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BACTERIOLOGICAL,PARASITOLOGICALAND MOLECULAR STUDIES ON SOME BACTERIALAND PARASITIC CAUSES OF ENTERITIS IN SMALL RUMINANTS

By

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ABSTRACT

Enteritis in small ruminants is a major field problem that causes great morbidity and mortality rates. Several agents are responsible for enteritis in small ruminants as bacteria (aerobic and anaerobic), parasites and viruses. In the present study, a total of 165 fecal samples and fecal swabs were collected from sheep and goats (107 sheep and 58 goats) belonging to farms at EL- Sharkia province during the period from November 2018 to May 2019. They were subjected to bacteriological and parasitological examinations. The obtained results showed that diarrhea was more common in young animals (lambs and kids) than in older ones. Fourteen sheep and nine goats were complicated cases and showed mixed infection with both bacteria and parasites. Isolated aerobic bacterial species from sheep included of *E.coli, Salmonella, Klebsiella pneumonia* and *Pseudomonas aeruginosa* that were isolated in prevalence rates of 27.6%, 7.1%, 3.1% and 1.0% respectively. On the other hand *E.coli, Salmonella, Klebsiella pneumonia* were isolated from goats in prevalence rates of 29.6%, 6.8%and4.5% respectively. Mixed infection in sheep and goats was 14.3%,20.5% respectively. The *E.coli* isolates belonged to the following serotypes O86 (2 isolates), O113 (2 isolates), O119 (2 isolates), O125 and O158 one isolate each, while the untypable isolates were 2. *Salmonella* typing resulted in *S. typhimurium* (3 isolates) and *S. enteritidis* (2 isolate). *C. perfringens* was isolated representing an incidence of 25.5% as 25/98 from sheep and 10/44 (22.7%) from goats. While 17 toxigenic strains of *C. perfringens* (68%) were recovered from sheep and 4 strains (40%) from goats, non-toxigenic strains were 8 (32%) and (6) (60%) from sheep and goats respectively. All toxigenic *C. perfringens* isolates belonged to type A. Results of parasitological examination revealed that *Giardia, cryptosporidium* and *coccidia* were detected in sheep samples in prevalence rates of 5.1%, 12.2% and 4.1% respectively. In goat, the incidence rates of *cryptosporidium* and *coccidia* were 11.4% and 4.5% respectively.

The results of antibiotic sensitivity test showed that all bacterial isolates were mostly highly resistanceto Ampicillin, Amoxicillin, penicillin, Trimethoprim/Sulphamethoxazole, Vancomycin, while they were mostly highly sensitive to Ceftriaxon and Ciprofloxacin. Application of PCR for detection of virulence *eaeA* gene of *E. coli* was detected in three strains of different serogroups (O113, O125 and O158). Also the virulence *stn* gene of *Salmonellae* was detected in *S. typhimurium* and *S. enteritidis* while the antibiotic-resistant of *blaTEM* gene of *E. coli* and *Salmonella* were detected by PCR and *blaTEM* and *Tet (A)* genes were detected in all the seven tested serotypes.

Virulence and antibiotic resistant genes of *Clostridium perfringens* detection by PCR showed that the *Clostridium perfringens* isolates harboured *CPA* virulence genes in all isolates by amplification of a 324bp Product. Two *C. perfringens* isolates were harboring erythromycin resistance gene the detected resistance gene *erm (B)* were detected in sheep samples and one isolate in goat by amplification of a 638bp. product Vancomycin resistance gene was detected in one isolate of sheep positive by amplification of a 732bp. product and not detected in goat isolates. Also tetracycline antibiotic resistance gene was not detected in all tested isolates.

Keywords:

Diarrhoea, *E. coli, Salmonella*, *Clostridium perfringens,Cryptosporidium*.

INTRODUCTION

Diarrhea is defined as an increased frequency, fluidity, or volume of fecal excretion. Feces may contain blood or mucous and be smelly. The color of feces could be abnormal. However, it is not possible to determine definitively the infectious organism through the color, consistency and the odor of the feces. A definitive identification requires a sample for microbiological analysis. In livestock, diarrhea is called scours. There are many causes of diarrhea including bacterial, viral, parasites and diet. **(Schoenian,2019).**

Diarrhea is a major problem in livestock worldwide, causing great economic losses due to deaths, poor growth rates and veterinary costs **(Weiss and Navas - Martin,2005).**

Bacterial enteritis in lambs is a serious disorder affecting weight gain resulting into economic losses, especially in young sheep and goats which are strongly affected by such condition leading to death due to malnutrition and dehydration. The costs associated with bacterial enteritis, including deaths, lost productivity and treatment have been estimated by $10-29 million annually. **(Slee and Buttom 1990, Stanger *et al.*, 2018 and Barwick *et al.*, 2019).**

Enterotoxigenic Escherichia coli (ETEC) and *Cryptosporidium parvum* are considered among

the most prevalent causative agents of enteritis in goats **(Gerald *et al.*, 1992).**

Themost important enteropathogensassociatedwithdiarrheain livestockinclude enterotoxigenic *Escherichiacoli*(ETEC),*Salmonella*speciesand *cryptosporidium*either alone or in combination **(Steiner *et al.*, 1997).**Other pathogens may also have a role in enteric diseases including: *Clostridium perfringens*, Giardia, Eimeria species, Campylobacter, *Klebsiella* and *Proteus* **(Muñoz *et al.*, 1996).**

Diarrhea in lambs is a complex multi-factorial disease involving the animal, environment, nutrition and infectious agents (bacterial, viral, and parasitic).Despite improvement in management practices, prevention, treatment strategies of diarrhea are still the most common and costly disease affecting neonatal small ruminants.

E. coli scours are an opportunistic disease associated with sloppy environmental conditions and poor sanitation. It is seen in lambs and kids less than ten days of age, but it is most common at 1 - 4 days of age. It usually presents itself as an outbreak in lambs and kids between the ages of 12 - 48 hours of age. It is also called "watery mouth," as it affects lambs salivating and having a cold mouth. Fluid therapy is the mainstay of therapy **(Schoenian, 2019).** *E .coli* is the main causative agent of white scour in goats **(Bhat *et al.*, 2008).**

Salmonella enterica is a facultative intracellular pathogen capable of causing disease in a broad range of host species **(Kaiser *et al.*, 2000).**The infection with *Salmonella* can take place at any age with more severity observed in neonates comparing with adult sheep and goats **(Ramaswamy *et al.*, 1992).**

Clostridium perfringens causing enteric diseases, generally called enterotoxaemia, in sheep and goats. This microorganism could be a normal inhabitant of the intestine in most animal species, but when the intestinal environment is altered by sudden changes in diet or other factors, *C. perfringens* proliferates and produces potent toxins that act locally or absorbed into the general circulation with usually devastating effects on the host. History, clinical signs, and gross post-mortem findings are useful tools for establishing a presumptive diagnosis of clostridial enterotoxemia in sheep and goats. Moreover, definitive diagnosis requires laboratory confirmation. Isolation of some types of *C. perfringens* (e.g., B and C) can be of diagnostic value but other types (e.g., A) are so commonly found in the intestine of normal animals. Definitive diagnosis of enterotoxaemia is carried through detection of *C. perfringens* toxins in intestinal contents **(Uzal and Songer, 2008).**

Clostridium perfringens Type A is the most frequently occurring Clostridium in mammals, birds and in the environment; it produces enteric disease generally mild, with minimal damage noted in the intestinal mucosa. In addition to enteritis, it produces gas gangrene and causes hemorrhagic abomasitis in young ruminants, often accompanied by severe diarrhea **(Miyashiro *et al.*, 2007).**

The most prominent clinical sign of cryptosporidiosis is diarrhoea lasting two to twelve days and also could be accompanied by anorexia, dehydration, reduced milk intake, growth retardation and stiffness **(Sevinc *et al*., 2005).** Transmission of Cryptosporidium, both within and between host species including humans, is through faecal or oral routes of the environmentally resistant oocysts **(Abebe *et al*., 2008).**

Giardiasis in domestic ruminant is an economically important disease as it causes acute or chronic diarrhea, dehydration and growth retardation in young animals **(Olson *et al.*, 2004).** Diarrhea associated with giardia infection in young animals is watery to pasty with mucus and lasts from several days up to 6 weeks **(Huetink *et al.*, 2002).**

Coccidosis is a protozoa parasitic disease that is a common cause of diarrhea in lambs and kids. It may also cause subclinical production losses **(Noha *et al.*, 2019)**

The aim of the present work study is to detect the bacteriological and parasitic causes of enteritis in small ruminants in some farms at El-Sharkia governorate, as well as their antibiogram profiles.

MATERIAL AND METHODS

Animals:

In the present study, a total of 165 animals (107 sheep and 58 goats) of different ages (1 -12 months) belonging to EL-Sharkia governorate were examined for bacteriological and parasitological investigations. All animals were clinically examined before sampling and the seasonal variation with the history of diarrhea was considered.

Samples:

A total of 165 diarrretic fecal samples and fecal swabs (107 sheep and 58goats) from some farms at EL-Sharkia governorate were collected. Samples were collected under complete aseptic condition in sterile disposable bags and swabs that were closed tightly and labeled by their age, place, and date of collection also clinical status of the animals. Then it were kept in an ice-box and sent to the laboratory as quick as possible for bacteriological and parasitological investigations.

Bacteriological examination:

Each sample was inoculated into nutrient broth for aerobic bacteria and cooked meat broth for *C. perfringens* and incubated aerobically and anaerobically respectively at 37^{o}C for 24 hours. A loopful from nutrient broth was streaked onto the following media blood agar, MacConkey agar, Edwards's agar, eosin methylene blue agar, mannitol salt agar and Salmonella-Shigella agar (SS) (Oxoid). A loopful from cooked meat broth was streaked onto neomycin blood agar (200 µg / ml). Plates were incubated aerobically and anaerobically respectively at 37 ^{o}C for 24 - 48 hr. The growing surface colonies were picked up, purified and re-inoculated into nutrient broth and cooked meat broth for further identification which was based on cultural, morphological and biochemical characteristics according to **Koneman *et al*.,(1997) and Quinn *et al.*, (2011).** Lecithinase activity of *C. perfringens* was done on egg yolk agar according to **Smith and Holden (1968).**

Antibiotic sensitivity test:

The test was done through disc diffusion method according to **Cruickshank *et al.*, (1975).** Briefly, 24- hour broth culture of pure bacterial isolates adjusted to McFarland tube No. 0.5, was streaked onto Mueller Hinton agar plates, left dry followed by antibiotic disc application. The plates were then incubated aerobically at 37^{o}C for 24 hours. The zones of inhibition of different antibiotic discs were measured according to **CLSI (2017).** The following antibiotic discs were used: Amikacin (AK 30 µg), Amoxicillin (AX 25µg), Ampicillin (Amp 15µg), Ceftriaxon (CRO µg 30), Ciprofloxacin (CIP 30µg), Erythromycin (E 15µg), Gentamicin (GN 30µg),Pencillin (P 10), Tetracycline (TE 30µg),Trimethoprim-Sulphamethoxazol (SXT 25µg), and Vancomycine (VA30 µg) (Oxoid).

Serotyping of *E. coli* and *Salmonella* isolates:

The *E. coli* isolates were confirmed biochemically. Ten random isolates of *E. coli* and five random *salmonella* isolates were subjected to serological identification using the slide agglutination test (antisera were purchased from Denka Seiken Co. LTD).**(Collee *et al.*, 1996).**

Parasitological examination:

Each fecal sample was examined for giardia spp. using direct wet smear with 1% Lugol's iodine and examined by light microscope **(Smith and Barlett, 1985).**

For staining the samples, Mallory's technique was adopted, in which the specimens were left in the stain at 37^{o}C for an overnight **(Souzan, 2005).**

A laboratory method for detection of cryptosporidium was carried out by examination of direct smears from fecal smears stained by modified Ziehl-Neelsen (MZN) technique **(Hendrix, 1998)**. The stained faecal smears were observed microscopically under oil immersion lens (X 1000 magnification).

All collected samples were examined for coccidia infestation using concentrated salt solution technique **(Soulsby, 1986).**

Polymerase chain reaction:

PCR was carried out on isolates of *E. coli* and Salmonella recovered from examined samples of sheep and goats.

DNA extraction:

DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with slight modifications. Briefly, 200 µl of the bacterial suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at $56^{O}C$ for 10 minutes. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then centrifuged and washed following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided with the kit.

Oligonucleotide Primers:

Primers used were supplied from **Metabion (Germany)** as listed in (Table 1).

PCR amplification:

For *eaeA* gene, primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix **(Takara, Japan)**, 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template.

Analysis of the PCR Products:

The PCR products were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the uniplex PCR product sand 30 µl of the duplex PCR products were loaded into each gel slot. A gene ruler 100 bp DNA ladder **(Fermentas, sigma)** was used to determine the fragment sizes. The gel was photographed by a gel documentation system **(Alpha Innotech, Biometra)** and the data was analyzed through computer software.

Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions of PCR on *E. coli and Salmonella* isolates.

Target gene	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)				Reference
			Secondary denaturation	Annealing	Extension	Final extension	
E. coli eaeA	ATGCTTAGTGCTGGTTTAGG GCCTTCATCATTTCGCTTTC	248	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	72°C 7 min	Bisi-Johnson *et al.*, 2011
Salmonella stn	TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC	619	94°C 30 sec.	59°C 40 sec.	72°C 45 sec.	72°C 10 min	Murugkar *et al.*, 2003
blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94°C 30 sec.	54°C 40 sec.	72°C 45 sec.	72°C 10 min	Colom *et al.*, 2003

***C. perfringens* toxin genes typing**

Resistance genes were detected for isolates viz: of *C. perfringens* erythromycin resistance genes *erm* (B), vancomycin resistance genes *van (A),*and tetracycline resistance genes *Tet (M).* Total genomic DNA from pure *C. perfringens* isolates was extracted using the QIAamp DNA mini-kit (Qiagen) according to the manufacturer's recommendations. Primers used were supplied from Metabion (Germany) as listed in (Table 2). PCR amplification and documentation was carried out as described above.

Table (2): Primers sequences, target genes, amplicon sizes and cycling conditions of PCR on *C. perfringens.*

Target gene	Toxtel	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
cpa	Alpha Toxin	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324	94°C 3 min.	94°C 1 min.	55°C 1 min.	72°C 1 min.	72°C 10 min.	Meer RR, Songer (1997)
iap	Iata	AAACGCATTAAAGCTCACACC CTGCATAACCTGGAATGGCT	293						Baums *et al.* 2004
cpb	Beta	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196						Moller and Ahrens (1996)
etx	Epsilon	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	655						Meer RR, Songer (1997)
vanA	Vancomycin resistance gene	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA ATTTGCTGATTTCGCTCG	732	94°C 2 min.	94°C 1 min.	54°C 1 min.	72°C 1 min.	72°C 10 min.	Duka *et al.*, 1995
ermB	Erythromycin	GAA AAG GTA CTC AAC CAA ATA AGT AAC GGT ACT TAA ATT GTT TAC	638	96°C 3 min.	96°C 30 sec.	55°C 1 min.	72°C 2 min.	72°C 10 min.	Luna *et al.* 2002
tet (M)	Tetracycline	GAA GCC CAG AAA GGA TTC /T GGT GTT TAT CAC GGA AGC /T GCA/T A	686	96°C 3 min.	96°C 30 sec.	55°C 1 min.	72°C 2 min.	72°C 10 min.	Miranda *et al.* 2003

RESULTS AND DISCUSSION

The clinical manifestations of sheep and goats affected with diarrhea was characterized by weakness, collapse, dyspnea, severe abdominal pain and nervous signs such as dullness, ataxia, incoordination and convulsive movement of the head and neck. Dehydration was shown in some cases.

Table (3): Bacteriological and parasitological examinations of diarrheic fecal samples of sheep and goats.

Animal species (No.)	Positive		Negative	
	No.	%	No.	%
Sheep (107)	98	91.6	9	8.4
Goats (58)	44	75.9	14	24.1
Total (165)	142	86.1	23	13.9

Percentage as calculated in relation to the number of examined samples.

Table (4): The prevalence of bacterial infection and parasitic infestation in relation to age in diarrhoeic sheep and goats fecal samples.

Animals	Causative	Age			
		1-3 Month	> 3-6 Month	>6-9 Month	>9-12 Month
Sheep (98)	Bacteria	46	13	10	8
	Parasites	9	8	4	_
	%	55 (56.1)	21 (21.4)	14 (14.3)	8 `(8.2)
Goats (44)	Bacteria	20	8	4	5
	Parasites	-	4	3	-
	%	20 (45.5)	12 (27.3)	7 (15.9)	5 (11.4)

Percentage calculated according to number of examined samples.

Table (5): Bacterial isolates of sheep and goats of diarrheic fecal samples.

Animals species	No. of examined samples	*C. perfringens*		No. of positive samples	Aerobic bacterial isolates							
					E. coli		*Salmonella*		*Klebsiella pneumoniae*		*Pseudomonas aeruginosa*	
		NO	%	38 (38.8%)	NO	%	NO	%	NO	%	NO	%
Sheep	98	25	25.5		27	27.6	7	7.1	3	3.1	1	1.0
Goat	44	10	22.7	18 (40.9%)	13	29.5	3	6.8	2	4.5	-	-

% calculated according to no .of examined samples.

Table (6): Lecithinase activity of *C. perfringens* isolates from sheep and goats.

Animals species	Lecithinase(+ve) toxigenic		Lecithinase(-ve) Non toxigenic	
	No.	%	No.	%
Sheep	17 (type A)	68	8	32
Goats	4 (type A)	40	6	60

% calculated according to the No. of samples examined.

Table (7): Parasitic infestation in sheep and goats fecal samples.

Parasites	Sheep (98)		Goat (44)	
	No	%	No	%
Giardia	5	5.1%	-	-
Cryptosporidium	12	12.2%	5	11.4%
Coccidia	4	4.1%	2	4.5%
Total	21	21.4%	7	15.9%

Table (8): Distribution of isolated parasitic species in sheep and goats according to age.

Parasite	Number of Sheep (98)				Number of Goat(44)			
	1-3	> 3-6	>6-9 M	> 9-12	1-3	>3-6	> 6-9	>9-12
Giardia	3 (3.1%)	1 (1.1%)			--	--	---	--
Cryptosporidium	4 (4.1%)	7 (7.1%)	4 (4.1%)		--	4 (9.1%)	3 (6.1%)	--
Coccidia	2 (2.1%)				--	--	--	--
Total	21 (21.4)				7 (15.9%)			

Table (9): Mixed bacterial infection and parasitic infestation of examined goats and sheep and fecal samples.

Animal species (No.of examined samples)	Mixed infection						Total	
	Aerobic & Anaerobic		Anaerobic & Parasites		Aerobic & Parasites			
	No	%	No.	%	No.	%	No.	%
Sheep (98)	11	11.2	_	_	3	3.1	14	14.3
Goat (44)	7	16.0	_	_	2	4.5	9	20.5

Table (10): Bacterial isolates and parasites in mixed infection of the examined sheep and goat faecal samples.

Species	Mixed isolates	No.	%
Sheep (98)	***E.coli +C. perfringens***	**5**	**5.1**
	Salmonella+C. perfringens	**3**	**3.1**
	Klebsiella pneumonia+C. perfringens	**1**	**1.0**
	E.coli +Coccidia	**3**	**3.1**
	p. aeruginosa +Giardia	**0**	**0**
	Salmonella +Cryptosporidia	**2**	**2.0**
	C.perfringens+Cryptosporidia	**0**	**0**
Total		**14**	**14.3**
Goat (44)	***E.coli +C. perfringens***	**4**	**9.1**
	Salmonella+C. perfringens	**3**	**6.8**
	Klebsiella pneumoniae +C. perfringens	**1**	**2.3**
	E.coli +Coccidia	**_**	**_**
	E.coli +Giardia	***1***	***2.3***
	Salmonella +Cryptosporidia	**_**	**_**
	C.perfringens+Cryptosporidia	**_**	**_**
Total		**9**	**20.5**

Table (11): Antimicrobial sensitivity testing of bacteria isolated from diarrheic cases.

Antibiotic	Isolates																			
	C.perfringens (35)				*E. coli* (48)				*Salmonella* (18)				*Klebsiella pneumonia* (7)				*Pseudomonas aeroginosa* (1)			
	Resistance		Sensitive		Resistance		Sensitive		Resistance		Sensitive		Resistance		Sensitive		Resistance		Sensitive	
	No.	%	No.	%	No.	%	No.	%	No.	%	No	%	No.	%	No	%	No.	%	No.	%
Erythromycin	26	74	9	26	7	14.6	41	85.4	9	50.0	9	50.0	2	28.6	5	71.4	0	0	1	100
Tetracyclin	27	77	8	23	3	6.3	45	93.7	7	38.9	11	61.1	5	71.4	2	28.6	0	0	1	10
Penicilline	31	88	4	12	48	100	0	0	16	88.8	2	11.2	7	100	0	1	0	0	1	100
Ceftriaxon	3	9	32	91	12	25	41	85.4	3	16.7	15	83.3	0	0	7	100	0	0	1	100
Vancomycine	24	69	11	31	44	91.7	4	8.3	17	94.4	1	5.6	2	28.6	5	71.4	0	0	1	100
Ciprofloxacin	3	9	32	91	48	100	0	0	12	66.7	6	33.3	2	28.6	5	71.4	1	100	0	0
Amoxicillin	28	80	7	20	43	89.6	5	10.4	18	100	0	0	6	85.7	1	14.3	1	100	0	0
Gentamycin	2	6	33	94	44	91.7	4	8.3	14	77.8	4	22.2	5	71.4	2	28.6	0	0	1	100
Amikacin	2	6	33	94	15	31.3	34	70.8	11	61.1	7	38.9	4	57.0	3	43.0	1	100	0	0
Ampicillin	28	80	7	20	35	72.9	13	27.1	9	50.0	9	50.0	4	57.0	3	43.0	0	0	1	100
Trimethoprim /Sulphamethoxazole	31	88	4	12	42	87.5	6	12.5	18	100	0	0	5	71.4	2	28.6	0	0	1	100

Table (12): Detection of virulence and anti-drug resistance genes in *E. coli* isolates

***E. coli* serotype**	***eaeA***	***blaTEM***
O86	-	+
O113	+	+
O119	-	+
O125	+	+
O158	+	+

Table (13): Detection of virulence and antidrug resistance genes in salmonella isolates.

***Salmonella* Sample**	***stn***	***blaTEM***
S. typhimurium	+	+
S. enteritidis	+	+

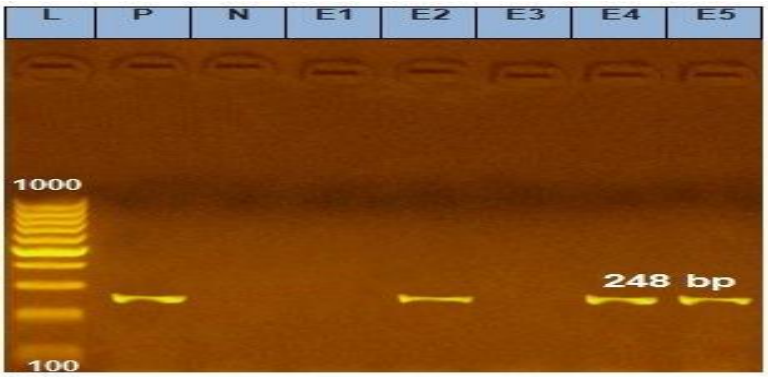

Photo (1): Agar gel electrophoresis results of PCR for detection of *eaeA gene* in *E. coli* showing amplified 248 bp (lanes 1-5). L: represent the molecular size marker (100pb ladder): N: Negative control. P: Positive control of *eae* gene (248 bp) Lanes: (2, 4, 5) were positive for *eae A* gene. Lanes: 1 and 3 were negative for *eaeA* gene.

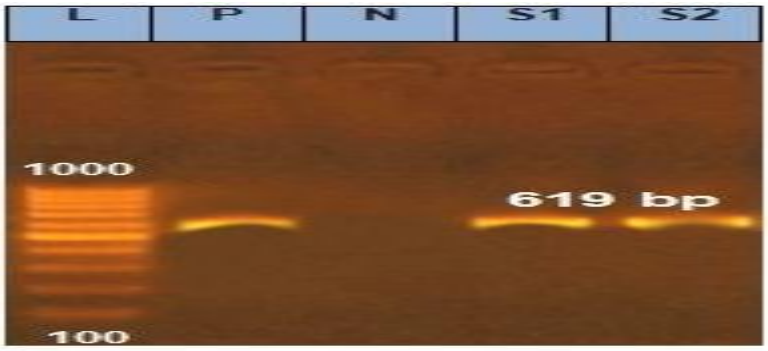

Photo (2): Agar gel electrophoresis results of PCR for detection of *stn* gene in *salmonella* isolates which amplified 619 bp lanes (1-2). L: represent the molecular size marker (100pb ladder): N: Negative control. P: Positive control of *stn* gene (619bp) Lane: (1,2) were positive for *stn* gene.

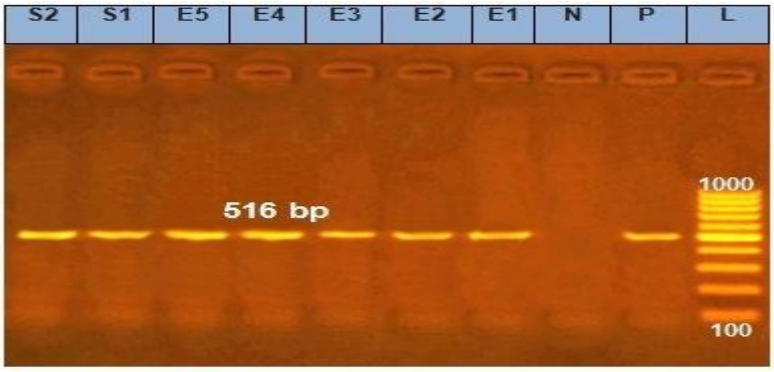

Photo (3): Agarose gel electrophoresis results of PCR for detection of *E .coli* and *salmonella bla TEM gene* Lane L: 100-1000 bp DNA ladder. N: Negative control, P: Positive control of *bla TEM* gene 516 bp Lanes: (1, 2, 3, 4, 5, 6, 7) were positive for *bla TEM* gene.

Table (14): Toxin typing and antibiotic resistance genes of *C. perfringens* isolates.

Bacterial isolates	Results						
	Antibiotic resistance genes			Toxinotyping genes			
	tetM	Van A	erm(B)	cpa	iap	cpb	Etx
S1	-	-	+	+	-	-	-
S2	-	+	+	+	-	-	-
S3	-	-	-	+	-	-	-
S4	-	-	+	+	-	-	-
S5	-	-	-	+	-	-	-
S6	-	-	-	+	-	-	-

S1, S2, S3 from Sheep **S4, S5, S6 from goats**

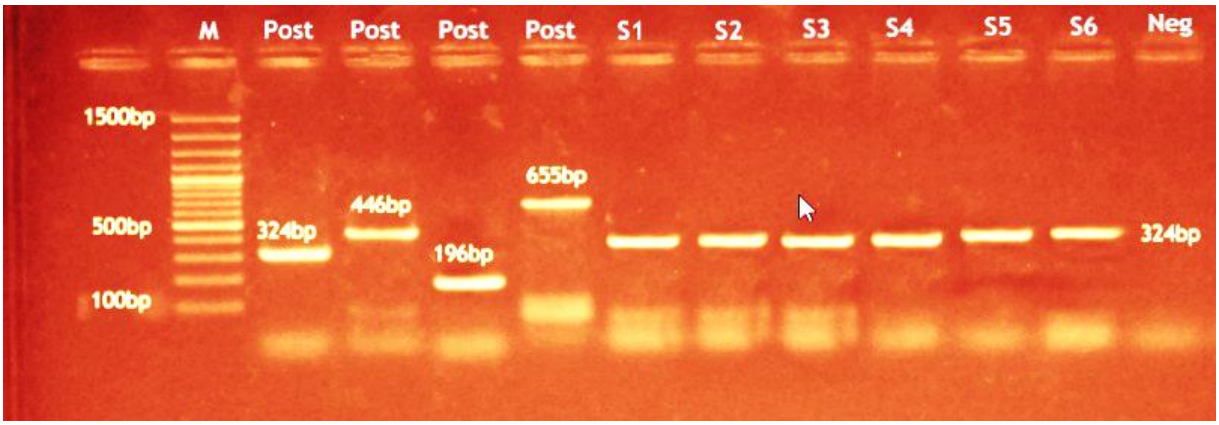

Photo (4): Electrophoresis profile of *C. perfringens* toxin genes typing *CPA* (324 bp) α toxin, *IAP* (446 bp) iota toxin, *cpb* (196 bp) beta toxin, *etx* (655 bp) epsilon toxin. Marker gene is a ruler thermo. All examined samples were α toxin producer.

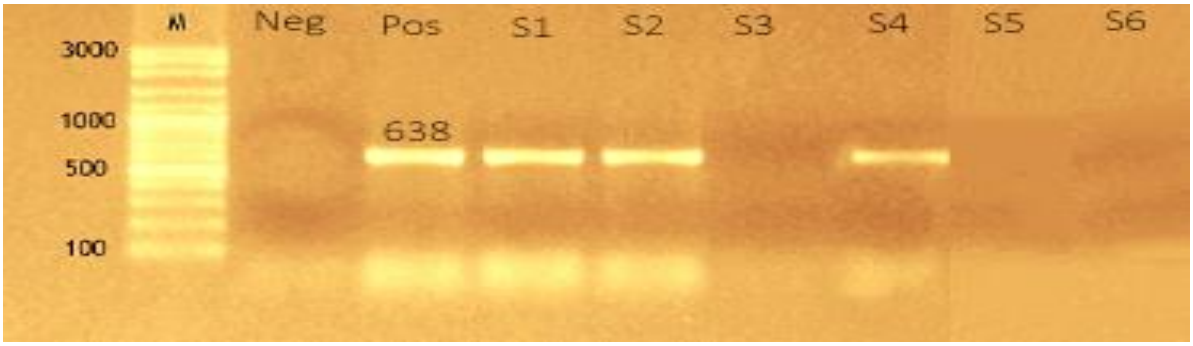

Photo (5): Electrophoresis profile of *C. perfringens* for erythromycin resistance gene *erm* (B), two sheep samples and one goat sample were positive *(S1 & S2) (S4)*. Marker DNA plus (Jena Bioscience).

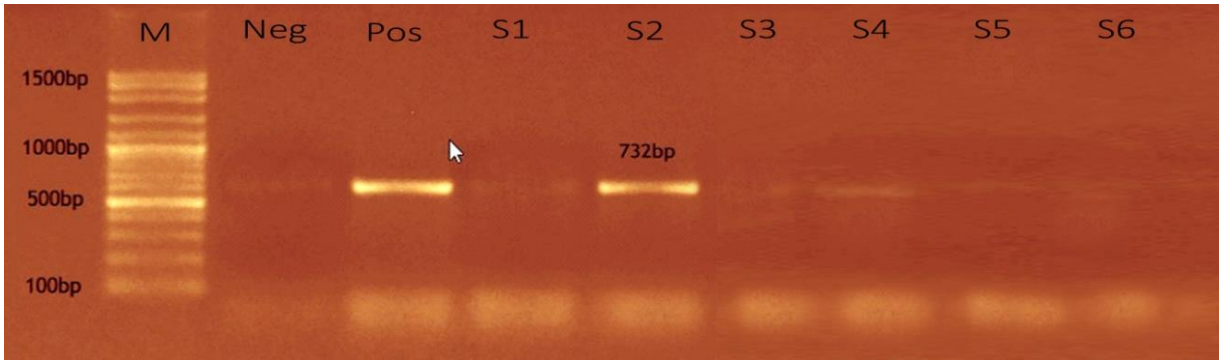

Photo (6): Electrophoresis profile of *C. perfringens* for vancomycin resistance genes *van (A)*, all isolates were negative except one sheep sample was positive (S2). Marker Gene Ruler (Thermo).

Photo (7): Electropherosis profile of *C. perfringens* for tetracycline resistance genes *Tet (M)*, all isolates of sheep and goats were negative. Marker DNA plus (Jena Bioscience).

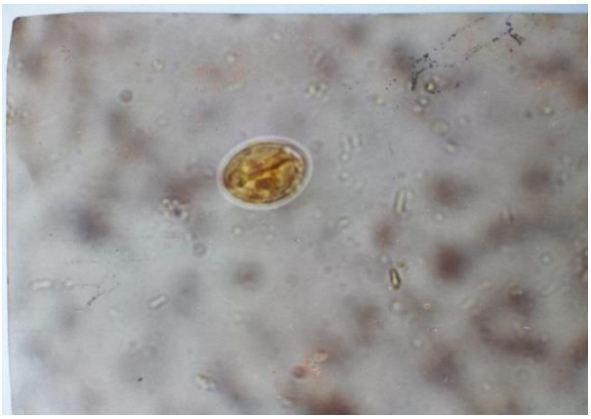

Fig. (8): Giardia cysts in a sheep faecal smear (x 100).

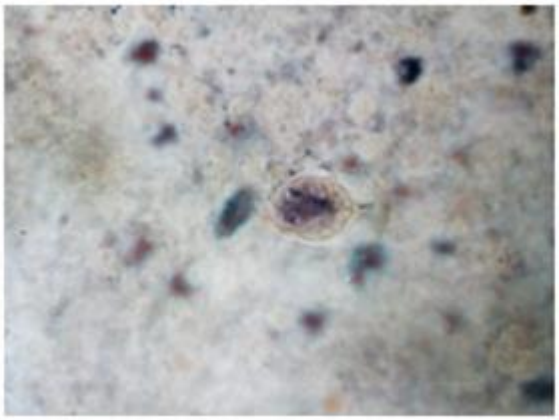

Fig. (9): Giardia trophozoites in sheep a faecal smear stained with Mallory (stainx1000).

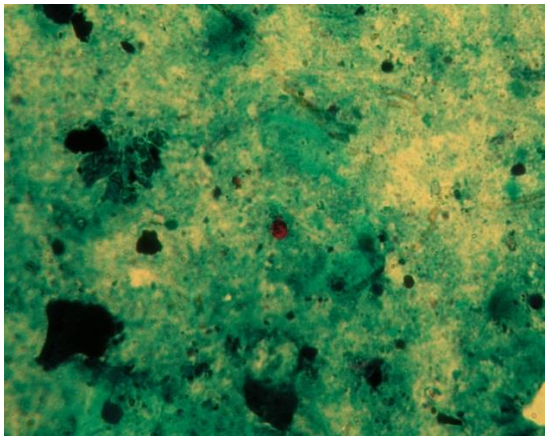

Fig. (10): Cryptosporidium oocysts in a faecal smear stained with Modified Zeihl Neelsen technique (MZN) x1000.

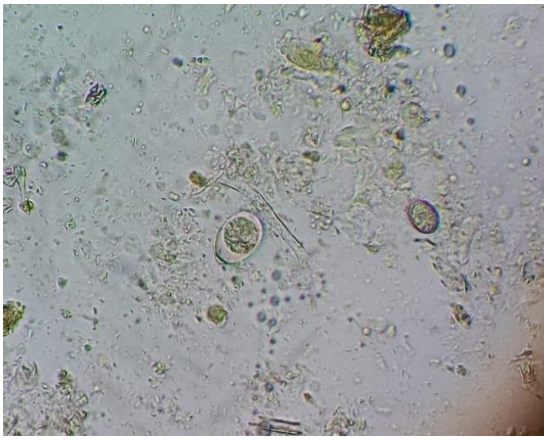

Fig. (11): Eimeria oocysts in a sheep faecal smear (x40).

Diarrhoea is a common symptom of the intestinal tract infection of neonatal lambs and goat kids with numerous accused agents including enteropathogenic bacteria such as *E*. coli and *Salmonella* **(Mohammed, 2011).** Enteritis is one of the major problems that adversely affect sheep and goats industry worldwide via either increased mortality rates of lambs and kids or via severe economic losses (reduced growth rates, antibiotic treatment cost, and weight loss).

The data collected in this study showed that bacterial infection and parasitic infestation in sheep was 91.6% and was in goat 75.9%, (Table 3). **Ershaduzzaman *et al.*, (2013)** reported that, the parasitic and bacterial affections in goat was 64%.

The prevalence of bacterial infection and parasitic infestation was high in neotates as compared to adult sheep and goats. This is because of the immature immune system in neotaes in comparison with adult sheep and goats. Kids and sheep aged between (1-3) months had high level of both bacterial and parasitic affections (Table 4). This is in agree with **Adesiyun *et al.*, (2001)** who reported that, the frequency of diarrhea and the prevalence of enteropathogens was higher among the young than older animals.

E. coli and Salmonella single infection rates were 27.6% and 7.1% respectively (Table 5). This result is nearly similar to that of **Nasr *el al.*,(2014)** who isolated *E. coli* (34.20%) and *Salmonella* (5.26%) from rectal swabs taken from lambs. *Salmonella* mainly affects older lambs and young lambs usually died suddenly without any symptoms **(Ahmed *et al.*, 2010).** Also **Zare *et al.*, (2014)** isolated *E. coli* (40%) and *Salmonella* (8%) from diarrhoeic lambs**.** Regarding goats isolation rates of *E.coli* and *Salmonella* were 29.5% and 6.8%, respectively (table5). This nearly agreed with results of **Mamunul *et al.*, (2007)** who isolated *E.coli* and *Salmonella* from rectal swab of goats as 25% and 5% respectively. Other aerobic bacterial isolates including *Klebsiella* spp. were 3.1% isolates from sheep samples, in agreement agreed with **Zare *et al.*, (2014) and Rada *et al.*, (2016)** who isolated *Klebsiella* spp. from 4% of samples. Higher isolation rate of *Klebsiella* from kids with enteritis (19.2%) was reported by **Abd El-Aty *et al.*, (2001)**. *Pseudomonas aeroginosa* was isolated only from sheep (1%).

The overall prevalence of *C. perfringens* in the examined fecal samples of sheep and goats was found to be 24.6% (35/142) (Table 6). These findings are in close agreement with the results of **Rahaman *et al.*, (2013),** who reported *C. perfringens* as 32.1% prevalent in sheep and goats. **Maqbool *et al.*, (2017)** found prevalence of *C. perfringens* in small ruminants to be 26%.The prevalence rate of *C. perfringens* in the examined diarrhoeic samples obtained from sheep was found to be 25.5%. Nearly similar results were obtained by **Maqbool *et al.*, (2017)**, **Fayez *et al.*, (2013) and Ozcan and Gurcay (2000)** isolated *C. perfringens* in incidence of 31%, 30.41% and 38.6% respectively from small ruminant fecal samples.

C. perfringens was isolated from goat fecal samples in an incidences of 22.7% as regarded by **Maqbool *et al.*, (2017)**, Higher incidences were obtained by **Khan *et al.*, (2019)** who detected *Clostridium perfringens* in unvaccinated goats and vaccinated one as 79.1% and 65.77% respectively. Lower figures were reported by **Ahsani *et al.*, (2011)** as (2.2%).*C. perfringens* toxigenic isolates occupied an incidence of 68% and 40% while non toxigenic isolates were 32% and 60% in sheep and goats respectively (Table 6).In the present study of parasitological

investigation showed that the microscopicall examination of fecal samples collected from 107 sheep and 58 goats revealed that the prevalence of giardia, cryptosporidium and coccidia were 5.1%, 12.2% and 4.1% respectively (Table 7). According to the age, the present study showed higher incidence 55 (61.8%) and 20 (45.5%) in young ages (1-3month) than in old ones (> 9-12 month) with a percentage of 8.2% and 5.1% in sheep and goat, respectively (Table 8). Higher incidences 25.5% and 13.1% of Giardia and cryptosporidium were recorded by **Geurden *et al.*, (2008)** in sheep and goats, respectively. Comparable results were recorded in other countries,**Abd-Elwahed, (1999)**, **Baraa *et al.*, 2017 and Dessi *et al*.,2020).**

The prevalence of infestation with cryptosporidium spp. in sheep were 40% in Egypt 46% in Iraq and 34.4% in Italy respectively. In contrary, lower incidence (2.50%) of cryptosporidium *s*pp. infestation was recorded by **Magdy *et al.*, (2014)** in Egypt. **Hiba and Haider (2018)** found the infestation of giardia in sheep and goats as 27.50% and 8.5% respectively. The higher rates (34.6% and 9.4%) were recorded in the age groups of ≤ 6 month, while the lower rates (23.8% and 6.8%) were recorded in the age groups of 6-12 month. In the present study, the prevalence of Eimeria species was 4.1% and 4.5% in sheep and goats respectively. This finding is lower than those reported in central region of Saudi Arabia **(Alyousif *et al.*, 1992),** Turkey **(Gül, 2007),** northeastern China **(Wang *et al.*, 2010)** These differences in prevalence may be due to various sanitation efforts in the management programs attempted by producers to control coccidiosis or due to differences in ecological condition **(O'Handley *et al.*, 1999).** Demographic factors may include age distribution of animals sampled, size of the farm, geographic location, herd size, and other species of animals present in the farm **Gow and Waldner, (2006)**. Management factors include general management (type of flooring, calf housing, and frequency and method of cleaning**) Maddox-Hyttel *et al.*, (2006).** Previous studies revealed that animals reared indoors especially under group housing were more likely to be infested with the parasites than those housed outside **Ruest *et al.*, (1998).**

Some management practices that reduce direct contact between animals such as separation of new born from the dam immediately after birth may aid in reducing the transmission of the cysts **(Wade *et al.*, 2000),** because adult animals are a potential source of parasites especially for neonates **(Castro *et al.*,2005).**

Our study proved that mixed bacterial and parasitic infections in sheep and goats samples is the main cause of sever morbidity and mortality rates particularly in young animals and

complication of cases treatment. Mixed infections were with *E. coli* and *C. perfringens* was (5.1%), *Salmonella* and *C. perfringens* (3.1%), *Klebsiella pneumoniae* and *C. perfringens* (1.0%), *E. coli* and *Coccidia* (3.1%), *Salmonella* and Cryptosporidia (2.0%) *P. aeruginosa* and *Giardia* and *C.perfringens* and *Cryptosporidia* were not detected. As showen (Table 10). Bacterial isolates recovered from goats were *E. coli* (29.6%), *Salmonella* (6.8%) and *Klebsiella pneumoniae* (4.5%) (Table 5). **Nasr *et al.*, (2014)** isolated bacteria from diarrheic lambs of which *E. coli* was the most predominant bacterial isolate other bacterial isolates were *salmonella*, clostridia, proteus species, *shigella*, *klebsiella*, in incidences (34.20 %), (5.26 %), (7.89 %), (13.10 %), (10.52 %) and (7.89 %), respectively. mixed infection was reportedin an incidenceof 21 %.**Singh *et al.*,(2018)**illusterated that incidence of *C. perfringens* was 15.13% in 0-1 month age group and 7.56% in 1-3month old group of neonatal goat kids. Antimicrobial therapy is considered as an important tool for treating bacterial infections in both humans and animals. The antibiogram of bacterial isolates (Table 11) show that most *E. coli* isolates were highly resistance to Ciproflxacin and pencillin (100%). *E. coli* was also resistant to Gentamycin and Vancomycine each (91.7%), Amoxicillin (89.6%) and Trimethoprim/Sulphamethoxazole (87.5%).The highest sensitivity was reported to Ceftriaxon and tetracycline (93.3%),erythromycin (85.4%) and Amikacin (70.8%). **Nasr *et al.*, (2014)** recorded that *E. coli* isolates were highly sensitive to chloramphenicol, marbofloxacin, enrofloxacin, gentamycin and Ceftriaxon but most of them were resistant to streptomycin, neomycin, tetracycline and amoxicillin. **Abdulaziz *et al.*, (2012)** recorded that all *E. coli* isolates from diarrhoeic lambs were highly sensitive to ampicillin, ciprofloxacin, ofloxacin and tobramycin (100% each). In this study, Salmonella isolates from sheep was found resistant to Amoxicillin and Trimethoprim / Sulphamethoxazole (100%), Vancomycine (94.4%), penicillin (88.8%), and gentamycin (77.8%), Salmonella isolates were highly sensitive to Ceftriaxon (83.3%). Concerning *Klebsiella pneumoniae,* they were highly resistant to pencillin (100%), amoxicillin (85.7%) followed by gentamycin (71.4 %), and sensivite to ceftriaxon (100%), followed by erythromycin, ciprofloxacin and Vancomycine was (71.4%).**Nasr*et al.*,(2014)** recorded that klebseilla was highly sensitive to chloramphenicol and marbofloxacin. Similar results were obtained by **Hussain *et al.*, (2018 a) and Hussain *et al.*, (2018 c)** *C. perfringens* type 'A' showed that ciprofloxacin and ceftriaxone were the most effective antibiotic according to the results based on the zone of inhibitions they produced**.** **Khan *et al.*, (2015)** tested different antibiotics for resistance profile of *C. perfringens* and

found that amoxicillin resistance of *C. perfringens*, isolated from different meat samples. **Khan *et al.*, (2019)** detected highest sensitivity for ceftriaxone followed by ciprofloxacin, similar to our results. We concluded from the antibiogram that differences may be due to strain variations and antibiotic tested. All bacterial isolates in this study shared their resistance to Ampicillin, Amoxicillin, penicillin, Trimethoprim / Sulphamethoxazole, Vancomycin, and their sensitivity to ceftriaxone and ciprofloxacin.

The virulence of recovered serotypes was mainly controlled via products of many virulence encoding genes such as *eaeA*. Intimin genes are defined as the main cause of lesions on intestinal cells are present mainly in enteropathogenic *E. coli* (EPEC) **(Kaper 1996)**. In this study, the virulence *eaeA* gene showed amplicons of 248 bp and was detected in *E. coli* serogroups (O113, O125 and O158) (Table 12 and photo 1). **Mohammad *et al.* (2011)** showed that the production of *eaeA* genes varied among the isolated serogroups. The production of *eaeA* gene recorded with O103, O18, O86, O26, O78, O111, O148 and one of the un-typed serogroup). *S. typhimurium* and *S. enteritidis* showed amplicons of 619 bp for *stn* gene as shown in (Table 13 and photo 2). The *bla TEM* antibiotic – resistance gene was detected by PCR at 516 bp size, in all tested isolates of *E. coli* and *Salmonella* (Table 12, 13 and photo 3). This agrees with studies of **Delmani *et al.* (2017).**

Multiplex PCR was applied for detection of *C. perfringens* toxin genes (*cpa*, *cpb*, *etx*) of isolates of sheep and goats (3 for each) where all the isolates were positive and amplified 324bp product was detected and consequently recorded as type A (Table 14 , photo 4). These results are in accordance with those obtained by **Hussain *et al.*, (2018c)** who found that all isolates of *C. perfringens* harboured alpha toxin (*cpa*) gene. Also **(Santana *et al.*, 2018)** recorded that *C. perfringens* type A isolates were positive only for alpha toxin encoding gene (*cpa*). Our results are also confirmed by the finding of **Hussain *et al.*, (2018b)** who showed that, the prevalence *C. perfringens* type A in sheep and goat was 60.45% and 70.06% Respectively. Moreover **Tutuncu *et al.*, (2018)** found that the prevalence *C. perfringens* type A was 65 % in small ruminants.

In conclusion, lambs diarrhoea is economically important health problem in sheep and goats which causes high mortality and morbidity. The bacterial causes of lamb diarrhoea is multiple with *E coli, Salmonella*, C. perfringens, giardia, cryptosporidium and coccidia altogether play

a vital role in results of this study will help to develop an effective treatment of diarrhoea against those organisms.

Bacterial isolates indicates the diverse nature of the causative organisms. Multidrug resistance genes were *bla TEM* and also *C. perfringens* resistance gene *Van* (A).

Hygiene mast is apply in farms, Bacteria and parastes in diarrhea stools of infected animals can be passed from animals to workers if hygiene or handwashing habits are not good.

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دراسات بكتريولوجية و طفيلية و جزيئية عن بعض المسببات البكتيرية و الطفيلية للالتهاب المعوى فى المجترات الصغيرة

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الملخص العربى

يعد الالتهاب المعوى فى المجترات الصغيرة من أهم المشاكل التى تواجة انتاج هذة الحيوانات والتى تسبب نسب عالية من النفوف وهناك مسببات عديدة للالتهاب المعوى فى للمجترات الصغيرة وتشمل البكتيريا و الطفيليات والفيروسات.

تم فى هذا العمل فحص عدد 165 عينة براز بواقع 107 من الاغنام و 58من الماعز من بعض المزارع فى محافظة الشرقية فى الفترة من نوفمبر 2018 الى مايو 2019 حيث تم اجراء الفحص البكتريولوجى و الطفيليات لهذه العينات. اثبتت نتائج البحث ان الالتهاب المعوى والاسهال اكثر فى الحيوانات الصغيرة عن البالغة. كما ان هناك 14 عينة من الاغنام و 9 عينة من الماعزبها عدوى مشتركة بين الطفيليات و البكتيريا.

اشتملت المعزولات البكتيرية من الاغنام على الاشيريشيا كولاى, سالمونيلا, الكلبسيلا نيمونى و السودوموناس ايروجينوزا بنسب مئوية 27.6% ,7.1 % ,3.1% و 1.0% على التوالى. بينما كانت نفس المعزولات ماعدا السودوموناس ايروجينوزا بنسب مئوية 29.6%, 6.8% و 4.5% على التوالى.

اظهرت نتائج الفحص السيرولوجى لبعض المعزولات عترات الاشيريشيا كولاى تنتمى الى المجموعات السيرولوجية O86 , O113, O119 , O125 و O158 بينما بعض معزولات السالمونيلا تنتمى الى المجموعات السيرولوجية سالمونيلا طايفى ميوريوم و سالمونيلا اينتريتيدس.

تم عزل ميكروب كلوستريديم بيرفرينجيز اللاهوائى من الاغنام و الماعز بنسبة مئوية 25.5% و 22.7% على التوالى. بينما كانت المعزولات السامة 17 و 4 (68% و 40%) من الاغنام و الماعز على التوالى. بينما كانت المعزولات الغير السامة بواقع 8 (32%) و 6 (60%) من الاغنام و الماعز على التوالى. جميع معزولات الكلوستريديم بيرفرينجيز تنتمى الى النوع (أ) .

اظهرت نتائج فحص الطفيليات ان عدد الطفيليات الاتية جياردیا , كريبتو اسبوريديم و كوكسيديان للاغنام 5.1% , 12.2% , و 4.1% على التوالى. بينما كريبتو اسبوريديم و كوكسيديان للماعز 11.4% و 4.5% على التوالى.

اظهرت نتائج اختبارات الحساسية ان غالبية المعزولات البكتيرية مقاومة لل Ampicillin , Amoxicillin , Sulphamethoxazole,penicillin Trimethoprim و ,Vancomycin بينما كانت حساسة.Ceftriaxon و Ciprofloxacin.

باجراء انزيم البلمرة المتسلسل للكشف عن الانتيمين جين لبعض معزولات الاشيريشيا كولاى عن وجوده فى 3 انواع سيرولوجية O113, O125 و O158 بينما تم الكشف عن *stn* للسالمونيلا طايفى ميوريوم و سالمونيلا اينتريتيدس. الكشف عن الجينات المقاومة للمضادات الحيوية للبيتالاكتام فى العترات المختبرة للأشيريشيا كولاى و السالمونيلا كانت جميعها ايجابية.

اظهرت نتائج الكشف عن جينات السموم لعدد 6 معزولات للكلوستريديم بيرفرينجيز بواقع 3 لكل من الاغنام و الماعز عن ايجابية جميع العترات المختبرة ايجابية *cpa* جين وتم تصنيفها على انها نوع (أ) بينما كانت الكشف عن الجينات المقاومة للمضادات الحيوية Vancomycin و Tetracycline ان جميع العينات المختبرة (6) سلبية لل Tetracycline جين بينما كانت عدد معزولة واحدة من الاغنام ايجابية لل Vancomycin جين وبالنسبة لجينات Erythromycin كان عدد 2 معزولة من الاغنام ايجابية *erm* وواحدة من الماعز سلبية.