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OCCURRENCE AND RISK FACTORS ASSOCIATED WITH PATHOGENIC *E. COLI* IN DIARRHEIC CALVES

By

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ABSTRACT

Bacteriological examination of faecal swabs collected from healthy (H) and diarrheic (D) calves, revealed isolation of 26 *E. coli* isolates from 56 calves with an incidence 46.4% Gram negative bacilli, non-sporulated. Then biochemical tests were done. Then serological test for representative number of samples from every farm identifying O1, O26, O44, O55, O115, O119, O125, O146, O151 and 2 samples were untypable. The samples were sensitive to Norofloxacin with 80.7% and resistant to Ampicillin and Cefotaxime with 100%.The PCR for 4 gene *omp*A, *stx1*, *stx2* and *eae*A.

INTRODUCTION

Neonatal calf diarrhoea (NCD) is one of the major health challenges in both beef and dairy cattle herds **(De la-Fuente *et al.*, 1999)**. Diarrhoea in neonatal calves is a complex multifactorial and dynamic disease with the balance between the host's resistance (i.e. active and passive immunity) and the pathogen pressure being cardinal **(Lorenz *et al.*, 2011)**. The aim of study is isolation and characterization of pathogenic *E. coli* associated with calf diarrhoea and trial to control the disease. Each strategy to prevent NCD should begin with a confirmed diagnosis and the setup of a farm interview. The herd anamnesis addressing young stock management creates a list with potential critical control points. Key questions in this anamnesis should focus on: colostrum management, housing and hygiene, feeding of the calves, periods of stress, and drug used **(Smith, 2012).**

MATERIAL AND METHODS

The samples were obtained from farms at El-Fayoum governorate, Egypt. 29 apparently healthy calves and 27 calves suffering from diarrhoea at different ages were selected from different private farms to collect faecal swabs. Samples were collected under complete aseptic condition. Faecal swabs were collected using sterile cotton swab and transported in ice box as

soon as possible to the lab. Faecal swabs were transferred to glucose solution 5 % and incubated for 18 hr. at 37 ºC. Swabs were streaked onto the surface of MacConkey's agar and incubated at 37 ºC for 24 hr. Typical *E. coli* colonies showed pink colonies according to **Kudra *et al.* (1997) and Quinin *et al.* (2002)**.*E. coli* was confirmed by biochemical tests according to **Murrary *et al.* (2003)** by the following tests as Oxidase, Urease, TSI, IMVC tests, and liquid stable antisera for the determination of "O" antigens for the serological identification of pathogenic *E. coli* **(Bioliography available on request)** in serological unit-Dokki. Antibacterial susceptibility testing of bacteria was carried out by Kirby-Bauer disk diffusion assay method using commercial disks in Mueller-Hinton. The PCR for 4 gene *omp*A, *stx1, stx2* and *eae*A in PCR unit-Dokki.

Table (1): Oligonucleotide primers sequences.

Reference	Amplified product	Primer Sequence 5'-3'	Gene
Ewers *et al*., 2007	**919 bp**	**AGCTATCGCGATTGCAGTG**	***omp*A**
		GGTGTTGCCAGTAACCGG	
Bisi-Johnson *et al*., 2011	**248 bp**	**ATG CTT AGT GCT GGT TTA GG**	***eae*A**
		GCC TTC ATC ATT TCG CTT TC	
Dipineto *et al.*, 2006	**614 bp**	**ACACTGGATGATCTCAGTGG**	***stx1***
		CTGAATCCCCCTCCATTATG	
	779 bp	**CCATGACAACGGACAGCAGTT**	***stx2***
		CCTGTCAACTGAGCAGCACTTTG	

DNA extraction: 20 µl QIAGEN protease were pipetted into the bottom of a 1.5 ml microcentrifuge tube. 200 µl buffer AL were added to the sample, mixed by pulse vortexing for 15 seconds. The mixture was incubated at 56˚C for 10 min. The 1.5 ml microcentrifuge tubes were centrifugated. 200 µl ethanol (96%) were added to the sample, and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifugated. The mixture from step 6 was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim, and centrifugated at 8000 rpm for 1 min. The column was placed a in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. 500 ml buffer AW1 were added and centrifugated at 8000 rpm for 1 min. Repeating with adding 500 ml buffer and centrifugated at full speed for 3 min. Repeating

with centrifugation at full speed for 1 min was done. The column was placed in a clean 1.5 ml microcentrifuge tube, and the filtrate was discarded. 100 µl buffer AE were added. The column was incubated at room temperature (15-25˚C) for 1 min, and then centrifugated at 8000 rpm for 1 min.

Preparation of PCR Master Mix:

1. Preparation of *eaeA* uniplex PCR Master Mix: according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit as the following components 12.5 µl Emerald Amp GT PCR master mix (2x premix), 4.5µl PCR grade water, 1µl Forward primer (20 pmol), 1µl Reverse primer (20 pmol), 6 µl Template DNA and 25 µl Total.

2. Preparation of multiplex PCR Master Mix: characterization according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit as the following components 25 µl Emerald Amp GT PCR master mix (2x premix), 10 µl PCR grade water, 1 µl each Forward primer (20 pmol), 1 µl each Reverse primer (20 pmol), 9 µl Template DNA, 50 µl Total.

Cycling conditions of the different primers during CPCR:

Temperature and time conditions of the primers during multiplex PCR according to specific authors and Emerald Amp GT PCR master mix (Takara) kit.: for *omp*A, *stx1* and *stx*2: Primary denaturation 94˚C for 5min. followed by 35 cycle of Secondary denaturation 94˚C for 30 sec, Annealing. 58˚C for 1 min, Extension 72˚C for 1 min followed by final extension 72˚C for 10 min and for eaeA: Primary denaturation 94˚C for 5min. followed by 35 cycle of Secondary denaturation 94˚C for 30sec, Annealing. 51˚C for 30sec, Extension 72˚C for 30sec followed by final extension 72˚C for 7min.

DNA Molecular weight marker:

The ladder was mixed by pipetting up and down. 6 µl of the required ladder were directly loaded. **Agarose gel electrophoresis (Sambrook *et al*., 1989) with modification:** Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask. 20µl of each uniplex PCR product and 40 µl of each multiplex PCR product negative control and positive control were loaded to the gel.

RESULTS

Identification of *E. coli*:

1. Clonial morphology:Typical *E.coli* showed pink colonies on MacConkey agar.
2. Under microscope: uniformly distributed Gram negative non-sporulated straight rods.

3. Biochemical identification: ve Urease was yellow,+ve Indole was red ring,+ve Methyl red was red color,-ve Citrate utilization was green,-ve TSI (acidic slant,acidic butt,-ve H2S, ± gas).

Serological identification of *E. coli* isolates: 14 isolates were analyzed serologically.

Table (2): Serological identification of some *E. coli* isolates.

	Farm1			Farm2				Farm3						
S	**158**	**164**	**310**	**1**	**6**	**11**	**12**	**6**	**9**	**14**	**19**	**21**	**17**	**23**
P	**2**	**2**	**2**	**1**	**6**	**1**	**2**	**1**	**3**	**3**	**1**	**2**	**Untypable**	
M	**O125**	**O55**	**O55**	**O26**	**O115**	**O26**	**O146**	**O119**	**O151**	**O151**	**O1**	**O44**	**Untypable**	

S: sample's Number **P: Polyvalent** **M: Monovalent**

Analysis of antimicrobial susceptibility testing for isolated *E. coli*:

The interpretation of inhibition zones test culture was according to guidelines of NCCL (2002), as following: Neomycin 1 of the isolates was sensitive(S),13 of the isolates were intermediate (I) and 12 were resistant(R), Ampicillin 26 were (R), Gentamicin 20 were (S), 1 was (I) and 6 were resistant, Kanamycin 14 were (S), 1 was (I) and 11 were (R), Cefotaxime 26 were resistant, Clindamycin 1 was (S) and 1 was (I), 24 were (R),Norofloxacin 21 were (S) and 5 were resistant and Sulphonamide-Trimethoprim 8 were (S), 2 were (I) and 16 were (R).

Detection of *omp*A, *stx1*, *stx2* and *eae*A gene:

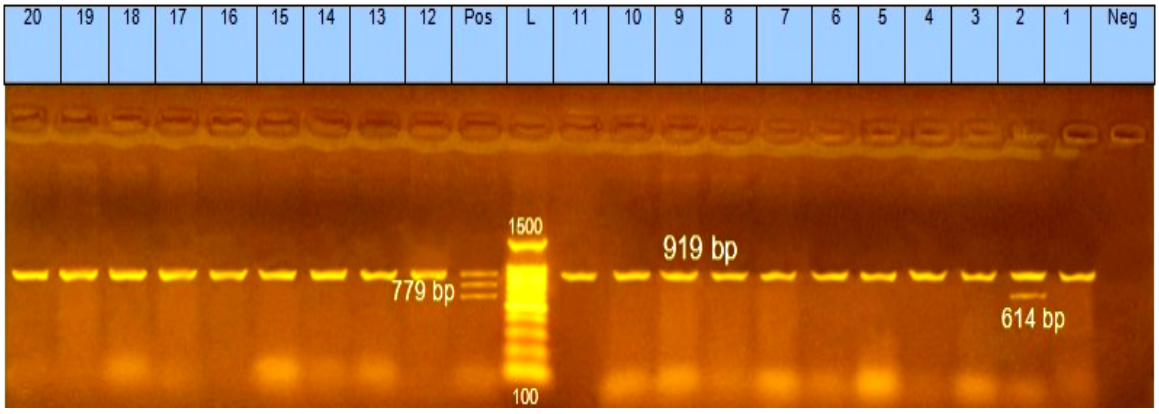

Fig (1): Agarose gel electrophoresis showing multiplex PCR with amplification of 919bp fragments for *omp*A gene of *E. coli* and amplification of 614bp fragments for *stx1* gene.

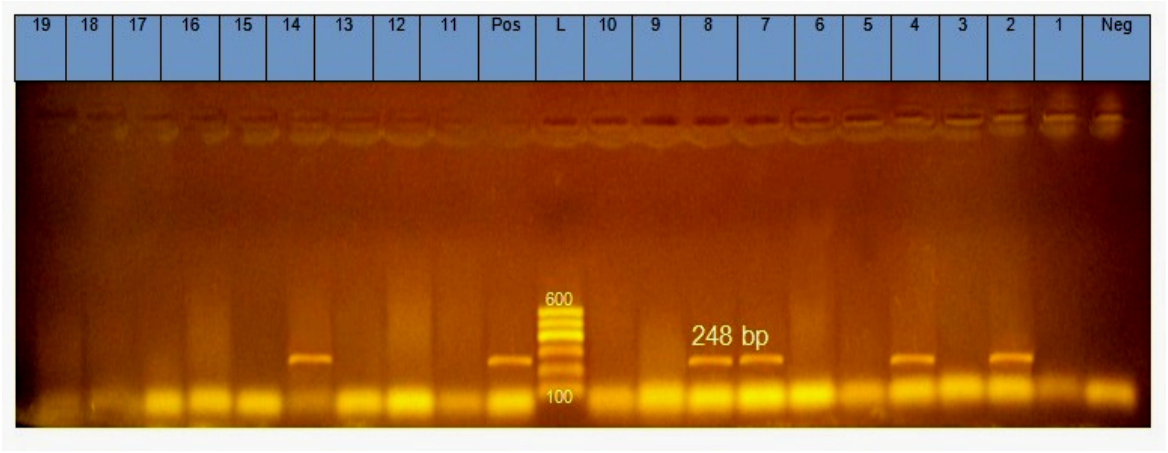

Fig (2): Agarose gel electrophoresis showing *eae*A uniplex PCR with amplification of 248bp fragments for *eae*A gene of attaching and effacing (AEEC).

PCR results:

PCR detect *omp*A gene in all 26 *E. coli* isolates multiplies a region of 919bp, *stx1* gene multiply regions of 614pb in sample (2) , *stx2* is in all 26 isolates, *eae*A gene in 5 faecal samples (2,4,7,8,14).

DISCUSSION

The incidence of calf scour had a great variation in literature. In this study 26 isolates recovered from faecal swabs were collected from 56 examined calves with an incidence of 46.4%. This percentage agrees to large extent with **Mosaad *et al*. (2008)**, who reported the percentage of *E. coli* in diarrheic Frisian calves was 48.4%. Serological testing showed that, the examined samples are serologically belonging to the following serovars O1, O26, O44, O55, O115, O119, O125, O146, O151 and 2 isolates were untypable. These results are closely similar to those obtained by **Mosaad *et al*. (2008),** who reported the predominant serovars were O1, O8, O25, O26, O55, O86, O125, O146, O151 and 2 isolates were untypable. The widespread use of antimicrobial agents would select for resistance and may have promoted the increasing frequency of STEC strain's multidrug resistance in bovines, perhaps greater, shedding which could lead to greater contamination of animal food products **(Zhao *et al*., 2001).** In this study, 100 % of the isolates tested were resistant to two or more of the antimicrobial agents tested (multidrug resistance), Ampicillin, Cefotaxime and Clindamycin showed the highest rates of resistance, 100 %, 100% and 92.3 %, respectively. In **Nizza *et al*. (2010)** study, Gram-negative bacteria strains carry overall high levels of resistance to penicillin G, leucomycin and neomycin (100 % of resistance), drug resistance

geographically depending on the degree of local use. In that study all 26 *E. coli* isolates were found to be positive for *omp*A gene of 4 virulence factors examined, One 3.8% *E. coli* isolate was found to be positive for *stx1* gene, none for *stx2* and 5 *E. coli* isolate was found to be positive for *eae*A gene, previous one *E. coli* isolate positive for *stx1* gene also carry *eae*A gene. All 26 *E. coli* isolates were negative for *stx2* gene. In **Carlos (2008)** study, 39 isolates of 230 (17%) of all examined samples, the PCR show 28 samples were positive *stx*1*,* 18 were positive *stx*2, 15.2% were positive to *stx*1*, stx*2 or both. 16 were positive *Ehly* gene and 12 were positive for *eae*A. The results confirm the importance of healthy and diarrheic calves as reservoir of shiga toxin producing *E. coli*.

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