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Genomic diversity and prevalence of Rotavirus in cow and buffalo calves in middle area of Iraq

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Abstract
A total of 335 suspected fecal sample were collected from calf of cattle and buffalo with age in between (3 days to 4 months) from middle area of Iraq between November 2016 to May 2017.
Immuno-chromatographic rapid tests (*FAST*est® Strips) were used for the detection of Bovine, rotavirus, which gave us 159 positive results, while when we took the 159 positive samples and tested by ELISA (cosabio) kit gave us 111 positive results. Extraction of RNA and PCR methods from these 111 positive samples for detection Rota virus using vp7, vp4 primers were done and gave us 19 positive samples, The nucleotide sequence of the VP7 gene of the strains which we got were X57852.1, X52650.1, KP013395.1 & AB457636.1 from the buffalo and cattle calf feces most closely identity to that American G10 P(11), Maxico, UK G10 P(5) and Iranian G10 bovine rotavirus genotype

Keywords: Rotavirus, genotype, calves, genotype

Introduction
Rotaviruses are the most common cause of neonatal diarrhea in calves [8], The virus is present in most cattle herds and typically causes diarrhea in calves up to 3 years. However, periodic asymptomatic re-infection and shedding occurs in older cows and calves [33], There are eight species of this virus, referred to as A, B, C, D, E, F, G and H. Young animals like calves are primarily infected by species A, B. Two surface proteins VP4 and Vp7 of rotaviruses, are important in serotype determination of rotavirus [15], and in inducing neutralizing antibodies and protective immunity, The two structural outer capsid proteins, VP7 (Glycoprotein) and VP4 (Protease sensitive protein) [22], Define as the G and P serotype/genotype of rotavirus, there are At least 27 G genotypes and 35 P genotypes have been identified.
The VP6 gene has been classified into 2 subgroups (SGI and SGII); SGII is the most prevalent and SGI is more commonly found in animals [34]. The virus is transmitted by the fecal-oral route also via surfaces and objects, contaminated food or water. Viral diarrhea is highly contagious. It infects and damages the cells that line the small intestine and causes gastroenteritis and causing dehydration, electrolyte disturbances, fever, shock and death, The diarrhea is caused by multiple activities of the virus. Malabsorption occurs because of the destruction of gut cells called enterocytes. This may result in decreased intestinal absorption of sodium, glucose, and water, and decreased levels of intestinal lactase, alkaline phosphatase, and may lead to isotonic diarrhea [17, 21].
The nucleotide sequences of the 11 RNA segments from different rotavirus strains are known and the complete nucleotide sequence [10].
Sequencing includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine—in a strand of DNA, The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery [14].
Sequencing helped us to determined the rotavirus ID in the NCBI and for detect the genotype identity in our governorates with other world rotavirus genotype by use sangar method that by send dsDNA and forward primers of VP7and VP4 for sequencing [16, 27].

Materials and Methods
Sample collection
A total of 355 fecal samples were collected between November 2016 to May 2017 from animal stations located in different governorates regions in middle of Iraq: (Baghdad, Dyala,

Salah aldden, Babil, Wasit) cattle and buffalo calf with acute diarrhea associated with fever and/or dehydration) were enrolled in the study. Stool specimens were tested for the presence of rotavirus antigen, fecal sample were centrifuged at 6000 rpm for 5 minutes to remove particulates, and stored the supernatant at -20ºC until use.

Immuno-chromatographic: (rapid tests): used (*ABON-FAST*est® Strips) The method described according to the protocol provided by the kit.

ELISA: The results were obtained from Chromatographic Immunoassay kits were tested by ELISA method to reveal the presence of rotavirus antigen, The method described according to the protocol provided by the kit and calculated the result according to the following equation:-

Cut-off value = the average value of $OD_{negative}$+ 0.10

The samples were considered as positive when OD_{sample} is more or equal cut-off value while it was considered as negative when OD_{sample} is less than cut-off value

Extraction of Rotavirus dsRNA from fecal Suspension

The method described according to the protocol provided by the kit manufactured by Qiagen.

Estimation RNA concentration and purity

The viral nucleic acid concentration and purity of the sample extracted from feces were estimated by using Nano drop (Acta/USA). 1µl of the extracted RNA was put in the machine. The purity was (~2.0) that means RNA is high purity according to Acta device protocol

Reverse Transcription (RT) for VP7 and VP4 Genes

The pure RNA which we revealed subjected to RT -PCR. to get cDNA. At first dsRNA was incubated at 56 °C for 5 minutes, A mixture of 8 µl of the extracted dsRNA with 2.5 µl of dimethyl sulfoxide,1 µl of VP7 antisense (VP7R) and 1 µl of VP7 sense primers (VP7F),same procedure used with VP4 primers, The method described according to the protocol provided by QIAGEN kit by utilize Omniscript and Sensiscript Reverse Transcriptases which included in the QIAGEN and provide highly efficient and specific reverse transcription, and also by utilize Hot Star Taq DNA Polymerase included in the QIAGEN OneStep RT-PCR kit which provides hot-start PCR for highly specific amplification. During reverse transcription, reactions were heated to 95 °C for 15 min to activate Hot Star Taq DNA Polymerase and to simultaneously inactivate the reverse transcriptases. The protocol provided by the kit [26].

Table 1: program of cDNA Qiagen.

No.	Step	Temperature	Time	Cycles
1	Reversetranscriptase (RT)activation	50 ºC	30 minutes	X1
2	Polymerase activation &RT inactivation (HotStarTaq)	95 ºC	15 minutes	X1
2	Denaturation	94 ºC	30 second	X35
3	Annyling	48 ºC	30 second	
4	Extenation	72 ºC	60 second	
5	Final-Extention	72 ºC	7 minutes	X1
6	Hold	4 ºC	-	-

[24].

Amplification of VP7 and VP4 Gene

For amplification of VP7 gene, forward (VP7F) and reverse (VP7R) primers, were used in the first round PCR to generate the 896 bp partial length VP7 gene product. For each sample, 5 µl of cDNA was added to a 0.5 ml PCR tube containing 2.5 µl of 10XPCR buffer [200 mM Tris-HCl, (pH8.3), 500 mM KCl], 0.75 µl MgCl2 2H2O (50 mM) 0.5 µl dNTP mixture [0.2 mM dATP, dACTP, dGTP, dTTP]; 1 µl of reverse primer (VP7R), 1 µl of forward primer (VP7F) is shown below and 14 µl sterile triple distilled water. Above mixture was mixed properly and given a small spin. Then subsequently 0.25 µl of Taq polymerase was added to each reaction tube. The tubes were placed in a thermal cycler and the PCR reaction was cycled 35 times maintaining the proper conditions. Same procedure was used for VP4 gene amplification by use VP4 forward (VP4F) and reverse (VP4R) primers which is shown below [22].

The primers which used in the tables below

Table 2: (the forward and reverse vp7 primer were we used, [14]

1	9CON 1F	VP7 Forward	ATGTATGGTATTGAATATACCAC	896 bp
2	VP7 R	VP7 Reverse	AACTTGCCACCATTTTTTCC	896 bp

Table 3: the forward and reverse vp4 primer were used [29].

1	CON3 F	VP4 Forward	TATGCTCCAGTNAATTGG	876 bp
2	CON 2 R	VP4 Reverse	ATTGCATTTCTTTCCATAATG	876 bp

Nucleotide sequencing

Amplified products of the VP7 and VP4 genes we obtained from conventional PCR, with the forward primers (VP7F), (VP4F) sent to (macrogen) Korean company for sequencing, direct sequencing of each amplicon was carried out using the Sanger dideoxynucleotide chain termination.

Results

Immuno-chromatographic rapid tests

The purpose of qualitative detection of Rotavirus in faeces specimen by chromatographic immunoassay (Monoclonal antibodies) to capture the presence of virus antigen. In our study samples were collected from 335 suspected animals gave us 159 positive infection rotavirus as in the table below.

Table 4: Results were obtained using Chromatographic Immunoassay kits for *rotavirus* in 335 fecal samples according to age of tested calves.

Region	AGE										
	1wk.	**2 wk.s**	**3 wk.s**	**4 wk.s**	**5 wk.s**	**6 wk.s**	**7 wk.s**	**8 wk.s**	**12 wk.s**	**14 wk.s**	**Total**
Dyala											
Positive	2	0	0	0	1	0	0	0	1	0	**4**
Negative	7	12	2	4	0	1	0	3	1	1	**22**
Total	9	12	2	4	1	1	0	3	2	1	**26**
Salahalden											
Positive	6	2	0	0	0	0	0	0	0	0	**8**
Negative	6	6	1	4	1	1	1	0	0	0	**27**
Total	12	6	1	4	1	1	1	0	0	0	**35**
Babel											
Positive	4	1	1	0	0	0	0	1	0	0	**7**
Negative	10	10	2	4	12	1	11	5	3	0	**57**
Total	14	11	3	4	12	1	11	6	3	0	**64**
Wasit											
Positive	19	10	11	10	5	8	8	10	4	0	**85**
Negative	3	2	0	0	2	3	2	2	10	10	**34**
Total	22	12	11	10	7	11	10	12	14	10	**119**
Baghdad											
Positive	13		10	9	8	4	5	2	5	0	**55**
Negative	2		3	2	2	0	5	3	11	9	**36**
Total	15		13	11	10	4	10	5	16	9	**91**

wk. = week

((Enzyme-linked Immunosorbent Assay (ELISA)

In the current study, all the 159 samples from chromatographic immunoassay were confirmed by ELISA tested, our study gave us 111 (70%) positive samples. The highest positive results were recorded in Wasit 71 positive 84 %, and the lowest 3 only were in Babil 42 %)from these 159 positive sample.

Conventional Polymerase Chain Reaction

In this study, PCRtechnique were used for detection the two outer layer protein'sVP7and VP4. All bovine stool, which had been tested using Chromatographic and ELISA, were confirmed by PCR and gave us 19 (45%) positive result

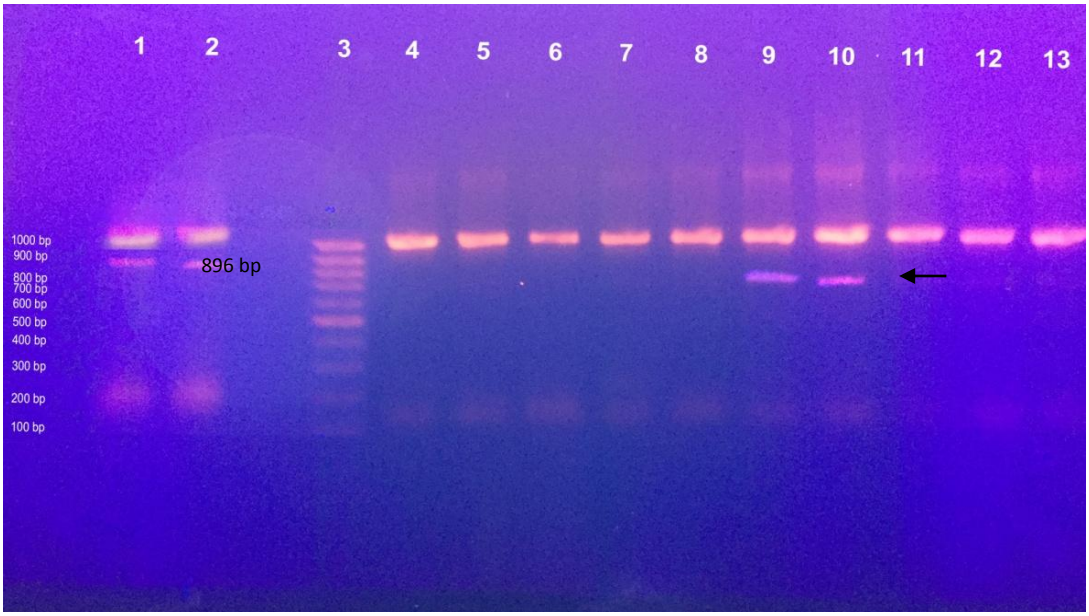

Fig 1: The gel electrophoresis of PCR products of VP7 (896bp) in and VP4 (876bp) on agarose gel with 100bp DNA ladder (line 1) at 70 voltage for (60) min. lane 1, 2: VP7 (896bp) and lane 9, 10: VP4 (876bp).

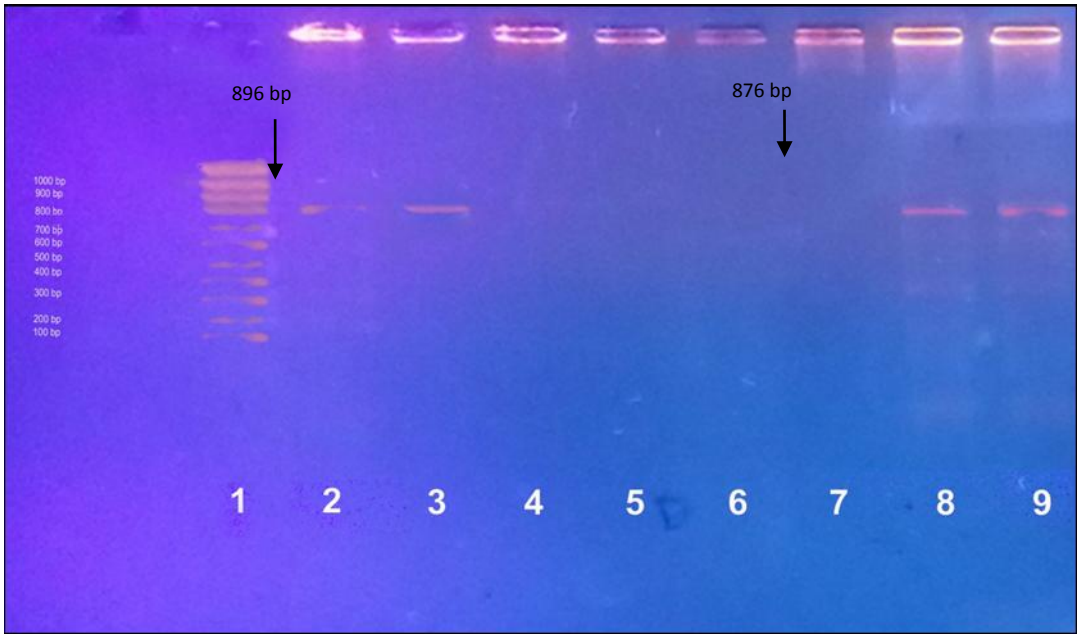

Fig 2: The gel electrophoresis of PCR products of VP7 (896bp) in and VP4 (876bp) on agarose gel with 100bp DNA ladder (line 1) at 70 voltage for (60) min. lane 2, 3: VP7 (896bp) and lane 8, 9: VP4 (876bp).

Sequencing and Genotyping of VP4 and VP7

In this study multiplex polymerase chain reaction technique and sequencing were used for genotyping of both VP7 and VP4. However we took the highest 42 titer from Elisa test to use it in molecular detection PCR technique reveal 19 positive result which were send for sequencing, the result of sequencing gave us 12 positive result were done in south Korean macrogen sequencing company, these are ID: X52650.1 (G10P [11]), KP013395.1 (G10), AB457636.1, (G10P [5]) and X57852.1 (G10P [11]) according to the Ncbi.

The nucleotide sequence of the VP7 gene of the strain were most closely related to that of a Iranian G10, American G10 P(11) and British G10 P(5) bovine rotavirus, from the cattle calf feces. Identity 98% to 97% G10P (11) were most frequently detected were 7 G10 P(11) in bovin samples genotype specially in Wasit provenance, That by six samples (four in Wasit, one in Dyala and Baghdad were X52650.1 which we found was identity 98% to Rotavirus A RVA/Cow-tc/USA/B223/1983/G10P [11] Sequence ID: LC133552.1 and we found two samples from calves in Wasit and Baghdad have the Sequence ID: KP013395.1 identity, partial cds to Rotavirus A isolate Markazi-G10 and identity 97% to Iranian Rotavirus A strain where three samples two from Baghdad and one in Babel with ID sequence AB457636.1 identity 99% to Rotavirus A strain RVA/Vaccine/USA/BRV-KC-1xUK/2009/G10P [5] segment 9 capsid glycoprotein VP7 (VP7) gene, Sequence ID: KC215545.1. four in Wasit, one in Dyiala and one from Baghdad were X52650.1 which identity 98% to Rotavirus A RVA/Cow-tc/USA/B223/1983/G10P [11].

Where in Wasit and Baghdad have the Sequence were ID: KP013395.1 identity, partial cds to Rotavirus A isolate Markazi-G10 and identity 97% to Iranian Rotavirus A strain and only one sample from Salah alden was ID: X57852.1 (strain B223) identity 98% to Rotavirus A RVA/Cow-tc/USA/B223/1983/G10P [11] VP7 gene for structural protein VP7, complete cds Sequence ID: X52650.1.

Discussion

Diarrhea is a leading cause of economic losses to the beef industry and major cause of calf mortality and morbidity during the first few weeks of life in most countries [27]. Most infected age of positive calves was between8-15 days of age [6].

Defining the map of calve diarrheal-causing entero pathogens allows for application of effective and targeted preventative measures. In middle area of Iraq, our study reported the prevalence incidence of the group A rotavirus in cattle and buffalo calves, Immuno-chromatographic rapid tests (FAS Test® Strips) which we used as a screening test gave as 159 positive from 335 fecal samples, the result revealed 71% (85 of 119 samples) were high positive in Wasit, but the lowest positive was in Dyala 15% (4 of 26 samples). The first week of age of infection gave us higher titer (44 positive case) which were the highest result from 159, the lowest infected age were 14 week and didn't give positive result, Most infected age of positive calves was between 3-15 days aged because the calf was born with no possesses natural antibodies to fight disease, high levels of serum estrogen and maternal-fetal cortisol produced at the end of pregnancy and calving have immunosuppressive effects on the cellular components that participate in the innate Immune Response (IR) [12]. In general previous evaluation studies suggested that rapid field test could be considered as a helpful tool for fast and effective diagnosis of common entero pathogens associated with neonatal calve diarrhea [11].

Thereafter we took 159 from the positive samples tested using ELISA as confirmatory tests to reveal the presence of rotavirus antigen. ElISA is more specific, more accurate, and rapid assay that have been used for detection of Rotavirus Antigen. Enzymes produce the signal and related to the detection reagents in fixed proportions to permit accurate quantification [1].

Chromatographic Immunoassay and ELISA are the simple and good standard methods for detection of rotavirus. These methods, however, require low cost equipment and simple experience, which is available in many laboratories. Some researchers for detecting rotavirus infection have used ELISA and PCR. These methods are expensive and requires long time if they have been used together [13].

The highest 42 positive titter of ELISA from fecal bovine samples were taken due to the molecular detection roots which were very difficult and high cost. These 42 ELISA higher positive titter agree with [11] who's got high selective result by ELISA. Then we utilize VP7 and VP4 primers for RT PCR and amplification which gave us 19 positive samples

(17%) because of the Iraqi hot climate and molecular lab difficulties, were these positive samples for molecular detection sent to sequencing and genotype our result gave us 12 positive.

In the present study we got ID sequence are KP013395.1, AB457636.1, X52650.1 and X57852.1 according to the NCBI, G10 were observed as a highly prevalent strain circulating in both buffalo and bovines calves, Specially among buffalo that agree with calves rotavirus Indian study [22] and 3 of P typing isolate were p [5] and that agree with Italian study [25].

Three isolates in this study were G10P [5] that agree with Indian project result [29]. in Our study the highest G and P genotype were G10P(11) in 7 isolates this agree with the other related regional researches [22].

Also the identity of sequence ID: LC133552.1, agree with [5] in Brazil which was 98% to our Sequence ID: X52650.1 in recent study.

Our study agree with the regional similar researches like Iranian study (Determination and distribution of the G and P genotypes of group A bovine rotavirus) were investigated on 386 fecal samples collected from calves with diarrhoea using a semi-nested RT-PCR typing assay, they got by ELISA. 75 positive samples were selected randomly and subjected to typing assay. G10 (50.6%) and P[11] (64%)) [4]. that may explain why G10 was the most distribution in our area.

While that different from turkey study which found genotype G1, G2, and G9, VP7 specificities and genotype P[4], P[6] and P[8] VP4 specificities and The most common strain was G2P[4] [3]. Also there is study in Saudi Arabia showed the distribution of serotype G1-G4, G9, G12, P [4], P [6], and P [8]. [19] these tow related studies showed genotype difference from our genotype.

Various hypotheses previously supported that BRVs may cross the host species barrier and circulate among neonates or adults and can cause zoonotic transmission and can infect human beings [35] like G10 P(11) was highly distributed and can transmit from animal to human and opposite by Indian project [16].

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