Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Molecular Characterization and Phylogenic Analysis of Rotavirus Isolated from Cattle in Sulaymaniyah, Iraq

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Abstract

BOVINE Rotavirus (BRV) is a major pathogen associated with acute gastroenteritis in animals and humans. This disease usually affects young animals, and as the animal ages, its susceptibility decreases. In both humans and animals, Bovine rotavirus (BRV) is an important pathogen associated with acute gastroenteritis. Approximately 18% of calves with diarrhea were found to be infected with BRV, with differences in prevalence among age groups, breeds, and geographic locations. Using a sterile swab, stool samples were taken directly from the anus of the calves and sent to the laboratory via virally transmitted media. The age, sex, breed, and location of the calves were noted using questionnaire sampling. RT-PCR successfully amplified the VP7 genes of the isolates, and the resulting genes showed an amplicon size of approximately 884 bp. In addition, phylogenetic analysis revealed that field virus sequences belonged to lineage II and clustering Iran and Turkish strains, with the highest identities ranging from 97.12%, and 96.82% to 97.17%, respectively. According to the study results, 18% of newborn calves were infected with rotavirus diarrhea in cows. Improving colostrum intake, vaccinating pregnant cows, and maintaining environmental hygiene are all important steps in reducing the incidence of rotavirus-related diseases in calves.

Keywords: Bovine Rotavirus, Cattle, Phylogenic analysis, RT-PCR, Sulaymaniyah.

Introduction

Bovine Rotavirus (BRV) is one of the main pathogens associated with acute gastroenteritis in animals and humans. The disease typically affects young animals, and as an animal ages, its susceptibility decreases. This is probably because of physiological changes in the animal or acquired immunity from prior exposures [1]. Symptoms of BRV infection include diarrhea, dehydration, and depression, and it typically affects calves between the ages of 15 and 45 days [2]. Additionally, it is linked to an increased incidence of infection and mortality in these calves [3,4]. The rapid infection spread results in extensive lining damage to the intestine which accelerates fluid loss and dehydration. Due to calf mortality, decreased productivity, higher treatment costs, and poor growth, rotaviral gastroenteritis causes large economic losses in the cattle industry [5]. Of all diarrheal agents, BRV is the only one that causes 62.5% of diarrhea outbreaks in dairy and beef herds [6]. Rotavirus infections occur from fall to spring. The virus exhibits seasonal patterns in temperate zones, with the colder months seeing the greatest epidemic peaks. Seasonality is less pronounced in tropical and subtropical environments [7]. Rotavirus infection prevalence varies with risk factors, including herd size and the quantity and timing of colostrum feeding [6]. According to [8], rotavirus is a non-enveloped viral particle with a diameter of 70-75 nm. It belongs to the Sedoreoviridae family Sedoreoviridae and the genus Rotavirus [9]. At least seven different genetic groups, or serogroups (A–G), comprise the Rotavirus genus. Rotavirus A (RVA) is the most diverse species in the genus regarding both genetic diversity and antigen diversity. The highest

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(Received 03 June 2025, accepted 10 July 2025)
DOI: 10.21608/ejvs.2025.391787.2888

frequency and pathogenicity of RVAs in humans and a wide range of animals make them the most significant. The 11 double-stranded RNA segments that comprise the RVA genome range in size from 16 to 21 kb. They are encased in a triple-layered virus particle that has an outer, inner, and core capsid [8,10]. The genome encodes six structural proteins (VP1-VP4, VP6, and VP7), as well as five or six non-structural proteins (NSP1-NSP6). To characterize the two outer capsid proteins, VP4 and VP7, which independently elicit neutralizing antibodies, a binary classification system similar to that used to classify influenza viruses has been established. As a result, RVA strains are divided into two categories: VP7 or G types (for glycoprotein), and VP4 or P types (for protease-sensitive) [11]. The virus primarily spread through interspecies transmission. Important mechanisms that drive the diversity of rotaviruses and allow for the emergence of new pathogenic strains with altered virulence are interspecies transmission and subsequent reassortments [12]. Transmission occurs through direct contact or the fecal- oral route [13]. In calves, rotavirus diarrhea is an acute disease with a 12- to 24-hour incubation period, or occasionally 18 to 96 hours. Fortunately, although morbidity is typically high, the majority of rotavirus infections are mild and self-limiting. Differences in rotavirus strain virulence, host age, immune status, inoculum dosage, occurrence of mixed infections, environmental stressors (housing, overcrowding, weather), and nutrition all influence the clinical disease variations seen in calves. These elements include fluid loss and metabolic acidemia, anorexia, profuse watery diarrhea, varying degrees of systemic dehydration, and the systemic effects of electrolyte imbalances. In extreme situations, cardiac arrest, dehydration, and electrolyte imbalances result in death. Because maternal antibodies pass through the placenta and into breast milk, rotavirus infections in infants under one month of age are often asymptomatic or mild [7]. The diagnosis of rotavirus laboratory is essential to manage and prevent the occurrence of the disease in calves. Since it can be difficult to identify the causal agents through a clinical examination, a laboratory test is a crucial diagnostic tool. Numerous tests, including polymerase chain reaction (PCR), antigen-captured enzyme-linked immunosorbent assay (Ag ELISA), virus isolation, and electron microscopy (EM), can be used to do this. According to [14], the diagnosis of rotavirus is typically predicated on the detection and separation of the virus from the feces [14, 15]. There is no specific treatment for rotavirus infections [15]. Therefore, the focus of treatment is on managing clinical symptoms and potential complications in addition to providing supportive care. Fluid replacement, acidosis correction, and electrolyte imbalance restoration are all dependent on fluid administration in livestock and companion animals. The most crucial elements of an effective rehydration solution are a sufficient sodium concentration and the right ratios of glucose to sodium [16]. The esophageal catheter can be used to administer fluids to young animals; intravenous administration is preferred for older animals [17]. Consequently, fluid administration, electrolyte management, and antiviral therapy are the most commonly used treatments for rotavirus infections [18]. Prevention and control strategies include:

- Improved colostrum intake (ensuring calves receive adequate antibodies).
- Enhanced sanitation (reducing environmental contamination).
- Surveillance and early detection (using accurate diagnostic methods) [19].
- Vaccination (especially for pregnant cows to enhance passive immunity in calves) [20–22].
- Developing new antiviral agents against BRV is essential for improving animal health [23].

Sulaymaniyah is one of the largest cities in Iraq and is located in the northeast. The city includes several neighborhoods and many villages, and the number of livestock is about 40 thousand heads. There have been various recent outbreaks of diarrhea in calves. However, definitive molecular characterization of the causative agent has not been followed by molecular techniques. In this study, we discovered and described the gene and performed a phylogenetic analysis of sequences with different isolates published in the GenBank databases.

Materials and Methods

Sample collection

The fecal samples were collected directly from 80 diarrheic calves from four different districts in Sulaimani province (Sharzoor, Khurmal, Halbjai Taza, and Kalar) from 10/2022-8/2023. The study area was located at 34-35°N and 45-46 °E (Figure 1). This region has four distinct seasons with different rainfall in autumn, winter, and early spring that ranged between 750 and 400 mm. This area has different habitats, such as a river, meadow pastures, and agricultural lands with high biological diversity. The animals were classified into three age groups (0-29 days, 30-59 days, and 60-90 days). The samples were collected directly from the diarrheic calf anal by sterile swab and transported through viral transport medium to the laboratory and the age; sex, breed, and location of the calves were recorded by using a questionnaire sampling.

RNA Extraction:

Viral RNA of the fecal samples was extracted using Genesaid (Genaid, Korea) following the manufacturer's guidelines. After precipitation, the RNA was dissolved in an eluted buffer. Then, this purified RNA was utilized for one-step RT-PCR.

Reverse transcriptase polymerase chain reaction (RT-PCR):

The RNA was used to amplify the bovine rotavirus gene by using primer set VP7-Forward (5' - ATG TAT GTT ATT GAA TAT ACC AC- 3') at positions 51-71 and VP7-Reverse (5' - AAC TTG CCA CCA TTT TTT CC- 3') at positions 914-933 was utilized to amplify a 884- of the VP7 gene [24, 25]. The one-step RT-PCR was carried out in a 0.2 ml PCR tube, comprising the following components: 10 µL of Genetbio SuprimeScript Premix (2x), 5 L of Korea, 5 µL of the extracted RNA, 1 µL each of forward and reverse primers (10 pmol) and 5 µL of ultrapure water, making up a total volume of 20 µL for the reaction. The amplification was performed using a thermal cycler from (BIO-RAD, USA). The PCR protocol involved an initial synthesis of cDNA at 50 ° C for 30 minutes, followed by an initial denaturation phase at 95 ° C for 10 minutes. Subsequently, 40 cycles were performed, consisting of denaturation at 95 ° C for 30 seconds, annealing at 59 ° C for 30 seconds, extension at 72 ° C for 35 seconds, and final extension at 72 ° C for 5 minutes.

The PCR products were analyzed using 1.5% agarose gel electrophoresis at 120 V for 40 minutes and viewed with a UV light trans illuminator after being stained with a safe dye (Gendirex).

Nucleotide Sequencing

VP7-amplified products derived from traditional PCR, utilizing VP7 forward and VP7 reverse primers, were forwarded to (Macrogen Co. Republic Korea), for sequencing. Each amplicon was directly sequenced through the Sanger dideoxynucleotide chain termination method.

Sequencing and Phylogenic Analysis

The resulting nucleotide sequence data were compared with the corresponding rotavirus sequences from the GenBank database. Multiple sequence alignments were performed using the Clustal-W method. Phylogenetic analyses were conducted using the neighbor-joining method and the Kimura-3 parameter using MEGAX software [26] . The reliability of branching was evaluated by the bootstrap test with 100 replicates [27, 28].

Statistical Analysis

The data were analyzed using SPSS version 25.0). and the Significance level of 0.05 was determined. The Chi-square test was employed to determine significant differences between infected species among the regions, breeds, and age. Additionally, multiple correspondence analysis was used to clarify the relationship between categorical variables. The odds ratio (OR) and relative risks (RR) were calculated to identify positive cases. These measurements were then compared against the evidence of the diseases.

Results

The P value between infected species among different locations was (P=0.825), the P value among breeds was (P=0.349), and the P value among different ages was (P=0.111). No significant differences were found in geographical locations, breeds, and ages.

The outcome of the present study showed that the infection of the animals with rotavirus differs according to breed, age, and location. The infection of rotavirus in the Friesian breed peaked at the highest 28% (OR: 2.5) than Local and Simmental breeds at 16% and 13% respectively. Regarding the geographical location, Khurmal had the highest prevalence which was 25% (OR: 1.9) while Kalar had 20%. The lowest infection percentage was in Halabja and Sharazoor which was 15% each. Rotavirus had different infectious statuses depending on the ages of the animals. Animals with the range of one month of age had the highest prevalence of 29% (OR: 5.1) whereas animals with two and three months of age had a prevalence of 18% and 0.7% respectively (Table 1).

Regarding the distributions of infections according to breeds, age, and geographical location, this study recorded that an association has been found between age, breed, and locations with the infection status. Meaning that all three breeds, four locations and three age groups had a relation with the infection. Moreover, we can say infection with rotavirus may be recorded in different levels with regards to breed, age, and geographical locations. (Fig. 2).

RT-PCR successfully amplified the VP7 genes of the isolates, and the resulting genes exhibited an amplicon size of approximately 884 bp (Figure 3). Through the analysis of the BLAST tool, the sequences obtained for genotype G10 were verified and compared with the existing sequences in the GenBank database. Subsequently, these genotype sequences were submitted to the GenBank database and assigned accession numbers OR786724 and OR786725.

Bioinformatics analysis

Nucleotide sequences of the VP7 genes of the two BRVs exhibited restricted diversity, showing close relatedness to each other, with DNA sequence identities ranging from 98. 63% and amino acid homology of 99.32%. Since this has not been conducted previously in Iraq, we cannot perform an accurate analysis of the variation of the VP7 gene.

Phylogenetic analysis of VP7 genes in our study, represented by strains named BRV/S/Iraq and BRV/S2/Iraq, indicated a correlation with strain TUR / KIRSEHIR and an Iranian strain (NZK6/Iran/2012), formed a cluster belonging to lineage II (Figure 4).

Furthermore, the findings revealed that the sequence of the VP7 gene of strains (BRV/S/Iraq and BRV/S2/Iraq) closely resembled Iranian strains (Khorasan-G10 KP013393.1) and Turkish strains (TUR/KIRSEHIR-OQ082576.1), with DNA identities ranging from 97.12%, and 96.82%-97.17%, respectively (Table 2).

Discussion

Calf diarrhea is a common and frequent disease in cattle rearing [10, 29], As a result of its causes, calf diarrhea can be broadly classified into two groups: infectious diseases, which include bacterial, viral, and parasitic infections, and non-infectious diseases, which include conditions linked to environmental discomfort, nutrition, or management [30, 31]. Viral diarrhea is the most dangerous and challenging to prevent among these causes [32]. Bovine rotaviral diarrhea in newborns is significant due to its economic consequences and widespread occurrence in veterinary and agricultural settings. The disease presents a serious risk to calves' health because it can cause malnutrition, dehydration, and, in extreme situations, higher death rates. Although some vaccines against bovine rotaviral diarrhea in newborns have been developed, these treatments' effectiveness is consistent and field prevention efforts are limited. For these reasons, it is crucial to develop a highly specific and sensitive test kit for diagnostic purposes [33]. One of the most common methods in molecular biology diagnosis is the PCR assay [33]. Bovine rotavirus (BRV) was detected in approximately 18% of diarrheal calves (Table 1). There were notable variations in the prevalence of BRV infection between different regions, breeds, and calves' ages. The overall prevalence of BRV of 18% among diarrheal calves is consistent with earlier research on rotaviral infection, which found that the infection was prevalent in Bangladesh at 18.3% [34]. The study result was found to be higher than those found by other studies, which included 16.7% in Ethiopia [35], 3.64% in Ethiopia [36], and 12.2% in China [32]. Interestingly, other countries were found to have a much higher prevalence of this viral infection than we did in our study: 63% in Argentina [37], 79.9% in Australia [21], 32.5% in Sudan [38], and 36% in Iraq [39]. Various spatial, temporal, and management-related factors could impact the prevalence of BRV infection. The year of the study, the variations in the study design, the sample size, the analytical approach, the sensitivity of the diagnostic tests used, environmental factors, the agricultural management practices used, the hygiene measures, and the geographic locations could all be contributing factors to the inconsistent results [36].

Our findings corroborate the earlier study's conclusion that calves in their first few weeks of life are more prone to infection than older calves [40]. Age-related variations in infection are likely caused by the enterocytes' loss of receptors [41]. In calves between the ages of 0 and 29 days, there were 8 (29%) cases of BRV, indicating a high prevalence of the virus (Table 1). The absence of passive immunity is a decline in natural immunity to infections [36], and insufficient maternal antibodies in the colostrum could be the cause of this [42]. Rotavirus infection was found in 5 (18%) of the calves aged 30-59 days. This could be related to the care and attention that the study area provides to newborn calves in this age range. The third age group (60-90 days) had the lowest prevalence of pathogens 2 (0.7%). This could be explained by the fact that when calves become three months old, their natural immunity to pathogens increases [43].

The animal breed plays a significant role in determining the immune response and severity of the disease; cross-bred calves are more likely to contract BRV than native calves [42]. The various animal breeds studied showed varying rates of infection (Table 1). Friesian was the most common (28%), followed by Simmental (16%) and local (11%), which was the least common.

According to this study, the prevalence of BRV infection varied by district, with Khurmal having the highest prevalence (25%), Kalar having the lowest (20%) and Sharazor and Halbjai Taza having the lowest infection rates (15%). This could be the result of variations in the surrounding area, calves' nutritional status, the type of animal raised, the use of hygienic practices in animal sheds, or the dairy farm's management [6].

Phylogenetic analysis revealed that the VP7 genes in our study, which were represented by the strains BRV/S/Iraq and BRV/S2/Iraq, correlated with the strain TUR / Kirsehir, and an Iranian strain (NZK6 / Iran / 2012), formed a cluster that belonged to lineage II (Figure 1) ،Therefore, both Iran and Turkey are expected to be the main sources of the disease. Furthermore, with DNA identities ranging from 96.82% to 97.17% and 97.12%, respectively, the results showed that the VP7 gene sequences of the strains (BRV/S/Iraq and BRV/S2/Iraq) closely resembled the Iranian and Turkish strains of BRV. These findings align with previous studies carried out on cattle in Bangladesh and Iran, which found that RVA is one of the leading causes of diarrhea in calves, along with BRV [6, 15].

Conclusion

Among calves with diarrhea in the Sulaymaniyah governorate, Iraq, the study effectively detected and characterized bovine rotavirus (BRV) strains. Reverse transcription-polymerase chain reaction

(RT-PCR) and bioinformatics were used in molecular analysis to enhance the VP7 gene of BRV isolates. The sequences produced showed a high degree of genetic similarity. Iraqi BRV strains and strains from Turkey and Iran belonging to the second lineage had a close relationship, according to the phylogenetic analysis. 18% of calves with diarrhea were found to be infected with BRV, with differences in prevalence between age groups, breeds, and geographic locations. Younger calves have been shown to be more susceptible to BRV infection, especially those between 0 and 29 days of age. These findings underscore the need for focused surveillance and prevention measures and advance our knowledge of the epidemiology of BRV. Reducing the incidence of BRV-associated diseases in calves requires policies such as vaccinating pregnant cows, improving colostrum transport, and improving environmental hygiene. To track the genetic diversity of BRV and create effective control strategies, ongoing research, and monitoring are crucial.

Acknowledgments

We would like to thank the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine of the University of Sulaymaniyah, Sulaymaniyah, for their support in conducting and processing all fecal samples. This project is funded by Sulaymaniyah University, Ministry of Higher Education and Scientific Research, Kurdistan Regional Government, Iraqi Kurdistan.

Funding statement

This study didn't receive any funding support

Declaration of Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Fig. 1. Geographical location of (Kalar, Halanjai Taza, Khurmal and Sharazoor) in Sulaymaniyah province on the map, where fecal samples were collected from calves for PCR tests.

(http://www.maphill.com/iraq/sulaymaniyah/panoramic-maps/savanna-style-map/)

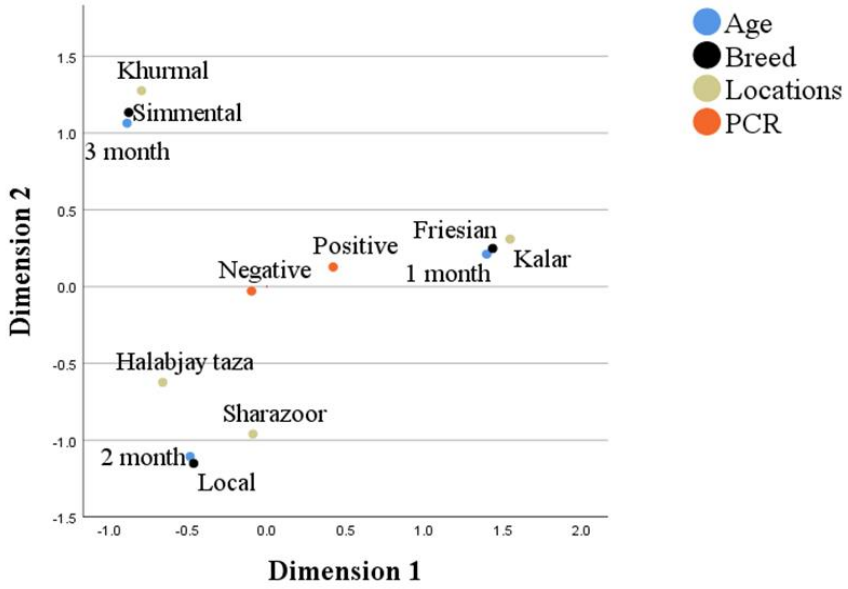

Fig. 2. Illustrates the distributions of age, breed, and geographical location associated with the infection status of rotavirus.

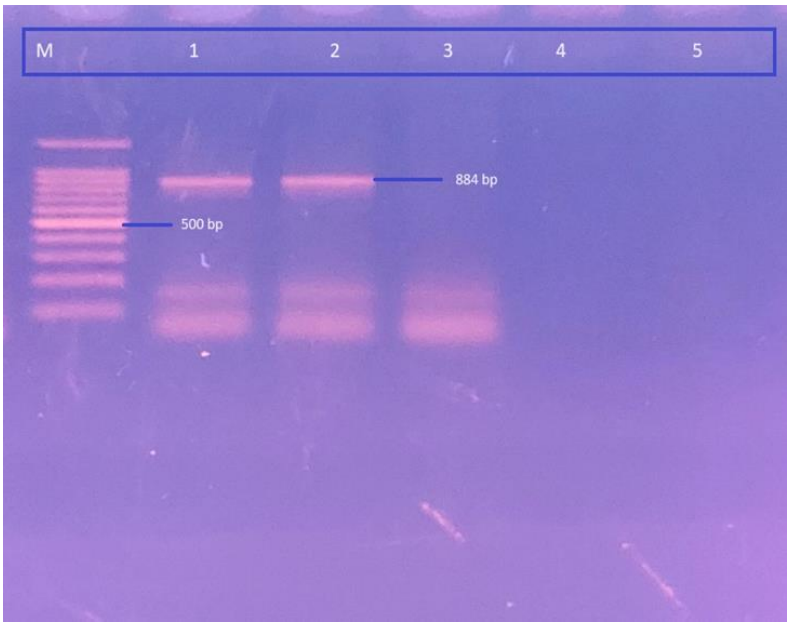

Fig. 3. The specific fragment of the VP7 gene (884bp) was amplified with the primer VP7-F/VP7-R. Lane M DNA marker (100bp), Lane 1-2, positive sample, Lane 3-5 negative sample.

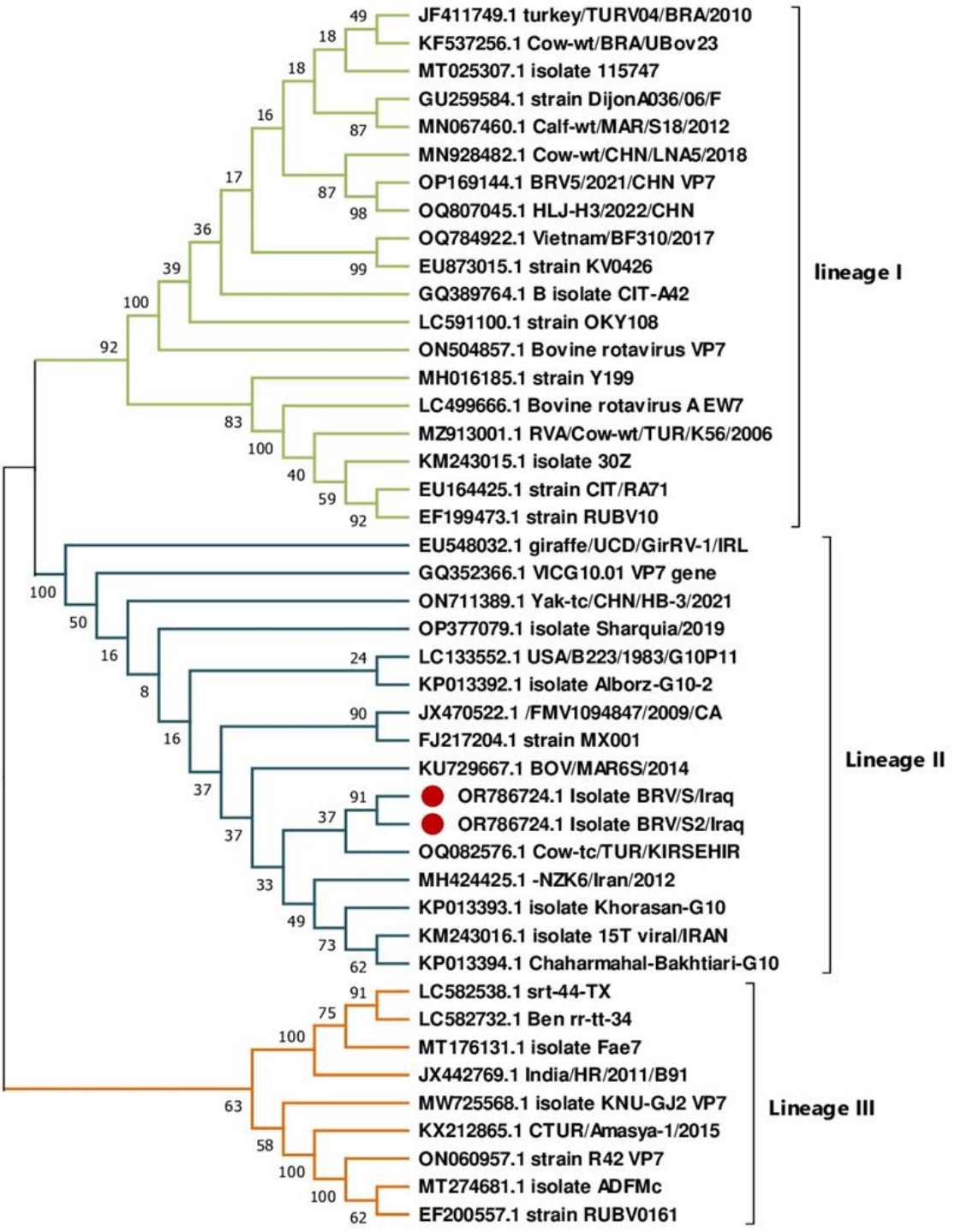

Fig. 4. Phylogenetic connections, determined by analysis of the VP7 gene segment of BRV, were established by comparing field strain isolates with reference strains. The neighbor-joining method with 1000 replicates, conducted using MEGA X software, revealed the relationships. BRV field virus variants are highlighted with a red square.

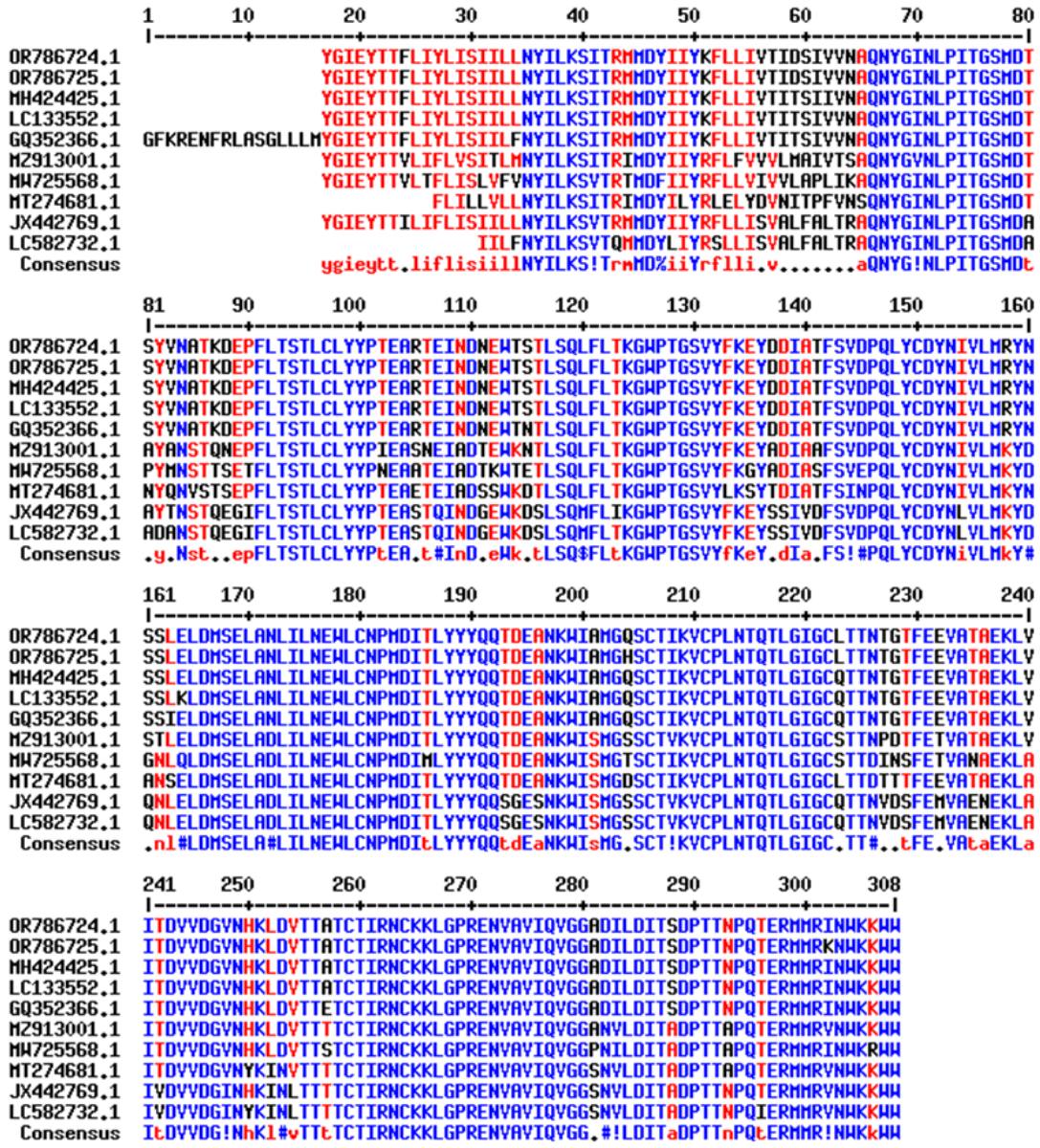

Fig. 5. Deduced amino acid alignment between field virus strains BRV of partial sequence VP7 gene with different strains at position (17-308).

TABLE 1. The prevalence rate of Rotavirus infection is determined by age, breed, and geographical location, with an odd ratio.

Location		No. of samples	Positive numbers	Percent positivity %	ODD [CI]	RR [CI]
	Kalar	20	4	20%	1.4 [0.27-7.3]	1.3 [0.3-5.2]
	Sharazoor	20	3	15%	Ref	ref
	Halabja	20	3	15%	Ref	ref
	Khurmal	20	5	25%	1.9 [0.4-9.3]	1.7 [0.5-6.1]
Breed	Fresian	25	7	28%	2.5 [0.6-9.9]	2.1 [0.7-6.4]
	Simmental	30	4	13%	1.2 [0.3-5.6]	1.2 [0.3-4.3]
	Local	25	4	16%	Ref	
Age	1	27	8	29%	5.1 [1-27.1]	3.8 [0.9-16.4]
	2	27	5	18%	2.4 [0.4 – 13.6]	2.2 [0.5-10.2]
	3	26	2	0.7%	Ref	

TABLE 2. DNA sequence identities of VP7 gene of the one field isolate of (BRV/S/Iraq) with different strains available in GenBank.

Accession No.	Strain/country	DNA identity	G-typing	Lineage
OQ082576.1	TUR/KIRSEHIR	96.82 - 97.17	10	II
MH424425.1	NZK6/Iran/2012	95.52	10	II
KP013393.1	Khorasan-G10	97.12	10	II
KM243016.1	Iran/isolate 15T	96.13-96.47	10	II
EU548032.1	1/IRL/G10	90.16-90.28	10	II
GQ352366.1	Autralia/VICG10.01	93.62	10	II
LC133552.1	USA/B223/1983	94.64-94.75	10	II
MZ913001.1	TUR/K56/2006	75.40	G6	I
OQ784922.1	Vietnam/BF310/2017	76.30-76.65	G6	I
OQ807045.1	HLJ-H3/2022/China	76.88	G6	I
GU259584.1	DijonA036/06/France	75.42	G6	I
MN067460.1	MAR/S18/2012/Moroco	75.42- 76.54	G6	I
MW725568.1	Korea/KNU-GJ2	75.65-75.87	G5	III
ON060957.1	China/ R42 VP7	74.74 74.87	G8	III
MT274681.1	India/ADFMc	74.24 74.71	G8	III
EF200557.1	India/RUBV0161	74.74 74.87	G8	III
LC582732.1	Iraq/Ben rr-tt-34	72.49 72.37	G1	III
JX442769.1	India/HR/2011/B91	74.03 73.80	G1	III

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التميز الجزيئي والتحليل النشوي لفيروس الروتا المعزول من الأبقار في السليمانية، العراق

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الملخص

فيروس الروتا البقري هو أحد مسببات الأمراض الرئيسية المرتبطة بالتهاب المعدة والأمعاء الحاد في الحيوانات والبشر. يصيب هذا المرض عادة الحيوانات الصغيرة ، ومع تقدم الحيوان في العمر ، تقل قابليته للإصابة به. وقد وُجد أن 18 ٪ من العجول المصابة بالإسهال مصابة بفيروس التهاب الكبد الوبائي ، مع وجود اختلافات في الانتشار بين الفئات العمرية والسلالات والمواقع الجغرافية. باستخدام مسحة معقمة، تم أخذ عينات البراز مباشرة من فتحة شرج العجول وإرسالها إلى المختبر عبر وسائط منقولة بالفيروس. تم تدوين عمر العجول وجنسها وسلالتها وموقعها باستخدام عينات الاستبيان. نجح تفاعل البوليميراز المتسلسل في تضخيم جينات ف 7 للعزلات. وأظهرت الجينات الناتجة حجم تضخيم يبلغ حوالي 884 بي بي بي. بالإضافة إلى ذلك، كشف التحليل السلالي عن أن تسلسلات الفيروس الحقلية تنتمي إلى السلالة الثانية وتجمع السلالات الإيرانية والتركية، حيث تراوحت أعلى نسبة تطابق بين 97.12% و96.82% إلى 97.17% على التوالي. ووفقًا لنتائج الدراسة، فإن 18% من العجول حديثة الولادة كانت مصابة بإسهال فيروس الروتا في الأبقار. إن تحسين نقل اللبأ، وتطعيم الأبقار الحوامل، والحفاظ على نظافة البيئة كلها خطوات مهمة في الحد من الإصابة بالأمراض المرتبطة بفيروس الروتا في العجول CCHF يمكن أن يُصاب البشر بالفيروس من خلال

الكلمات الدالة: فيروس الروتا البقري، الماشية، التحليل الفيليوجيني، تفاعل البوليميراز المتسلسل، السليمانية.