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Original Research Article

Monitoring and molecular characterization of multidrug resistant enteropathogenic *E. coli* in dairy calves and their environment

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ABSTRACT

This study was performed to investigate the frequency and the distribution of antimicrobial resistance and resistant genes in enteropathogenic *Escherichia coli* (EPEC) isolated from both calves and their environment. Fecal samples (n=136) were collected from calves, besides 270 environmental samples for isolation and identification of EPEC. 50 *E. coli* isolates comprising 6 serogroupgs were recovered and tested against 12 antimicrobials comprising 4 different groups and 3 disinfectants with characterization of resistance genes. Results revealed that *E. coli* was isolated in the highest percentage from diarrheic calves (68.3%) followed by apparently healthy and environment (56.7 and 56.6 %, respectively). Six serogroups of *E. coli* were identified from diarrheic calves with the highest percentage of O_{26} (27.8 %) followed by O_{159} and O_{55} (16.7 and 16.6 %, respectively).The tested isolates showed high rates of resistance to tetracycline, ampicillin, and sulfamethoxazole-trimethoprim whereas the highest rates of susceptibility were found to enrofloxacin and neomycin. Meanwhile the highest bactericidal effect against *E. coli* isolates from calves and environment was exhibited by Virkon® S (80 % and 70 %, respectively) compared to 80 % and 50%, respectively for TH^{4+} and 60 and 70 %, respectively for iodine. *E. coli* isolates were found to include the following genes bla_{TEM}, *qacED1*, *dfrA*, *tetA*, and *sul1*. In conclusion, the high prevalence of multidrug resistant *E. coli* provided insights about the possibility of dairy calves to act as source of resistance genes in the environment that pose health risk for humans and animals.

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1. Introduction

Calf diarrhea is one of the most economic and pervasive concern in veterinary industry all around the world. It constitutes substantial costs in terms of calf mortality, veterinary costs, and loss in calf value. Calf diarrhea is a disease complex characterized by acute undifferentiated diarrhea in young calves up to 3 months of age. It is a multifactorial disease, where not only the causative pathogenic agent affects its outcome but also calf age, management, and environmental factor (Bruning-Fann and Kaneene, 1992). *E. coli* and Salmonella are considered the most common and economically important causes of diarrhea in young calves (Muktar *et al.*, 2015). Shigella, Klebsiella and *C. perfringens* have also been identified among the bacterial causes of calf diarrhea. The prevalence of each pathogen and the disease incidence can vary by farm location, management practices, and herd size (Acha *et al.*, 2004).

Although *E. coli* is potentially pathogenic, it is a normal inhabitant of the gastrointestinal tract of worm blooded animals. *E. coli* can be responsible for the transfer of resistance genes that reflect the selective pressure from use of antimicrobials in treatment practices of populations (Van den Bogaard and Stobberingh, 2000). Monitoring enteric *E. coli* is essential as indicators for antimicrobial resistance in animal populations (Franklin *et al.*, 2001).

Bacterial antimicrobial resistance is an emerging and serious public health concern especially in veterinary medicine, due to the possible transfer of resistance genes between animal and human strains (Martinez and Baquero, 2002). The husbandry and management practices followed in several dairy farms contribute to the high frequency of antimicrobial resistance, such as raising calves in calf barns with low level of hygiene exposing them to pathogens and disease with subsequent use of antimicrobials. Calves housed separately to control infection are fed milk or milk replacer, which may contain antibiotics. Another popular practice in the veterinary medicine is using sub-doses of antibiotics as growth promoters (Svensson *et al.*, 2003**)**.

In Egypt, overuse and/or abuse of antimicrobials are common in stockbreeding, which possess high risks of antimicrobial-resistance contaminations. Knowledge of used antibiotics and local antimicrobial resistance patterns are essential to guide empirical and pathogen-specific therapy and critical for optimal decisions regarding infection control policies and help assessing the magnitude of the resistance problem (Doyne *et al.*, 2004).

The overriding purposes of this study was to monitor the frequency and distribution of multi-drug resistant strains of *E. coli* associated with calf diarrhea and their environment and some genes responsible for their resistance in a dairy herd.

2. Materials and methods

2.1. Study site and animal population

This study was carried out in a private dairy farm located in Beni-Suef district during the period from September 2014 till April 2016; it included 183 dairy cows of different production stages besides 83 calves of different ages (1 week to three months). Calve were housed separately from adult herd in completely sheltered yards each containing 25 calves with earthy floor. Water was provided ad libitum from common water trough. Newly born calves were separated from their dams soon after birth and receive colostrum for the first three days of age from buckets ad libitum followed by milk replacer that contain neomycin antibiotic as a growth promoter and concentrates for elderly calves. No special attention was paid for cleaning and disinfection of milk equipment or buckets. The general hygienic conditions prevailing in the farm was fair.

2.2. Structure questionnaire

A questionnaire was designed and used to collect data at both the individual animal and farm levels, including the following information (numbers of cows, numbers of un-weaned calves, calves age, calf management (time for cow-calf separation, feeding , use of calf group feeder and rearing systems), routines for antimicrobial treatment, number of calves with diarrhea in the herd, calf mortality.

2.3. Study design

A cross-sectional study was carried out to investigate the prevalence and outline the most predominant bacterial agents causing diarrhea in calves by different cultural, biochemical and serological techniques, and to test their antimicrobial sensitivity *in-vitro* and then characterize resistance genes in calves and their surrounding environment. Samples were collected monthly from calves and their surrounding environment using stratified sampling technique throughout the study period.

2.4. Collection of samples

A total number of 136 fresh fecal samples were collected directly under aseptic condition from the rectum of both apparently healthy and diarrheic calves using sterile rectal swabs that were kept in sterilized containers and preserved on ice to be transferred to the

lab of animal hygiene in the Faculty of Veterinary Medicine, Beni-Suef University. Meanwhile, 270 environmental samples including individual milk samples, water samples, trough swabs, swabs from attendants' hands, soil samples that were collected from different sites particularly from the wetted area with high organic load and flies samples that were collected using baited jug traps (NMC, 1999; APHA, 1989; Clegg *et al.*, 1983 and Watson *et al.*, 1994). Samples were properly identified and immediately sent to the lab for further microbiological examination.

2.5. Isolation and identification of *E. coli*

All samples were diluted in phosphate buffered saline at 37°C for 24hrs for isolation of *E. coli* then loopful was cultured on MacConkey's agar (Oxoid; CM0115) and incubated at 37°C for 18-24hrs. Lactose positive colonies were cultured on Eosin Methylene Blue (Oxoid; CM 69) and incubated at 37C for 24hrs. Metallic green colonies were selected and sub cultured on nutrient agar for identification. Gram staining technique was applied and Gram negative short bacilli were selected and the following biochemical tests were applied TSI, indol, citrate utilization, urease and methyl red tests according to Collee *et al*. (1996) as well as RapID® (Remel; USA) kits which were used for *E. coli* confirmation.

2.6. Serogrouping of *E. coli*

Forty two isolates of *E. coli* (16 form calves and 26 from environment) were serogrouped by slide agglutination test using standard polyvalent and monovalent *E. coli* antisera (Edwards and Ewing, 1972)

2.7. *In-vitro* antimicrobial susceptibility testing

The efficacy of 4 different classes of antimicrobials (tetracyclines, β- lactamases, sulphonamides and phenicols) and 3 different disinfectants; TH^{4+} 0.5% (SoGeVal, France), Virkon®S 1% (Antec international TD, UK) and iodine 5% (Oxoid, USA) were tested against 50 *E. coli* isolates (20 from diarrheic calves and 30 from their environment).

2.7.1. Antibiotic sensitivity testing (CLSI, 2012)

Fifty *E. coli* isolates from calves and their environment were cultivated on Muller-Hinton agar under aerobic condition (at 37°C for 24hrs) for testing their susceptibility to 12 commonly used antibiotics (ampicillin 10µg, amoxicillin 10µg, chloramphenicol 30µg, oxytetracycline 5µg, sulfamethoxazole/trimethoprim 25µg, tetracycline 30µg, neomycin 30µg, florofenicol 30µg, penicillin 10µg, erythromycin 15µg, enrofloxacin 5µg and cefoxitin 30µg).

2.7.2. Disinfectant sensitivity testing

The same *E. coli* isolates subjected to antibiotic sensitivity were screened for susceptibilities to the following disinfectant TH^{4+} 0.5% (SoGeVal, France), Virkon® S 1% (Antec international TD, UK) and iodine 5% (Oxoid, USA) at different exposure times (5 min., 10 min., 15 min. and 30 min.) using broth macro dilution test Standard strain of *E. coli* ATCC 25922 was applied as a quality control positive organism (Costa *et al.*, 1998).

2.8. Detection of antimicrobial resistance genes using PCR

PCR was applied on 6 multidrug resistant (MDR) *E. coli* isolates (3 from calves and 3 from the environment) that showed resistance to 3 or more different antimicrobial agents, for detection of following 8 resistance genes *tetA*(A) (responsible for resistance to tetracyclines), bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1}$ (responsible for resistance to β-lactams), *Sul1* (responsible for resistance to sulfonamides), *dfrA* (responsible for resistance to trimethoprim), *floR* (responsible for resistance to phenicols), and *qacED1* (responsible for resistance to quaternary ammonium compound, QAC) **(Table 1)**.

2.8.1. DNA extraction

All *E. coli* isolates were cultured on Peptone water (at 37°C for 24 hrs). One hundred and fifty microliters of cultured PW media were added to four hundred microliters from sterile distilled water. All components were boiled for 12 min. This suspension was frozen and then centrifuged at 14000 rpm for 14 min.

2.8.2. PCR screening for antimicrobial resistance genes

The *E. coli* strains were grown in 500 µl LB broth overnight, and 20 µl of the culture was transferred to 200 µl lysis buffer [0.1 M Tris-HCl (pH 8.5), 0.05% Tween 20, and 0.24 mg/ml proteinase K]. The samples were incubated at 60C for 1 hour and subsequently heated at 97C for 15 min (Lanz *et al.*, 2003). The PCR primers are compiled in Table (1). The multiplex PCRs were all performed with a total 25µl reaction mixture and a Qiagen multiplex PCR kit (Qiagen, Shanghai) with 1 µl Qiagen multiplex PCR master mixture, 1× Q-solution, and 1× primer mixture according to the manufacturer's instructions. The PCRs were performed as follows: 1 cycle of 4 min at 95°C; 35 cycles, each consisting of 1 min at 95°C, 1 min at annealing temperature, and 1 min at 72°C; and 1 cycle of 7 min at 72°C list of primers used showed in **Table (1).**

2.9. Statistical analysis

Data were recorded using the Microsoft excel spread sheet then prepared for analysis. The prevalence of diarrhea in dairy farms and frequent distribution of bacteria in both animals and environmental samples were calculated by the use of non-parametric tests (Chi-square test, K independent sample) using statistical package for social sciences (SPSS, version 20.0).

Table 1 Oligonucleotide primers sequences of antimicrobial resistance genes in *E. coli*

Primer	Sequence	Amplified product	Reference
TetA(A)	*5′- GGTTCACTCGAACGACGTCA-3′* *5′- CTGTCCGACAAGTTGCATGA-3′*	576 bp	Randall *et al.* 2004
Sul1	*5′- CGG CGT GGG CTA CCT GAA CG-3′* *5′- GCC GAT CGC GTG AAG TTC CG-3′*	433 bp	Ibekwe *et al.*, 2011
dfrA	*5′- AGC ATT ACC CAA CCG AAA GT-3′* *5′- TGT CAG CAA GAT AGC CAG AT-3′*	817 bp	Huovinen *et al.*, 1995
floR	*5′- TTTGGWCCGCTMTCRGAC-3′* *5′- SGAGAARAAGACGAAGAAG-3′*	494 bp	Doublet *et al.*, 2003
qacED1	*5′- TAA GCC CTA CACAAA TTG GGA GAT AT-3′* *5′- GCC TCC GCA GCG ACT TCCACG-3′*	362 bp	Chuanchuen *et al.*, 2007
bla$_{TEM}$	*5′- ATCAGCAATAAACCAGC-3′* *5′- CCCCGAAGAACGTTTTC-3′*	516 bp	Colom *et al.*, 2003
bla$_{SHV}$	*5′- AGGATTGACTGCCTTTTTG-3′* *5′- ATTTGCTGATTTCGCTCG-3′*	392 bp	
Bla$_{OXA-1}$	*5′- ATATCTCTACTGTTGCATCTCC-3′* *5′- AAACCCTTCAAACCATCC-3′*	619 bp	

3. Results

3.1. Frequency of bacterial isolates from calves

The frequency of bacteriological isolation from fecal samples of both diarrheic and healthy calves were 45/49 (91.8%) and 27/87 (31.03%), respectively, and with a total prevalence of 72/136 (52.9%). A total of 93 bacterial isolates were recovered (30 and 63 from healthy and diarrheic calves, respectively). *E. coli* was the most prevalent isolates from fecal samples of examined calves (60/136; 64.5%). Moreover *E. coli* was the most prevalent isolates recovered from both diarrheic and apparently healthy calves (68.3 and 56.7% respectively) (at X2= 122.28, P <0.0001), followed by *Klebsiella* (16.1%), *C. perfringens* (11.8%), *Shigella* (4.3%), and finally *Salmonella* (3.2%) (Table 2).

3.2. Frequency of bacterial isolates from environment

Out of 270 environmental samples 128 were bacteriologically positive (47.4%) of which 150 bacterial isolates were recovered. *E. coli* was the most prevalent isolates (68 isolates with a prevalence rate 45.3%) followed by *Klebsiella*, *Salmonella*, *Shigella* and *C. perfringens* (32.0, 10.7, 7.3 and 4.6 %, respectively**)** (at X2= 487.63, P<0.001**)** (Table 3). Among the 68 *E. coli* isolates obtained from environmental samples the highest percentage was recovered from soil (66.0%) followed by flies (47.8%), water trough (39.1%), attendant's hands (38.5%) and feed manager (30.8%), while the least percentage was recovered from milk bucket (18.8%).

3.3. Serogrouping of *E. coli* obtained from calves and their environment

Concerning serogrouping of E. coli isolates obtained from both calves and their environment, it was found that O26 was the most prevalent serogroup (31%) followed by O55 (19%), O159 (16.78%), O111 (14.3%) then untyped stains (9.5%) and O127 (7.1%) and finally O103 (2.4%). Belonging the calves isolated E. coli serogroups, O26 was the most prevalent (31.3%), followed by O159 and O55 (18.8% for each) then O111

and untyped stains (12.5% for each) then O103 (6.3%) while O127 was not detected. On the other hand, among E. coli serogroups obtained from environmental samples O26 was the most frequently detected followed by O55, O111, O159, O127 and untyped (30.8, 19.2, 15.4, 15.4, 11.5 and 7.7 %, respectively) while O103 was not detected (**Table 4).**

Table 2 Frequent distribution of bacteria isolated from calves in the examined farm

Calves	Total examined (No.)	Positive isolation No. (%)	Bacterial isolates (No.)	Bacterial isolates (%)*				
				E. coli	*Salmonella*	*Shigella*	*Klebsiella*	*C. perfringens*
				No. (%)	No. (%)	N. (%)	No. (%)	No. (%)
Apparently healthy	87	27 (31.0)	**30**	17 (56.7)	ND	ND	5 (16.7)	8 (26.7)
Diarrheic	49	45 (91.8)	**63**	43 (68.3)	3 (4.8)	4 (6.3)	10 (15.9)	3 (4.8)
Total	136	72 (52.9)	**93**	60 (64.5)	3 (3.2)	4 (4.3)	15 (16.1)	11 (11.8)

X^2= 122.28, P <0.0001 ND: not detected * % were calculated according to No. of the corresponding bacterial isolates

Table 3 Frequent distribution of bacteria isolated from calf's environment in the examined farm

Samples		Total examined (No.)	Positive isolation No. (%)	Bacterial isolates (No.)	Bacterial isolates (%)				
					E. coli	*Salmonella*	*Shigella*	*Klebsiella*	*C. perfringens*
					No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Milk	**Sample**	**30**	ND	ND	ND	ND	ND	ND	ND
	Bucket	**30**	17 (56.7)	16	3 (18.8)	ND	1 (6.3)	12 (75.0)	ND
Water	**Source**	**30**	ND	ND	ND	ND	ND	ND	ND
	Trough	**30**	23(76.7)	23	9 (39.1)	ND	1 (4.3)	11 (47.8)	2 (8.7)
Feed	**Sample**	**30**	2 (6.7)	2	ND	1 (50.0)	ND	ND	1 (50.0)
	Manager	**30**	11 (36.7)	13	4 (30.8)	2 (15.4)	2 (15.4)	3 (23.1)	2 (15.4)
Attendants hands		**30**	26 (86.7)	26	9 (38.5)	2 (11.5)	1 (3.8)	12 (46.2)	ND
Flies		**30**	19 (63.3)	23	11 (47.8)	5 (21.7)	2 (8.6)	5 (26.1)	ND
Soil		**30**	30(100.0)	47	31 (66.0)	5 (10.6)	4 (8.5	5 (10.6)	2 (4.3)
Total		**270**	128 (47.4)	150	68 (45.3)	16 (10.7)	11 (7.3)	48 (32.0)	7 (4.7)

X^2= 487.63, P<0.001 ND: not detected * % were calculated according to No. of the corresponding bacterial isolates

3.4. Antibiotic sensitivity testing

Concerning *In-vitro* sensitivity of 50 *E. coli* isolates recovered from calves (*n*= 20) and their environment (*n= 30)* against 12 antimicrobials **(Table 5)**. *E. coli* isolated from calves were highly sensitive to enorofloxacin and neomycin (80.0% and 60.0%, respectively) and they showed intermediate sensitivity towards chloramphenicol, florofenicol (70.0% for each) and erythromycin (50%). Complete resistances (100.0%) were detected against the β-lactamases (ampicillin, amoxicillin, and penicillin), tetracyclines (tetracycline, oxytetracycline), cefoxitin and sulfamethoxazole-trimethoprim.Meanwhile environmental strains of *E. coli* showed low level of sensitivity toward enrofloxacin (20%) and chloramphenicol (10.0%) and intermediate sensitivity to neomycin and erythromycin (50.0 and 30.0 %, respectively) and complete resistance (100%) to the rest of antibiotics (at X^2= 30.66, P< 0.001).

3.5. Disinfectant sensitivity testing

Referring to the bactericidal effect of the 3 types of disinfectants at different exposure times against *E. coli* isolated from calves and their environment. *E. coli* isolated from calves (*n*= 20) showed the highest

bactericidal effect after 30 min. of exposure to both TH^{4+} 0.5% and Virkon ® S 1% followed by 15 min (70.0 and 60.0%, respectively) and the least activity was after 5 min. of exposure (40%). Meanwhile iodine 5% showed the highest bactericidal activity after 30 min. (60.0 %) and the least was after 5 min. (20.0%). On the other hand environmental strains (*n= 30)* showed the highest sensitivity to Virkon ® S 1% after contact time 30 and 15 min (70.0 and 50%, respectively), followed by iodine 5% after 30 and 15 min contact time (70.0 and 40.0%, respectively); while the least bactericidal activity was exhibited by TH^{4+} 0.5% after 30 and 15 min contact time (50 % for each) (at X^2= 17.79, P< 0.001) **(Table 6)**.

3.6. Polymerase chain reaction for detection of antimicrobial resistance genes

Table 4 Serogrouping of *E. coli* isolated from diarrheic calves and their environment

Isolates sites	No. of tested isolates	*E. coli* serogroups						
		O_{26}	O_{111}	O_{103}	O_{127}	O_{159}	O_{55}	Untyped
		No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Calves	16	5 (31.3)	2 (12.5)	1 (6.3)	ND	3 (18.8)	3 (18.8)	2 (12.5)
Environment	26	8 (30.8)	4 (15.4)	ND	3 (11.5)	4 (15.4)	5 (19.2)	2 (7.7)
Total	42	13 (31)	6 (14.3)	1 (2.4)	3 (7.1)	7 (16.7)	8 (19)	4 (9.5)

ND: not detected

Table 5 In-vitro sensitivity of *E. coli* isolated from calves and their environment against different antimicrobials

Tested antibiotic (µg)	Calves *E. coli* isolates (*n*=20)			Environment *E. coli* isolates (*n*=30)		
	R	I	S	R	I	S
Enrofloxacin (10)	-	20.0	80.0	60.0	20.0	20.0
Neomycin (30)	-	40.0	60.0	50.0	50.0	-
Chloramphenicol (30)	-	70.0	30.0	40.0	50.0	10.0
Flroofenicol (30)	-	70.0	30.0	100.0	-	-
Erythromycin (15)	50.0	50.0	-	70.0	30.0	-
Tetracycline (30)	100.0	-	-	100.0	-	-
Oxytetracycline (5)	100.0	-	-	100.0	-	-
Penicillin (10)	100.0	-	-	100.0	-	-
Ampicillin (10)	100.0	-	-	100.0	-	-
Amoxicillin (10)	100.0	-	-	100.0	-	-
Sulfamethoxazole/trimethoprim (25)	100.0	-	-	100.0	-	-
Cefoxitin (30)	100.0	-	-	100.0	-	-

X^2= 30.66, P< 0.001; R: resistant; I: intermediate; S: sensitive

The results of PCR of MDR *E. coli* isolates recovered from calves and environment revealed that bla_{TEM} gene was the most prevalent found in all isolates (100%) followed by *qacED1* found in 5 isolates (83.3%); 3 from calves and 2 from environment, and *dfrA* genes which was found in 4 isolates (66.7%); 2 from both calves and environment, then *tetA* and *sul1* which were detected in 3 isolates (50% for each); both have 2 from calves and 1 from environment. Then, bla_{SHV} found in 2(33.3%); one from both calves and environment, while both bla_{OXA-1} and *floR* genes were found in 1 (16.7%) from calves **(Table 7 and Fig. 1, 2, 3 and 4)**.

Table 6 In-vitro sensitivity of *E. coli* isolated from calves and their environment against three types of disinfectants at different exposure times

Contact time / Disinfectants	Calves *E. coli* isolates (*n*=20)				Environment *E. coli* isolates (*n*=30)			
	5min	10min	15min	30min	5min	10min	15min	30min
TH^{4+} (0.5%)	40.0	60.0	70.0	80.0	R	30.0	50.0	50.0
Virkon® S (1%)	40.0	50.0	60.0	80.0	20.0	40.0	50.0	70.0
Iodine (5%)	20.0	40.0	50.0	60.0	R	20.0	40.0	70.0

X^2= 17.79, P< 0.001; R: resistant

Table 7 Prevalence of resistance-associated genes among the examined *E. coli*.

Tested gene	*E. coli* isolates (n=6)	
	Positive	
	No.	%
tetA	**3**	**50**
blaTEM	**6**	**100**
bla_{SHV}	**2**	**33.3**
bla_{OXA-1}	**1**	**16.7**
sul1	**3**	**50**
dfrA	**4**	**66.7**
floR	**1**	**16.7**
qacED1	**5**	**83.3**

% was calculated according to Number (n.) of examined isolates

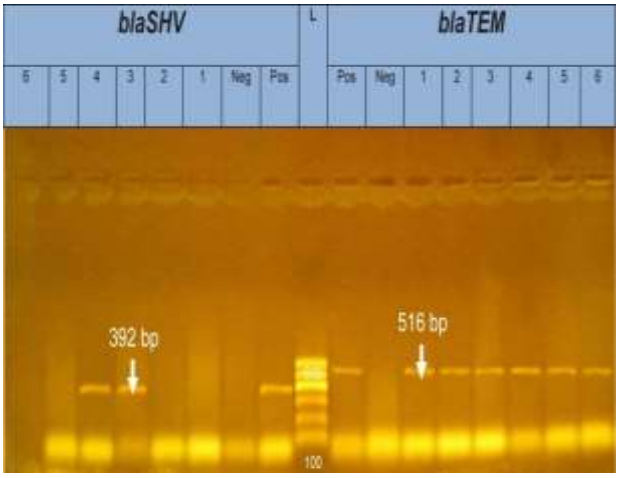

Fig. 1 PCR amplification of the 392 and 516 bp fragments of *bla_{SHV}* and *bla_{TEM}* genes, respectively from 6 *E. coli* (lane 1-6), Pos (control positive), Neg (control negative) L: DNA Ladder.

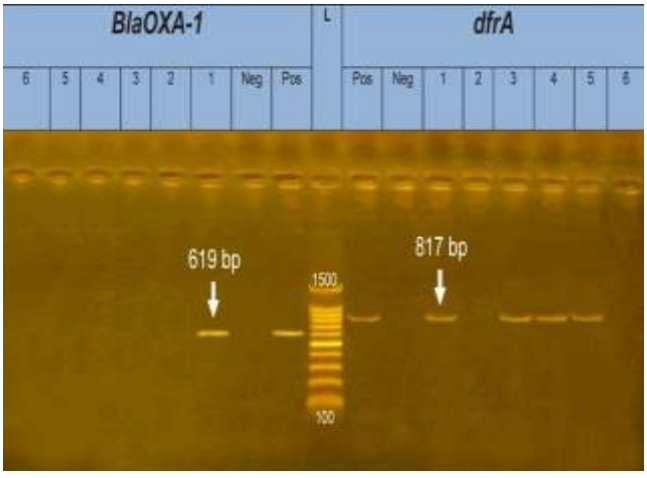

Fig. 2 PCR amplification of the 619 and 817 bp fragments of *Bla_{OXA-1}* and *dfrA* genes, respectively from 6 *E. coli* (lane 1-6), Pos (control positive), Neg (control negative) L: DNA Ladder.

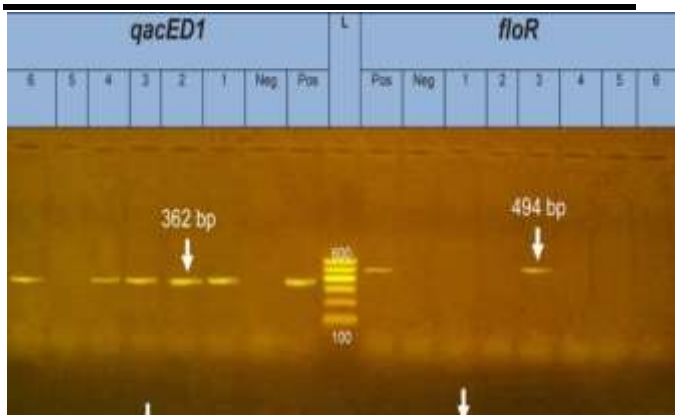

Fig. 3 PCR amplification of the 362 and 494 bp fragments of *qac*$_{ED1}$ and *floR* genes, respectively from 6 *E. coli* (lane 1-6), Pos (control positive), Neg (control negative) L: DNA Ladder.

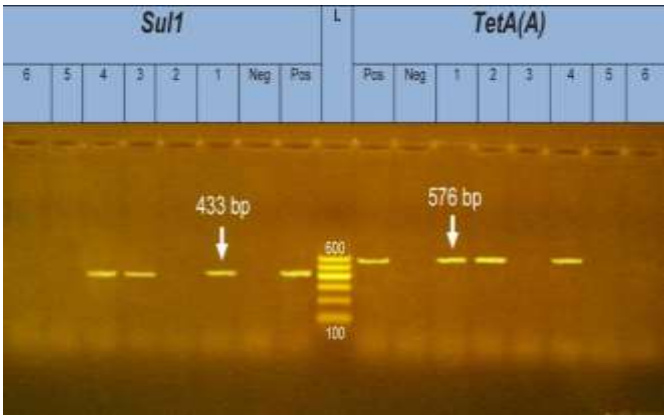

Fig. 4 PCR amplification of the 433 and 576 bp fragments of *sul1* and *TetA*(A)genes, respectively from 6 *E. coli* (lane 1-6), Pos (control positive), Neg (control negative) L: DNA Ladder.

4. Discussion

Calf diarrhea remains the most important cause of morbidity and mortality in young calves especially in their early life (Constable, 2004). Among the bacterial enteropathogens causing calf diarrhea is enteropathogenic *E. coli* (EPEC) that is considered the most economically important pathogen (Acha *et al.*, 2004).

In the present study, a high frequency was obvious of bacterial isolates that were obtained from fecal samples of both apparently healthy and diarrheic calves. These results might be attributed to low level of hygiene prevailing in the farm where the study was applied in and no routine cleaning or disinfection to milk utensils or equipment used for feeding and drinking the calves which favor the growth of such bacteria causing diarrhea. These obtained results are in harmony with that detected by Atawa *et al.*, (2012) who demonstrated that the positive fecal samples for bacteriological isolates were (79.5%). Herrera-Luna *et al.*, (2009) recorded that the frequency and percentage of bacteriologically positive samples were above 100.0% due to concurrent infection with different microorganisms whereas *E. coli* followed by *C. perfringens*, *Klebsiella* spp. and *Proteus* spp. were isolated at (18.9, 10.0, 3.3, 1.1%, respectively). Concerning, the distribution of bacterial isolates revealed that the most predominant diarrhea causing bacteria in calves was *E. coli* (68.3%) in the examined farm. These are in coincide with that obtained by China *et al.*, (1998) and Harbby, (2002) who found out that *E. coli* was the most predominant bacteria isolated from diarrheic calves. Furthermore, Raihan *et al.*, (2014) who found out that frequency distribution of bacterial isolates from diarrheic calves included two types of bacteria *E. coli* (28%) and *Salmonella* (8.8%). El-Hamamy *et al.*, (1999) demonstrated that the predominant isolates from diarrheic calves were *E. coli* (52.5%), *Enterobacter aerogense* (15%), *proteus vulgoris* (12.5%) and *Salmonella* spp. (5%). On the contrary Haschek *et al.*, (2006) found out that the most frequently isolated pathogen from diarrheic calves was bovine coronavirus (25.7%) while *E. coli* was the second highest frequent isolated enteropathogen with (17%).

In calves' environment, the frequent distribution of enteropathogenic bacteria and their sero-grouping revealed that *E. coli* was the predominant isolated bacterial pathogen beside *E. coli* O_{26} was the most frequently detected serogroup which might be contributed to contamination of the calves' environment with feces which contain *E. coli* predominantly as well as the large herd size that is accompanied with high stocking density with subsequent lower degree of hygiene and less time for individual calf care, these results were to some extent coincides with that obtained by Aragan, (2012) and Hessain, (2012) who highlighted that the poor hygienic conditions of the water source

used play a role in transmission pathogenic bacteria-induced mastitis, enteritis, and calf diarrhea, and Emmanuel *et al.*, (2011) who isolated *Proteus* spp., *Pseudomonas* spp., *Bacillus* spp., *E. coli*, *Salmonella* spp. and *Shigella* spp. (70.21, 59.13, 58.33, 58.37, 47.02 and 21.63%, respectively) from soil at dumpsites in the farm, and to some extent with Mohammed, (2016) who found that *E. coli* (56.7 %) followed by *Salmonella* spp. (26.7 %), *Streptococcus faecalis* (23.3 %), *Shigella flexneri* (16.7 %), *Proteus* spp. (16.7 %), and *Klebsiella pneumonae* (10.0 %) were the most isolated bacteria from surface water. On contrary, Minj and Behera, (2012) isolated *E. coli. Salmonella* sp., *Shigella* sp. *Klebsiella* sp. and *Pseudomonas* sp. from milk samples suggesting the possible contamination of the udder of adult cows from their surrounding environment. Also Plews *et al.*, (1981) isolated Klebsiella and *Enterobacter* sp. from soil samples from calves' environment beside *E. coli* and in the same time *E. coli* couldn't be isolated from clean soil which suggests that calves are the source of soil contamination with bacterial enteropathogens. Meanwhile, Vicente *et al.* (2005) pointed out that water is an important source of STEC (Shiga toxin producing *E. coli*) in the farm and he isolated *E. coli* O_{113} (1.9%) from the drinking water. Mohammed *et al.*, (2016) declared that *E. coli* serotypes O_{91} (41.17 %), O_{145} (29.41 %), and untyped (poly I–III) (29.41 % each) were the most predominant serogroups obtained from flies in cattle farms. Rahn *et al.*, (1997) isolated *E. coli* O_{119} from calf manager together with high rates from *E. coli* O_{157} was also detected in calf managers and water bowels suggesting the potential transmission of infection from animal to animal. Concerning, *E. coli* serogroups isolated from soil samples in this study including *E. coli* O_{26}, O_{111}, O_{127} and O_{55}. And Plews *et al.*, (1981) isolated *E. coli* O_{26}, O_{55}, O_{111}, O_{119}, O_{124}, O_{125} and O_{128} serogroups from soil sample which are similar to those isolated from calves indicating that contaminated soil may act as a source of infection to the healthy calves if being raised on.

Concerning serogroups recovered from fecal samples of diarrheic calves illustrated in Table (4) it was found that *E. coli* O_{26} was the most prevalent serogroup isolated from fecal samples of diarrheic calves and this is similar to that detected by Badouei *et al.*, (2010) who isolated O_{157}, O_{111} and O_{26} serogroups from diarrheic and non-diarrheic calves and the most common serogroup detected was O_{26} (18.4%). Similarly, Lee *et al.*, (2008) isolated O_{26} and O_{111} from diarrheic and healthy calves (14.4% and 12.5%, respectively) suggesting that O_{26} and O_{111} consider the main cause of diarrhea in calves. Also Atawa *et al.*, (2012) who serogrouped *E. coli* isolated from diarrheic calves as O_{157}, O_{111}, O_{125}, O_{119}, O_{26} and O_{128} and untyped (17.9, 9.5, 11.6, 15.8, 12.6, 8.4 and 3.2% respectively). Also, Thin *et al.*, (2011) isolated different serogroups of E. coli and the most predominant serogroups were O15, O20, O103 and O157 in Vietnam. Antimicrobial sensitivity tests showed that E. coli isolated from both calves and their environment showed high sensitivity to enrofloxacin and neomycin, intermediate sensitivity to chloramphenicol and florophenicol; and complete resistance to β-lactams, tetracycline and sulphamethoxazole-trimethoprime. These obtained results agreed to some extent with Abd-Elrahman, (2011) who recorded that E. coli isolates from calves were mostly sensitive to marbofloxacin followed by enrofloxacin (96.07 and 88.23%, respectively), and mostly resistant to penicillin, neomycin, erythromycin, streptomycin, tetracycline and chloramphenicol. Sadiek and Sohair, (1999), El-Gaml et al. (2001) and Aba-Alkhalil and El-Naenaeey, (2003) all indicated that enrofloxacin and ciprofloxacin were the most efficient antibiotics in treatment of calf diarrhea. Also, Adetunji and Isola, (2011) indicated that E. coli isolated were resistant to nitrofuration, tetracycline, ampicillin, gentamicin, ciprofloxacin and chloramphenicol, and Mohammed et al., (2016) revealed that the antimicrobial resistance pattern of Gram-negative bacteria proved that E. coli was resistant to tetracycline (92.9 %). On the contrary EL-Seedy et al., (2016) reported that E. coli isolates were sensitive to marbofloxacin, spectinomycin and neomycin only and resistant to the majority of tested antibiotics in Egypt.

Referring to the efficiency of 3 different types of disinfectants against *E. coli* isolated from both calves and the environment, it was revealed that TH^{4+} 0.5% and Virkon ® S 1% were the most efficient disinfectants at exposure time 30 min, while iodine 5% was the least efficient disinfectant at the same exposure time. The

more the contact time increase the more the efficiency of the disinfectant will increase. These findings were in harmony with those found by Fawzia *et al.,* (2013) that found *E. coli* isolates were sensitive to TH^{4+} 0.2% and Virkon s 1% using disc diffusion method (86.7%) while by pits method (93.3%) at the same time iodine proved to have lower efficiency which might be attributed to its need to long contact time. Gehan *et al.,* (2009) recorded that TH^{4+} is the most powerful disinfectant due to synergism between QAC and glutaraldehyde. In contrast to the present data Gasparini *et al.,* (1995) found that Virkon®S was effective against *Pseudomonas aeruginosa* and *E. coli* and they were resistant to QAC while Saha *et al.,* (2009) found that iodine showed moderate efficiency against *S. aureus* and *E. coli* than *Klebsiella* sp. *S. typhi* while *S dysenteriae* was the only sensitive microorganism to iodine, reciprocally Mohammed, (2014) who indicated that iodine 0.5%had the lowest lethal effect (0.0%) against all the isolated bacteria after 15 seconds exposure time and it's efficiency increased to be moderate when exposure time increased to 5 min against *E. coli* isolates with (50%) efficiency.

Results concerning the distribution of resistance genes in *E. coli* isolates showed that all of eight's targeted resistance genes (*tetA*(A), *dfrA, sul1*, *floR*, bla_{TEM}, bla_{SHV}, bla_{OXA-1} and *qacED1*) were detected in all of *E. coli* from calves and their environment and this is similar to that detected by Nelson *et al.,* (2014) who detected sul1 and *tet*(A) genes in 73.0% *E. coli* isolates obtained from diarrheic calves and Momtaz *et al.,* (2013) who detected *sul1* and *tet*(A) genes in 82.78% and 51.63%, respectively. Kucken *et al.,* (2000) reported that *qacED* gene that is responsible for resistance to QAC was detected in 10.0% of *E. coli* isolates which is unlike the present finding where this gene was detected in 83.3% of the tested isolates. Also Zou *et al.,* (2014) reported that *qac-ED1* was detected in 7.0% of *E. coli* isolates. For β-lactams resistance genes (bla_{TEM}, bla_{SHV} and bla_{OXA}) the current results were to some extent similar to Jiang and Zhang, (2013) who reported that *TEM* gene was identified in 84.6% of tested isolates while *SHV* and *OXA* were not detected 0% of the tested isolates while *floR* gene that was indicated in 20 isolates which is responsible for cross resistance to florphenicol and chloramphenicol which is banded antibiotic but yet resistance to it still detected that indicates unauthorized and miss use.

5. Conclusion

Calves can act as a source of contamination to the environment with multiple antimicrobial resistant E. coli which poses high risk for human and animal populations. Regular screening of antibiotics sensitivity before actual application on animals is essential to reduce the possibility of dissemination of resistance genes.

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