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BACTERIAL DIARRHOEA IN NEWLY BORN CALVES IN MENOUFIEA GOVERNORATE

ELHAM I. ATWA*, EMAN M. SHARAF* and EMAN M. ZAKARY**
* Bacteriology Department Animal Health Research Institute of Egypt Shebin El- Kom branch.
** Biotechnology Department, Animal Health Research Institute of Egypt.

ABSTRACT

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Out of 200 examined rectal swabs of diarrheic calves, 159 samples 79.5% were positive for bacteriological isolates. The recovered bacterial species were *E. coli, S. typhimurum, Campylobacter sp, Corynebacyerium sp, P. aeruginosa* and *C. perfringens types A and B* with incidence of 47.5, 9, 7.5, 6, 4 and 5.5%, respectively. Typing of *C. perfringens* by intradermal inoculation test in albino Guinea pig revealed that the incidence of toxigenic and non toxigenic strains were (81.9% and 18.1%) respectively. *C. perfringens* type A was the most predominant to type B with the incidence of 45.5% and 36.4% respectively. The serological serotyping of 95 *E. coli* isolates revealed the following serotypes K99 (21.1%), O157 (17.9%), O111 (9.5%), O125 (11.6%), O119 (15.8%), O26(12.6%), O128 (8.4%) and un typed *E. coli* (3.2%). In vitro sensitivity of the recovered *E.coli* isolates to different antimicrobial agents shows that, *E. coli* isolates were highly sensitivite to enrofloxacin, flumequine and tetracycline. In contrast, these isolates were found to be resistant to ampicillin, erythromycin, gentamicin, lincomycin and penicillin-G. PCR panel could help diagnosticians rapidly determine the causative agents for bovine diarrhea in the early stages of disease and help practitioners initiate appropriate treatments or interventions quickly. Using PCR technique to improve the results, in case of *E coli* O 157 for *rfb* gene the molecular bands appeared at 259 bp, *E coli* K 99 gene *F41* it appeared at 314 bp, while in *Salmonella typhmiurium flic* gene at 559 bp .

Keyword: *Diarrhea, calves, E. coli, Salmonella C. perfringens*

INTRODUCTION

Diarrhea is a major problem in livestock production in Egypt and throughout the world (Farid *et al.,* 2001 and Ibrahim, 2007). Enteritis in newborn calves causes high morbidity and mortality, leading to significant economic losses in Egypt (Novert and Hammad, 2001 and Ashraf, 2007).

Diarrhea is a well-known clinical sign in neonatal animals. Its aetiology is complex involving management, environmental, nutritional, physiological variations and variety of pathogens including bacteria, viruses, protozoa and intestinal parasites are described as important agents causing diarrhea (either separately or in combination) in buffalo calves (Snodgrass *et al.,* 1986; Roberts, 1993; El-Ghari *et al.,* 1994 and Prescott *et al.,* 2008).

Enterotoxigenic E. coli (ETEC) and *Salmonella* are known to be the most common and economically important agents (SJ Achá *et al.,* 2004) but other bacteria, e.g. *Campylobacter* spp. *Clostridium* sp, have also been identified as the cause of enteric disease and diarrhea in calves (Schoenians, 2006; Prescott *et al.,* 2008 and Myers, 1984). The 2 latter groups also contain important human pathogens that may cause outbreaks of food-borne diseases (De Rycke *et al.,* 1986) and thus are of high public health importance. In acute neonatal diarrhea, an important disease of calves, 4 micro-organisms in particular, are of widespread occurrence and proven enteropathogenicity: rotavirus, coronavirus, cryptosporidia and enterotoxigenic *E. coli* (Snodgrass, 1986).

Diarrhea due to *E. coli* is one of the most common diseases of young calves (Uhde, 2008), despite vaccination programs and management measures, necessitating treatment with antibiotics and fluid therapy (Gyles, 1993). *E. coli* diarrhea in newborn calves (9–10 days of age) is usually characterized by watery white or yellowish diarrhea, rapid onset and time course, and high mortality. In affected calves, diarrhea typically begins within 36–72 hours of birth,

and affected calves die within 2–3 days. Some calves die several hours after appearing healthy and free of diarrhea. Calf scours is not a single disease entity; it is a clinical syndrome associated with several diseases characterized by diarrhea. Regardless of the cause, absorption of fluids from the intestine is altered, leading to life-threatening electrolyte imbalances. The scouring calf loses fluids, rapidly dehydrates, and suffers from electrolyte loss and acidosis. Infectious agents may cause initial damage to the intestine, but death from scours usually results from dehydration, acidosis, and loss of electrolytes. Identification of infectious agents that cause scours is essential for implementation of effective preventive and treatment measures (Radostits *et al.*, 2007).

E. coli populations are divided into serotypes and serogroups on the basis of antigenic composition (somatic or O antigens, flagellar or H antigens, and capsular or K antigens; (Campos, 2004). In one study, the most common *E. coli* serotypes isolated from diarrheic fecal samples were O119, O111, O126, and O78 (Dean-Nystrom *et al.*, 1997; Tamaki *et al.*, 2005 and Badouei *et al.*, 2010) isolated O157:H7, O111 and O26 serotypes of *E. coli* strains from 297 fecal samples, from 200 diarrheic and 97 non-diarrheic calves. The most predominant serogroup was O26 (18.4%). Current treatment regimens for the treatment of neonatal calf diarrhea center on antimicrobial therapy and fluid therapy.

Salmonellosis is one of the major foodborne diseases. Due to its endemic nature, high morbidity and association with a wide range of foods, this zoonotic disease is of high public health concern (Aarestrup *et al.*, 2007; Alizadeh *et al.*, 2007 and Kottwitz *et al.*, 2008).

Salmonella infections occur worldwide in both developed and developing countries and are a major contributor to morbidity and economic costs (Antoine *et al.*, 2008).

Several rapid and sensitive methods have been developed for identification of *Salmonella* serovars from clinical samples (Zahraei *et al.*, 2007). Especially *S. typhimurium* causes diarrhea in calves 2-12 wk old. *Salmonella typhimurium* is the most frequently isolated serovars from foodborne outbreaks throughout the world (Herikstad *et al.*, 2002).

Salmonellae produce enterotoxins but are also invasive and produce inflammatory change within the intestine. In calves, infection commonly progresses to a bacteremia.

Targeting *fliC* gene specific for *S. typhimurium*. This gene is suitable for PCR targeted for detection of *Salmonella* Serovars. (Malorny *et al.*, 2003). Investigation of *Salmonella* infection in calves was the major strategy of our work in several localities, as well as to detect the most accurate sensitive and rapid diagnostic assay among different diagnostic procedures.

PCR is a useful diagnostic tool because it is quick, specific, sensitive, and relatively inexpensive. A PCR which detects genes (Stone *et al.*, 1994 and China *et al.*, 1996). The identification and differentiation of specific gene via PCR amplification of virulence-associated genes commonly found in these *E. coli* strains. Primers specific for genes encoding the fimbrial subunits of *K99, E coli* O 157 and *S.typhimurium*.

The aim of this work is the evaluation of the bacteriological aetiology of diarrhoea in newly born calves. Also, antimicrobial sensitivity of most isolated bacteria in diarrheic fecal samples to overcome the problem and reduce losses. On the other hand, using polymerase chain reaction (PCR) test to substitute the traditional methods and rapid diagnosis.

MATERIALS and METHODS

Collection of samples:
Rectal swabs were collected from (200) diarrheic calves using sterile cotton swabs, at different farms of Menoufiea, Gharbia and Qaliubiya Governorates in Egypt, to evaluate the performance of the PCR panel in comparison with other laboratory procedures routinely for the same target agents bacterial culture for *E. coli* K99, *E coli* O 157 bacterial culture and serotyping for *Salmonella*, and microscopic observation.

Samples were transferred directly to the laboratory in a separate clean sterile plastic bag, in an ice box and kept in retail package under complete aseptic condition without delay and subjected to required investigations.

Bacteriological examination:
1- Isolation and identification of *E. coli*:
All samples were inoculated into tubes of freshly prepared nutrient broth and incubated aerobically at 37°C over night, followed by subculturing onto MacConkey's agar and eosin methylene blue agar plates for 24-48 hours at 37°C. Lactose positive colonies were confirmed as *E. coli* according to Gershwin (1990); Koneman *et al.* (1992) and Quinn *et al.* (1994).

Suspected colonies grown were picked on nutrient agar slopes and incubated at 37°C for 24 hours, then kept in refrigerator at 4 °C for further identifications according to (Edwards and Ewing, 1972).

2- Isolation of *Salmonella*:
All samples were inoculated into tubes of Selenite-F and Tetrathionate broths and streaked out onto MacConkey and brilliant green agar after overnight incubation at 37°C. Suspected colonies were subjected to biochemical testing according to (Adyin *et al.*, 2001 and Echeita *et al.*, 2002). Slide agglutination test was used for identification according to the Kauffmann-White Schema (Zahraei *et al.*, 2007; Nori and Thong, 2012). Fnally identification of *Salmonella typhimurium* was done according to Waltner-Toews *et al.* (1986).

3- Isolation of *Campylobacter*:
Small portion of feacal samples was suspended in 0.85% saline, filtered through 0.45 mm Milipore filter papers. Filters were then cultured in Preston broth (Oxoid) and incubated overnight at 37°C. Cultures were then inoculated onto Preston agar plates and incubated for 48 h in an atmosphere of 5% oxygen, 10% CO2 and 85% nitrogen. Suspected colonies were identified based on their motility, hydrolysis of sodium hippurate and sensitivity to cefalotin and nalidixic acid.

Each faecal sample was also cultured onto 5% sheep blood agar, incubated at 37°C for 24 h and inspected for the presence of other bacterial pathogens, e.g. *Bacillus* spp., *Corynebacterium* spp., *Pseudomonas aeruginosa.*

4- Isolation of *Clostridium perfringens*:
A loopful from a small piece of the feacal samples, was inoculated into tubes of freshly prepared Robertson's cooked meat medium at 37°C for 24 hours which had been boiled for 10 minutes and cooled rigidly prior to inoculation. Loopful from each tubes was streaked onto the surface of 10% sheep blood agar, then incubated anaerobically at 37°C for 24 hours. The plates were examined for characteristic colonies of *Clostridium perfringens.*

Subcultures from suspected colonies in cooked meat broth were made for further biochemical identification according to Koneman *et al.* (1992). Typing of *C.perfringens* isolates was done by the intradermal inoculation test in guinea pigs according to Koneman *et al.* (1992).

5- Serological identification of *E. coli* isolates:
This was carried out according to Orskov and Orskov (1984). Slide agglutination test was done for demonstration of surface antigen, first using polyvalent antisera then for further identification by monospecific antisera, previously described (Ewing, 1986).

6- Susceptibility of *E.coli* isolates to various chemotherapeutic agents:
The isolates of *E.coli* were tested in vitro sensitivity to different antimicrobial agents by the disc diffusion method described by NCCLS (2004). The following antibiotic discs were used; chloramphenicol (30 mg), lincomycin (15mg), spectinomycin (200mg), amoxicillin (25 mg), norfloxacin (10 mg), neomycin (30 mg), gentamycin (30mg) and doxycycline (30 mg).

7- Molecular technique:
Nucleic acid extraction
Nucleic acids of all target agents were simultaneously extracted from specimens by use of a commercial nucleic acid isolation kit as described in the manufacturer's manual. In brief, 0.01 M phosphate buffered saline (pH 7.4) was added to each sample to make 30% fecal homogenates. After centrifugation for 1 min at 100 × g to pellet larger-size particles, 175 µl of the supernatant of each sample was carefully transferred into clean microcentrifuge tubes. Using a Fermentas kit (Gene JET Genomic DNA purification kit #K0729. The extracted total nucleic acids in the elution plate were stored in −80°C until used for PCR reaction.

Primers were chosen from published sequences with the aid of the Primer Select software (DNASTAR Inc, Madison, Wis.). Table 1 includes the bacteria, the primer sequences and the position of the primer. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa).

PCR technique
PCR is a useful diagnostic tool because it is quick, specific, sensitive, and relatively inexpensive. A PCR which detects genes according to Stone *et al.* (1994) and China *et al.* (1996).

The identification and differentiation of specific gene via PCR amplification of virulence-associated genes commonly found in these *E. coli* strains. Primers specific for genes encoding the fimbrial subunits of *K99, E coli O 157* and *Salmonella typhimurium.*

The 20-µl PCR mixture contain the specific primer, and 5 µl bacterial DNA. Samples were amplified in a PCR thermal cycler (Perkin-Elmer) under the specific conditions.

PCR design and amplification conditions for *E coli O157 gene rfb*
rfb O157, PCR assays for the detection of O157, H7 antigens s were screened by specific primers forward 5'- CGGACATCCATGTGATATGG -3' and the reverse 5'-TTGCCTATGTACAGCTAATCC-3' PCR condition and thermal cycles were similar to Paton and Paton (1998). The amplification conditions included 25 cycles of a denaturation step at 94 °C for 30 s, primer annealing at 50 °C for 45 s, and extension at 70 °C for 90 s. The extension time was ramped for an additional 3 s per cycle and a final extension step of 10 min at 70 °C was performed.

PCR design and amplification conditions for *K99 gene F 41*

20 µl of mix which contain the specific primer forward 5' TATTATCTTA GGTG GTATGG, 3' and the reverse primer 5' GGTATCCTTTAGCAGCAGTATTTC, 3' according to Paton *et al.* (1993) and add a 5 µl of samples and then using thermocycles Bekman with this condation The amplification conditions included 25 cycles of a denaturation step at 94 °C for 30 s, primer annealing at 50 °C for 45 s, and extension at 70 °C for 90 s. The extension time was ramped for an additional 3 s per cycle and a final extension step of 10 min at 70 °C was performed.

PCR design and amplification conditions for *S. Typhimurium flic* gene

The oligonucleotide primers for PCR were synthesized according to Cohen *et al.* (1996a); Cohen *et al.* (1996b) and Jacb (2005). Considering reported nucleotide sequence of the *flic* gene (EMBL and Gen Bank accession number M18283 and sequence name F2M47) of *S. Typhimurium.* The 20-mer forward primer *(Flic)*,5'-TATTATCTTAGGTGGTATGG 3', reverse primer *(Flic)*, 5'-ACTCTTGCTGGCGGTGCGACTT -3', gene of *S. typhimurium.* The 559 bp fragment was specifically amplified using this set of primers. From pure cultures or genomic DNAs of *Salmonella* strains, amplification of *flic* gene was achieved on the thermal cycler as follows Initial denaturation at 94°C for 5 min., followed by 35 cycles of (denaturation at 94°C for 1 min, annealing 55°C for 1 min and extension at 72°C for 1 min). Final extension was carried out at 72°C for 10 min as reported by Sambrook *et al.* (1989).

DNA Products were electrophoresed in 2% agarose gel (FMC Bioproducts, Rockland, Maine) for 1 h at 100 V, stained with ethidium bromide, and photographed under UV light. Each experiment contained negative controls with all reagents except template DNA.

Table 1: Primer used in PCR for *E coli O 157* , *E coli K 99* and *S. Typhimurium.*

Virulence factor	Primer sequence 5'-3'	Size of product (bp)	Reference
O157*(rfb)*	CGGACATCCATGTGATATGG TTGCCTATGTACAGCTAATCC	259	(Paton and Paton, 1998)
K99 *(F41)*	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	314	(Paton *et al.*, 1993)
S. *Typhimurium.* (*fliC*)	CGGTGTTGCCCAGGTTGGTAAT ACTCTTGCTGGCGGTGCGACTT	559	(Cohen *et al.*, 1996a and Cohen et al., 1996b)

RESULTS

Results in table 2, shows the bacterial isolates from examined rectal swabs of diarrheic calves. Out of 200 samples 159 samples (79.5%) were positive for bacteriological isolates.

Recovered bacterial species from faeacal samples were *E. coli, S. typhimurum, Campylobacter, Corynebacyerium, P. aeruginosa* and *C. perfringens* with incidence of 47.5, 9, 7.5, 6, 4 and 5.5%, respectively.

The results in table 3, illustrate Typing of *C. perfringens* isolates by intradermal inoculation test in Guinea pig and shoued that the incidence of toxigenic and non toxigenic strains were 81.9 and 18.1% respectively. Type A predominant to type B with the incidence of 45.5 and 36.4% respectively.

The results in Table 4, show the serotyping of 95 *E. coli* isolates, and revealed that the *following serotypes:- K99* (21.1%), *O157* (17.9%), *O111* (9.5%), O125 (11.6%), O119 (15.8%), O26(12.6%), O128 (8.4%) and un typed *E. coli* (3.2%%).

In vitro sensitivity of the recovered isolates of *E.coli* to different antimicrobial agents was done. Table 5 shows that, *E. coli* isolates were highly sensitivite to enrofloxacin, flumequine and tetracycline. In contrast, these isolates were found to be resistant to ampicillin, erythromycin, gentamicin and penicillin-G.

From the Fig 1, good agreement in the test results was observed between the PCR panel and the traditional diagnostic methods, the results reached 97 % to *salmonella* using PCR from the samples directly, 94 % to *E coli k99* and *E coli O157* the results reach to 95%. We can record that the molecular characterize of *K 99 (gene F41)* the molecular bands appeared at 314 bp compared with the molecular marker, but the *E coli O 157* for *rfb* gene the molecular bands appeared at 259 bp. While the *Flic* gene of *Salmonella typhimurium* molecular bands appeared at 559 bp.

Table 2: Incidence of bacteria in the examined faecal samples.

Bacterial isolates	No.*	Single		Mixed		Total	
		No.	%	No.	%	No.	%
E. coli	200	86	43%	9	4.5%	95	47.5%
S. typhimurium	200	13	6.5%	5	2.5%	18	9%
Campylobacter	200	9	4.5%	6	3%	15	7.5%
Corynebacterium	200	8	4	4	2	12	6
P. aeruginosa	200	6	3	2	1	8	4
C.perfringens	200	7	3.5	4	2	11	5.5
Total	200	129	64.5	30	15	159	79.5

% Calculated according to the number of tested samples
No.*: Number of tested samples

Table 3: Typing of *C. perfringens* isolated from faecal samples

Typing of C. perfringens	No.	%	No. of non toxigenic isolates	%	Total number of isolates
C .perfringens Type (A)	5	45.5	2	18.1	11
C.perfringens Type (B)	4	36.4			

% Calculated according to the total number of isolates

Table 4: Prevalence of *E. coli* serotypes in faecal samples from diarrheic calves.

Serotypes	No.	%
K99	20	21.1
O157	17	17.9
O111	9	9.5
O125	11	11.6
O119	15	15.8
O26	12	12.6
O128	8	8.4
Un typed	3	3.2
Total	95	100

Table 5: Susceptibility of *E.coli* isolates to different antimicrobial agents.

Chemotherapeutic agents.	Tested *E.coli* serotypes																	
	K99			O157			O111			O125			O119			O26		
	NO. (20)*	%	AA	NO. (17)*	%	AA	NO. (9)*	%	AA	NO. (11)*	%	AA	NO. (15)*	%	AA	NO. (12)*	%	AA
Ampicillin	3	15	R	3	17.6	R	0	0	R	3	27	R	0	0	R	2	16.7	R
Chloramphenicol	14	70	IS	12	70.6	IS	6	66.7	IS	9	81.8	S	9	60	IS	8	66.7	IS
Enrofloxacin	19	95	S	16	94.1	S	9	100	S	10	90.1	S	15	100	S	12	100	S
Erythromycin	4	20	R	2	11.8	R	1	1.1	R	0	0	R	2	22.2	R	1	8.3	R
Flumequine	15	75	S	9	52.3	IS	4	44.4	IS	6	54.5	IS	13	86.7	S	10	83.3	S
Gentamicin	3	15	R	2	11.8	R	1	11.1	R	1	9.1	R	2	13.3	R	1	8.3	R
Lincomycin	13	65	IS	8	47.1	IS	3	33.3	R	3	27.3	R	8	53.3	IS	3	25	R
Penicillin-G	0	0	R	0	0	R	1	11.1	R	2	18.1	R	0	0	R	1	8.3	R
Spectinomycin	14	70	IS	10	58.8	IS	6	66.7	IS	7	63.6	IS	9	60	IS	7	58.3	IS
Streptomycin	13	65	IS	7	41.2	R	5	55.6	IS	7	63.6	IS	8	53.3	IS	3	25	R
Tetracycline	16	80	S	11	64.7	IS	7	77.7	S	7	63.6	IS	10	66.7	S	7	58.3	IS

No. Number of sensitive isolates. *. Number of isolates.
%: Percentage of sensitive isolates in relation to total isolates.
AA: Antibiogram activity. S: sensitive.
IS: intermediate sensitive. R: resistant.

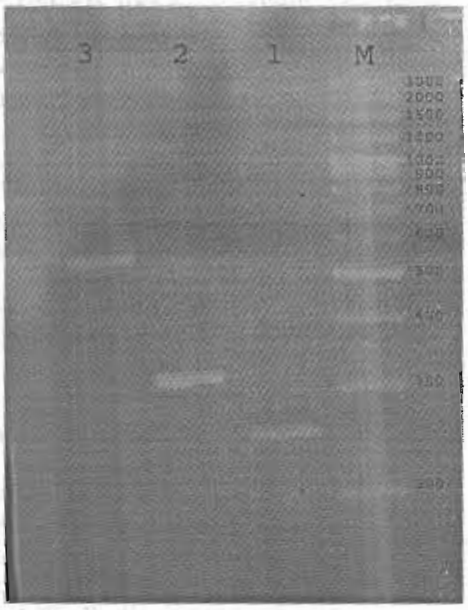

Fig. 1: Ethidium bromide stained 2% agarose gel electrophoresis showed the amplified fragment

M marker 100 bp staudard (# SM 0323) Fermantas.
Lane 1 : *E coli O 157 gene rfb*
Lane 2 :*E coli K 99 gene F 41*
Lane 3 : *S. Typhimurium gene flic*

DISCUSSION

Neonatal calf diarrhea is considered one of the most serious constraints of animal production. The incidence of calf diarrhea occurs all over the year with some increase in calving seasons.

The results in Table 2, show that, the total bacteria isolated from faeacal samples were *E. coli, S.typhimurium, Campylobacter, Corynebacyerium, P. aeruginosa* and *C. perfringens* with incidence of 47.5, 9, 7.5, 6 , 4 and 5.5%, respectively. Our findings are similar to previous data that found the *E. coli* was the most common bacteria isolated from fecal samples of diarrheic calves, (China *et al.,* 1998 and Harbby, 2002). Also the results agreed with that obtained by El-Hamamy *et al.* (1999), who recorded that cultures of swabs from diarrheic calves revealed that, the predominant isolate was *E-coli* (52.5%), *enterobacter aerogense* (15%), *proteus vulgoris* (12.5%) and *Salmonella* spp. (5%). Also other results (Ibrahim, 2007) revealed that 7 of 150 samples were positive for *E-coli* with an incidence of 24.66%, *E. coli K99* were isolated from 13 samples, (Quigley *et al.,* 1995) recorded that out of 150 samples 22.7% were positive for *Campylobacter, Salmonella* which were isolated in 22 samples with an incidence of 14.66% . The same results are obtained by Novert and Nawal (2002), who detected *E –coli* K99 antigen in 13 fecal samples (23.2%) out from 56 fecal samples obtained from untreated diarrheic calves aged from one day up to 3 weeks of age, using the traditional culture method. Also same results of supported (Bendali *et al.,* 1999a), who concluded that *E-coli* was isolated from 20.3% diarrheic fecal samples out of 3080 fecal samples obtained from neonatal calves with diarrhea, it appeared during first days of life.

Different clostridia species cause intestinal disorders and enterotoxaemia in various animals species including neonatal calves demonstrated that, in neonatal calves enterotoxaemia is defined as a sudden death syndrome with lesions of hemorrhagic enteritis (Manteca *et al.,* 2000), the infectious etiology has not been identified, although *C.perfringens* is often regarded as responsible (Popoff, 1990).

The results in table 3, show the Typing of *C. perfringens* by intradermal injection of Guinea pig revealed that the incidence of toxigenic and non toxigenic strains were 81.9 and 18.1% respectively. Typing toxigenic strains of *C. perfringens* revealed that the type A was the most predominant than type B with the incidence of 45.5% and 36.4% respectively, these results agrees with that obtained by Haschek *et al.* (2006), who found that prevalence of *C .perfringens* was 9.1% in collected feces of 230 calves with and without diarrhea during the winter period 2004/2005 in 100 Austrian farms (Styria and Lower Austria), a higher prevalence of infection was reported by Haschek *et al.* (2006), who examined fecal samples taken from 344 calves aging between one day to 4 months old from dairy farms in Fayoum, Kafr El-sheikh and Beharia Governorates, 293 of them were apparently healthy and 51 of them were diarrheic. Microbiological examination revealed that, the isolation of *C. perferingens* was 71.32%, 59.65% and 57.01% from the apparently healthy calves and 96.15%, 88.89% and 87.5% from the diseased diarrhoeic calves in Fayoum, Kafr El-sheikh and Beharia Governorates respectively. The highest percentage of isolations was in 1 day–1-week-old calves as it reached 80.61%. (Harbby, 2002), examined fecal swabs collected from 200 calves showed that 150 diarrhoeic, 20 apparently healthy and 30 intestinal samples, aged from one day to 12 weeks old. *C.perfringens* were detected in 66.7%.

The results in Table 4 shows that serological serotyping of 95 *E. coli* isolates, revealed that the *E. coli* were typed as *K99* (21.1%), O157 (17.9%) ,O111 (9.5%) , O125 (11.6%), O119 (15.8%), O26(12.6%), O128 (8.4%) and untyped *E. coli* (3.2%%) . These findings are similar to previous data stated that the most common *E. coli* serotypes isolated from diarrheic fecal samples was O119, O111, O126, and O78 (Tamaki *et al.,* 2005). Similarly, (Badouei *et al.,* 2012). Isolated O157:H7, O111, and O26 serotypes from 297 fecal samples from 200 diarrheic and 97 non-diarrheic calves. The most common serogroup was O26 (18.4%). The same results were obtained by John *et al.* (2007) who isolated EHEC O26 and EHEC O111 from diarrheic and non-diarrheic young calves from 115 different farms. Of the 257 calves with diarrhea, 37 (14.4%) and 32 (12.5%) was found positive EHEC O26 and EHEC O111,respectively. Suggesting that EHEC O26 and O111 are possible causes of the disease in infected neonatal calves. The high percentage of bacterial isolation in winter months may be attributed to increased relative humidity which activate microorganisms and increase number of births which facilitated contamination and spread of infection.

Results in table 5 shows that , the in vitro sensitivity of the recovered isolates of *E.coli* to different antimicrobial agents were highly sensitivite to enrofloxacin, flumequine and tetracycline. In contrast, these isolates were found to be resistant to ampicillin, erythromycin, gentamicin ,lincomycin and penicillin-G. The present results agreed with those of Sadiek and Sohair (1999), who studied antibiotic sensitivity of fecal samples from diarrheic calves and found that enrofloxacin was the antibiotic of choice for most bacterial isolates (*E. coli, Salmonella* species, *Klebsiella* species, and *Proteus* species). In another study, in vitro sensitivity testing of isolated bacteria from the feces of diarrheic calves showed

that ciprofloxacin, enrofloxacin, and gentamicin were the most effective drugs (El-Gaml *et al.*, 2001). Recent work has also shown that ceftiofur and enrofloxacin were highly efficient antibiotics in treatment of neonatal calf diarrhea, as indicated by antimicrobial sensitivity tests (Aba-Alkhalil and El-Naenaeey, 2003). In addition, most *E. coli* isolates from diarrheic calves had been shown to be resistant to kanamycin, gentamycin, chloramphenicol, tetracycline, and ampicillin (Sato *et al.*, 2005).

Showed that the molecular characterized of *K 99 (gene F41)* the molecular bands appeared at 314 bp compared with the molecular marker (Fig, 1), on the other hand *E coli O 157* the *rfb* gene the appeared at 259 bp. While the *Flic* gene of *salmonella Typhimurium* molecular bands appeared at 559 bp. *S. typhimurium flic* gene has been cloned and sequenced (Nicols *et al.*, and Swenson *et al.*, 1994). Specific primers were designed by Cohen *et al.* (1996a) and Cohen *et al.* (1996b) which had the ability to amplify region of the *flic* gene from *Salmonella* strains but not from *E. coli.* These results agreed with the results of Jones *et al.* (1988); Segall and Lindberg (1993) and Seleim *et al.* (2004). All *Salmonella* strains either standard or isolated from fecal samples were positive with PCR and the 559 bp PCR product was observed on agarose gel electrophoresis. This results confirms the result of Cohen *et al.* (1996a) and Cohen *et al.* (1996b). All bacteriologically positive fecal samples were positive with PCR and the specific PCR product (559 bp fragment).

Our results indicate that the PCR amplification of the *flic* gene sequence of *S. typhimurium* could be used as a target sequence for rapid and sensitive method for direct detection of *Salmonella* serovars in the fecal samples of diarrheic and contact apparently normal calves.

Diarrhoea due to the *Escherichia coli O 157 gene rfb and K 99 gene F 41* is one of the most frequent bacterial diseases in neonatal calves and the predominant pathogen cultured from calves with septicaemia (Lofstedt *et al.*, 1999). *E coli* was the bacterial agent cultured with the highest frequency from diarrhoeic and healthy calves in our study, a finding that agrees with results of Bendali *et al.* (1999a) and Garcia *et al.* (2000).

E. coli belonging to the O157 serogroup have been mainly isolated from cattle which are considered a natural reservoir of these bacteria (Armstrong *et al.*, and Dombek *et al.*, 2000). As demonstrated in the present study, none of the O157-positive isolate the *rfb* gene as tested by PCR. *E. coli* O157:H7- strains have been isolated from cattle and have emerged as important etiological factor of hemorrhagic colitis. As demonstrated in the present study, that are characteristic for *E. coli* isolates pathogenic for humans. Therefore, these bacteria can be considered potentially virulent microorganisms for man. The genetic analysis of selected O157:H7- isolates, performed with random amplification of DNA sequences, revealed that bacteria possessing the same but also different virulence-associated factors represented related clonal lineages. the obtained result agreed with that of Snodgrass *et al.* (1986), as the isolated *E.coli* with an incidence of (50.76%) . lethal *E.coli* was found in 6/32 samples (West *et al.*, 2007).

Therefore, the PCR panel could help diagnosticians rapidly determine the causative agents for bovine diarrhea in the early stages of disease and help practitioners which initiate appropriate treatments or interventions quickly. Study was supported in part by funding from Iowa calf scour Fund. It was found that the PCR is relatively rapid and highly sensitive (Singer *et al.*, 2006).

CONCLUSION

The aim of the present study was to determine the association between *E. coli* and *Salmonella* (one of the most important enteropathogenic bacteria causing calf diarrhea). Serotyping of *E.coli* were identified in percentage infection of *Salmonella* and *E.coli* was detected in the newly developed PCR described in the current study is specific and more sensitive than other traditional diagnostic methods and drastically decreases turnaround time, labor, and cost. Therefore, the PCR panel could help diagnosticians to determine rapidly the causative agents for bovine diarrhea in the early stages of disease and help practitioners initiate appropriate treatments or interventions quickly.

Study was supported in part by funding from Iowa calf scour fund. It was found that the PCR is relatively rapid and highly sensitive (Singer *et al.*, 2006).

The PCR method was also highly sensitive, as it correctly detected all genes of interest in 100% of the strains and isolates containing them. This assay could be useful in diagnostic situations for identification and characterization of *E. coli* isolated from calves with diarrhea. These results highlight the usefulness of the multiplex PCR for the rapid detection of the two serotypes of *Salmonella* from field samples especially after pre-enrichment on RV media. Moreover, detecting *S. typhimurium* and *S. enteritidis* by this assay was carried out within two days opposed to five to six days by the bacteriological and serological methods (Moussa *et al.*, 2012).

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المسببات البكتريولوجية للأسهال في العجول حديثي الولادة في محافظة المنوفية

الهام إبراهيم عطوة ، ايمان محمود فريد شرف ، ايمان مجدى زكاري

تم فحص ٢٠٠ عينة مسحات شرجية من عجول مصابة بالاسهال. اتضح ان عدد ١٥٩ عينة بنسبة (79.5%) ايجابة للفحص البكتريولوجي .تم عزل ميكروب الاشريشيا كولاى والسالمونيلا والكامبيلوباكتر والكورينى باكتريم والسودموناس اريجينوزا والكلوستريديم بيرفرنجيز نوع ا وب بنسبة ٤٧.٥ ، ٩ ، ٧.٥، ٦، ٤ ، و ٥.٥% علي التوالي . وبتصنيف الكلوستريديم بيرفرنجيز عن طريق الحقن داخل الجلد فى الارانب البيضاء وجدت نسبة السام والغير سام كانت (٨١.٩ و ١٨.١%) على التوالى ، كما تبين ان نوع (ا) اكثر عزلا من نوع (ب) بنسبة ٤٥.٥ و ٣٦.٤% على التوالى. وباجراء التصنيف السيرولوجى للاشريشيا كولاى المعزولة تم تصنيف الاتى (9.5%) O111, (17.9%) O157 (21.1%) K99 O125 (11.6%), O119 (15.8%), O26(12.6%), O128 (8.4%) , وغير مصنفة بنسبة ٣.٢ %. تم استخدام اختبار الحساسية للمعزولات البكتيرية للاشريشيا كولاى ووجد انها اكثر حساسية لأنروفلاكسين ثم الفلوماكين والتتراسيكلين بالمقارنة الى مقاومتها لكلا من الامبيسلين والاريثرومايسين والجينتاميسين واللنكوميسين وبنسلين ج. وباستخدام اختبار انزيم البلمرة المتسلسل لتحديد السبب فى الاسهال فى المراحل المبكرة بطريقة سريعة وحديثة وتحديد الاشريشيا كولاى *rfb* O 157 و *الاشريشيا كولاى* K 99 جين *F41* وكذلك السالمونيلا *flic* وجد انة الجين يظهر عند ٢٥٩ و ٣١٤ و٥٥٩ على التوالى.