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Co-Infection of Bovine Rotavirus and *E. coli* K99 in Neonatal Calves: Insights into Humoral and Cellular Immune dynamics

Ahmed F. Afify[*], Rabab T. Hassanien[*], Hammad O. Hammad[**], Heba E. Farhan[**], Abeer S. Hafez[***], Asmaa M. Badawy[***], Wessam Youssef[****], El Saber A.E[*****]., and Momtaz A. Shahein[*]

[*]Virology Research Department, Animal Health Research Institute, Agriculture Research Center (ARC), 12618 Dokki, Giza, Egypt
[**]Bacteriology Research Department, Animal Health Research Institute, Agriculture Research Center (ARC), 12618 Dokki, Giza, Egypt
[***]Immunology Research Department, Animal Health Research Institute, Agriculture Research Center (ARC), 12618 Dokki, Giza, Egypt
[****]Biotechnology Research Department, Animal Health Research Institute, Agriculture Research Center (ARC), 12618 Dokki, Giza, Egypt
[*****]Department of Clinical Pathology, Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh 13736, QG, Egypt

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ABSTRACT

Neonatal calf diarrhea (NCD) is one of the most serious diseases affecting dairy herds in many parts of the world. This disease leads to high morbidity and mortality causing significant economic losses. This study was aimed to determine the incidence of Bovine Rotavirus (BRV) and Enterotoxigenic E.coli (ETEC) in new born calves in Egypt to understand the consequences of these pathogens on the immune status and the biochemical alterations related to these infections. Out of 100 sampled diarrheic calves, 8% were positive for BRV, with 6% of them having ETEC co-infection. In E. coli isolates, resistance genes like blaTEM and qnrA showed spread of multidrug resistance. Calves suffering from the infection were found to have considerably lowered immunoglobulins (IgG, IgM, IgA), but had raised levels of lysozyme and Nitric oxide especially in the co-infected group. There was also an electrolyte imbalance, increased serum proteins and elevated values of pro-inflammatory cytokines (IL1 β, IL6 and TNF α). These results highlight the immune antagonism and systemic inflammation that is a feature of BRV and ETEC co-infections in NCD indicating a potential need to change antimicrobial strategies, include better diagnostic techniques to control the spread of the infections and enhance the health of the calves .

Corresponding author: Ahmed F. Afify, Virology Research Department, Animal Health Research Institute, Agriculture Research Center (ARC), 12618 Dokki, Giza, Egypt
Email address:
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INTRODUCTION :

Neonatal calf diarrhea (NCD) is a significant global disease accompanied by high morbidity, mortality, growth delays, and economic losses **(Lojkić et al. 2015).** Various enteric pathogens contribute to NCD, including viral agents such as bovine rotavirus (BRV), bovine coronavirus (BCoV); parasitic agents like Cryptosporidium parvum and Eimeria species; and bacterial agents such as Escherichia coli K99 (ETEC) and Salmonella species **(Gillhuber et al. 2014 and Scharnböck et al. 2018).**

The primary cause of calf mortality in Egypt is NCD **(Younis et al. 2009).** The condition can result from various infectious and non-infectious factors. Age of the calves positively influences susceptibility to infections. During the first fourteen days of age calves are vulnerable to BRV infections, from the 5th to the 20th days of age to BCoV and during the first four days of life to ETEC **(Brunauer et al. 2021).**

Several factors, including dam and calf immunity, environmental conditions, and management practices, influence the occurrence and emergence of NCD **(Cho and Yoon 2014).** Rapid and accurate diagnosis is crucial due to the swift progression of calf diarrhea and the involvement of multiple pathogens or factors. Dead or stressed bacteria can be identified by PCR even in the presence of antibody-antigen complexes **(Blanchard 2012).** This makes PCR especially useful for diagnosing mixed infections of BRV and ETEC, where precise identification of multiple pathogens is critical for effective treatment.

The presence of fimbrial antigens like F5 and enterotoxins facilitate the colonization of ETEC in the intestinal tract leading to watery diarrhea (**Andrade et al. 2012).** Detecting of E. coli resistance genes such as blaTEM and aadA1 is crucial for confirming virulence and assessing antibiotic susceptibility **(Belete et al. 2022, Pokharel et al. 2023 and Naderi et al. 2024).** Newborn calves' susceptibility to viral and bacterial infections can be attributed to their immature immune systems **(Sobhy et al. 2020).** Variations in cattle susceptibility to infections and immune responses are considerable **(Reid et al. 2021)**. The natural immunity plays a crucial role in early immune responses and determines outcomes of bacterial and viral infections **(Thacker et al. 2007).** The strength and durability of acquired immune responses are mainly depending on Innate immune responses **(Kasturi et al. 2011).** Serum immunoglobulin concentration in dairy cattle indicates immunity against pathogenic microorganisms **(Mendonsa 2011).** In cattle, IgG and IgM are essential for virus and toxin neutralization, bacterial agglutination, and opsonization **(Maunsell and Chase 2019).**

The pro-inflammatory cytokines (IL-1α, IL-1β, and IL-18) are synthesized as precursor proteins . They have an essential role in the way of diagnosis and treatment of inflammatory conditions **(Dinarello 2011)**. The pro-IL-1α could serve as a main damage-associated molecular pattern (DAMP) , produced from dead or damaged cells, capable of triggering strong inflammatory responses **(Suwara et al. 2014).** Production of IL-1β and IL-18 from monocytes and macrophages take place in response to pathogen-associated molecular pattern (PAMP) / (DAMP) that are recognized by pathogen recognition receptors (PRRs) **(van de Veerdonk et al. 2011).**

Accurately differentiating between bacterial and viral infections is essential for appropriate treatment and can prevent excessive morbidity and mortality. Monitoring host immune responses may serve as a fast and dominate diagnostic tool for infectious diseases.

This study aims to explore the impact of infection with ETEC and/or BRV on neonatal calves in relation to the immune response (Innate and specific). By characterizing profiles of lysozyme , nitric oxide, immunoglobulins levels and pro- inflammatory cytokines. we aim to elucidate the mechanisms underlying the host immune response to these mixed infections, thereby providing insights into their pathogenesis and informing more effective treatment strategies.

MATERIAL AND METHODS :

Sample collection and preparation:

Fecal and serum samples were collected from 100 diarrheic calves with an average age ranging from one to 45 days in Al-Fayoum governorate, between May 2023 and June 2024. Fecal samples were kept at 4 °C for bacterial investigation and at −80 °C for viral analysis. Serum samples were kept at −20 °C until examination.

Detection of BRV antigen in fecal samples:

Il was performed using indirect sandwich ELISA Kit **(Monoscreen Ag Elisa®, Bio-X Diagnostics, SA., Belgium)**.

RNA extraction:

IT was performed for Molecular confirmation of BRV by using the QIAamp viral RNA Mini kit **(QIAGEN, GmbH, Germany)** following the manufacturer's recommendations. Briefly, sample suspension was allowed to be incubated with AVL lysis buffer and carrier RNA at room temperature for 10 min. Then, samples were washed and centrifuged after addition of 100% ethanol. Nucleic acid Elution was achieved by elution buffer.

RT-PCR for molecular confirmation of BRV:

Primers targeted to NSP5 gene of Bovine Rotavirus are supplied from **Metabion©, Germany ,table (1)**. The reaction was performed in a Biometra thermal cycler with reverse transcription at 50°C for 30 min and a primary denaturation at 95°C for 5 min. Then 35 cycles of extensions at 94°C, 60°C and 72°C for 30 sec., 45 sec. and 45 sec. respectively. The final step of extension was at 72 °C for 10 min. **(Schroeder et al. 2012).** The PCR products were separated by electrophoresis on 1.5% agarose gel **(Applichem, GmbH, Germany)**. The fragment sizes was measured by a gelpiolt 100bp DNA **ladder (QIAGEN, Gmbh, Germany)**. The gel was photographed and the data was analyzed through computer software **(Alpha Innotech, Biometra)**.

Isolation and identification of E. coli :

It were applied on MacConkey's agar and Eosin methylene blue (EMB) agar (Oxoid, UK). Morphological and biochemical identification of pink colonies on MacConkey's agar and metallic green sheen colonies on (EMB) were performed as described by **Quinn et al. (2011)**.

Antibiogram E. coli isolates :

It was performed on Mueller-Hinton agar plates by disk diffusion method (Oxoid, UK) following the Clinical and Laboratory Standards Institute **(CLSI 2020)** guidelines. A panel of 15 antibiotic disks was used: Cefotaxime (30 µg), trimethoprim/sulfamethoxazole (25 µg), Ampicillin (10 µg), amoxicillin (10 µg), erythromycin (15 µg), vancomycin (15 µg), amikacin (30 µg), streptomycin (30 µg), gentamycin (10 µg), Ciprofloxacin (5 µg), Norfloxacin (10 µg), cefadroxil (30 µg), and Tetracycline (10 µg), Cefoperazone + Sulbactam (10 µg), Meropenem (10 µg),Sparfloxacine (5 µg).

DNA extraction:

IT was applied for Molecular detection of E. coli virulence and Antibiotic resistance genes by using **QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany)** following the manufacturer's instructions. Briefly, mixture of proteinase K, lysis buffer AL and sample suspension were incubated. The lysate was exposed to washing and centrifugation after addition of absolute ethanol. Elution buffer AE was used to elute nucleic acid.

Molecular detection of E. coli virulence and Antibiotic resistance genes:

Following DNA extraction, PCR amplification was achieved using specific primers from **Metabion, Germany**, **Table (2)** . Electrophoresis was used to analyze PCR products **(AppliChem, GmbH ,Germany)**. Sizes of fragment were estimated using DNA ladders, Gene Ruler 100bp ladder **(Fermentas, Germany)** and Gel Pilot 100 bp ladder **(QIAGEN GmbH, Germany)**.The gel was photographed. Data analysis were performed through **(Alpha innotech and protein simple software, biometra).**

Detection of immunological parameters:

Lysozyme Assay:

Activity of Lysozyme was measured according to **Schultz (1987**). Breifly, by dissolving 500 mg/L Micrococcus lysodeikticus and 1% agarose in 0.06 M PBS (pH 6.3). The logarithmic curve of standard lysozyme was used to determine lysozyme concentrations.

Nitric oxide (NO) levels:

The concentration of NO was calculated from a standard curve created using NaNO2 **(Lee et al. 2011) .** Equal volumes of Griess reagent and serum samples were incubated at 25°c for 10 min in an ELISA plates. The absorbance was measured at 550 nm .

Serum immunoglobulins (IgG, IgM and IgA) concentrations:

It were estimated by commercial kit of Single Radial Immunodiffusion (SRID) **(RID Kits, WMRD, Pullman, Wash.,USA).**

Levels of pro-inflammatory cytokines (IL1 β, IL-6 and TNF-α) in Serum:

The parameters were measured with a Bovine ELISA kit **(R&D systems, Minneapolis, MN, USA).**

Detection of some biochemical parameters in serum:

Total proteins (TP) and albumins (Alb) were evaluated using commercial kits of biodiagnostic . CAT.No. 2020 and 1010 respectively. Globulins (Glob) were calculated by subtracting albumins from total proteins . Sodium (Na) was measured according to **Trinder (1951)** whereas, chloride (Cl) was assessed according to the method of **Schales and Schales (1941)**. Levels of potassium (K) were determined as described by **Sunderman and Sunderman (1958)**. Concentrations of calcium (Ca) and inorganic phosphorus (ip) were measured according to **Gindler and King (1972) and El-Merzabani et al. (1977)** respectively. Magnesium was estimated according to **Nazifi et al. (2003).**

Statistical analysis :

A analysis of the obtained data were performed using one-way ANOVA test by SPSS-14 software according to **Norusis (2006).**

RESULTS:

BRV Detection:

The overall detection rate of Bovine Rotavirus (BRV) using ELISA was 8% (8/100 samples). Positive samples primarily came from calves aged between six to 11 days.

E. coli K99 Detection and Co-Infection:

Out of 65 E. coli diarrheic calves samples, eight (12.3%) were tested positive for the K99 gene. RT-PCR on BRV-positive samples identified six cases of co-infection with E. coli K99. Additionally, these samples exhibited multiple antibiotic resistance genes: all eight samples carried the blaTEM gene, seven had the qnrA gene, six carried the aadB gene, and five had the Sul1 gene.

Antimicrobial Susceptibility:

Antibiotic susceptibility testing showed that cefoperazone plus sulbactam, meropenem, amikacin, sparfloxacin, ciprofloxacin, and gentamicin were the best antibiotics against E. coli isolates, with efficacy rates ranging between 65% and 90%. Less effective antibiotics included vancomycin, trimethoprim/ sulfamethoxazole, amoxicillin, ampicillin, clarithromycin, and spiramycin. Intermediate efficacy was noted for cefotaxime, doxycycline, amoxicillin plus clavulanic acid, and ampicillin plus sulbactam.

Grouping of Infected Calves:

For evaluation of immunological and biochemical parameters, we divided the infected calves into three main groups according to results of virological and bacteriological analysis beside the non-infected group:

- Group 1: Control (non-infected) calves.
- Group 2: E. coli K99-infected calves.
- Group 3: BRV-infected calves.
- Group 4: Co-infected calves (BRV and E. coli K99).

Levels of lysozyme and nitric oxide:

The obtained data revealed a noticeable high values of lysozyme and NO concentrations ($p \leq 0.05$) among the three infected groups compared to the control group (Group 1). Specifically, Group 4 exhibited a notably higher increase in both lysozyme and nitric oxide levels than Groups 2 and 3, as illustrated

in Figures 4 and 5.

Immunoglobulins Levels:

The results convey lowering values of IgG, IgM, and IgA in the three infected groups compared to the control. Such decrease was very prominent and highly significant ($p \leq 0.05$) in case of IgM, and IgA in relative to IgG. There were no any significant differences between the three infected groups (table 4).

Levels of IL1 β, IL-6 and TNF-α:

Increases in IL1 β, IL6, and TNF α were observed in the E. coli, BRV-infected groups and co-infected group ($P \leq 0.05$) relative to the control. However, IL6 and TNF α levels were lower in BRV-infected calves compared to those infected with E. coli and co-infected group, while IL1 β levels were higher in BRV-infected calves and co-infected group than in those infected with E. coli (table 5).

Serum Protein Levels:

A prominent increases ($P \leq 0.05$) in total proteins and albumins levels were recorded in all infected groups compared to the control group. No changes were observed in globulins levels across any group (table 6).

Electrolyte Levels:

Electrolyte analysis indicated significant lower values ($P \leq 0.05$) in sodium (Na), calcium (Ca), phosphorus (Ph), chloride (Cl), and magnesium (Mg) levels, while potassium (K) levels were highly up in all infected groups in comparing with the control. Group 4 exhibited the most pronounced alterations (table 7).

DISCUSSION :

Neonatal calf diarrhea is a complex, multifactorial disease, significantly impacting calf health and dairy industry economics through high morbidity and mortality rates **(Gomez and Weese 2017).** This study examined the dominance of co-infections in diarrheic calves, specifically BRV and E. coli K99, and assessed their effects on immunological and biochemical responses. Based on the results, infected calves were categorized into four groups: control (non-infected), E. coli K99-infected, BRV-infected, and co-infected with both pathogens.

The detection rate of BRV was 8%, a notably lower prevalence than the 32.5% and 36% previously reported by **Ali et al. (2011) and Hassan et al. (2014**), respectively. This difference could reflect regional variances in exposure or herd immunity. The average age of infected calves (8.5 days) and higher infection frequency in the first and second weeks of life are in agree with **Bartels et al. (2010),** who attributed reduced BRV-related diarrhea in older calves to increasing natural resistance. This pattern suggests that early-life infections prompt immune responses critical to pathogen clearance.

RT-PCR identified six cases of co-infection in BRV-positive samples, while 12.3% of E. coli isolates were positive for the K99 gene. The existence of multiple resistance genes among these isolates, including blaTEM, qnrA, aadB, and Sul1, supports previous findings by **Güler et al. (2008)** and **Uysal et al. (1992),** highlighting the significant role of E. coli as a causative agent in calf diarrhea. These findings underscore the challenge in managing E. coli infections due to increasing antimicrobial resistance, which complicates treatment and requires tailored interventions.

The susceptibility results align with prior studies, showing that cefoperazone plus sulbactam, meropenem, amikacin, sparfloxacin, ciprofloxacin, and gentamicin are most effective against E. coli isolates, with resistance observed against vancomycin, amoxicillin, ampicillin, clarithromycin, and spiramycin. Intermediate efficacy observed for cefotaxime and doxycycline suggests a narrower, gram-negative-targeted therapy as most effective in reducing collateral damage to gut flora **(Joachim and Constable 2009).** The need for pre-treatment diagnostics and controlled antimicrobial usage is emphasized, particularly in line with protocols suggested by **El-Azzouny et al. (2020)** and **Francisco et al. (2019).**

The elevated lysozyme concentration in the infected groups underscores the protective role of the innate immune response, which involves the recruitment of macrophages and natural killer cells to boost lysozyme secretion

(Gandhi et al. 2017).

Moreover, the significantly elevated nitric oxide levels in diarrheic infected calves align with findings by **Nahed et al. (2022).** Similarly, **Borghan et al. (2007)** observed an increase in mRNA inducible nitric oxide synthase (iNOS) and metabolites of nitric oxide in rotavirus-infected mice.

The reduction in IgG, IgM, and IgA levels across all infected groups aligns with the studies by **Al-Alo et al. (2018)** and **Balikci and Al (2014).** Reduced immunoglobulin levels could result from insufficient passive transfer or immunoglobulin translocation to the gut to counteract infection **(Athanasiou et al. 2019),** potentially weakening the immune response and making calves more susceptible to concurrent infections.

Elevated IL1 β, IL6, and TNF α levels observed in the E. coli, BRV infected groups and co-infected group correlate with **Gilbert et al. (2013),** who found that these cytokines serve as inflammatory markers in neonatal infections. The higher IL6 and TNF α levels in E. coli infections and in mixed infection compared to BRV infections suggest a more robust inflammatory response in bacterial infections. IL-6's role in initiating the acute-phase response **(Pritts et al. 2002)** could be instrumental in understanding the inflammatory processes triggered by these pathogens.

Elevated total protein and albumin levels in infected groups likely result from dehydration due to diarrhea, as indicated by **Piccione et al. (2009).** This increase supports observations by **El-Sissi et al. (2020)** and underscores dehydration's role in altering serum protein concentrations. Dehydration-induced serum protein increases may also indicate the body's compensatory response to fluid loss, which is common in diarrheic calves.

Electrolyte imbalances, including decreased Na, Ca, Ph, Cl, and Mg, along with increased K levels, suggest significant fluid and electrolyte shifts due to diarrhea. These findings align with those by **El-Seadawy et al. (2020) and Ghanem et al. (2012),** who discussed electrolyte depletion and compromised cell membrane permeability associated with severe diarrhea. The elevated K levels result from shifts from intracellular to extracellular compartments during fluid loss, and reduced renal function may further contribute to these changes **(Dratwa-Chalupnik et al. 2012 and Singh et al. 2014)**.

CONCLUSION :

This study highlights the complexity of neonatal calf diarrhea, demonstrating the presence of mixed (co) infections involving Bovine Rotavirus (BRV) and enterotoxigenic E. coli(K99) in Egyptian dairy herds. Findings revealed distinct age-related susceptibility, with BRV and E. coli K99 infections peaking in the first two weeks of life. Co-infections were associated with altered immunological and biochemical parameters, including decreased immunoglobulins, electrolyte imbalances, and elevated cytokine levels (IL1 β, IL6, TNF α), indicative of a robust inflammatory response.

The existence of resistance genes (blaTEM and qnrA) among E. coli isolates underscores the challenge of effective therapeutic intervention and the urgent need for targeted antimicrobial protocols to combat resistance. Immunological findings point to a weakened humoral response, as evidenced by reduced immunoglobulin levels, which may compromise immunity and increase vulnerability to secondary infections.

Overall, these results emphasize the importance of early diagnostic screening and judicious antimicrobial use, alongside optimized rehydration and supportive care to manage calf diarrhea. Improved biosecurity, vaccination strategies, and responsible antimicrobial stewardship are essential to control disease spread and improve calf health and survival outcomes in affected regions.

Recommendations:

More investigations are needed to compare between mixed and single infection that cause

diarrhea in new born calves.

Special care should be taken in consideration to assure arrival of adequate amount of immunoglobulins from dam to calves through colostrum.

Infusion with rehydrate during diarrhea is needed to overcome electrolyte imbalance. Similar results of total serum protein and albumin between infected groups may be referred to that E.coli and BRV are gastroenteric pathogens affect the same organs

List of Tables

Table 1. Primers sequences, target gene, amplicon size for RT-PCR for detection of BRV

Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	Reference
NSP5	GAT ATT GGA CCA TCT GAT TCT GCT TCA AA GAA ATC CAC TTG ATC GCA CCC AA	151 bp	Schroeder et al. 2012

Table 2. Primers sequences, target genes, amplicon sizes and cycling conditions for detection of E. coli genes

Target bacteria	Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondar denaturation	Annealing	Extension		
E. coli	*K99*	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	314	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Franck et al. 1998
	blaTEM	ATCAG-CAATAAACCAGC CCCCGAAGAACGTTTTC	516	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Colom et al. 2003
	qnrA	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	516	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Robicsek et al. 2006
	aadB	GAGCGAAATCTGCCGCTCTGG CTGTTACAACGGACTGGCCGC	319	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Frana et al. 2001
	Sul1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Ibekwe et al. 2011

Table 3. Gene detection results in E. coli isolates from calve diarrhea

Gene	Number of E. coli strains and resistance genes	Positive percentage (%)
K99	8	8/65 = 12.3%
blaTEM	8	8/8 = 100 %
qnrA	7	7/8 = 87.5 %
aadB	6	6/8 = 75 %
Sul1	5	5/8 = 62.5 %

Table 4. Levels of serum immunoglobulin's (IgG, IgM and IgA) in all groups

Groups	IgG (mg/ml)	IgM (mg/ml)	IgA (mg/ml)
Group1	7.49±0.04 b	1.18±0.12 b	2.35±0.16 b
Group2	6.93±0.03 a	0.73±0.01 a	1.35±0.02 a
Group3	7.05±0.07 a	0.71±0.01 a	1.29±0.01 a
Group4	6.95±0.01 a	0.69±0.01 a	1.15±0.04 a

Data were presented as mean ± standard error. Different small letters indicate significant between groups in the same column at p ≤0.05.

Table 5. Levels of Pro-inflammatory cytokines (IL1β , IL 6 and TNF-α) in all groups

Groups	IL1β (pg/ml)	IL6 (pg/ml)	TNF-α (pg/ml)
Group 1	90.81±3.37 a	10.74±.9 a	24.93±1.4 a
Group 2	254.03±6.51 b	65.02±1.7 b	175.59±2.06 b
Group 3	486.99±6.46 c	28.4±1.18 c	145.44±3.56 c
Group 4	523.15±9.7 d	78.99±3.7 d	199.48±3.13 d

Data were presented as mean ± standard error. Different small letters indicate significant between groups in the same column at p ≤0.05

Table 6. Serum protein profile in control and diarrheic calves

Groups	Total proteins (gm/dl)	Albumins (gm/dl)	Globulins (gm/dl)	A/G ratio
Group1	6.12±0.07 b	3.52±0.21 b	2.6±0.14	1.4±0.16 a
Group2	7.44±0.2 a	4.73±0.17 a	2.71±0.03	1.7±0.04 a
Group3	7.36±0.2 a	4.94±0.15 a	2.42±0.06	2.6±0.24 b
Group4	7.1±0.06 a	4.5±0.18 a	2.6±0.15	1.8±0.2 a

Data were presented as mean ± standard error. Different small letters indicate significant between groups in the same column at p ≤0.05.

Table 7. Serum minerals and electrolytes in control and diarrheic calves .

Parameters	Group1	Group2	Group3	Group4
Na (mEql)	150.35±0.14 b	141.717±2.4 a	143.147±1.2 A	129.39±0.58 C
Ca (mg/dl)	9.29±0.09 b	8.28±0.03 a	8.37±0.04 a	7.84±0.009 c
Ph (mg/dl)	5.92±0.01b	5.11±0.07 a	4.81±0.1a	3.87±0.2 c
K (mEq/l)	3±0.01c	3.44±0.01a	3.4±0.04 ab	3.31±0.04 b
Cl (mEq/l)	84.34±1.3 b	70.04±0.9 a	67.83±0.7 a	56.43±0.8 c
Mg(mg/dl)	3.4±0.08 b	2.62±0.06 a	2.4±0.11a	1.93±0.19 c

Data were presented as mean ± standard error. Different small letters indicate significant between groups in the same column at $p \leq 0.05$

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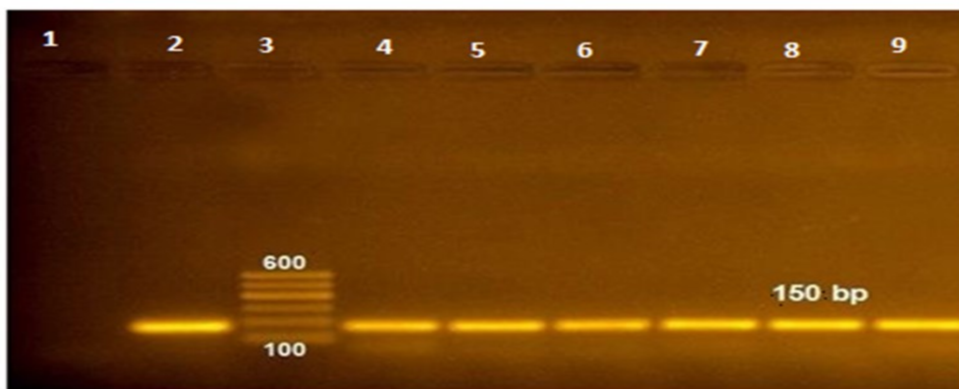

Fig.1: RT-PCR amplified products of NSP5 gene at 150 bp (lane 1: negative control, lane 2: positive control, lane 3: Gel pilot 100bp ladder (Qiagen, Gmbh, Germany) and lane 4-9 positive samples)

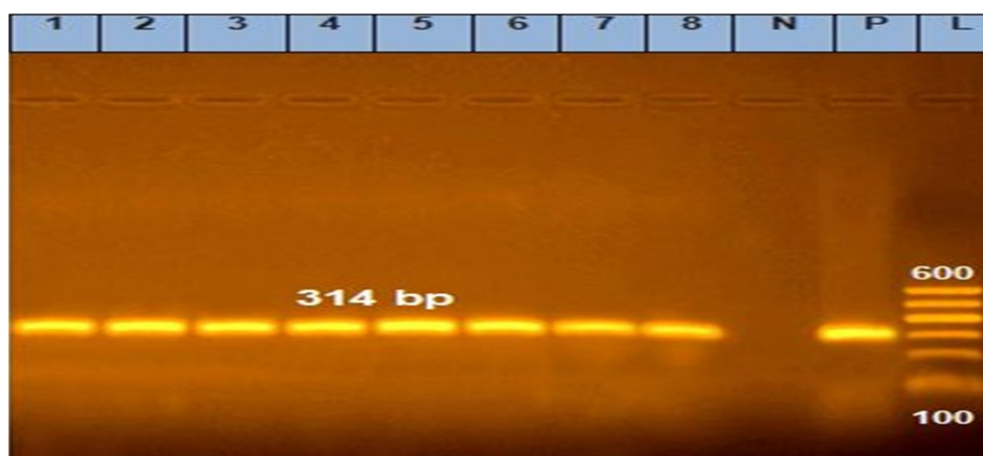

Fig. 2: PCR results of the E. coli isolates using E. coli K99 primers showing positive amplification of 314 bp of K99 which indicates the presence of the K99 gene in 8 of the tested isolates (P: Positive control, N: Negative control, lanes 1-8: positive samples).

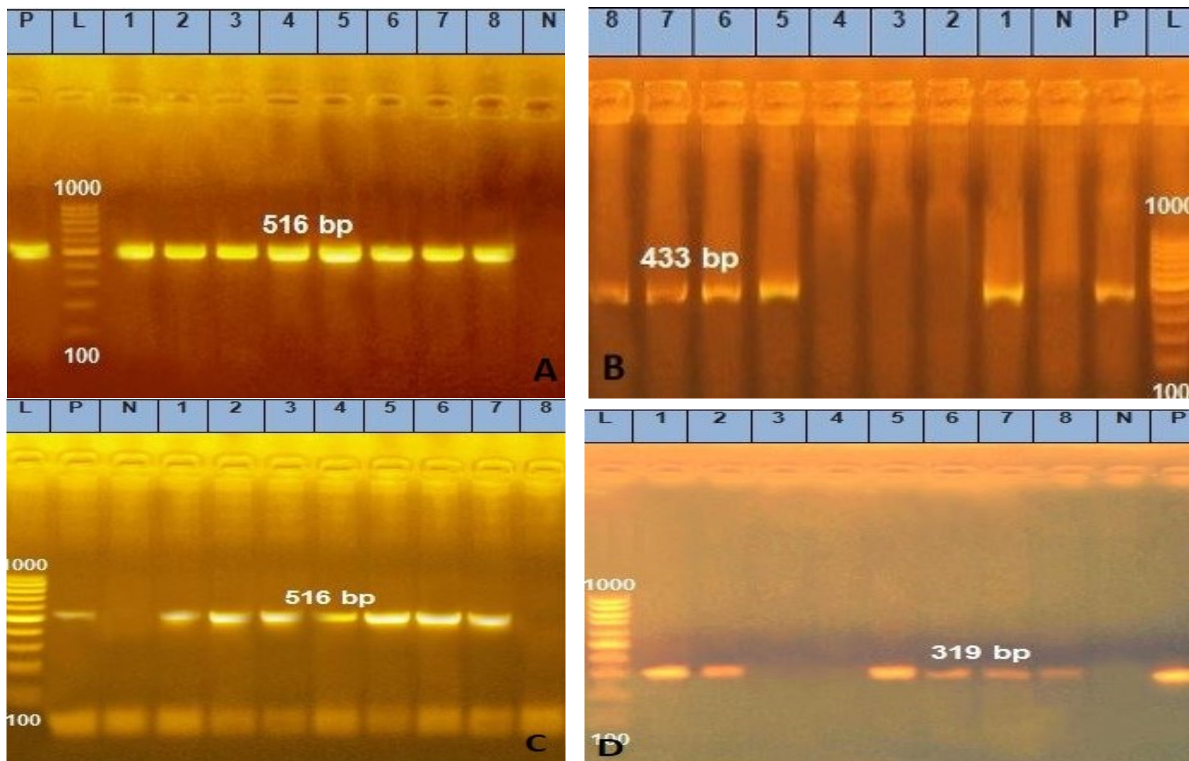

Fig.3: PCR detection of antibiotic-resistant genes in Escherichia coli isolates, A: showing amplification of 516 bp representing blaTEM. 8 isolates are positive for blaTEM gene, P-positive control, N-Negative control, B: showing amplification of 433 bp representing sul 1 . 5 isolates are positive for sul1 gene, P-positive control, N-Negative control, C: showing amplification of 516 bp representing qnrA, 7 isolates are positive for qnrA gene. P-positive control, N-Negative control, D: showing amplification of 319 bp representing aadB , 6 isolates are positive for aadB gene, P-positive control, N-Negative control

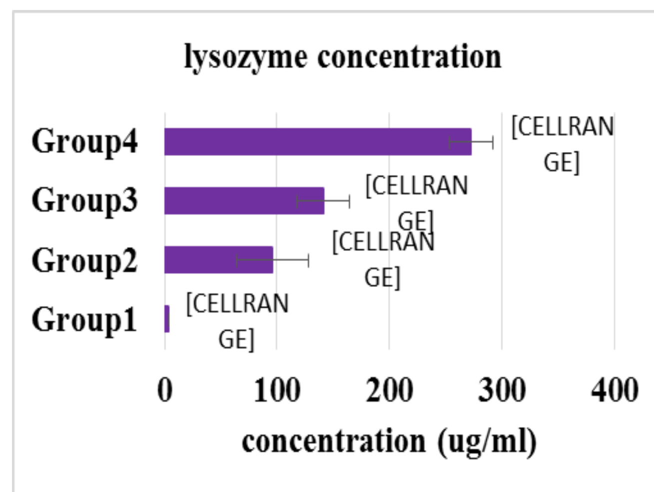

Fig. (4): Effect of diarrhea on lysozyme concentration.
Data were presented as mean ± standard error. Different small letters indicate significant between groups at $p \leq 0.05$.
Group1: control, Group2: Ecoli (K99) infected group, Group3:Rota virus infected group
Group4: Mixed infection (Ecoli K99 +Rota virus)

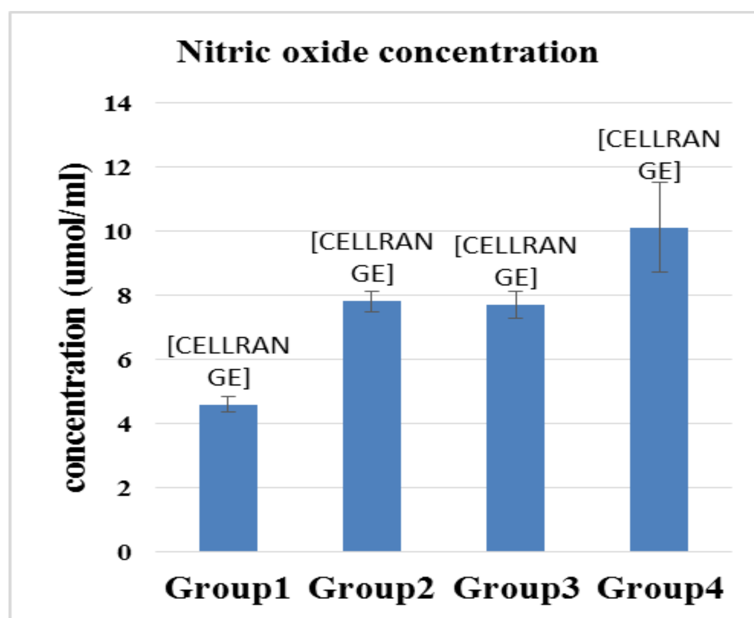

Fig. (5): Effect of diarrhea on Nitric oxide concentration.
Data were presented as mean ± standard error. Different small letters indicate significant between groups at $p \leq 0.05$.
Group1: control, Group2: Ecoli (K99) infected group, Group3:Rota virus infected group
Group4: Mixed infection (Ecoli K99 +Rota virus).

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