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Haemato-biochemical alterations caused by diarrhea in Friesian calves infected with *Escherichia coli* and *Salmonella* with detection of their virulence genes.

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ABSTRACT

Diarrhea is a common fatal disease of neonatal calves. There are many causes of diarrhea while *Salmonella* and *E. coli* are the main bacterial causes. One hundred blood and fecal samples were collected for this study; 80 from diarrhetic calves and 20 from apparent healthy calves (the control group). Blood samples were collected from each animal for hematological and biochemical assessment. An increase in total RBCs count, Hb content, total WBCs count, neutrophil percent, hematocrit value, ALT, AST, urea, creatinine, and K level while, total proteins, albumin, globulin Na and Cl decreased in diseased calves when compared to control group. Fecal Samples were bacteriologically examined. Seventy-two bacterial isolates were recovered, they included 22 *Salmonella* (27.5%) and 50 *E. coli* (62.5%). Four serogroups were identified for *Salmonella* including Enteritidis (54.5%), Typhimurium 31.8%, Newport (13.6%), and (9.1%) Anatum. Out of 50 *E. coli* isolates; 10 serogroups were identified included O26, O103, O127, O119, O86, O111, O157, O44, O158 and O78. Molecular identification of *Salmonella* strains revealed that *InvA*, *hilA*, *fimA*, and *sopB* genes were detected in all *Salmonella* serovars (100%), *mgtC* (90%), *ssaQ* and *stn* (86%) each, and *spi4R* (81%). *E. coli* strains revealed that the *16SrRNA* gene was detected in all identified *E. coli* strains (100%), *Sta* and *eaeA* (88%) each, *Stx1*(84%), *Vt2e* and *F41*(76%) each, *Stx2* (64%).

INTRODUCTION

Calves have an essential role in the future of animal wealth. They represent a principal source of high-quality protein required for the rapidly increasing population **(Ahmed and Ghada, 2007).**

The first week of life for newly born calf is considered a critical period because it is associated with a high mortality rate (10%). Diarrhea is an important cause of mortality during this

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age. The prevalence of diarrhea ranges from 15 to 20% in calves less than one month **(Vandeputte et al. 2010).** It is commonly attributed to *Salmonella spp., Escherichia coli (E. coli), Clostridium perfringens* type C, bovine rotavirus group A, bovine corona virus, and *Cryptosporidium spp.* **(Raihan et al. 2014).** *Salmonella* and *E. coli* are considered as the main pathogenic organisms isolated from diarrhetic calves leading to changes in nutrient transport mechanisms and severe gastrointestinal lesions **(Tarabees et al. 2021).**

Multiple factors predispose to diarrhea in newborn calves including vaccination programs, umbilical cord problems, the number of calves in the herd, and colostrum feeding. Other risk factors that affect the incidence and severity of the disease are the animal's age, the season, poor cleaning, farm type, shelter construction, and inadequate disinfection in shelters **(Berber et al. 2021).**

Salmonella spp. is a gram-negative rod-shaped facultative anaerobic bacteria belonging to the family of *Enterobacteriaceae*. Manifestations of *Salmonella* infection usually appear in calves between 4 and 28 days after birth **(Holschbach and Peek, 2018).** It is transmitted through water, food, or direct contact with diseased animals. Signs of the disease in calves include fever, recumbency, loss of appetite, and diarrhea which sometimes contains mucus or blood **(Nikkhah et al. 2023).**

Colibacillosis is considered an important infection in farm animals produced by pathogenic serotypes of *E. coli* **(Hassan et al. 2013).** *E. coli* is a gram-negative rod-shaped, non-spore-forming, and motile or non-motile facultative anaerobic bacteria related to the family of *Enterobacteriaceae* **(Muktar et al. 2015).** The clinical picture of Colibacillosis in calves includes dullness, depression, lethargy, and loss of appetite. The feces are semisolid to watery with a bad offensive odor. They are greenish to yellowish white and occasionally blood-stained. Mild to moderate dehydration may occur in diseased animals **(Singh et al. 2014).** Death from diarrhea is mainly from acidosis, dehydration, and loss of electrolytes. This dehydration in calves is followed by hyponatremia, hyperkalemia, and hypochloremia **(Mostafa et al. 2018).** The diarrhea in calves is mainly associated with an increment in the concentration of liver enzymes and kidney function tests **(Shehta et al. 2022).**

The most important hematological changes caused by acute infection occur in leukocytes in the form of neutropenia or neutrophilia **(Santos et al. 2002) (Taylor et al. 2017).**

Many enteropathogens producing diarrhea lead to alterations in enzyme activity, severe intestinal lesions, and nutrient transport mechanisms or a combination of these effects **(Wudu et al. 2008).**

Salmonella expresses a variety of virulence factors that are responsible for the organism's pathogenicity. It includes phase-variable flagella polymorphic surface carbohydrates, multiple fimbrial adhesins, and well-structured mechanisms for invasion and survival in host macrophages. It includes about 200 genes carried on the five chromosomal pathogenicity islands (SPI-1 to SPI-5) on *Salmonella* chromosomes which are important for virulence **(Thabet, 2023).** The *invA* gene is present in *Salmonella* isolates and is responsible for invading the epithelial tissue of the animal's intestine **(Borriello et al. 2012).** It is used as a confirmatory gene for *Salmonella* **(Truit et al. 2018).**

The ability of *E. coli* to cause severe diseases in humans is due to its ability to secrete verocytotoxins (*VT1* and *VT2*), shiga toxins (*Stx1* and *Stx2*), and intimin (*eae*) **(Kargar and Homayoon, 2015).** The *E. coli* strains that have the *eaeA* gene and do not give *stx1* and *stx2* genes, were known as Enteropathogenic *E. coli* **(Al-gammal et al. 2020).**

This study was conducted to assess the clinical, hematological, and biochemical changes in diarrhetic Friesian calves. Also, it was aimed to isolate and identify *Salmonella* and *E. coli* with molecular detection of their virulence genes by PCR technique.

MATERIALS AND METHODS

Animals:

This study was conducted from January to April 2024 and included one hundred Friesian calves recruited from different farms in Sohag governorate. Eighty calves were suffering from diarrhea and 20 calves were apparently healthy as a control group. Their ages ranged from 7-14 days with a body weight between 30-45kg. The calves lived in small bins near their mothers. They fed milk from their mothers twice daily, in the morning and at the end of the day. Each calf fed on two teats. They fed on the colostrum of their mothers. These calves defecated 4 or 5 times daily. They lost the suckling reflex. Their mothers had good health and received the vaccines for lumpy skin disease, FMD, and three days sickness. These calves were treated with nanazoxide syrup (30 ml daily for two days), in addition to sulfademadine, neomycin, pectin, and tannic acid powder mixture (a teaspoonful in water twice daily for two days).

This work was done according to the requirements of the Medical Research Ethics Committee of Faculty of Medicine, Sohag University under IRB Registration number Soh-Med-24-01-06PD.

Clinical examination:

Clinical examination of each calf included respiratory rate, pulse rate, and body temperature according to (**Constable et al. 2017).**

Sampling:

Blood and fecal samples were collected from each calf. Ten milliliters (ml) of blood were collected from the jugular vein. Each blood sample was divided into two tubes, 5 ml each. The first tube was coated with EDTA for hematological analysis. The second tube was a plain tube without EDTA, for biochemical analysis. Fecal samples were collected from the rectum of diseased calves after evacuation on the second and third day of the appearance of clinical signs. Then they were stored in sterile plastic cups, identified, and transmitted to the laboratory of the Animal Health Research Institute, Sohag in an icebox within one hour for bacteriological examination of *E. coli* and *Salmonella.*

Hematological analysis:

The hematological examination was carried out to estimate the red blood cells count (RBCs), hematocrit value (HCT), hemoglobin (Hb), white blood cells count (WBCs), and neutrophil using an automated hematology analyzer (Celltac a model no. MEK-6500k).

Biochemical examinations:

Serum was used for the estimation of total proteins and albumin according to the technique described by (**Titez 1990 and 1994**) and serum globulins were determined by subtracting the albumin values from those of total proteins. Liver function tests including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were measured according to **(Breur, 1996 and Young, 1990).** Kidney function parameters including urea and creatinine were assessed according to (**Young, 2001and Titez, 1986).** Serum electrolytes (Na, K, and Cl) were measured according to **(A.O.A.C, 2015).** Biochemical parameters were estimated using the automated chemistry analyzer (Aptio, Siemens, ADVIA® 2400).

Bacteriological examination:

One gram of each fecal sample was enriched in buffered peptone water and then incubated at 37° c for 24 hrs.

For isolation of *Salmonella* strains, 0.1 ml from the enriched sample was inoculated into 10 ml Rappaport Vassiliadis broth and incubated overnight at 37° c for 24 hrs, then a loopful from Rappaport Vassiliadis broth was streaked into brilliant green agar (BG) xylose-lysine and deoxycholate agar (XLD) and incubated at 37°c for 24 hrs according to **Collee et al. (1996)**. Typical *Salmonellae* colonies on XLD agar appeared as red colonies with black centers. On the brilliant green, *Salmonella* appeared as pinkish-white or red colonies.

For isolation of *E. coli* strains, a loopful from the enriched specimen on a buffered peptone water was inoculated into McConkey's agar and Eosin methylene blue (EMB) then incubated at 37°c for 24 hrs according to **Collee et al.**

(1996). *E. coli* colonies on MacConkey's agar appeared as pink or red colonies lactose fermenter. On EMB appeared as bluish black colonies with a green metallic sheen.

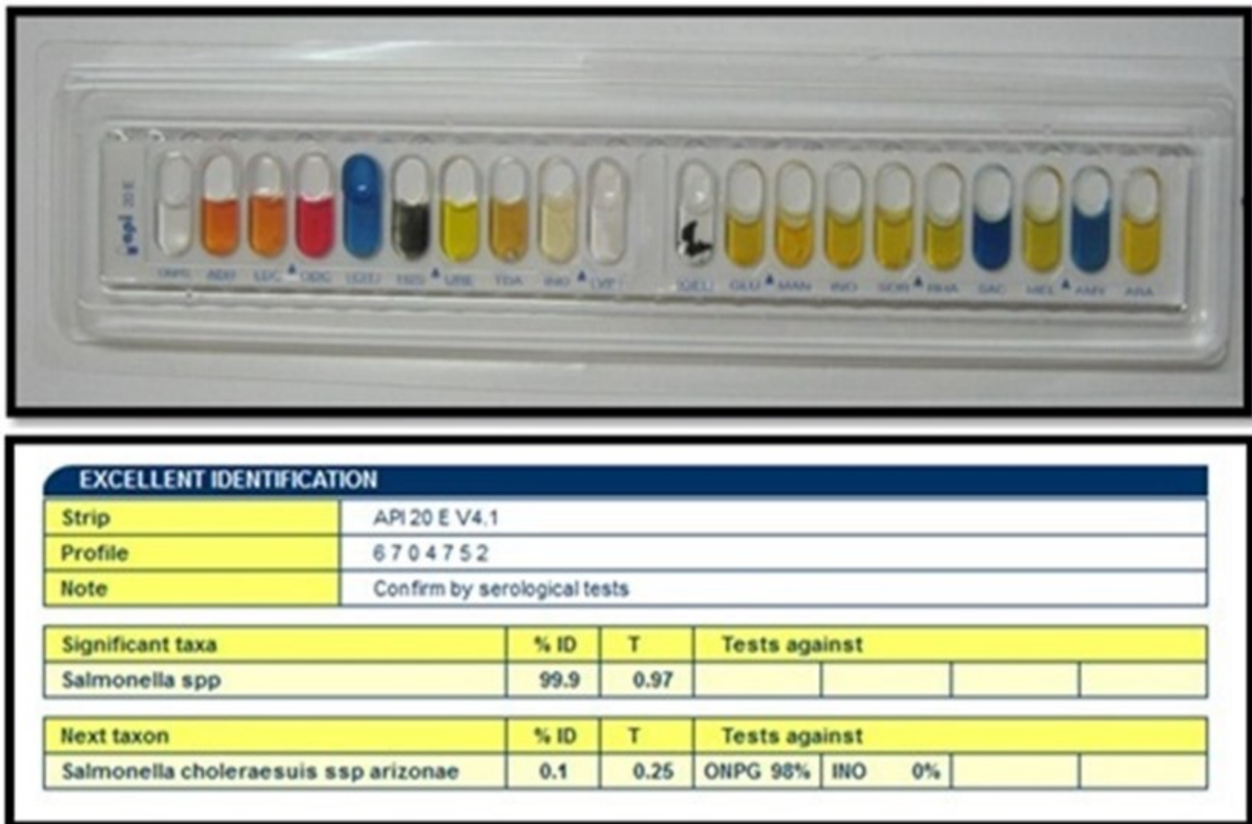
7. Identification of *Salmonella* and *E. coli:*

7.1. Microscopic examination:

Typical colonies for *Salmonella* and *E. coli* were examined under the microscope by using of Gram stain.

Biochemical identification:

Suspected colonies of *Salmonella* and *E. coli* were confirmed by the API 20E test. A Strip of API 20E test was inoculated for each isolate and then incubated for 24 hours at 37° c. Positive results were examined for each of the 20 biochemical tests. By using of profile manual or calling the API voice (APIWEB™), the seven-digital number was calculated, and the organisms were identified (figure 1 and figure 2) **(Mitham and Rasha, 2018)**



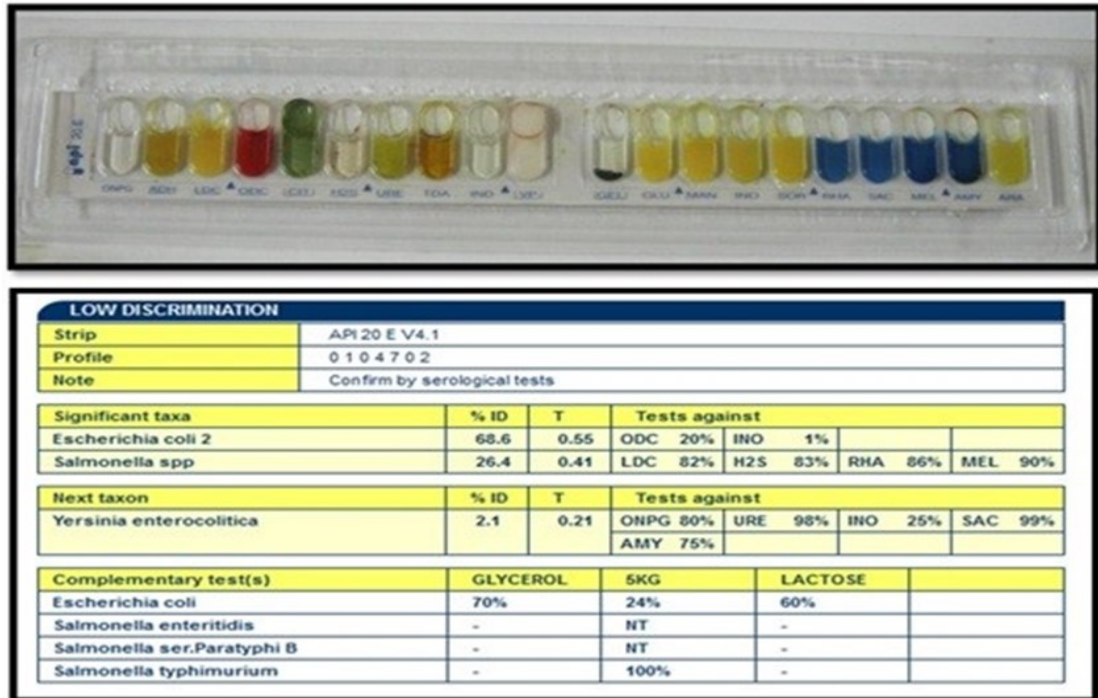
EXCELLENT IDENTIFICATION

Strip	API 20 E V4.1
Profile	6 7 0 4 7 5 2
Note	Confirm by serological tests

Significant taxa	% ID	T	Tests against			
Salmonella spp	99.9	0.97				

Next taxon	% ID	T	Tests against			
Salmonella choleraesuis ssp arizonae	0.1	0.25	ONPG 98%	INO 0%		

Figure 1: *Salmonella* identification on API 20 E



LOW DISCRIMINATION

Strip	API 20 E V4.1
Profile	0 1 0 4 7 0 2
Note	Confirm by serological tests

Significant taxa	% ID	T	Tests against			
Escherichia coli 2	68.6	0.55	ODC 20%	INO 1%		
Salmonella spp	26.4	0.41	LDC 82%	H2S 83%	RHA 86%	MEL 90%

Next taxon	% ID	T	Tests against			
Yersinia enterocolitica	2.1	0.21	ONPG 80%	URE 98%	INO 25%	SAC 99%
			AMY 75%			

Complementary test(s)	GLYCEROL	5KG	LACTOSE	
Escherichia coli	70%	24%	60%	
Salmonella enteritidis	-	NT	-	
Salmonella ser.Paratyphi B	-	NT	-	
Salmonella typhimurium	-	100%	-	

Figure 2: *E. coli* identification by API 20 E

Serological identification:

Slide agglutination test to identify *Salmonella* isolates was done according to **Grimont and Weill (2007),** while for *E. coli* isolates, it was performed according to **Ørskov and Ørskov (1984).**

Molecular identification of the suspected *Salmonella* and *E. coli* isolates and detection of virulence genes:

8.1. DNA extraction and amplification:

DNA extraction of *Salmonella* and *E. coli* isolates was done by QIAamp DNA Mini kit (Qiagen, Germany, GmbH). In brief, 200 µl of the sample suspension were incubated with 20 µl proteinase K and 200 µl lysis buffer, respectively at 56 °C for 10 min. Next, 200 µl of 100% ethanol were added to the lysate. Washing and centrifugation of samples were done according to the manufacturer's instructions. Nucleic acid was eluted with 100 µl of the provided elution buffer. Primers used for *E. coli* and *Salmonella* in this work were provided by **Metabion (Germany)** as shown in tables 1 and 2, respectively.

Molecular identification of *Salmonella* isolates was performed using virulence genes *hilA* **(Yang et al. 2014),** ***fimA*** **(Cohen et al. 1996),** ***STn*** **(Murugkar et al. 2003),** ***invA*** **(Oliveira et al. 2003),** ***ssQ*** **(Soto et al. 2006),** ***spi4R*** **(Sanchez-Jimenez et al. 2010),** ***sopB*** **(Soto et al. 2006) and** ***mgtC*** **(Sanchez-Jimenez et al. 2010)** virulence genes for *Salmonella* (table 1).

E. coli subjected for molecular identification using *16S rRNA* gene **(Tivendale et al. 2004)** and virulence genes; *Vt2e* **(Orlandi et al. 2006),** ***Sta*** **(Lee et al. 2008),** ***STX2*** **(Dipineto et al., 2006),** ***F41*** **(Franck et al. 1998),** ***eaeA*** **(Bisi-Johnson et al. 2011),** ***STX1*** **(Dipineto . 2006) (table 2).**

Table 1. Salmonella Primers sequences, target genes, amplicon sizes, and cycling conditions.

Target gene	Primers sequences	Amplified segment	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
hilA	CATGGCTGGTCAGTTGGAG CGTAATTCATCGCCTAAACG	150 bp	94˚C 5 min.	94˚C 30 sec.	60˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Yang *et al.*, 2014
invA	GTGAAATTATCGCCAC-GTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Oliveira *et al.*, 2003
fimA	CCT TTC TCC ATC GTC CTG AA TGG TGT TAT CTG CCT GAC CA	85 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Cohen *et al.*, 1996
Stn	TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC	617 bp	94˚C 5 min.	94˚C 30 sec.	59˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Murugkar *et al.*, 2003
ssaQ	GAATAGCGAATGAAGAGCGTCC CATCGTGTTATCCTCTGTCAGC	677bp	95º c 2 min.	95ºc 1 min.	58º c 1 min	72ºc 1 min	72ºc 5 min	Soto et al., 2006
sopB	GATGTGATTAATGAA-GAAATGCC GCAAACCATAAAAACTACAC-TCA	1170bp	95º c 2 min.	95º c 1 min.	53º c 1 min.	72º c 1 min.	72º c 5 min.	Soto et al., 2006
mgtC	TGACTATCAATGCTCCAGTGAA ATTTACTGGCCGCTATGCTGTTG	655bp	95º c 2 min.	95º c 1 min.	54º c 1 min.	72º c 1 min.	72º c 5 min.	Sánchez-Jiménez et a, 2010
spi4	GATATTTATCAGTCTATAACAGC ATTCTCATCCAGATTTGATGTTG	1269bp	95º c 2 min.	95º c 1 min.	51º c 1 min.	72º c 1 min.	72º c 5 min.	Sánchez-Jiménez et a, 2010

Table 2. *E. coli* Primers sequences, target genes, amplicon sizes, and cycling conditions.

Target gene	Primers sequences	Amplified segment	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
16S rRNA	GCTGACGAGTGGCGGACGGG TAGGAGTCTGGACCGTGTCT	253 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Tivendale *et al.*, 2004
Stx1	ACACTGGATGATCTCAG-TGG CTGAATCCCCCTCCATTATG	614 bp	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Dipineto *et al.*, 2006
Stx2	CCATGACAACGGACAG-CAGTT CCTGTCAACTGAGCAGCAC-TTTG	779 bp	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
eaeA	ATGCTTAGTGCTGGTTTAGG GCCTTCATCATTTCGCTTTC	248 bp	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Bisi-Johnson *et al.*, 2011
STa	GAAACAACATGACGG-GAGGT GCACAGGCAGGAT-TACAACA	229 bp	94˚C 5 min.	94˚C 30 sec.	57˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Lee *et al.*, 2008
Vt2e	CCAGAATGTCAGA-TAACTGGCGAC GCTGAGCACTTT-GTAACAATGGCTG	322 bp	94˚C 5 min.	94˚C 30 sec.	57˚C 40 sec.	72˚C 40 sec.	72˚C 7 min.	Orlandi *et al.*, 2006
F41	GCATCAGCGGCAGTATCT GTCCCTAGCTCAG-TATTATCACCT	380 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	72˚C 7 min.	Franck *et al.*, 1998

Analysis of the PCR Products:

At room temperature, the products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer using gradients of 5V/cm. For gel analysis a gene ruler 100 bp ladder (Fermentas, thermo, Germany), gelpilot 100 bp and 100 bp plus ladders (Qiagen, Gmbh, Germany), and Genedirex 50 bp DNA ladder RTU, Cat. No. DM012-R500 were used to determine the fragment sizes. The gel was photographed (Alpha Innotech, Biometra) and analyzed by computer software.

Statistical Analysis:

The obtained data of hematobiochemical parameters were statistically analyzed to calculate mean, standard deviation, and P values≤ 0.5 using the independent sample T-test, SPSS program according to Sendecor and Cochran, (1980).

RESULTS

Clinical examination:

The clinical examination of the eighty diarrhetic calves recorded anorexia in 75 calves (93.7%), staggering movement in 60 calves (75%), sunken eyes in 18 calves (22.5%), dehydration mild to moderate, diarrhea was detected in all examined calves (100%). The stool color varied from dark yellow to green with an offensive odor.

There was an increment in the levels of temperature, pulse rate, and respiratory rate in diarrhetic calves which were 39.7^{o} c, 121beats/ minute, and 40.7 breath/ minute, when compared to control calves which were 38.4^{o}c, 94.6 beats/ minute, and 33.8 breath/minute; respectively at the level of P value≤ 0.5 (table 3).

Table 3. Clinical findings in diarrhetic and normal calves.

Parameters	Control	Diseased
Temperature ^{o} C	38.4± 0.3	39.7±0.57**
Pulse rate beats/minute	94.6± 6.4	121±3.3*
Respiratory rate breath/minute	33.8± 3.4	40.7± 2.3*

*Significant at P≤ 0.05 **Significant at P ≤0.001

Hematological results:

This work revealed a significant increase in the levels of total RBCs count, and Hb content which were 7.8± 1.4 x10^{6}/ml and 11.1±0.8 gm% when compared to the control group which were 6.5± 1 x10^{6}/ml and 8.4± 0.3; respectively. There was a significant increase in the level of total WBCs count, neutrophil percentage, and hematocrit value which were 14± 2.510^{3}/ml, 71± 0.8and 35.3± 3.7 when compared to the control group which were 7.7± 1.110^{3}/ml, 57± 0.9% and 30.4±1.5; respectively when P value ≤ 0.5 (table 4).

Table 4. Hematological in diarrhetic and normal calves.

Groups Parameters	Control	Diseased	P value
RBCs count (10^6/ml)	6.5± 1	7.8± 1.4*	0.029
Hematocrit value	30.4±1.5	35.3± 3.7*	0.045
Hb content (gm%)	8.4± 0.3	11.1± 0.8**	0.001
WBCs count (10^3/ml)	7.7± 1.1	14± 2.5**	0.001
Neutrophil %	57± 0.9	71± 0.8**	0.001

*Significant at P≤ 0.05 **Significant at P ≤0.001

Biochemical results:

The result of the current study revealed that there was a significant decrease in the levels of total proteins, albumin, Na, and Cl in diseased calves which were (6± 0.1& 3± 0.2 g/dl), 123± 1.4 mmol/l& 77± 0.6 mg/l when compared to control one which was (7± 0.2& 3.8± 0.13 g/dl), 148± 15 mmol/l& 95.2± 0.9 g/dl; respectively while, there were a significant increase in ALT, AST, urea, creatinine and K level in diarrhetic calves which were (76.5± 0.8, 116.7± 1.6 u/l), (45±2, 1.6± 0.19 mg/dl) and 5.3± 0.09mmol/l when compared to control calves which were (61± 0.8, 84± 0.8 u/l), (26± 0.7, 0.5± 0.07mg/dl) and 4.5± 0.06 mmol/l; respectively and non-significant change in globulin level in diseased calves (3± 0.1 g/dl) when compared to control (3.2± 0.1 3g/dl) at the P value ≤ 0.05 as showed in (table 5).

Table 5. Biochemical examination

Groups Parameters	Control	Diseased	P value
Total proteins g/dl	7± 0.2	6± 0.1**	0.001
Albumin g/dl	3.8± 0.13	3± 0.2**	0.001
Globulin g/dl	3.2± 0.13	3±0.1	0.067
ALT u/l	61± 0.8	76.5± 0.8**	0.001
AST u/l	84± 0.8	116.7± 1.6**	0.001
Urea mg/dl	26± 0.7	45±2**	0.001
Creatinine mg/dl	0.5± 0.07	1.6± 0.19**	0.001
Na (mmol/l)	148± 15	123± 1.4**	0.001
K (mmol/l)	4.5± 0.06	5.3± 0.09**	0.001
Cl (mg/l)	95.2± 0.9	77± 0.6**	0.001

*Significant at P≤ 0.05 **Significant at P ≤0.001

Bacteriological examination:
Out of 80 collected fecal samples from diarrhetic calves; 72 bacterial isolates were recovered which included 22 *Salmonella* (27.5%) and 50 *E. coli* (62.5%).

Serotyping of isolated *Salmonella* species:
The 22 isolates of *Salmonella* were serotyped as 10 *Salmonella* Enteritidis (45.5%), 7 *Salmonella* Typhimurium (31.8%), 3 *Salmonella* Newport (13.6%) and 2 *Salmonella* Anatum (9.1%) (table 6).

Table 6. Serotyping of *Salmonella* species isolated from Friesian diarrhetic calves.

Identified strains	Group	Antigenic structure		Frequency (%)
		H	O	
Salmonella Enteritidis	D_1	g, m: 1, 7	1, 9, 12	10 (45.5%)
Salmonella Typhimurium	B	i, 1, 2	1, 4, 5, 12	7 (31.8%)
Salmonella Newport	C_2	e, h; 1,2	6, 8	3 (13.6%)
Salmonella Anatum	E_1	e, h: 1, 6	3, 10	2 (9.1%)

Serotyping of *E. coli* strains:
Ten serogroups were identified. The serogroups were O26 (18%), O103 (18%), O127 (16%), O119 (14%), O86 (10%), O111 (8%), O157 (6%), O44 (4%), O158 (4%) and O78. (2%). The O26 and O103 were the most prevalent isolates (18%) each (table 7).

Table 7. Serotyping of *E. coli* strains isolated from Friesian diarrhetic calves.

Serogroup	No (%)
O 26	9 (18%)
O 103	9 (18%)
O 127	8 (16%)
O 119	7 (14%)
O 86	5 (10%)
O 111	4 (8%)
O 157	3 (6%)
O 44	2 (4%)
O158	2 (4%)
O 78	1 (2%)

Molecular identification of virulence genes of *Salmonella* and *E. coli* strains:
Molecular identification of *Salmonella* strains revealed that InvA, *hilA*, *fimA*, and *sopB* genes were detected in all *Salmonella* serovars (100%), *mgtC* (90%), *ssaQ* and *stn* (86%) each, and *spi4R* (81%) (table8).

Molecular identification of *E. coli* strains revealed that the *16SrRNA* gene was detected in all identified *E. coli* strains (100%), *Sta* and *eaeA* (88%) each, *Stx1*(84%), *Vt2e* and *F41* (76%) each, *Stx2* (64%) (table8).

Table 8. Virulence genes in identified *Salmonella* and *E. coli*

Virulence genes in identified *Salmonella*			Virulence genes in identified *E. coli*		
Gene	Incidence	%	Gene	Incidence	%
InvA	22	100%	*16SrRNA*	50	100%
hilA	22	100%	*eaeA*	44	88%
fimA	22	100%	*Sta*	44	88%
sopB	22	100%	*Stx1*	42	84%
mgtC	20	90%	*Vt2e*	38	76%
stn	19	86%	*F41*	38	76%
ssaQ	19	86%	*Stx2*	32	64%
spi4R	18	81%			

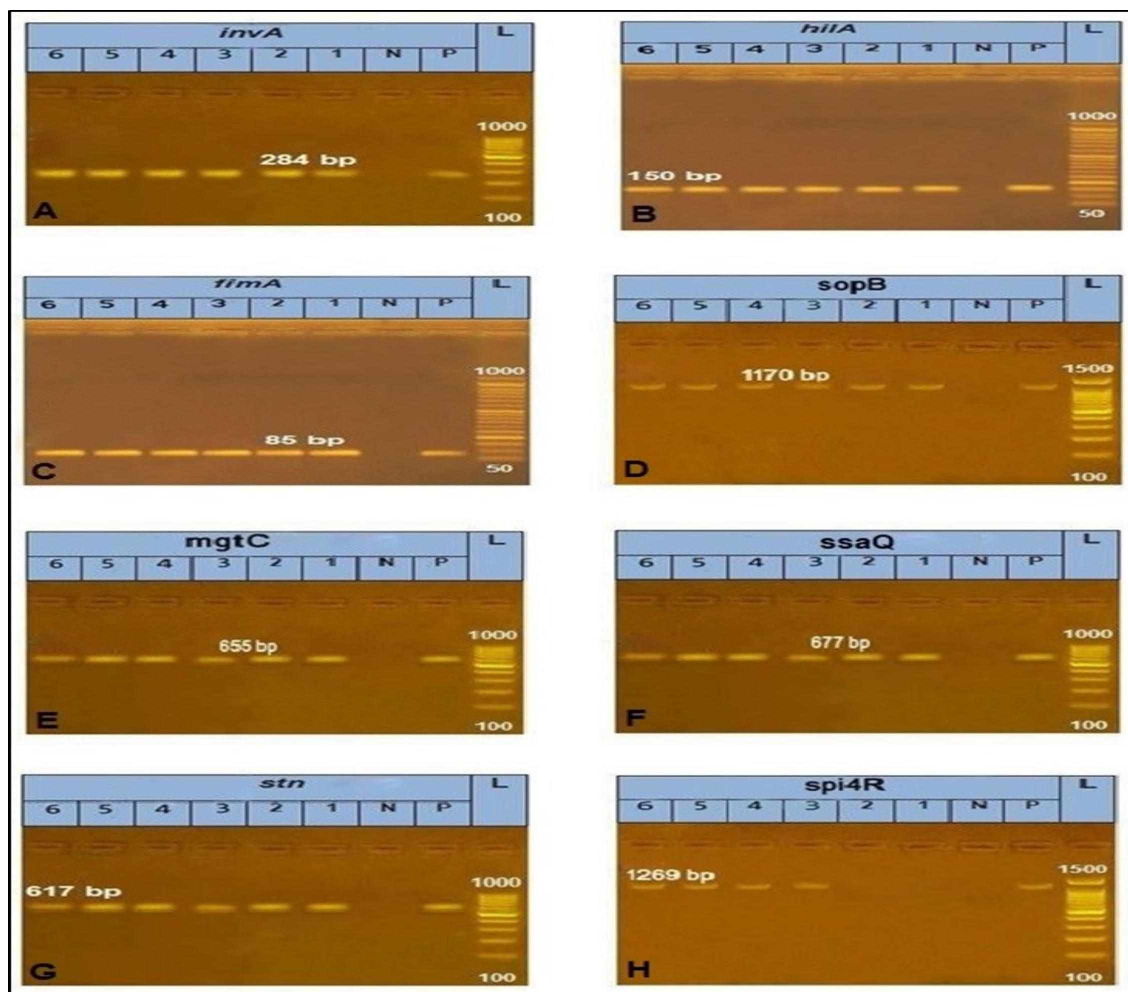


Figure (3): Gel electrophoresis and PCR amplification for *Salmonella* virulence genes. Lane (L): DNA marker, lane (P): positive control, lane (N): negative control, (bp) base pair.
(A): 1, 2, 3,4,5,6 lanes are positive for *the InvA* gene (284 bp). (B): 1, 2, 3,4,5,6 lanes are positive for *hilA* gene (150 bp). (C): 1, 2, 3,4,5,6 lanes are positive for *fimA* gene (85 bp). (D): 1, 2, 3,4,5,6 lanes are positive for *sopB* gene (1170 bp). (E): 1, 2, 3,4,5,6 lanes are positive for *mgtC* gene (655 bp). (F): 1, 2, 3,4,5,6 lanes are positive for *ssaQ* gene (677bp). (G): 1, 2, 3,4,5,6 lanes are positive for *stn* gene (617bp). (H): 3,4,5,6 lanes are positive, while 1,2 lanes are negative for the *spi4R* gene (1269 bp).

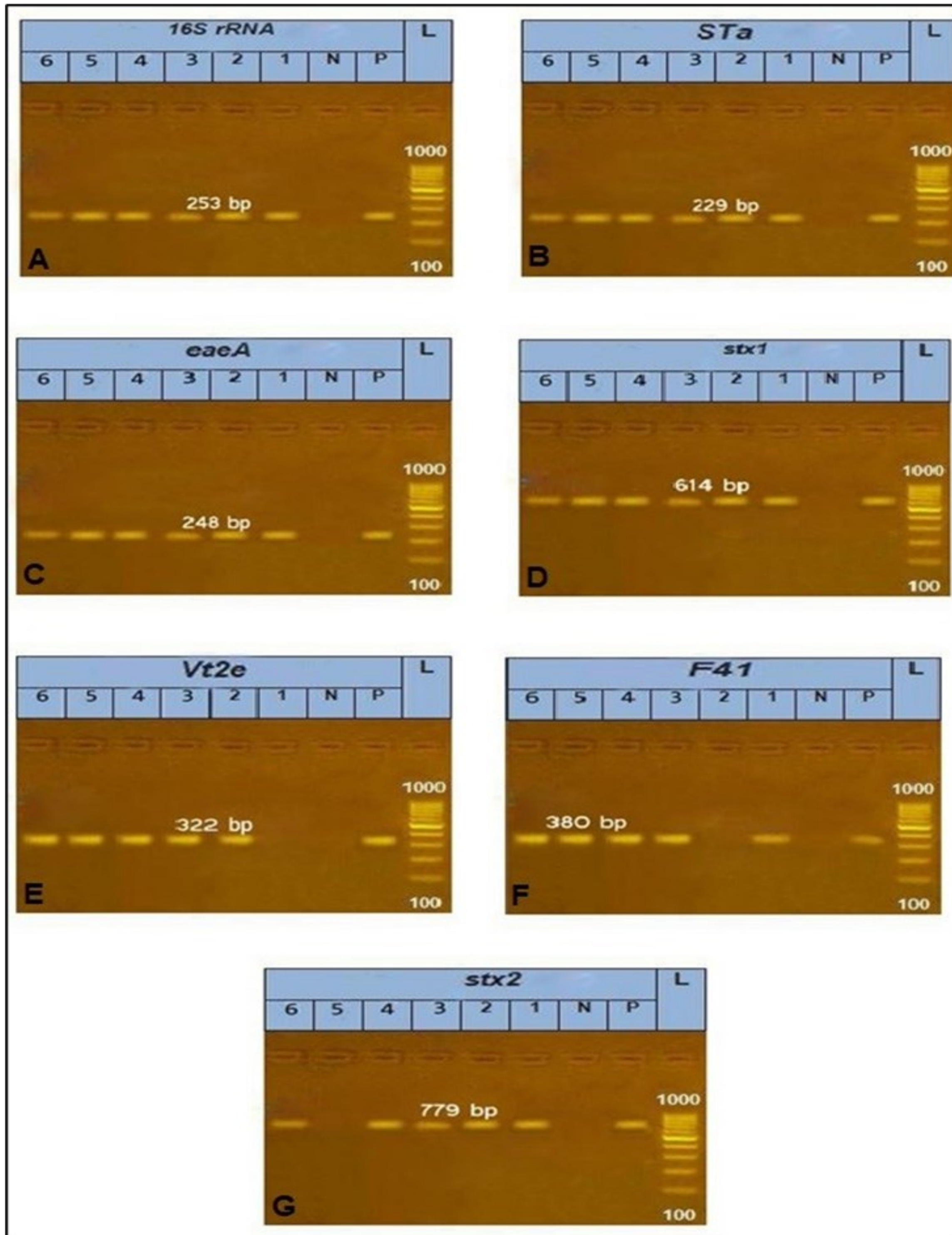


Figure (4): Gel electrophoresis and PCR amplification for *E. coli* virulence genes. Lane (L): DNA marker, lane (P): positive control, lane (N): negative control, (bp) base pair.
(A): 1, 2, 3,4,5,6 lanes are positive for *16SrRNA* gene (253 bp). (B): 1, 2, 3,4,5,6 lanes are positive for the *Sta* gene (229 bp). (C): 1, 2, 3,4,5,6 lanes are positive for *eaeA* gene (284 bp). (D): 1, 2, 3,4,5,6 lanes are positive for *Stx1*gene (614bp). (E): 2, 3,4,5,6 lanes are positive, while 1 lane is negative for *Vt2e* gene (322 bp). (F): 1,3,4,5,6 lanes are positive, while 2 lane is negative for *F41*gene (380 bp). (G): 1,2,3,4,6 lanes are positive, while 5 lane is negative for *Stx2*gene (779 bp).

DISCUSSION

Diarrhea causes high morbidity and mortality rates that can reach more than 50% of total calves' deaths. The clinical examination of the eighty diarrhetic calves in this study recorded anorexia in 75 calves (93.7%), staggering movement in 60 calves (75%), sunken eyes in 18 calves (22.5%), dehydration mild to moderate, diarrhea was detected in all examined calves (100%). The stool color varied from dark yellow to green with an offensive odor.

These results are nearly similar to that reported by **Shehta et al. (2022**) who proved the presence of staggering movement in 76%, anorexia in 94%, sunken eyes in 82%, dehydration in 76%, and diarrhea in 100% of examined calves. Also agreed with that mentioned by **Ghanem et al. (2012), Constable et al. (2017), Özkan et al. (2011), and El-Seadawy et al. (2020).**

Sunken eyes and dehydration due to high losses of electrolytes and water during diarrhea **(Torche et al. 2020).**

In this research, we isolated and identified *Salmonella spp*. and *E. coli* from diarrhetic calves. *E. coli* bacteria is able to adhere to the microvilli apical portion that fuse with one another and become atrophic leading to malabsorption and indigestion. In the case of *Salmonella* infection, there is an increase in stimulation of secretion of active chloride with inhibition of absorption of sodium which ends with the drawing of water tissue to the gut and by occurrence of diarrhea **(Radostits et al. 2007).**

The clinical examination of diarrhetic calves proved an increment in the temperature, pulse, and respiratory rate. These results agreed with that reported by **Leal et al. (2008) Ghanem et al. (2012); Abdel Khalek et al. (2012) and Alsaad et al. (2012)** and disagreed with that reported by **Malik et al. (2012)** who reported a non-significant change in temperature in calves suffering from diarrhea.

Increased body temperature in diarrhetic calves may be attributed to microbial infection responsible for diarrhea. **(Walter, 2012).**

The increase in respiratory rate is considered a compensative polypnea in response to acidosis that happened to diarrheic calves to eliminate the excess of CO_2 to the normal pH level. Tachycardia compensates for the hypovolemia that occurs due to dehydration happens to calves suffering from diarrhea **(Scott et al. 2004) (Bleul and Gotz 2013).**

The result of the current study revealed that there was a significant increase in the level of total RBCs count, and Hb content in diseased calves in comparison with the control group.

These results came in accordance with what was reported **by Fatma et al. (2007) and Malik et al. (2012)** who concluded that Hb was 12.5 and 11.5 g/dl and R.B.Cs count was 8x 10^6 and 8.5 million/ cm^3 in diarrhetic calves when compared to healthy control which was 11and 10 g/dl and R. B.Cs was 6.5x 10^6 and 8 million/ cm^3; respectively and disagreed with that reported by **Shehta et al. (2022)** who proved that RBCs count and Hb content in diarrhetic calves were 7.3x 10^6/ ml and 8.8g/dl decreased than control one which were 9x10^6/ ml and 11.4 g/dl; respectively.

The increase in the RBCs count may be related to hem concentration due to the water loss combined with diarrhea, hypovolemia, and hemoconcentration due to dehydration **(Mostafa, 2018).**

The increase in Hb content can be attributed to thirst and the decrease of water content in the vascular space of diarrhetic calves associated with dehydration **(Singh et al. 2014).** Hemoglobin is related to the rate of transported oxygen in the bloodstream of calves. This result agreed with previous studies **(Fatma, 2007, Abdel Khalek, 2012 and Song et al. 2020)** which were 11.4, 10.7, and 14 g/dl when compared to control calves which were 9.7, 10.3, and 11g/dl; respectively.

The obtained results of this study revealed an increase in the hematocrit value in diseased calves when compared to control ones. This result agreed with that illustrated by **Song et al. (2020) and Shehta et al. (2022)** who proved that the hematocrit value in diarrhetic calves was 44% and 37% when compared to control ones which was 31.7and 31.5%; respectively. Hematocrit gives information about the overall blood volume also, it increases at

the first period of birth and then decreases with the calf's age **(Malheu, 2007). Song (2020)** concluded that the increase in the hematocrit value of calves with diarrhea means dehydration resulting from a high loss of moisture combined with diarrhea.

The results of this study revealed an increase in the total leukocytic count and absolute increase in neutrophils in diseased calves compared to control ones. These results are in accordance with those reported by **Seifi et al. (2006) and Rasha et al. (2015)** who proved an increment in the total leucocytic count and relative increase in neutrophil percentage. This result could be attributed to the body's defense mechanism against infection, inflammation lesions in the digestive system, or due to hemoconcentration due to dehydration combined with diarrhea. The neutrophil increased in case of bacterial infection such as *E. coli* infection **(Akyuz et al. 2022).**

Our results proved that there was a significant decrement in the concentrations of total proteins and albumin in diarrhetic calves when compared to healthy control ones. These results came in accordance with that reported by **Shehta et al. (2022).** This reduction may result from the increase of protein parameters excretion in the intestinal lumen with diarrhea **(Constable et al. 2016 and Choi et al. 2021).**

There was a significant increase in the levels of ALT and AST in diarrhetic calves compared to healthy control ones. These results match that reported by **Ghanem et al. (2012)** who proved an increment in the levels of ALT and AST which were 78 and 124 Iu/l when compared to control ones which were 64 and 85 Iu/l. This result may be related to chronic inflammation of the gastrointestinal tract and affection of the liver of diarrhetic calves. **(Chernecky and Berger, 2013).** Liver enzymes may increase due to toxic effect of abnormal digestion or bacterial toxins **(Ashraf, 2007).**

There was a significant increase in the values of urea and creatinine in diarrhetic animals when compared to the healthy group. These results agreed with that reported by **Ghanem et al. (2012) and Singh et al. (2014)** who reported that there was a significant increase in urea and creatinine in diarrhetic calves when compared to control ones and also agreed with **Santos et al. (2002) and Akyuz et al. (2022).**

Such increase in urea and creatinine levels may be due to a deficit in glomerular filtration rate (renal blood perfusion) thus decreasing urine formation or may be related to excessive urea production by body proteins catabolism in some toxic conditions **(Ashraf, 2007).** This increase in serum urea and creatinine was also related to dehydration due to diarrhea. Hypovolemia due to dehydration, excessive fluid loss, and decreased fluid intake lead to plasma solutes concentration with a proportionate increase in urea and creatinine **(Dratwa-Chalupnik et al. 2012 and Singh et al. 2014).**

Our results proved that there was a significant reduction in Na and Cl and there was a significant increase in K level in diarrheic calves when compared to control ones. These data agreed with that reported by **Ghanem et al. (2012) and Singh et al. (2014).**

This decrease in Na and Cl levels in diseased calves may be due to high loss of these ions associated with an increase in their intestinal secretion, diarrhea, and failure of reabsorption of gastric H+ and Cl- ions by the small intestine villi **(Radiostitis et al. 2007).**

The increase in the K concentration occurs due to increased potassium retention by diseased calves' kidneys or cellular damage that happens in diarrhetic calves **(Fisher et al. 1971).**

Out of 80 fecal samples collected from diarrhetic calves; 72 isolates of bacteria were recorded which included 22 *Salmonella* (27.5%) and 50 *E. coli* (62.5%).

Salmonella was nearly similar to that proved by **El-Azzouny et al. (2020) and Shehta et al. (2022)** who proved that *Salmonella* prevalence in diarrhetic calves was 30% and 24%; respectively and more than those of other studies in Egypt **(Seleim et al. 2004: 17.5% and Youssef and El-Haig, 2012:**

18.66%) while higher than that reported by **Haggag and Khaliel (2002)** (4%) and **Younis et al. (2009)** (4.09%). On the contrary, this result was lower than that reported by **Moussa et al. (2010)** (43.53%).

The incidence of *E. coli* is nearly similar to that reported by **Shehta et al. (2022):** 62%, Osman et al. (2013): 63.6% and more than that reported by **Azzam et al. (2006):** 5.4%, **El-Shehedi et al. (2013):** 35.83% and Hassan (2014): 50%.

Variations in the prevalence rates of *E. coli* and *Salmonella* in calves with diarrhea could be attributed to the species, geographical area, breeding system, environment, immunity status, age of calves, and hygienic measures during the management of calves and Enterohaemorrhagic *E. coli* (EHEC) mainly occurs due to ingestion of contaminated water and food **(Cho and Yoon, 2014).**

The 22 isolates of *Salmonella* were serotyped as 10 *Salmonella* Enteritidis (45.5%), 7 *Salmonella* Typhimurium (31.8%), 3 *Salmonella* Newport (13.6%) and 2 *Salmonella* Anatum (9.1%). In this study, *Salmonella* Typhimurium and Enteritidis were the most prevalent serotypes. These results came in accordance with that reported by **Seleim et al. 2004; Younis et al. 2009; Moussa et al. 2010 and Youssef and El- Haig, 2012** in Egypt and reports from other countries by Smith-Palmer et al. (2003) in Scotland. On the contrary to previous results obtained by El-Seedy et al. (2016) who found that *Salmonella* Enteritidis was (60.9%) and *Salmonella* Typhimurium (30.4%). **Reda and Mohamed. (2013)** reported that *Salmonella* Anatum (8%) which is nearly similar to our result.

The frequency of isolation of *Salmonella* serovars differs from one area to another due to the differences in calves' hygienic measures and management in addition to the environmental and geographical differences **(Veling et al. 2002).**

Salmonella Enteritidis and Typhimurium are considered as non-host-adapted serovars and so they establish a “carrier state” in the recovered animals. Infection with a non-host-adapted *Salmonella* leads to transient shedding of bacteria for about 3–16 weeks. Contaminated water and food are the most common sources of infection. Approximately 40% of fish or bone meals may be contaminated with *Salmonella*. In some herd outbreaks, human sewage has also been a source of infection. Carcasses of birds and rodents also can spread infection **(Donaldson et al. 2006).**

The most prevalent serogroups of *E. coli* identified in our study were O26(18%), O103 (18%), O127(16%), O119 (14%), O86 (10%), O111(8%) and O157 (6%).

These findings are nearly similar to that reported by **Elseedy et al. (2020)** who proved that the serogroups of *E. coli* isolates were O103 and O26 were the most prevalent groups (17.7% each) followed by O127 (14.6%) and O119 (13.6%). These findings were similar to those obtained by **El-Shehedi et al. (2013)** and **Osman et al. (2013).** Concerning other countries; Tamaki et al. (2005) in Japan concluded that the most common *E. coli* serotypes were O119, O111, O126, and O78 isolated from animals with diarrhea. Similarly, Badouei et al. (2010) reported that O26 (18.4%) serotypes from diseased and non-diseased calves were the most common serogroups. **Fouad et al. (2022)**proved that the most common serogroups of *E. coli* isolated from diarrhetic calves were O55 (14) followed by O25 (11) and O111 (10), O119 (8), O126 (8), O78 (5), O157 (3), O186 (2) and O128 (2).

Salmonella invA gene is one of the most famous PCR target sequences and its amplification has been known as an international standard used for detection of *Salmonella* **(Malorny et al. 2003).** It encodes a protein in the bacterial inner membrane that is responsible for the invasion of the host epithelial cells **(Bell et al. 2016).** The results of PCR applied on 22 isolates of *Salmonella* to determine the virulence *invA* gene revealed that all the *Salmonella* tested isolates showed positive results with PCR assay using oligonucleotide primer that amplified a 284 bp fragment and these results were matched with that obtained by **Soli-**

man (2014) and El-Seedy et al. (2016) who proved that the virulence *invA* gene recovered in all *Salmonella* serogroups.

The gene *sopB* was found in all isolates and these results agreed with that reported by **El-Azzouny et al. (2020)** who proved the presence of *sopB* in all isolates (100%).

Our results proved the presence of *fimA* in all isolates and *stn* in (86%) of isolates and these results were like that obtained by **Chaudhary et al. (2015)** who proved that out of 37 *Salmonella* isolates, all isolates contained virulence genes (*fimA, stn, invA*). **Ownagh et al. (2023)** proved that 50 fecal samples (11.90%) were positive for *fimA, stn,* and *invA* genes isolated from buffalo.

Our results proved the presence of *ssaQ* (86%), *spi4R* (81%), *sopB* (100%), and *mgtC* (90%). These data are nearly similar to **Salama et al. (2023)** who proved that most isolates of *Salmonella* had 5 virulence genes (*invA, ssaQ, sopB, spi4R,* and *mgtC*) with a percentage of 71.4%. It is known that *invA* and *sopB* are primarily responsible for calf diarrhea caused by *Salmonella* while *ssaQ* is responsible for the survival of the organism intracellularly.

Molecular identification of *E. coli* strains revealed that the *16SrRNA* gene was detected in all identified *E. coli* strains (100%), *Sta* and *eaeA* (88%) each, *Stx1*(84%), *Vt2e* and *F41* (76%) each, *Stx2* (64%)

Shiga-toxin producing *E. coli* is considered an important group of zoonotic human pathogens **(Caprioli et al. 2005).** These strains are carried in cattle without clinical signs and shed in their feces, which leads to their spread among cow herds **(Tauxe, 1997).** Shiga toxins can inactivate the host cell ribosomes and make inhibition of protein biosynthesis. The occurrence of the *stx1, eaeA,* and *stx2* genes together have an epidemiological significance; the combination of these genes leads to an increment in the ability of *E. coli* to cause severe illness in humans **(Kudva, et al. 1998).** These results are similar to that reported by **El-Azzouny et al. (2020)** who concluded that out of ten *E. coli* isolates examined by PCR, there were *stx1*: 8 (80%) and stx2: 9 (90%) while *eaeA* found in all isolates (100%). **Salama et al. (2023)** proved the presence of *eaeA* in all isolates (100%), *stx1* (16.7%), and *stx2* (11%) in isolates of *E. coli,* and these results were lower than that reported by Franz et al. (2007) who concluded that the low-input conventional Dutch dairy farms were positive for all Shiga toxin genes included *stx1, stx2,* and *eaeA.* Our results were higher than those reported by **Nguyen et al. (2011)** who proved that Shiga toxin genes were found in 177 isolates (51.3%) from calves with diarrhea in Vietnam.

Concerning the *sta* gene which was found in 88% of the *E. coli* isolates in this study. This gene is important for the occurrence of watery diarrhea in calves as it activates the guanylate-cyclase system in diseased calves. This result is higher than that published by **Borriello et al. (2012)** in Italy who concluded the absence of the *sta* gene in diarrheic bufalo calves with *E. coli.* **Algammal et al. (2020)** concluded that *sta* was found in 8.9%, 8% with *It and f41,* and 4% with *It* of *E. coli* isolates from calves at different farms in El-Sharqia Governorate, Egypt suffered from diarrhea. **Beutin et al. (1989)** studied hemolysin production of *E. coli* strains and found an association between enterohaemolysin and verotoxin production in 89% of *E. coli* strains belonging to nine different serotypes. A suggestion was raised that enterohaemolysins may complement the effects of Shiga toxins enhancing their virulence **(Nataro and Kaper, 1998).**

According to our study, the *vt2e* gene was detected in 76% of all isolates. This result was higher than that illustrated by **Bendary et al. (2022)** who proved the absence of the *vt2e* gene in all isolates from calves.

The *f41* gene is linked to the occurrence of diarrhea in calves and warrants an important function in the pathogenesis of enterotoxigenic *E. coli* **(Bisi-Johnson et al. 2011).** Our result is increased than that reported by **Algammal et al. (2020)** who concluded that the *f41* gene was found in 10% with the *It* gene and in 3% with *Itand sta* genes of *E. coli* isolates from calves at different farms in El-Sharqia Governorate, Egypt which have diarrhea.

The variation in the presence of virulence genes, enterotoxin genes, and shiga toxin genes

between studies and our results may be attributed to the size of the sample, the variation in handling of collected samples, the geographical origin of samples, the number of examined strains and the type of the virulence genes detected by PCR (**Franz et al. 2007).**

CONCLUSION

Diarrhea in calves is associated with multiple changes in the hemato-biochemical parameters that could be used as an important indicator of diarrhea. *Salmonella* and *E. coli* are important causes of diarrhea in calves which is with high morbidity and mortality rates. Molecular surveillance represents a rapid, sensitive, and reliable procedure that can effectively detect Salmonella and *E. coli* virulence genes.

RECOMMENDATIONS

After conducting this study, the following recommendations should be considered:

Improvement of clinical laboratory procedures for diagnosis of *Salmonella* and *E. coli* using specific and sensitive detection techniques to protect the consumers.

Implementation of hygienic and sanitation measures of farm animals.

Application of hygienic and control measures to prevent transmission of animal pathogens to humans through milk and meat.

Prompt treatment of calves suffering from diarrhea to avoid dehydration and death.

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