 samples positive for corona, in diarrheic fecal samples . our results were closed to result of (14) who found calves younger than 3 months the last researchers mentioned that calves mainly of 1– 21 days old were affected with a percentage of 58.7% and 7.8% respectively .

The result of infection rate shows that the bovine rotavirus and coronavirus in infected calves with age of 5– 22 days, were 40% and 6.57%, respectively this result agreeed with another study **(14)** who reported the presence of BRV in 10% of fecal samples of dairy calves younger than 3 months .

Conclusion and Recommendation

Conclusion

1. Rota viral syndrome Diarrhea is considered as one of the most important diseases in neonatal calves ,because mortality, cost of treatment.
2. The virus is recorded in Iraq in wide spread in different area .
3. RT.PCR assay is more sensitive and more .

Recommendation

1. Good hygiene and management should be care .
2. Provided another methods to like real time PCR for detection the viral causes of diarrhea.
3. Detection the mixed infection causes of diarrhea.