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FACTORS AFFECTING CALF ENTERITIS INFECTION CAUSED BY SALMONELLAE AND *ESCHERICHIA COLI*

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ABSTRACT

Calf diarrhea is a major economic concern in bovine industry all around the world. Therefore, the present study was designed to investigate seasonal and age variations in the prevalence of *E. coli* and *Salmonella* species in diarrheic calves and their virulence genes, pathotypes, serogroups and antibiogram. Bacteriological examination of 120 fecal samples collected from diarrheic calves less than three months of age in dry and rainy seasons showed that, 71 (59.5%) and 36 (30%) were positive for *E. coli* and *Salmonella species*, respectively while 26 (21.66%) had mixed *E. coli* and *Salmonella* species infection. The most prevalent *E. coli* serogroups were O111 and O26 and *Salmonella* serovars were *S. typhimurium* and *S. enteritidis*. Isolation frequency of *E. coli* was significantly higher than *Salmonella* species isolated from diarrheic calves in different ages and seasons (P value=0.004). *E. coli* and *Salmonella* species was statistically significant higher in rainy season than dry season. PCR investigation of six virulence determinants among the MDR *E. coli* isolates revealed that *fim*H and *iss*, were the most prevalent (100%), followed by *sxt*2 (90%), *sxt*1 (80%), *hyl*A (60%) and *eae*A (40%). All examined multiple drug resisitant (MDR) *Salmonella species* isolates harbored both *inv*A and *sop*B virulence genes. *E. coli* and *Salmonella* species isolates showed a high sensitivity rate for amikacin (100%), and ciprofloxacin (78.8% and 77.7%), respectively but showed resistance against amoxicillin and tetracycline. The most commonly detected resistance gene was *tet*A gene. Finally, strict management and hygienic measures should be taken in rearing neonatal calves especially in rainy seasons.

***Keywords**:* Neonatal calf diarrhea - *E.coli- Salmonellae* - virulence and resistance genes.

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INTRODUCTION

Calf diarrhea (CD) is one of the most common problems in young animals, causing huge economic and productivity losses to bovine industry worldwide (Cho and Yoon, 2014). In Egypt, neonatal calf diarrhea (NCD) continues to be the 1st cause of calf mortality, which ranges between 27.4% and 55% of the total deaths in young calves (Younis *et al.*, 2009). The economic losses occur not only from mortality but also from other costs including treatment, diagnostics, labor intervention and decreased number of herd as well as subsequent chronic ill thrift and impaired growth performance (Bazeley, 2003). NCD is a multifactorial syndrome including pathogen (infectious NCD) as well as non-infectious factors related to the animal (immunological and nutritional status), the environment or the management (Izzo *et al.*, 2011). Because of the multifactorial nature of NCD, it is difficult to be controlled effectively (Cho and Yoon, 2014). The number of cases of diarrhoea is normally higher during the rainy seasons, from October/November to March than during the dry seasons, from March to October Achá *et al.* (2004).

In spite of the complex etiology of calf diarrhea, the involvement of bacterial pathogens is still responsible for more than 50% cases of diarrhea in neonatal calves (Kumar *et al.*, 2012) Among bacteria, enterotoxigenic *Escherichia coli* (ETEC) and Salmonellae are the most economically important pathogens (Achá *et al.*, 2004), also other bacteria have been found as causes of enteric disease and NCD, e.g. *Clostridium* species (Cho *et al.*, 2010). Identification of the possible causative agent in outbreaks of diarrhea is important to allow targeted preventative measures, such as vaccination, and identification of possible risk factors or sources of infection (Izzo *et al.*, 2011). *Salmonella enterica* colonizes the digestive system of both adult cattle and calves, but the infection is more common and often causes severe symptoms in 10-day to 3-month-old calves (Fossler *et al.*, 2005). On the other hand, *E. coli* K99+ causes a watery diarrhea, dehydration, and weakness in 1- to 4-dayold newborn calves (Acres *et al.*, 1977). Younger age and low colostrum feeding calves were significantly associated with *E. coli* isolation (Ashenafi and Tesfaye, 2016). Diarrhea due to Salmonella infection is watery and mucoid with the presence of blood and fibrin (Fossler *et al.*, 2005). Salmonella pathogenicity island SPI-1 and SPI-5 including *inv*A and *sop*B genes are known to influence the type III secretion system, and are mainly responsible for *Salmonella* induced diarrhea in calves (Treuer and Haydel, 2011).

Infected cattle and carriers can serve as source of infection for other animals or even human through food-borne routes or direct contact and so the determination of Salmonella strains in fecal samples is not only important for the diagnosis of salmonellosis, but also essential to identify the carriers (Warnick *et al.*, 2003).

E. coli classified into enterohemorrhagic (EHEC), enterotoxigenic (ETEC), necrotoxigenic (NTEC), enteroinvasive (EIEC), enteropathogenic (EPEC) and attaching and effacing *E. coli* (AEEC) pathotypes Kaper *et al.* (2004). The diarrhea in calves is commonly caused by enterotoxigenic *Escherichia coli* (ETEC), the most common type of colibacillosis in neonatal calves (Nagy

and Fekete, 2005). Two of the more prominent virulence factors identified for ETEC strains are (i) expression of fimbrial (pili) antigens that enables the bacteria to adhere to and to colonize the luminal surface of the small bowel and (ii) elaboration of one or more enterotoxins that influence the intestinal secretion of fluids (Holland, 1990). Unlike ETEC, Enteropathogenic *E. coli* (EPEC) strains do not produce toxins, but habor an outer membrane protein, intimin, which mediates the intimate attachment of bacteria to the enterocyte, causing typical attaching and effacing (A/E) intestinal lesions (Law, 2000). One feature EHEC and EPEC have in common is the causation of intestinal epithelial lesions known as A/E (Moxley and Smith, 2010). EHEC strains are a subset of STEC strains (Nguyen *et al.,* 2011).

The most common cause of NCD is ETEC strains that produce the K99 (F5) adhesion antigen (*E. coli* K99+) and heat-stable (*STa* or *STb*) and/or heat-labile (LT1 or LT2) enterotoxins (Kaper *et al.,* 2004). Another virulence factors including Shiga toxin 1 (*stx*1) and Shiga toxin 2 (*stx*2), the protein intimin (*eae*) and enterohaemolysin or enterohaemorrhagic *E. coli* haemolysin (*ehly*) which are related to the pathogenesis of STEC strains (Law, 2000).

Furthermore, it appears that *E. coli*, particularly serogroups O26, O111and O118 are virulent to cattle, particularly young calves (Wieler *et al.,* 1998). *E. coli* O26 has been isolated from clinical cases of hemorrhagic diarrhea in young and neonatal patients (Pearson *et al.,* 1999).

Antimicrobials are commonly used in treatment of diseased calves either orally or parentally. Some commonly used antimicrobials as sulbactam, ampicillin, neomycin, cephalosporin, tetracycline and sulphonamide- trimethoprim mixture have been used (Kumar *et al.,* 2010). However, in the last decade or so the development of drug resistance and the presence of multi drug resistant pathogenic bacteria are major problems in treating bacterial diseases (Anita *et al.,* 2013).

Therefore, the present study was planned to assess seasonal and age variations in the prevalence and serogroup distribution of *E. coli* and *Salmonella* species in fecal sample of diarrheic calves and investigate their susceptibility to commonly used antimicrobials.

MATERIAL AND METHODS

Sampling and bacteriological examination.

A total of 120 fecal samples from diarrheic calves were collected randomly from September 2018 to October 2019. Seasons of samples collection (rainy and dry) and age of diarrheic calves (one week to 3 months) were recorded during sampling. Fecal samples were taken using sterile rectal swabs. All samples were transferred in an ice box to the laboratory and cultured in the same day or stored at 4ºC and cultured within 3 days.

Isolation and identification of *E. coli* and *Salmonella species* among the collected samples were confirmed based on their morphology, cultural and biochemical tests using standard bacteriological procedures described by Quinn *et al.* (2002) and (ISO 6579, 2002),

respectively. Serological identification of *E. coli* and *Salmonella species* isolates was conducted at Serology Unit, Animal Health Research Institute, Dokki, Giza, Egypt using commercial antisera (Difco, Detroit, MI, USA) according to the manufacturer's instructions.

Antimicrobial susceptibility testing.

In vitro susceptibility of all *E.coli* and *Salmonella* isolates to commonly used antimicrobial drugs was tested by the Kirby-Bauer standard agar disk diffusion technique as described earlier (Bauer *et al.,* 1966), using Mueller Hinton agar and commercial antibiotic disks (Oxoid, Basingstoke, Hampshire, England, UK). The tested antibiotics and their concentrations in µg/disk were as following: amoxicillin (AX;10), spectinimycin(SPT:100), amikacin(AK:30), ceftriaxone (CTX; 30), colistin(CT:10), tetracycline (TE; 30), sulfamethoxazole/trimethoprim (SXT; 25), streptomycin (S; 10), gentamicin (CN; 10), and ciprofloxacin (CIP; 5). The inhibition zones, in millimeters, were measured in duplicate and scored as sensitive, intermediate, and resistant categories in accordance with the critical breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011).

Polymerase chain reaction procedure

DNA extraction:

DNA extraction from bacterial samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations.

Oligonucleotide Primer:

Primers used were supplied from Metabion (Germany). Primer sequences, amplicon size and references are listed in table (1).

PCR amplification:

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp 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. *E. coli* virulence genes (*stx1, stx2, hly*A and *eae*A) were performed in Multiplex DNA amplification reaction. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR Products:

The products of PCR 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 products was loaded in each gel slot. Gelpilot 100 bp (Qiagen, Germany, GmbH) 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.

Statistical analysis.

The SPSS (Statistical Package for the Social Sciences) software (Ver. 24) and Chi-square test were used to study the statistical relationship between the incidence of a bacterium in various ages, and seasons and also between the frequency of various virulence genes, antibiotic resistance genes and serogroups. P-value < 0.05 was considered statistically significant.

RESULTS

Occurrence of *E. coli* and *Salmonella species* among the examined samples.

Out of 120 fecal samples collected in both dry and rainy seasons from diarrheic calves between 1 week and 3 month of age, 71 (59.1%) and 36 (30%) were characterized as *E. coli* and *Salmonella* species, respectively. From examined samples 26 (21.66%) had mixed *E. coli* and Salmonella species infection as represented 11/60 (18.33%) in dry and 15/60 (25%) in rainy seasons). Prevalence of *E. coli and Salmonella* species in the tested samples were listed in (Table 2).

Effect of seasonal and age variations on *E. coli* and *Salmonella* species isolated from diarrheic calves.

The results of *E. coli* and *Salmonella* species prevalence in fecal swabs obtained from diarrheic calves showed that *E. coli* and *Salmonella species* significantly higher in rainy season 40/60(66.6%), 22/60(36.6%) than dry season 31/60(51.6%), 14/60 (23%), respectively also, their isolation rate significantly different according to age of diarrheic calves.

E. coli isolates were significantly higher occurrence in diarrheic calves with age <2 month 54/76 (71%), than diarrheic calves with age 2-3months 17/44 (38.6%), irrespective of seasons. On the other hand, *Salmonella* spp. isolates were significantly lower occurrence in diarrheic calves with age <2 month 16/76(21%) than diarrheic calves with age 2-3months 20/44 (45.4%), irrespective of seasons.

Totally, *E. coli* isolates were significantly higher than *Salmonella* species isolates in diarrheic calves with different age and seasons (p-value=0.004) (Table 2).

E. coli were serogrouped to O111, O26, O119, O124, O17 and O8. The most common serogroups were O26 and O111 (35.2% and 32.3 %, respectively), followed by O124, O119, O17 and O8. There was different between the pathotypes of *E.coli* in different seasons as we found that in dry season the prevalence of EHEC and EPEC pathotypes were 64.5% and 35.4% while in rainy season EHEC, EIEC and ETEC pathotypes were 70%, 20%, and 10%, respectively (Table 3).

Serotyping results revealed that *Salmonella* species belonged to 4 different serovars. *S. typhimurium* was accounted for 61.11% of total Salmonella isolates and others serotypes were 38.88% included: *S. enteritidis* (25%), *S. infantis* (11.1%) and *S. essen* (2.8%) as listed in table (3).

Antimicrobial sensitivity test among *E. coli* and *Salmonella* species isolates.

Antibiogram results illustrated the presence of multi-drug resistance in most of *E. coli* and *Salmonella species* isolates recovered from diarrheic calves against the ten used antibiotics. Interestingly, *E. coli* isolates were resistant to most of the tested antibiotics as amoxicillin, colistin, tetracycline, streptomycin, gentamycin sulfamethoxazole/trimethoprim and spectinomycin with percentage of (100%, 100%, 83% , 81,6%, 77.4%, 73.2%,and 67,6%, respectively) while all examined *Salmonella* species isolates exhibited absolute resistance (100%) to them except tetracycline (88.8%), streptomycin (80%) and gentamycin (69.4%). On the other hand amikacin and ciprofloxacin were found to be the effective drugs for *E.coli* and *Salmonella* that showed sensitivity rate 100% for amikacin and in case of ciprofloxacin (78.8% and 77.7%), respectively as listed in table (4).

Virulence and resistant genes among *E. coli* and *Salmonella species* isolates.

Ten MDR *E. coli* isolates were examined by PCR to detect **6** virulence genes (*fim*H, *iss*, *stx1, stx2, eae*A,, and *hly*A) (Fig. 1-3) (Table 5). All isolates were positive for both *fim*H and *iss* genes (100%). Amongst isolates that carried the *stx* genes, 8 (80%) were *stx1*, 9 (90%) were *stx2* and 4(40%) were have both *stx1/stx2*. Moreover, *hly*A gene was detected in 60% of isolates. Only 4 *E. coli* isolates carried *eae*A gene (40%) that includes 3 and 1 isolates in dry season and rainy season, respectively. Our results illustrated that 40% of the analyzed *E. coli* isolates harbored all 6 examined virulence genes and 20% of isolates had at least three virulence genes.

Screening the presence of *tet*A, *aad*B, *aac*C genes in the most MDR *E. coli* by PCR technique revealed that distribution of resistance genes among 10 phenotypically resistant *E. coli* were as the following: all examined isolates were positive for *tet*A genes with percentage of (100%) (Fig. 4) while 3 isolates were positive for *aac*C (30%) (Fig. 5) and *aad*B gene were not detected.

All examined ten MDR *Salmonella species* isolates had both *inv*A and *sop*B virulence genes (100%) (Fig. 6 and 7). Interestingly, the results of *tet*A gene (100%) (Fig. 8) but, *aadB* and *aac*C genes were not detected.

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

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
stx1	ACACTGGATGATC TCAGTGG CTGAATCCCCCTC CATTATG	614	95°C 3 min.	95°C 20 sec	58˚C 40 sec.	72˚C 90 sec.	72˚C 5 min.	**Dhanashree and Mallya, (2008)**
Stx2	CCATGACAACGG ACAGCAGTT CCTGTCAACTGAG CAGCACTTTG	779						
eaeA	GTGGCGAATACTG GCGAGACT CCCCATTCTTTTTC ACCGTCG	890	95°C 3 min.	95°C 20 sec	58˚C 40 sec.	72˚C 90 sec.	72˚C 5 min.	**Mazaheri *et al.*, (2014)**
hlyA	ACGATGTGGTTTA TTCTGGA CTTCACGTGACCA TACATAT	165						**Fratamico *et al.*, (1995)**
iss	ATGTTATTTTCTG CCGCTCTG CTATTGTGAGCAA TATACCC	266	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Yaguchi *et al.*, (2007)**
*fim*H	TGCAGAACGGAT AAGCCGTGG GCAGTCACCTGCC CTCCGGTA	508	94˚C 5 min.	94˚C 30 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Ghanbarpour and Salehi, (2010)**
*inv*A	GTGAAATTATCGC CACGTTCGGGCAA TCATCGCACCGTC AAAGGAACC	284	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Olivera *et al.*, (2003)**
*sop*B	TCAGAAGTCGTCT AACCACTC TACCGTCCTCATG CACACTC	517	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Huehn *et al.*, 2010)**
*aad*B	GAGCGAAATCTGC CGCTCTGG CTGTTACAACGGA CTGGCCGC	319	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	**Frana *et al.*, (2001)**
*aac*C	GGCGCGATCAAC GAATTTATCCGA CCATTCGATGCCG AAGGAAACGAT	448	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Lynne *et al.*, (2008)**
*tet*A*(A)*	GGTTCACTCGAAC GACGTCA CTGTCCGACAAGT TGCATGA	576	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Randall *et al.*, (2004)**

Table 2: Occurrence of *E. coli* and *Salmonella* spp. in diarrheic calves.

Season/Age (n) *	*E. coli* (%)**	*Salmonella spp*. (%)**	*E. coli* and *Salmonella spp*.(mixed)
Dry season <2 month(38)	24 (63.15%)	6 (15.78%)	5(13.15%)
2-3 months (22)	7 (31.81%)	8 (36.36%)	6(27.27%)
Total of dry season (60)	31 (51.6%)	14 (23%)	11 (18.33%)
Rainy season <2 month(38)	30 (78.94%)	10 (26.31%)	6(15.78%)
2-3 months (22)	10 (45.45%)	12 (54.54%)	9(40.90%)
Total of rainy season (60)	40 (66.6%)	22 (36.6%)	15(25%)
Total of two seasons (120)	71 (59.16%)	36 (30%)	26(21.66%)
Mean± SE* **	17.75±5.51	9±1.29	6.50±0.86

* Significant differences in isolation rate according to seasons and ages diarrheic calves (P> 0.05).
** Significant differences between isolation rate of *E. coli* and *Salmonella species* in diarrheic calves (P <0.05).*** mean ± stander error

Table 3: serological identification of *E. coli* and *salmonella* species isolated from diarrheic calves in different seasons.

Isolates(n)	Season/ Age							
	Dry season				Rain season			
	<2month		2-3 months		<2month		2-3 months	
E.coli (71)	**EHEC** (14)	O111 (5)	**EHEC** (6)	O111 (3)	**EHEC** (21)	O111 (12)	**EHEC**(7)	O111 (3)
		O26 (9)		O26 (3)		O26 (9)		O26 (4)
	EPEC (10)	O17 (3)	**EPEC** (1)	O17	**EIEC** (6)	O124	**EIEC** (2)	O124
		O119 (7)			**ETEC** (3)	O8	**ETEC** (1)	O8
Salmonella (36)	*S.typhimurium* (5)		*S.typhimurium*(6)		*S.typhimurium* (5)		*S.typhimurium* (6)	
					S. enteritidis(4)		*S. enteritidis*(5)	
	S.infantis (1)		*S.infantis* (2)		*S.infantis* (1)		*S. essen*(1)	

EHEC (Enterohemorrhagic Escherichia coli), EPEC (Enteropathogenic Escherichia coli), ETEC (enterotoxigenic Escherichia coli, EIEC (Enteroinvasive Escherichia coli).

Table 4: Antimicrobial susceptibility profiles of *E.coli* and *Salmonella* spp. isolates.

Antimicrobial agent	*E. coli* (71)		*Salmonella* spp.(36)	
	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)
amikacin(AK)	71 (100%)	0 (0%)	36 (100%)	0 (0%)
ciprofloxacin (CIP)	56 (78.8%)	15 (21.1%)	28 (77.7%)	11 (30.5%)
ceftriaxone (CTX)	37 (52.1%)	34 (47.8%)	0 (0%)	36 (100%)
spectinimycin(SPT)	23 (32.3%)	48 (67.6%)	0 (0%)	36 (100%)
sulfamethoxazole/trimethoprim (SXT)	19 (26.7%)	52 (73.2%)	0 (0%)	36 (100%)
gentamicin (CN)	16 (22.5%)	55 (77.4%)	11 (30.5%)	25 (69.4%)
streptomycin (S)	13 (18.3%)	58 (81.6%)	7 (19.4%)	29 (80.5%)
tetracycline (TE)	12 (17%)	59 (83.1%)	4 (11.1)	32 (88.8)
amoxicillin (AX)	0 (0%)	71 (100%)	0 (0%)	36 (100%)
colistin(CT)	0 (0%)	71 (100%)	0 (0%)	36 (100%)

Table 5: Distribution of virulence and resistant genes of *E. coli* and *Salmonella species* from diarrheic calves.

Isolates/genes(n)	Dry season	Rainy season	Total (%)
E. coli* (10)** ***stx1	4	4	8(80%)
stx2	5	4	9(90%)
***eae*A ***	3	1	4(40%)
***hly*A**	3	3	6(60%)
iss	5	5	10(100%)
fimH	5	5	10(100%)
tetA(A)	5	5	10(100%)
aacC **	1	3	4(40%)
aadB	ND	ND	ND
Salmonella spp.* (10)** ***invA	5	5	10(100%)
sopB	5	5	10(100%)
tetA(A)	5	5	10(100%)
aacC	ND	ND	ND
aadB	ND	ND	ND

* Significant differences according to seasons in presences of *eae*A gene (P> 0.05).
****** Significant differences according to seasons in presences of *aac*C gene (P> 0.05).

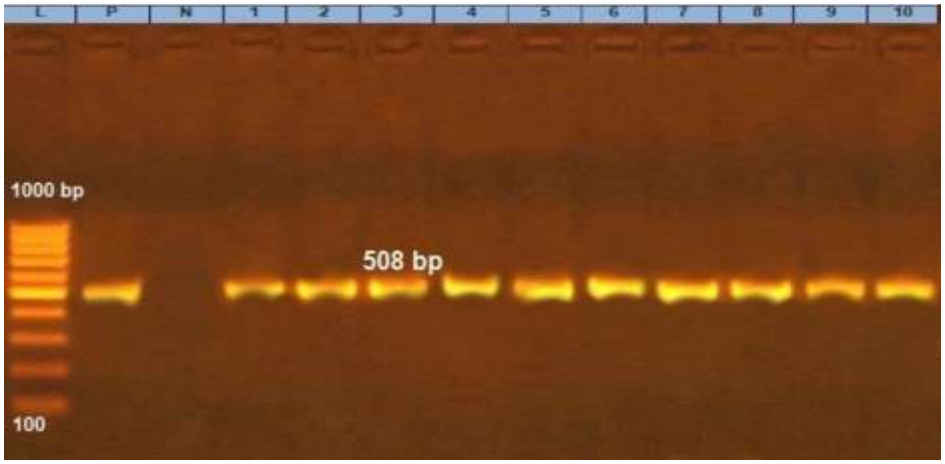

Figure 1: PCR amplicons of ***fimH*** gene of *E.coli*. Lane L: 100-bp ladder; lane Neg.: negative control; lane Pos.: positive control; lanes 1–10: positive isolates at 508 bp amplicons.

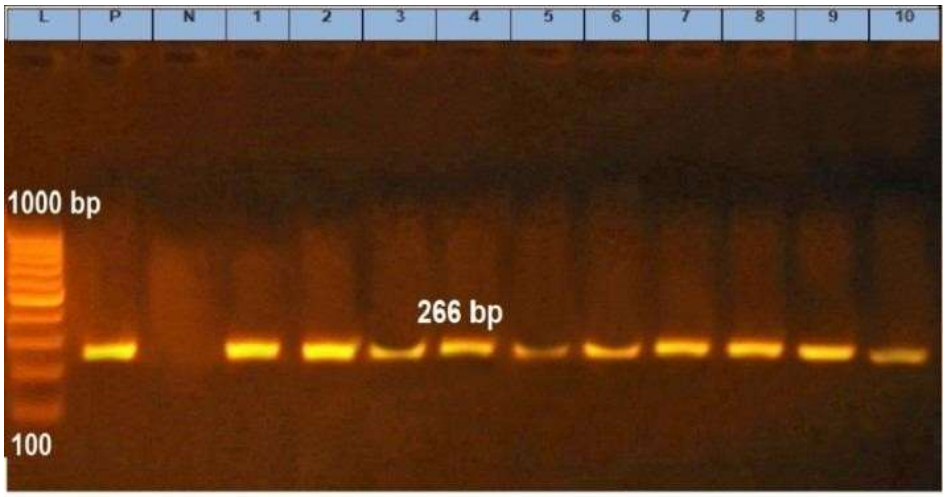

Figure 2: PCR amplicons of *iss* gene of *E.coli*. Lane L: 100-bp ladder; lane Neg.: negative control; lane Pos.: positive control; lanes 1–10: positive isolates at 266 bp amplicons.

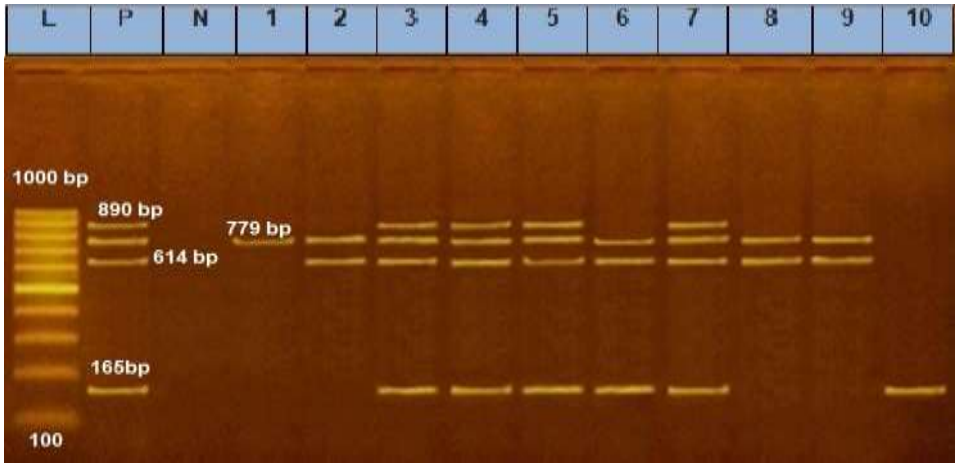

Figure 3: Multiplex PCR of *stx*1 (614 bp), *stx*2 (779 bp), *eae*A (890 bp) and *hly*A (165 bp) virulence genes of *E. coli.* L:100-bp ladder; lane Neg.: negative control; lane Pos.: positive control

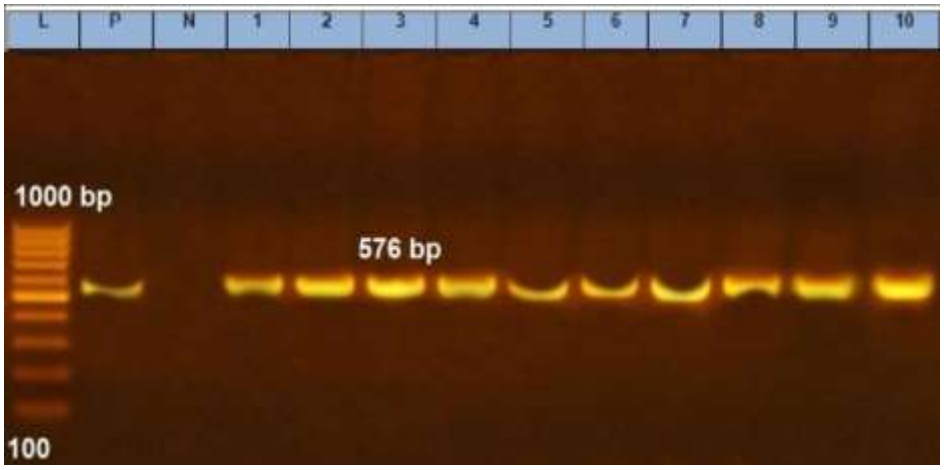

Figure 4: PCR amplicons of ***tetA*(A)** gene of *E.coli*.. Lane L: 100-bp ladder; lane Neg.: negative control; lane Pos.: positive control; lanes 1–10: positive *E.coli* isolates at 576 bp amplicons.

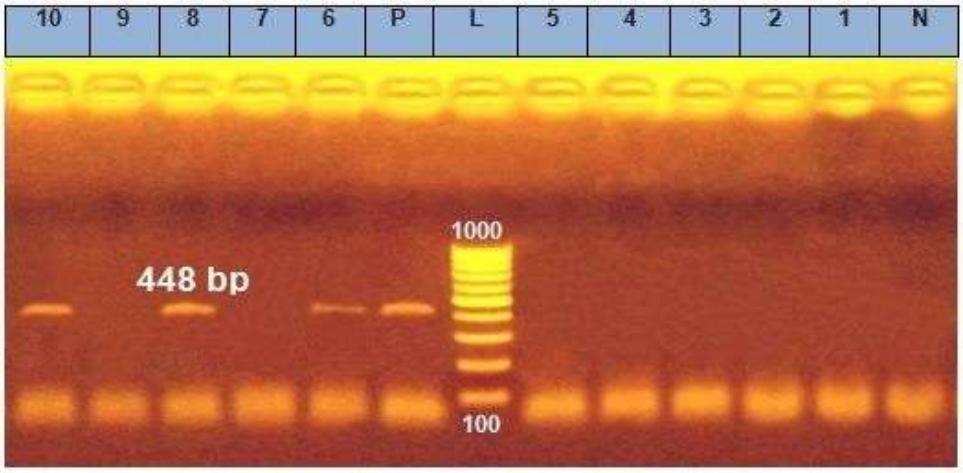

Figure 5: PCR amplicons of *aacC* gene of *E.coli*.. Lane L:100-bp ladder; lane Neg.: negative control; lane Pos.: positive control; lanes 6,8,10: positive *E.coli* isolates at 448 bp amplicons.

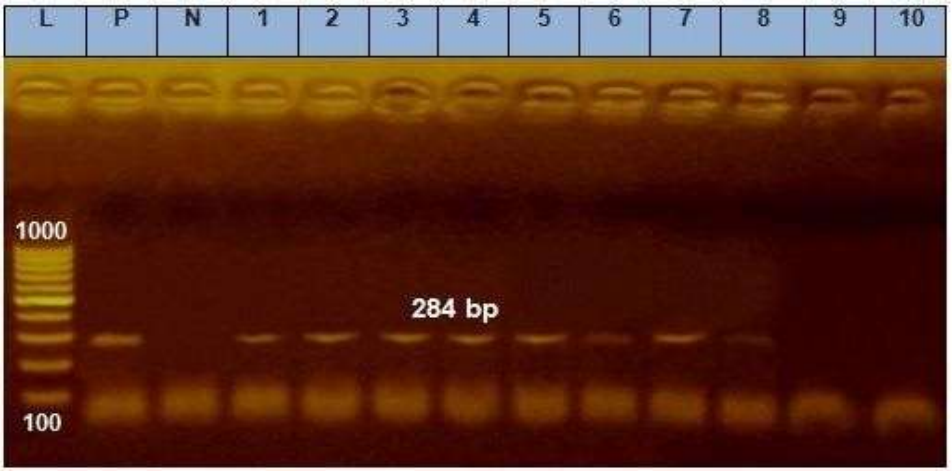

Figure 6: PCR amplicons of ***invA*** gene of *salmonella*. Lane L:100-bp ladder; lane Neg.: negative control; lane Pos.: positive control; lanes 1–8: positive salmonella isolates at 284 bp amplicons; lane 9-10: negative isolates

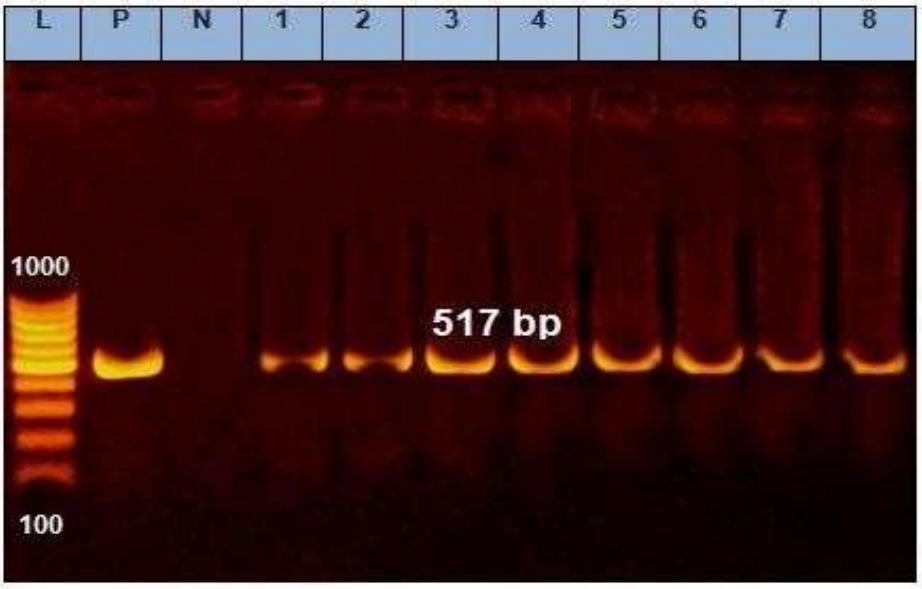

Figure 7: PCR amplicons of sopB gene of salmonella. Lane L:100-bp ladder; lane Neg.: negative control; lane Pos.: positive control; lanes 1–8: positive salmonella isolates at 517 bp amplicons

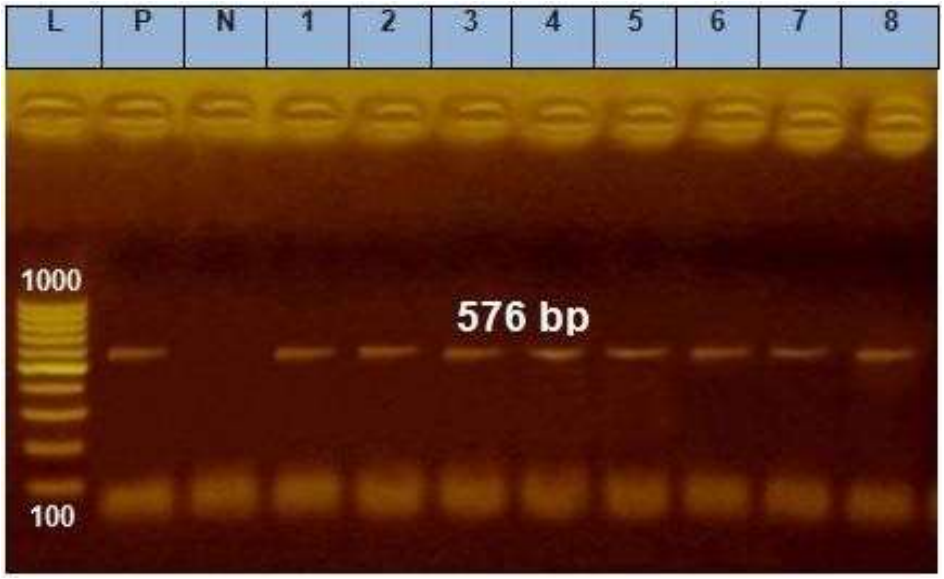

Figure 8: PCR amplicons of tetA(A) gene of salmonella. Lane L:100-bp ladder; lane Neg.: negative control; lane Pos.: positive control; lanes 1–8: positive salmonella species isolates at 576 bp amplicons

DISCUSSION

Neonatal calf diarrhea (NCD) remains as one of the most important problems encountered by livestock industry, causing great economic losses. Calves are at greatest risk of diarrhea within the first month of life and the incidence of diarrhea decreases with age (Garcia *et al.*, 2000).

Herein, the prevalence of *E. coli* and *Salmonella* in neonatal diarrheic calf was 59.5% and 30%, respectively. From examined samples 18.33 % and 25% had mixed *E. coli* and *Salmonella* species

infection in both dry and rainy season, respectively.

The prevalence of *E. coli* in the current study had nearly coincided with the findings of Osman *et al*. (2013) (63.6%), and Hassan (2014) (50%), while higher than those of Azzam *et al.* (2006) (5.4%) and El-Shehedi *et al*. (2013) (35.83%), and lower than that obtained by Ibrahim, (1995) (100%). In comparison to other countries, the present results were similar to that mentioned by Hemashenpagam *et al*. (2009) in India (75%), while it was higher than those obtained by Joon and Kaura (1993) in India (23%), Viring *et al.* (1993) in Sweden (11.5%), Steiner *et al*. (1997) in Germany (42%).

E.coli isolation rate in rainy season was significant higher than dry season. Similar finding obtained by Masoud *et al.* (2014) who found that prevalence of *E.coli* in samples collected in the winter had (54.92%), but in summer (2.53%). One possible explanation for the high prevalence of *E. coli* strains in calves in winter is that climatic variables such as heat, rain and thunderstorms, together with variable barometric pressure may have affected the autonomic nervous systems. These variables could affect immunity, thus making calves more susceptible to infections. Alternatively, Norheim *et al.* (1985) stated that the higher prevalence of *E. coli* strains in winter may be related to the fact that the mean serum IgG1 concentrations were low in winter born calves and increased during the spring and summer.

We found that in case of calves less than 2 month age had the highest incidence of *E. coli* (71%), while the 2-3 months age calves had the lowest incidence (38.6%) similarly to previous mentioned by Masoud *et al.* (2014). Similarly, another study found that *E. coli* is the most common agent causing diarrhea in calves during the first week of life (Pereira *et al.*, 2011 and Wagdy *et al.*, 2016).

Serogrouping of *E. coli* isolates showed that six O-serogroups were identified; out of them O26 and O111 were the most prevalent groups (35.2% and 32.3 %, respectively) followed by O124, O119, O17 and O8. These findings were also nearly similar to that obtained by Wieler *et al*. (1998) who revealed that *E. coli*, particularly serogroups O26, O111 and O118 are virulent to cattle, particularly young calves. Also, Pearson *et al.* (1999) mentioned that *E. coli* O26 has been isolated from clinical cases of hemorrhagic diarrhea in young and neonatal patients. EHEC Pathotype was the most common pathogenic *E.coli* isolates (67.6%) obtained from examined diarrheic calves, while, higher than stated by Pereira *et al.* (2011) (38%). On contrary to previously recorded by Nataro and Kaper (1998) who found the most common cause of neonatal diarrhea is ETEC stains. Interestingly, we found that in dry season the prevalence of EHEC and EPEC pathotypes were 64.5% and 35.4%, respectively. EHEC, EIEC and ETEC pathotypes in rainy season were 70%, 20%, and 10%, respectively. The previous study by Nasr-Eldin *et al.* (2018) characterized pathogenic *E.coli* and the common detected pathogroups were EPEC and EHEC, ETEC and EIEC. Conversely, another study by Borriello *et al.* (2012) reported infrequent occurrences of ETEC strains.

The prevalence of *Salmonella* in the present study (30%) was similar to those of other studies in Australia by Izzo *et al.* (2011) (23.8%), while higher than that

reported by Riad *et al.* (1998) (18.2%); Seleim *et al.* (2004) (17.5%); Youssef and El-Haig, (2012) (18.66%); Haggag and Khaliel, (2002) (4%), and Younis *et al*. (2009) (4.09%). On contrary, much higher prevalence was reported by Moussa *et al.* (2010) (43.53%) and Akam *et al.* (2004) (66.6%).

In our study the differences in prevalence rates of *Salmonella* and *E.coli* isolated from diarrheic calves in comparison to the previous studies could be explained in the light of species and geographical locations and hygienic measures, and these factors significantly influence the prevalence of salmonellosis in calves (Younis *et al.*, 2009).

The diarrheic calves in both dry and rainy season with age <2 months had the lower *Salmonella* species isolation rate (21%) than diarrheic calves with age 2-3 months (45.4%) that in accordance with finding of Wagdy *et al.* (2016) and Achá *et al.* (2004) whom stated that *E. coli* and *Salmonella* are the most common identified pathogens in scouring calves less than 2 months of age.

Of interest, *S. typhimurium* (ST) and *S. enteritidis* (SE) were the most prevalent serotypes with percentages 61.1% and 25%, respectively. This result supported by many previous reports in Egypt (Seleim *et al.*, 2004; Younis *et al.*, 2009; Moussa *et al.*, 2010 and Youssef and El-Haig, 2012) and reports from the other countries by Smith-Palmer *et al.* (2003) in Scotland and Rothenbacher (1965) in the USA. On contrary to previous obtained by El-Seedy *et al.* (2016) who found that *S. enteritidis* (60.9%), *S. typhimurium* (30.4%).

Antimicrobial resistance of *Salmonella and E.coli* is particularly worrisome in view of its potential to extend into the human food chain, posing a challenge to public health. The data from the present study showed widespread resistance of *E. coli* and *Salmonella* species isolates against the tested antibiotics especially against amoxicillin and colistin (100%) followed by tetracycline (83% and 88.8%, respectively) that in consistence with previous related studies on antimicrobial resistance (De Verdier *et al.*, 2012 and Call *et al.*, 2008). On the other hand, they were sensitive to amikacin (100%) followed by ciprofloxacin (78.8%, 77.7%, respectively) as reported previously by El-Seedy *et al.* (2016). Similar findings have been found by Pereira *et al.* (2011) who found that the most prevalent multidrug resistant pattern within all isolates was tetracycline-ampicillin-streptomycin-sulfamethoxazole-trimethop. Call *et al.* (2008) attributed the epidemiology of resistant *E. coli* in calves to be multifactorial, complex that influenced by co-selection due to linkage of resistance genes. Totally, most of isolates were resistant to at least 6 of the tested antimicrobial agents, making them MDR. This finding was in agreement with that reported by Pereira *et al.* (2011). This is not surprising in view of the high level of resistance observed for almost all the *Salmonella* and *E.coli* in this study, representing a significant disease burden in Egypt. This resistance may be attributed to indiscriminate use of antibiotics at recommended doses or at subtherapeutic doses as feed additives to promote growth, and as chemotherapeutic agents irrespective of etiological agents (Kumar *et al*., 2010 and Verma *et al*., 2007).

The use of PCR-based technology to identify virulence genes has become widely adopted to distinguish pathogenic

E. coli strains from normal gut flora. Concerning *E.coli*, PCR was applied on 10 isolates representing all the recovered serogroups for screening some virulence genes, all *E.coli* isolates were harbored both *fim*H and iss genes. Similar prevalence has been found by De Verdier *et al.* (2012). Our findings contradicted to that recorded by Wagdy *et al.* (2016) who found that *iss* gene was not detected in any isolates while *fimH* were positive in all isolates (100%). Nguyen *et al.* (2011) reported that 31.30% of *E. coli* strains of diarrheic calves had one of the fimbrial genes.

Amongst isolates that carried the *stx* genes: (80%) were *stx1*, (90%) were *stx2* and (40%) were have both *stx1/stx2*. Similarly, high percentages (40% or more) of *stx* gene in *E. coli* strains (STEC) have been reported in Brazil by Salvadori *et al.* (2003) and in India by Arya *et al.* (2008). However, Osek *et al.* (2000) has reported a lower rate (less than 10%) of STEC in diarrheic calves. Only 4 *E. coli* isolates carried *eae*A gene (40%) includes three isolates in dry season and one rainy season, respectively that higher than reported by Natalia *et al.* (2015) who found that *eae*A gene was (12.8%).

Interestingly, the *hly*A gene was detected at high percent (60%) as it considered pathogenic virulence factor, supported our results Wieler *et al.* (1992) who mentioned that enterohemolysin *(hly*A) gene can be used as a diagnostic marker because its presence is strongly correlated with Shiga toxin. Nataro and Kaper (1998) stated that the role of enterohemolysin *hly*A gene in causing diarrhea in calves has not been demonstrated, but may be compliment the effects of Shiga toxin.

We found that the EPEC pathoytpe were expressed both *sxt*1 and *sxt*2 beside *iss* and *fim*H genes but most of the virulence profiles were compatible with EHEC strains and almost equally distributed in the both dry and rainy seasons. While, Natalia *et al.* (2015) found that most of the virulence profiles were compatible with ETEC strains.

Our results illustrated that 40% of the analyzed *E. coli* isolates harbored all 6 examined virulence genes and 20% of isolates had at least three virulence genes. The successful combinations of virulence factors have persisted to become specific *E. coli* pathotypes that are capable of causing disease in healthy individuals Kaper *et al.* (2004). Another study recorded that (30.1 %) were found to be positive for at least one of the virulence genes Natalia *et al.* (2015).

PCR was applied on 10 isolates of *Salmonella* species to determine the virulence *inv*A gene that encodes a protein in the inner membrane of bacteria, which is responsible for invasion to the epithelial cells of the host. Malorny *et al.* (2003) mentioned that *inv*A gene has been recognized as an international standard for detection of Salmonella. The results revealed that all tested isolates harbored *inv*A gene (100%). These results were similar to those obtained by Soliman (2014). With respect to PCR screening of *sop*B gene in *Salmonella* species isolates found that all isolates had *sop*B gene which similar to results obtained by Rahman, (2006). This study revealed that there was association between virulence genes and antimicrobial resistance of the tested *E. coli* and *Salmonella species* isolates, similar to previous study by Wagdy *et al.* (2016).

Of interest, PCR screening for some resistant genes among phenotypically MDR *E. coli* isolates showed that all isolates were positive for *tet*A genes with percentage of (100%) while 3 isolates were positive for *aac*C (30%) and *aad*B gene not detected. In case of examined MDR *Salmonella species*, harbored *tet*A(A) gene (100%) but, *aad*B and *aac*C genes were not detected The prevalence of *tet*A gene was relatively in accordance with phenotypic antimicrobial susceptibility in *E. coli* isolates as mentioned before by Chopra and Roberts (2001). In case of examined MDR *Salmonella species*, *aad*B and *aac*C genes were not detected on contrary to previously study by Masoud *et al.* (2014) who found that genes encode resistance to streptomycin, sulfonamide, gentamicin, ampicillin and trimethoprim antibiotics, were the most common antibiotic resistance genes in the diarrheic calves.

The discrepancies between genotypic and phenotypic antimicrobial sensitivity results for streptomycin and gentamicin in *E.coli* and *Salmonella* spp. were justified by the possibility of carrying other drug resistance genes or harboring some other extra-chromosomal genetic elements or antimicrobial resistance by other resistance mechanism as efflux pump (Jouini *et al.*, 2009). The wide distribution of *tet*A gene across gram-negative genera, including *Escherichia and Salmonella*, indicates that it is highly likely that horizontal transfer of tetracycline resistance occurs (Chopra and Roberts, 2001).

CONCLUSION

This work provides updated information on the molecular characterization of MDR pathogenic *E. coli* and *salmonella* spp. isolates obtained from diarrheic calves. In rainy season pathogenic *E. coli* and *Salmonella species* were higher isolation rate than dry season. Furthermore, EHEC (O26) and *S. Typhimurium* were most predominant serogroups. Almost of pathogenic isolates were resistances to commercial used antibiotics. Therefore, we advise for routine bacteriological screening of fecal samples from diarrheic calves followed by testing for antimicrobial susceptibility that are important to formulate a suitable treatment against *E. coli* and *Salmonella species*, to reduce or prevent the losses. Application of strict hygienic measures in rearing calves especially in rainy season and calves age less than 2 months to decrease infections.

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العوامل المؤثره على عدوى الاسهال فى العجول المسببه بالسالمونيلا و الايشيرشيا كولاى

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يعتبر الإسهال فى العجول مصدر خسائر اقتصاديه كبيره في صناعة الماشيه في جميع أنحاء العالم. لذلك ، تم تصميم هذه الدراسة للتحقيق من مدى تاثير التغيرات الموسميه والعمريه في معدل انتشار الايشيريشيا كولاى و السالمونيلا E. coli)و Salmonella) في العجول التى يظهر عليها الإسهالات. و ايضا دراسه جينات الضراوة وأنماط السلالات وقياس درجه الحساسية للمضادات الحيويه لكلا من الايشيريشيا كولاى و السالمونيلا. تم جمع ١٢٠ عينة براز من العجول المصابه بالاسهال فى عمر اقل من ثلاثة اشهر فى المواسم المختلفه (الجافة والممطرة). بعد الفحص البكتريولوجي ، كانت ٧١ (٥٩,٥٪) و ٣٦ (٣٠٪) إيجابية لكلا من الايشيريشيا كولاى و السالمونيلا على التوالي و ٢٦ (٢١,٦٦٪) كانت مختلطة بعدوى الايشيريشيا كولاى و السالمونيلا. فوجد ان أنماط السلالات الأكثر انتشارا من ميكروب الايشيريشيا كولاى هي O111 و O26 و السالمونيلا كانت S. Typhimurium و S. enteritidis.و كانت معزولات الايشيريشيا كولاى الاكثر عددا بشكل ملحوظ عن معزولات السالمونيلا في العجول بمختلف الأعمار والمواسم (قيمة P = 0.004). كما ان نسبه عزل الايشيريشيا كولاى و السالمونيلا كانوا أعلى بشكل ملحوظ في موسم الأمطار عن موسم الجفاف. جميع عزلات الايشيريشيا كولاى كانت ايجابية بالنسبة لـجينات الضراوه fimH و iss و٩٠%و ٨٠%و ٦٠%و ٤٠٪ لجينات *Stx2 stx1* *hly*A, *eae*A على التوالى وجميع معزولات السالمونيلا كان لديها جينات الضراوة invA و sopB. أظهرت معزولات الايشيريشيا كولاى و السالمونيلا معدل حساسية عالي للأميكاسين (١٠٠٪) وسيبروفلوكساسين (٧٨,٨٪ و ٧٧,٧٪) على التوالي ولكن أظهرت مقاومة ضد الأموكسيسيلين والدوكسيسيكلين والاستربتوميسين والجنتامايسين.و كان جين tetA الأكثر شيوعًا من بين جينات المقاومه التى تم قياسها. وأخيرًا ، ينبغي رفع مستوى الوعي حول الإجراءات الصحية والنظرية الصارمة خاصة في المواسم الممطرة في تربية العجول حديثي الولادة.