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Bacteriological and molecular study of *Salmonella* spp and rotavirus isolated from diarrheal calves

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Abstract

Neonatal calf diarrhea represents an essential health challenge for livestock care because of its profound financial loss and extensive death rates among neonatal calves. A purpose of this research was to separate and identify bacterial and viral pathogens, explicitly focusing on the Detection of Salmonella spp. and rotavirus, from diarrheic fecal samples of calves less than three months old. Three hundred diarrheic fecal samples were gathered from different cattle farms throughout Babylon Province in Iraq. The research involved bacterial isolation using various media types, followed by confirmation of the presence of Salmonella spp, through biochemical identification tests by Viyk2. The detection method for rotavirus involved the use of reverse transcription polymerase chain reaction (RT-PCR) to evaluate the highly conserved VP6 gene. The tested samples revealed that rotavirus infected 5% of the specimens, as confirmed by protein band analysis of the 237 bp VP6 gene fragment through gel electrophoresis. VP6 gene expression levels dropped significantly in positive samples, as determined by quantitative RT-PCR analysis; however, testing revealed different viral loads. The study of VP6 sequences with SNP detection revealed various gene mutations that resulted in both functional and non-functional changes in protein configuration. The biological classification of VP6 sequences produced two genetically divergent clusters, indicating the different evolutionary rates of rotavirus strains, similar to those of Salmonella spp. Isolates successfully succeeded in testing. Molecular and microbiological diagnostic methods prove vital to understand the causes of calf diarrhea. The reduction of enteric infectious burden in calves depends on both persistent surveillance and specific intervention approaches such as vaccination and antimicrobial care practices.

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Introduction

Neonatal calf diarrhea is still a significant reason for illness and death among very young calves. Despite advanced studies and the identification of many causes, calf pneumonia is still the most common reason for death in newborn calves (1). Although the main risk factors are now understood, the rate of diarrhea-related calf deaths has not decreased significantly. Salmonella infections in young cattle remain a concern internationally, as they cause significant mortality, lead to reduced growth, and pose a risk of human disease (2). Although one or two main infectious agents are often highlighted as the primary cause of calf diarrhea, the condition usually arises due to the combined effect of multiple interconnected risk factors (3,4). Diarrhea is a significant problem affecting newborn calves, resulting

in substantial economic losses for livestock production worldwide. Because diarrhea affects people in various ways, it causes significant economic issues (5). Multiple factors contribute to the development of this condition, as it results from the interactions between infectious agents, environmental factors, and the immune status of the calf (6). Rotavirus is the leading viral pathogen for neonatal diarrhea, which often exacerbates its impact through co-infection with Salmonella spp. (7,8). The combined effects of viral and bacterial infections increase the mortality rate and disease prevalence in calves, thereby creating significant challenges for both herd management and productivity (9). Rotaviruses are double-stranded RNA viruses that belong to the Reoviridae family and lack viral envelope structures (10). Neonatal calves experience the most commonly occurring rotavirus infections that belong to group A (11-13). Pathogenic assessments of rotavirus and epidemiological reasoning utilize the VP6 gene, which contains an evolutionarily conserved inner protein that enables essential diagnostic approaches (14-16). The exact identification of rotavirus and the VP6 gene has become possible through the use of reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR, allowing scientists better to understand its impact on neonatal diarrhea (17,18). Salmonella spp. Along with bacterial pathogens, they also play a significant role in calf diarrhea (19). After Salmonella infects neonatal calves, it causes them to develop severe diarrhea, accompanied by high fever and dehydrating symptoms (20). Salmonella exhibits its ability to cause disease primarily through virulence factors, including type III secretion systems and enterotoxins, which result in intestinal damage and an impaired immune response (21). Studies conducted in recent times have shown how multidrug-resistant Salmonella strains have evolved into treatment-resistant strains, creating new challenges to public health (22,23). Diagnosing Salmonella and rotavirus in feces from individuals with diarrhea is a crucial step in implementing effective control strategies. Widespread diagnostic methodologies based on bacterial culture of Salmonella and enzyme-linked immunosorbent assay (ELISA) examination of rotavirus often reveal limitations in sensitivity and time efficiency. Modern pathogen detection benefits significantly from two molecular diagnostic techniques: quantitative PCR and next-generation sequencing (NGS). The analysis using these techniques delivers both fast and precise results, enabling scientists to explore genetic diversity and epidemiological patterns that support their research on vaccines and disease prevention strategies (24).

The analysis investigates both Salmonella spp. Identification from neonatal calf diarrheal feces, as well as the presence of the rotavirus VP6 gene in these specimens. The research employs a combination of molecular diagnostics and traditional methods to gain detailed insights into the causes of neonatal diarrhea.

Materials and methods

Ethical approval

All study animals' blood samples were treated and handled in accordance with the required biosafety and security protocols. Before commencing this study, the Ethics and Scientific Committee in the Department of Microbiology at the College of Veterinary Medicine at Al-Qasim Green University approved the research protocol (No. 5534, 14/7/2024).

Samples collection

We collected 300 fecal samples from young calves under three months old who showed signs of diarrhea. The researchers attracted the required four sterile gloves and collection tubes, then extracted fresh fecal material directly from the calf rectums, which showed symptoms of diarrhea. The samples intended for molecular analysis were cooled with ice to maintain a temperature of -80 degrees Celsius, while the bacterial isolation samples were kept at 4 degrees Celsius. Rapid test cassette kits from a commercial supplier enabled the initial screening for rotavirus using the CTK Biotech Inc. (USA) Rota-Adeno Ag Combo Rapid Test (Cassette format). The provided buffer solution was used to mix the stool samples before homogenization, followed by a room-temperature equilibration stage that lasted between 15 and 30 minutes at 37 °C. According to the manufacturer's guidelines, the test evaluation required up to 20 minutes after adding 2-3 drops of the stool-buffer solution into the sample cassette well. All calves were younger than three months and were examined in three age groups. Calves were included in the study, exhibiting increased stool frequency (more than three times a day) and abnormal stool consistency. An established system was used to measure the level of diarrhea: Soft-formed stools and very few signs of illness are part of the mild category. Semi-liquid to liquid stool, showing signs of dehydration. Serious: Watery stools along with significant problems such as weakness, sunken eyes, and a rapid heartbeat.

Bacterial Isolation and identification

The process of creating a uniform suspension required 1 g of feces to be blended in 9 mL sterile phosphate-buffered saline solution adjusted to pH 7.4. The mixture was vortexed and then centrifuged at 500 × g for five minutes before collecting the supernatant for subsequent tests. Bacterial culture of the supernatant was performed on Nutrient Agar for overall bacterial development and Salmonella-Shigella (SS) Agar for the isolation of Salmonella spp. and Shigella spp. Isolation. The test plates received inoculation, followed by aerobic incubation at 37°C for 24-48 hours. Gram staining allowed for the identification of bacteria before proceeding with tests that verified specific species identities.

Rotavirus RNA Extraction and Detection

Total RNA extraction from rotavirus-positive samples was performed using the Total RNA Mini Kit, following the manufacturer's instructions (Geneaid Biotech Ltd., Taiwan).

cDNA Synthesis and PCR Amplification of the VP6 Gene

The synthesis of cDNA material proceeded within a reaction volume of 20 microliters, which consisted of 1 microliter of Random Primer N9, combined with 10 microliters of 2× ES Reaction Mix, one microliter of RT Enzyme Mix, and five microliters of extracted RNA. The enzyme inactivation required a 5-minute treatment at 85°C following 35 minutes at 42°C and 11 minutes at 25°C. PCR was used to amplify the VP6 gene from Group A rotavirus using primers designed to detect conserved genomic sequences. The two primers targeting the 237 bp fragment consisted of the forward primer (5'-GTTGCGTGATGTTCAAATGG-3') and the reverse primer (5'-AGCTGACGGAGCGACTACAT-3'). Designed by Primer3 GitHub Repository: https://github.com/primer3-org/primer3. The reaction mixture consisted of qPCR Master Mix (10 µL), both primers (0.5 µL), and synthesized cDNA (5 µL), with nuclease-free water added to bring the final volume to 20 µL. Initial denaturation was performed at 95°C for 1 minute, followed by 45 cycles consisting of 95°C for 15 seconds, 60°C for 30 seconds, and then an extension step. Gel Electrophoresis. Testing of PCR products was performed using 1.5% agarose gel electrophoresis. The gel analysis contained a mixture of 5 µL PCR product with loading dye, together with a DNA size marker ladder in individual gel wells. Observers processed the test samples through electrophoresis at 100 V for 45 minutes before applying the DNA dye stain for examination under UV light. The analysis using gel electrophoresis revealed the correct 237 bp VP6 gene fragment, proving that rotavirus RNA was successfully present within the tested samples.

Statistical analysis

All analyses were performed using JMP® 16.1 software (8). The Chi-square test was used to assess the association between the presence of different pathogens, the age of calves, the severity of diarrhea, and the occurrence of bacterial vs. viral infections. The results were substantial, with a $P<0.05$.

Results

Out of the 300 fecal samples, only 5% contained microbial pathogens (a mix of bacterial and viral), while the rest (95%) tested negative for infectious agents. Salmonella spp. was grown using a selective technique on SS agar and a general method on nutrient agar, where the former produced black colonies due to the release of H_2S gas. Further confirmation of the suspected Salmonella isolates was done by biochemical profiling on the VITEK 2 system. Specific regions of group A rotavirus were targeted using RT-PCR, and the presence of rotavirus was detected through screening of the VP6 gene. In positive rotavirus samples, a 237 bp amplification band was clearly visible, and the assay's accuracy was confirmed by the presence of clear fluorescence in the test results and the positive control (Figures 1-4). The analysis based on quantitative RT-PCR demonstrated reduced VP6 gene expression levels in positive samples, as Sample 1 had a 4-fold reduction and Samples 2 and 3 displayed successive reductions of 16-fold then 64-fold compared to the positive control A large number of diagnostic kits (Qiagen, Thermo Fisher or Bio-Rad) come with a ready-made positive control containing rotavirus RNA or DNA with the VP6 gene (Table 1). The examined viral RNA load exhibited a pattern that could be explained by different infection stages, together with partial expression inhibition. A color-coded nucleotide pattern appeared in the alignment results comparing VP6 DNA with its protein translation sequence (Figure 5).

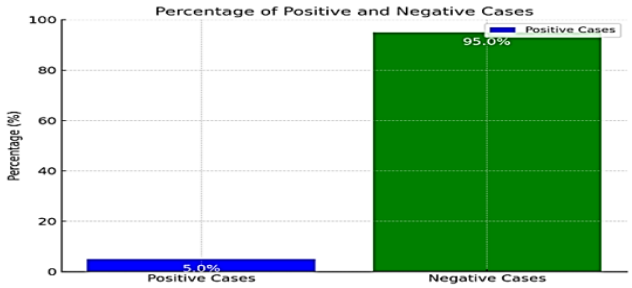

Figure 1: The chart shows that out of 300 tested samples, 5% were found to contain mixed microbial pathogens, while the rest showed no signs of contamination. It was observed that clinically apparent diseases were uncommon among the diarrheic cattle included in the investigation.

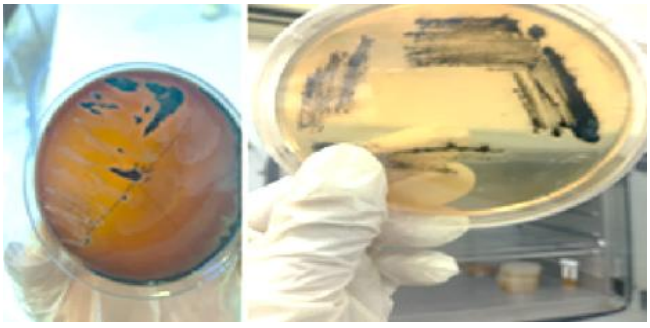

Figure 2: Isolation of *Salmonella spp.* on selective and general media. Black-colored Salmonella-Shigella (SS) agar colonies indicated the presence of Salmonella spp. Growth due to hydrogen sulfide (H_2S) production in the left panel. Non-selective growth of *Salmonella spp*. appears as white-colored colonies on nutrient agar plates, which allows further biochemical testing and molecular analysis.

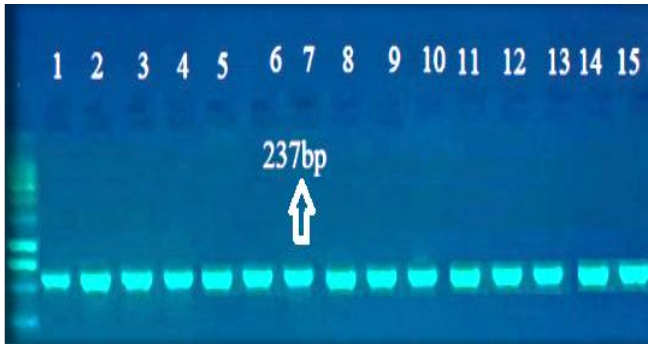

Figure 3: Gel electrophoresis of RT-PCR amplification of the VP6 gene from rotavirus-positive calf fecal samples. Gel electrophoresis of RT-PCR amplification of the VP6 gene from rotavirus-positive calf fecal samples. Lane 1: 100 bp DNA ladder. Lanes 2-15: amplified VP6 gene products (237 bp) from individual fecal samples. All samples show clear bands at the expected size, confirming the successful detection of the VP6 gene. The DNA ladder serves as a size reference, and amplification products were visualized under UV light after staining with an intercalating dye.

The expression level of the VP6 Gene Sample 1 decreased by 4-fold, therefore reaching only 25% of the positive control level. This data shows that the VP6 gene expression continues at reduced levels. The data from VP6 Gene Sample 2 revealed a 16-fold reduction, which indicated its VP6 expression was limited to 6.25% of the control levels. Both potent genetic expression inhibition and minimal viral substance levels seem likely based on this data. The data revealed the most significant expression reduction in VP6 Gene Sample 3, as this pattern reached only 1.56% of the control level, following a 64-fold reduction (Table 1). The assessment of the VP6 gene revealed five single-nucleotide polymorphisms, including one stop codon mutation, one silent mutation, and three missense mutations (such as Ser→Leu and Gly→Asp) (Table 2). The detected mutations could change structural or functional properties or stability characteristics of the protein. Due to the nonsense change in the DNA sequence, the resulting VP6 protein lacks proper structure and becomes nonfunctional.

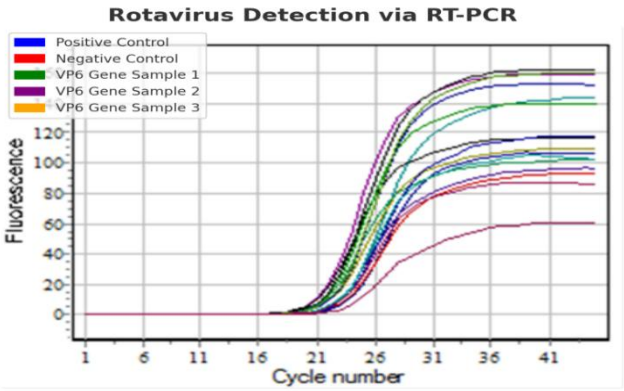

Figure 4: The detection of rotavirus by RT-PCR resulted in the graphical representation of amplification curves. The blue outcome indicates the proper functioning of both the reaction conditions and reagents, as demonstrated by the positive control. The negative control red curve confirms that neither contamination nor non-specific amplification is present. The successful amplification of the rotavirus VP6 gene is evident from three amplification curves, representing VP6 gene samples 1, 2, and 3, measured at green, purple, and orange wavelengths, respectively.

Table 1: Fold change data in a table format

Sample	Fold Change
Positive Control	1.000
VP6 Gene Sample 1	0.250
VP6 Gene Sample 2	0.0625
VP6 Gene Sample 3	0.015625

Table 2: SNPs and Their Effects on the VP6 Gene of Rotavirus

Sequence No.	SNP Position	Reference Nucleotide	Mutant Nucleotide	Amino Acid Change	Effect	Significance	Accession Number
Seq1	35	C	T	Ser → Leu	Missense Mutation	Alters hydrophobicity, potential structural impact	PQ899760
Seq2	120	G	A	Gly → Asp	Missense Mutation	May affect protein stability	PQ899761
Seq3	215	A	T	Stop codon	Nonsense Mutation	Truncated protein, potential loss of function	PQ899762
Seq4	300	T	C	None	Silent Mutation	No significant effect	PQ899763
Seq5	450	A	G	Lys → Arg	Missense Mutation	Affects charge distribution, possible functional impact	PQ899764

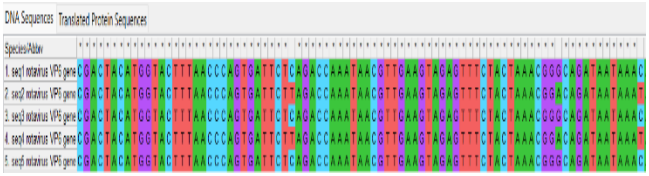

Figure 5: Alignment of DNA sequences and their corresponding translated protein sequences for the VP6 gene of rotavirus.

The phylogenetic evaluation of VP6 sequences yielded two distinct groupings. Pairwise sequence analysis revealed strong genetic correspondence between sequences 2 and 4, with a bootstrap value of 100% (Figure 6). At the same time, sequences 3 and 5 created a different grouping. The Phylogenetic analysis showed that Sequence 1 was genetically distant from the other groups, indicating evolutionary separation (Figure 6).

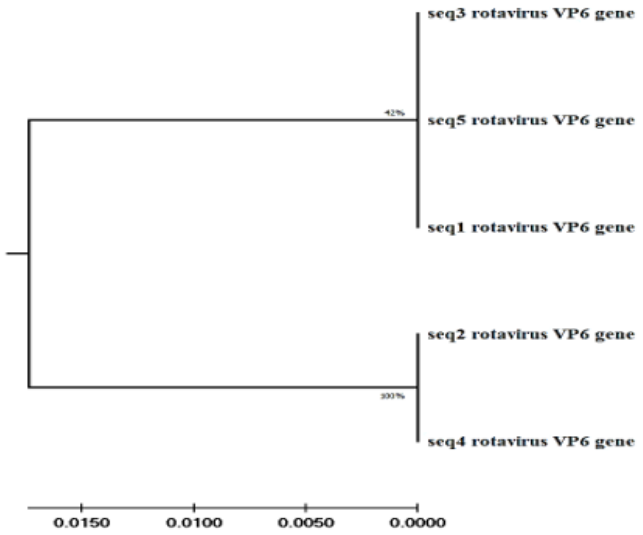

Figure 6: Phylogenetic tree of the rotavirus VP6 gene sequences (seq1, seq2, seq3, seq4, and seq5). The tree shows evolutionary relationships among the examined sequences. Two distinct clusters emerge in the analysis, where seq3 and seq5 form one group, and seq1 remains separated from them with negative bootstrap support. The second cluster combines seq2 and seq4, exhibiting a high level of sequence similarity as indicated by a substantial bootstrap value of 100%. The genetic divergence between sequences is indicated by the scale bar, which shows the level of their separation. The value of Bootstrap shows how specific each grouping analysis is at each point in the tree.

Analysis and significance

The SNPs that fall under the missense category resulted in amino acid transformations that could modify both the structure and operational properties of the VP6 protein. For example, the conversion from hydrophobic to hydrophilic character leads to protein misfolding and prevents correct interactions. Mutations in function-maintaining areas may disrupt viral assembly mechanics, rendering them less detectable to the immune system. Premature stop codons created by nonsense mutations result in a truncated VP6 protein that loses its functionality, likely inhibiting rotavirus replication and affecting disease immunity. Silent mutations never alter amino acid sequences, but they occasionally affect mRNA stability or mRNA processing. Viral nucleotide polymorphisms positioned within VP6 regions that maintain sequence similarity tend to trigger functional changes in rotavirus proteins.

Two main clusters appear on the rotavirus VP6 gene phylogenetic tree with variable levels of genetic relationship between them. The first clustering group shows seq1 together with seq3 and seq5, where seq3 and seq5 demonstrate a moderate bootstrap value of 42% but seq1 shows greater distance. The genetic analysis indicates that seq2 and seq4 share extensive similarity since their bootstrap value reaches 100%. Genetic sequence data reveal that seq2 and seq4 exhibit low genetic distance, while the other sequences demonstrate different degrees of evolutionary separation. The findings indicate that seq2 and seq4 belong to the same genetic subgroup, along with varying members of the group represented by seq3 and seq5. Sequence 1 demonstrates an evolutionary divergence position that suggests evolutionary differences at the VP6 gene level. These research results demonstrate the diverse genetic structure of VP6 rotavirus genes across different viral strains, as well as their impact on virulence levels, immune evasion, and epidemiological tracking. Ongoing genetic monitoring of novel virus strains should continue due to their capacity to track both emerging viral strains and their disease-related properties.

Discussion

The evaluation of the VP6 rotavirus gene from diarrheic young calves (three months old) demonstrates how multidimensional methods provide insight into rotavirus molecular epidemiology, bacterial infections, and genetic differences among viruses. The analysis of VP6 gene phylogenetic relations revealed two divergent sequences that exhibited a weaker genetic relationship compared to other, more closely related rotavirus Group A genetic sequences. Their lower rate of infection could reflect the reduced ability of the two divergent rotavirus variants to spread because of genetic changes in VP6 (25). When Leu replaces the amino acid Ser and a termination signal appears early, it may stop VP6 from functioning in viral assembly and detection by the immune system. Due to these mutations, the virus may experience difficulty in multiplying or evading the immune system, which helps keep its spread under control (26). Recent research has shown that variations in the VP6 gene

are associated with specific rotavirus types and can indicate variations in rotavirus strains by region (27). Observations from SNP analysis of the VP6 gene in this study revealed that a missense mutation changed Ser to Leu, potentially affecting protein formation, and a nonsense mutation introduced a premature stop codon, which could impair the protein's function. The new findings suggest that regions of the VP6 gene that remain unchanged are crucial for the virus's replication, its ability to trigger an immune response, and its interaction with the host immune system (28). Moreover, the quantitative reverse transcription PCR (qRT-PCR) fold change data consistently showed a drop in VP6 expression in samples from infected animals (29). It is possible that the virus's strength is hitting the right amount, or that the host has activated immune responses to block the virus (30). Research confirms that different VP6 gene sequence clusters match specific rotavirus genotypes and geographical regions (31). The SNP evaluation identified two significant mutations where the Ser→Leu shift might modify protein structure, and one sequence featured a premature stop signal that could potentially cause functional impairment. The targeted selection of VP6 regions, which remain stable because mutations in these elements affect viral replication, immunogenic potential, and host responses (32). The fold change assessment revealed that VP6 gene expression decreased steadily, suggesting either a reduction in viral particles in the bloodstream or the influence of factors, including host defense mechanisms (33).

Studies on fecal samples reveal the complex nature of calf enteric infections, as Salmonella spp. and Shigella spp. are often found together (34). Black pigmentation of Salmonella colonies on Salmonella-Shigella (SS) agar was attributed to hydrogen sulfide production, while biochemical testing confirmed their microbial identity. Multiple studies have demonstrated that bacterial co-infections with rotavirus result in more severe diarrhea in livestock populations, with similar trends also observed in calf populations (35). Salmonella species enzymatic activities have been biochemically characterized (36) through investigations of hydrogen sulfide production and lactose fermentation, which have been used to identify species types (37). Salmonella identification plays a crucial role in selecting appropriate treatments, as typhoidal Salmonella typically requires antibiotics, whereas nontyphoidal strains often resolve on their own, except in cases of compromised immunity (38).

The researchers successfully amplified the 237 bp VP6 gene fragment in RT-PCR tests to determine rotavirus RNA in fecal samples for diagnostic purposes. The clear products detected by agarose gel electrophoresis confirmed that PCR maintains high levels of reliability for this experiment. The findings align with veterinary medical international standards for rotavirus diagnostics and molecular analyses used at both animal and human research facilities regarding zoonotic virus potential (39). The findings from this study indicated that rotavirus and Salmonella spp. were present in 5% of the tested samples, and the rest of the samples displayed no virus or bacterial presence 95%. Another factor contributing to the lower rotavirus prevalence may be related to differences between regions, environmental factors, and the time of sample acquisition. Scientific research from various parts of the world indicates that rotavirus occurs in 3% to 20% of diarrhea cases in calves; however, the prevalence rises in areas where there is no widespread vaccination process. The study results provide important insights into VP6 genetic patterns, as well as clinical and environmental factors, which enhance vaccine development and control methods. The development of vaccination targeting stable VP6 regions holds potential to decrease rotavirus-induced morbidity in farm calves, as other areas have already proven successful. The investigation of rotavirus prevalence in Karbala and Basrah provinces, together with human-animal rotavirus genetic reassortment results, has enhanced knowledge of Iraqi virus molecular epidemiology (40). Monitoring rotavirus strains through genetic testing remains essential because it reveals the varying viral patterns that influence both animal and human wellness. Future research should analyze complete rotavirus sequences to identify new genetic elements that affect the virus's strength and ability to infect host species (41). The analysis of VP6 sequences revealed the presence of clusters, indicating that circulating rotavirus strains exhibit genetic differences. Therefore, regular monitoring of viral genes and analysis of their genomes are necessary to track changes in pathogens, design new methods for detecting infections, and enhance strategies for controlling diseases in animals and humans.

Conclusions

The study concludes that mixed infections, including both viruses and bacteria, can cause neonatal calf diarrhea. Rotavirus was detected in 5% of the samples, and analysis of the VP6 gene revealed the presence of different genetic strains with mutations that may affect the virus's structure and its recognition by the immune system. Additionally, the detection of Salmonella spp. Confirms its role in worsening the severity of diarrhea through co-infection. These findings underscore the need for comprehensive diagnostic approaches and targeted control strategies to manage calf diarrhea effectively.

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Conflict of interest

There is no conflict of interest.

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دراسة بكتيرية وجزيئية لجراثيم السالمونيلا وفيروس الروتا المعزولة من براز العجول المصابة بالإسهال

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الخلاصة

الإسهال الوليدي في العجول يمثل تحديًا صحيًا رئيسيًا في مجال رعاية الماشية نظرًا لما يسببه من خسائر مالية فادحة وارتفاع معدلات الوفيات بين العجول حديثة الولادة. كان الهدف من هذه الدراسة هو فصل وتحديد السالمونيلا وفيروس الروتا من عينات برازية مأخوذة من عجول مصابة بالإسهال والتي تقل أعمارها عن ثلاثة أشهر. تم جمع ٣٠٠ عينة برازية من مزارع ماشية مختلفة في محافظة بابل، العراق. شملت الدراسة عزل البكتيريا باستخدام نوعين مختلفين من الأوساط الزراعية، تلاها تحديد السالمونيلا بناءً على نتائج جهاز الفايتيكا. أما الكشف عن فيروس الروتا، فقد اعتمد على استخدام تقنية تفاعل البلمرة المتسلسل العكسي لتحديد جين VP6، وهو جين محفوظ بدرجة عالية. أظهرت النتائج أن فيروس الروتا أصاب ٥% من العينات، وتم تأكيد وجوده من خلال تحليل شريط البروتين باستخدام الترحيل الكهربائي، والذي كشف عن قطعة وراثية بحجم ٢٣٧ زوجًا قاعديًا من جين VP6. كما أظهرت التحليلات انخفاضًا ملحوظًا في مستويات تعبير الجين VP6 في العينات الإيجابية وفقًا لاختبارات تفاعل البلمرة المتسلسل العكسي، مما أشار إلى اختلافات في الحمل الفيروسي. كشف تحليل الطفرات النقطية في جين VP6 عن وجود طفرات جينية متنوعة أدت إلى تغييرات وظيفية وغير وظيفية في بنية البروتين. وأظهرت الدراسة أن التصنيف البيولوجي لتسلسلات VP6 أنتج مجموعتين وراثيتين متباعدتين، مما يعكس اختلاف معدلات التطور بين سلالات فيروس الروتا. كما تم عزل سلالات السالمونيلا بنجاح. تثبت الأساليب التشخيصية الجزيئية والميكروبيولوجية أهميتها في فهم أسباب الإسهال في العجول. ويعتمد تقليل العبء المرضي للعدوى المعوية في العجول على المراقبة المستمرة وتطبيق استراتيجيات التدخل المحددة، مثل التطعيم والممارسات السليمة لاستخدام المضادات الحيوية.