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CAMEL-CALVES DIARRHOEA INSTIGATED BY *Escherichia coli* AND DETECTION OF SOME VIRULENCE-ASSOCIATED MARKERS

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SUMMARY

Bacteriological examination of internal organ specimens including intestine, liver, spleen, lungs and kidneys (45) and faecal samples (77) from necropsied and diarrhoeic camel-calves respectively, that had severe episodes of diarrhoea, revealed the isolation of *Escherichia coli* from 33/45 (73.3%) of the necropsied specimens and 58/77 (75.3%) of the faecal samples. Eleven serogroups were detected, with high prevalence of O3 (19.8%), O11 (17.6%), O80 (16.5%). Two enterohaemorrhagic, enterotoxigenic O157 isolates (2.2%) were also detected one from each group of specimens. *E. coli* isolation from faecal specimens revealed wider serogroups range as O91, O125 and O29 were additionally detected from faecal samples and not from the organ specimens. Investigating of some virulence markers of the isolates revealed that the haemolytic activity was expressed significantly higher ($p<0.01$) than the rest of investigated markers as 43/91 (47.3%) were haemolysin producers. The detection of the VT2 gene was revealed in 34/91, (37.4%), Shiga-like toxin production in 26/91, (28.6%) and Congo red binding affinity was expressed in 31/91 (34.1%) of the isolates. The highest percentage of hydrophobic activity was (81.2±5.7) by the O163 isolates, whereas the lowest activity (40.2±3.2) was recorded by the O125 isolates. It was observed that the highest expression of virulence markers was among the isolates of O157 (2 isolates), O26 (3 isolates) and O163 (2 isolates), as these isolates expressed full range of the investigated virulence markers. The rest of *E. coli* isolates from other serogroups revealed abridged virulence criteria. Antibiotic sensitivity testing of *E. coli* revealed highest sensitivity against gentamicin (94.4%), trimethoprim sulphamethazole (91.2%), streptomycin (87.9%) and cephalosporine (84.6%). It was concluded that highly virulent *E. coli* strains induced infection in camel-calves, and their treatment with the recommended antibiotics could curb the episode of the diarrhoea in few days.

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INTRODUCTION

E. coli infection in camel-calves has a great economic magnitude for camel farms and breeders. In certain cases, the mortality rate can be 40% with the possibility of reaching 100% if the medical intervention was delayed (Fowler et al., 1998; Schwartz and Dioli 1992; Wernery and Kaaden 2002). Clinical signs observed on infected camel-calves were mostly signs of dysentery, abdominal pain, diarrhoea, dehydration, fever (40-41°C), anorexia and general malaise. In dead necropsied animals, catarrhal enteritis, congestion in the intestine with enlarged oedematous mesenteric lymph nodes. Also petechial haemorrhages and oedema may be observed on the meningese, metritis, mastitis, tissue abscesses were also recorded (Chauhan et al., 1987; Haenichen and Wiesner 1995 and Wernery and Kaaden 2002). Among the wide range of *E. coli* pathogenic factors were shiga-like toxin production which is produced by enterotoxigenic *E. coli* group. There are different subclasses of shiga-like toxins that are encoded by a set of genes with a relative homology. The cytotoxic effect of the Shiga-like toxins was demonstrated in-vitro by many researchers on Vero cell cultures (Pabst et al., 2000). Surface structure proteins, porins and iron regulated proteins were considered a major virulence factor (Gyles, 1994). Some of these virulence proteins can bind to Congo red (Crb+), which can be easily detected as red coloured colonies in special culture media (Silveira et al., 2001). Hydrophobic proteins are also another factor that enhances *E. coli* attachment to the mucosal membrane, inistigating the primary step in pathogenesis. Treatment of *E. coli* infection can be curbed with different antibiotic therapy as streptomycin, gentamicin, trimethoprime sulfamethazole, sulfadiazine, tetracyline (Jin, 1985; Cid et al.,1996 and Wernery and Kaaden 2002).

The objective of this study was to bring more attention to camel-calves diarrhoea induced by *E. coli* as one of the crucial disease problem, different serotypes involved, their correlated virulence markers encountered as well as the potential treatment.

MATERIALS AND METHODS

Investigation of animals and collection of samples.

This study was carried out on 77 diarrhoeic camel-calves aged from 1-12 months. Animals had history of diarrhoea. The symptoms observed were profuse watery and in some cases tinged with blood diarrhoea, fever (40-41°C), anorexia, and in progressive cases the animals were recumbant. Also 45 internal organs (intestine, liver, spleen, lungs and kidneys) collected from another 9 camel-calves, died shortly after suffering from the previous symptoms. Specimens were collected during the period from February 2003 up to July 2003 from farms in Giza and Matrouh Governo-

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rate. All samples were collected in separate sterile plastic bags, transferred immediately to the laboratory in ice box and examined bacteriologically.

Isolation and Identification of *E. coli.*

For isolation and identification of *E. coli*, specimens were cultured directly onto blood agar (10% defibrinated sheep blood) and MacConkey agar plates, incubated at 37°C for 24hrs. From each plate 5 suspected colonies were confirmed morphologically, culturally and biochemically by conventional methods (Quinn et al.,1994), and further confirmed by API-20 diagnostic strips, (Bio Meriaux, France). Isolates were then serologically identified by slide agglutination test using polyvalent and monovalent somatic (O) and flagellar (H) antigens (Difco Laboratories, USA) according to the method of Ewing, (1986).

Haemolysin production of *E. coli.*

The haemolysin production of *E. coli* was determined by its ability to lyse erythrocytes. Isolates were subcultured onto 10% sheep blood agar plates and incubated at 37°C for 24h. Hemolysin production was detected by the ability to haemolyse sheep erythrocytes which was verified by the presence of a clear halo haemolytic zone around the haemolysine-producing colonies (Quinn et al., 1994).

Detection of VT2 gene by PCR assay.

E. coli isolates were suspended in 300ml lysis buffer (10mMTris-HCl pH8.0, 1% Triton X-100, 0.5% Tween 20 and 1mM EDTA) and heated at 99°C for 10min, then centrifuged at 1000xg for 2 min to sediment the bacterial debris. The supernatant which contained the *E. coli* DNA, was transferred to microfuge tubes and used for PCR (Olsson et al., 2000). Small aliquites from each bacterial DNA extract (2µl) were amplified in 50µl reaction mixtures containing 200µM of each deoxynucleoside triphosphates, 250nM of VT2F and VT2R primers (Table1), and 1 unit Taq polymerase (Boehringer GmbH, Mannheim, Germany) 10mM Tris HCl (pH8.3), 50mM KCl, 2mM MgCl2 , 0.1% gelatin, 0.1% Tween 20. PCR mixtures were subjected to 35 cycles, each cycle was 1min denaturation at 95°C, 2min annealing at 65°C and 1.5min extension at 72°C. The time for extension was increased to 2.5min at the last 10 cycles. PCR products were electrophoresed on 2% agarose gels with marker 1-kb DNA ladder (New England Biolabs, Inc.). Gels were stained with ethidium bromide and visualized under ultraviolet light (Paton and Paton 1998 and Reischl et al., 2002).

Table 1: PCR specific primer pair for *E. coli* VT2 gene.

Primer	Sequence	Target sequence location	Target size
VT2F	5`GGCACTGTCTGAAACTGCTCC3`	From 603-857 nucleotide of the VT2	255bp
VT2R	5`TCGCCAGTTATCTGACATTCTG3`		

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In-Vitro Production of Shiga-like toxins.

E. coli isolates were grown into casamino acid-yeast extract fluid media for 12h. at 41°C. Bacterial pellets were removed by centrifugation at 10,000xg for 10min. The supernatant which contained the Shiga-like toxins was filtered with millipore membrane filter 0.22µm. Then 100µl of the filtrate were added to confluent monolayer of Vero cells, then incubated at 37°C in 5% CO_2 atmosphere and evaluated for 72h. for cytopathic effect (CPE) (Blanco et al., 1996 and Bonardi et al., 1999).

Detection of Congo red binding (Crb) surface protein.

The medium used for determination of Congo red binding affinity of the *E. coli* isolates was trypticase soy agar (TSA), supplemented with 0.003% Congo red dye (Sigma) and 0.15% bile salts. Each isolate was cultured onto a separate plate and incubated at 37°C for 24hrs. After 24hrs incubation, the cultures were left at room temperature for 48hrs to facilitate reading of results. Invasive *E. coli* were identified by their ability to take up Congo red dye and produced red colonies due to aquiring the Congo red dye by their surface structure proteins. The negative isolates appeared as colourless colonies (Silveira et al., 2001).

Detection of hydrophobic surface protein activity.

E. coli isolates were tested for their hydrophobic activity by their adherence to n-hexadecane as a method of detecting the amount of surface hydrophobic proteins. Isolates were grown into brain heart infusion broth (BHI) for 18 hrs at 37°C., then were pelleted by centrifugation at 4000xg for 10min. *E. coli* was then washed and resuspended in PBS, pH 7.5 and adjusted photometrically to 0.5 absorbance at 540nm. Equal volume of *E. coli* suspension (0.8ml) and n-hexadecane (Sigma) were thoroughly mixed by vortexing for 30seconds, then left to settle at room temperature. *E. coli* hydrophobic activity was measured according to the method of Wibawan et al., (1992).

Antimicrobial sensitivity testing.

E. coli isolates were tested for their invitro sensitivity to the following different antimicrobial agents including, Gentamicin (10µg), Kanamycin (30µg), Chloramphenicol (30µg) , Tetracycline (30µg) Neomycin (30µg), Streptomycin (10µg) Trimethoprim sulphamethazole (25µg), Penicillin (10units), Ampicillin (10µg), Cephalosporine (30µg), Novobiocin (30µg), Norfloxacin (10µg) and Bacitracin (10µg). The method used was the agar disc diffusion method (Bauer et al., 1966).

Statistical analysis.

Statistical analysis of the results was carried out using the "*t* " test as described by Snedecor and Cochran (1967).

RESULTS

Bacteriological examination of internal organ

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specimens (45) as well as the faecal samples (77) collected from necropsied and diarrhoeic camel-calves respectively revealed positive isolation of *E. coli* from 33/45 (73.3%) of the necropsied specimens and 58/77 (75.3%) of the faecal samples. Eleven different serotypes were detected from both groups of specimens (Table 2) with high prevalence of O3 (18 isolates, 19.8%), O11 (16 isolates, 17.6%) and O80 (15 isolates, 16.5%). Two enterohaemorrhagic enterotoxigenic O157 isolates (2.2%) were detected once from each necropsied camel-calves and the other from faecal samples. Five untypable isolates were also revealed. *E. coli* isolation from faecal specimens from diarrhoeic camel-calves revealed wider serotype range as O91, O125 and O29 were isolated only from these specimens, but were not isolated from dead camel-calves (Table 2).

Investigating some virulence markers of the isolates revealed that the haemolytic activity was expressed significantly higher ($p<0.01$) than the other investigated markers as 43/91 (47.3%) were haemolysin producers. The highest haemolytic activity was expressed by O157, O26 and O29 as all isolates (100%) were haemolytic to sheep erythrocytes as expresed by a clear wide zone of haemolysis on the blood agar plates. The detection of the VT2 gene was revealed in 34/91, (37.4%), Shiga-like toxin production in 26/91, (28.6%) and Crb affinity was expressed in 31/91 (34.1%) of the isolates. It was observed that, all isolates of the serogroup O26 (4) and O157 (2) were 100% positive for the possession of the VT2 genes as revealed by the production of 255bp band in the VT2-PCR assay (Figure 1). Other serogroups as O91, O125, and O29 were lacking the VT2 gene, which is the major gene for Shiga-like toxins production. The detection of the VT2 gene was also demonstrated in vitro by the shiga-like toxins production effect on the vero cell cultures. It was noticed, that the frequency of the phynotypic demonstration of cytopathic effect (CPE) on Vero cells was significantly ($p<0.01$) lower than the genotypic expression, as only 26/91 (28.6%) of the isolates induced CPE on the Vero cells against 34/91 (37.4%) which actually possessed the VT2 gene. Only one isolate of the serogroup O91 had CPE on Vero cells and did not possess the VT2 sequence as demonstrated by PCR (Table 3).

Investigating the Crb affinity of the isolates, revealed that 31/91 (34.1%) were Crb-positive (Crb+). Low Crb affinity was recorded in isolates of the serotype O80 (3/15, 20%) as well as the untypable isolates (1/5, 20%). Isolates of the serotype O157 (2) were Crb+ (100%) as well as the one isolate of the serotype O29 was Crb+. There was no correlation between Crb affinity and the hydrophobic properties of the isolates. The highest percentage of hydrophobic activity of *E. coli* isolates was (81.2$\pm$5.7) recorded by the O163, whereas the lowest hydrophobic activity (40.2$\pm$3.2) was recorded by the O125 isolates (Table 3).

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It was observed that the highest expression of virulence markers was among the *E. coli* isolates of O157 (2 isolates), O26 (3 isolates) and O163 (2 isolates), as these isolates expressed full range of the investigated virulence markers (100% positive for each of haemolysin production, possession of VT2, expression of CPE on Vero cells and Crb affinity). Also the three serotype groups demonstrated relatively high percentage of hydrophobic activity (63.1±7.5), (60.8±3.5) and (81.2±5.7) respectively. Most of the rest of *E. coli* isolates from other serogroups revealed either one or two virulence criteria .

Antibiotic sensitivity testing of *E. coli* isolates revealed that the highest sensitivity was against gentamicin (94.4%), trimethoprim sulphamethaxzole (91.2%), streptomycin (87.9%) and cephalosporin (84.6%) (Table 4). These antibiotics when recommended for therapy, the episode of diarrhoea ceased in few days and most animals recovered completely.

Table 2: Isolation of *E. coli* isolation from dead and diarrhoeic sheep

Animal condition	Specimen/number	*E. coli* isolation	*E. coli* Serotype (number in parenthesis)
Total isolation from Dead cases	Internal organs (45)	33/45 (73.3%)	O3(7), O11(7), O80(5), O4(3), O2(3), O163 (3); O26 (2); O157 (1); Un*(2)
clinical cases with diarrhoea	Faecal samples (77)	58/77 (75.3%)	O3(11), O80(10), O11(9), O4(7), O2(5), O 163 (4); O91(3); O26 (2); O125(2);O29 (1) O157 (1); Un*(3)
Total	122	91/122 (74.6%)	O3(18), O11(16), O80(15), O4(10), O2(8), O163 (7); O26 (4); O91(3); O125(2); O157(2); O29 (1) Un* (5)*

* Un = untypable isolates

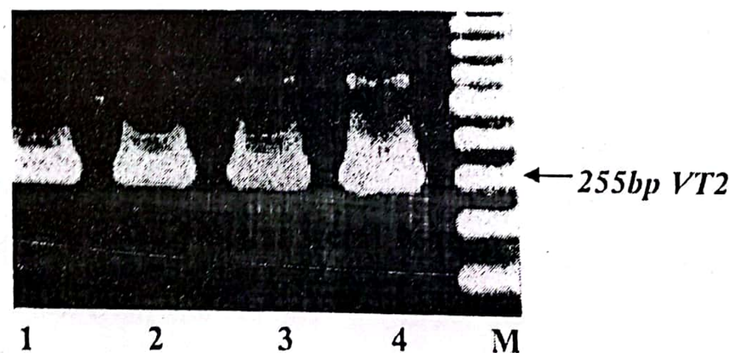

Figure 1: Results of PCR after electrophoretic analysis in 2% agarose, lane 1,2,3 and 4 reveals positive amplification of 255bp VT2 gene band from DNA extracts of *E. coli* isolates. Lane M is 1-kb DNA ladder (New England Biolabs, Inc.).

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(Table 3): Different virulence-associated markers in *E. coli* isolates in correlation to serotype.

Serotype/ number	Haemolytic activity	PCR VT2 positive	Shiga-like toxin positive	Congo red binding (Crb)	Hydrophobic protein (%)
O3(18)	8/18 (44.4%)	7/18 (38.8%)	6/18 (33.3%)	5/18 (27.8%)	(56.6±7.3)
O11(16)	5/16 (31.3%)	5/16 (31.3%)	4/16 (25%)	6/16 (37.5%)	(74.5±3.9)
O80 (15)	6/15 (40%)	4/15 (26.7%)	2/15 (40%)	3/15 (20%)	(48.2±5.2)
O4(10)	4/10 (40%)	2/10 (20%)	1/10 (10%)	3/10 (33.3%)	(63.0±6.4)
O2(8)	3/8 (37.5%)	3/8 (37.5%)	1/8 (12.5%)	3/8 (37.5%)	(42.4±3.1)
O163(7)	5/7 (71.4%)	5/7 (71.4%)	4/7 (57.1%)	2/7 (28.6%)	(81.2±5.7)
O26 (4)	4/4 (100%)	4/4 (100%)	4/4 (100%)	3/4 (75%)	(60.8±3.5)
O91(3)	2/3 (66.7%)	0/3 (0%)	1/3 (33.3%)	1/3 (33.3%)	(53.3±4.0)
O125(2)	1/ 2 (50%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	(40.2±3.2)
O157(2)	2/2 (100%)	2/2 100%	2/2 (100%)	2/2 (100%)	(63.1±7.5)
O29 (1)	1/1 (100%)	0/1 (0%)	0/1 (0%)	1/1 (100%)	(71.3)
Un(5)	2/5 (40%)	2/5 (40%)	1/5 (20%)	1/5 (20%)	(66.2±4.1)
Total	43/91 (47.3%)	34/91 (37.4%)	26/91 (28.6%)	31/91 (34.1%)	(60.1±5.4)

* mean (± SD)

(Table 4): In Vitro sensitivity of *E. coli* isolates to different antimicrobial agents.

Antimicrobial agents	Conc.	Number of *E. coli* sensitive	% of sensitivity
Gentamicin	10µg	86/91*	94.5%
Trimethoprim sulphamethazole	25µg	83/91	91.2%
Streptomycin	10µg	80/91	87.9%
Cephalosporin	30µg	77/91	84.6%
Tetracycline	30µg	71/91	78%
Chloramphenicol	30µg	63/91	69.2%
Ampicillin	10µg	54/91	59.3%
Norfloxacin	10µg	46/91	50.5%
Neomycin	30µg	41/91	45.1%
Bacitracin	10µg	37/91	40.7%
Kanamycin	30µg	32/91	35.2%
Novobiocin	30µg	29/91	31.9%
Penicillin	10 units	27/91	29.7%

* No. positive/total

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DISCUSSION

Camel breeding and rearing is very promising and profitable farm activity that can help to fill the gap of animal protein shortage in Egypt. Yet camel farms face many disease problems among which is camel-calves diarrhoea which can incur heavy losses to the farmers and breeders if the veterinary aid was posponed and procrastinated (Fowler et al., 1998 and Wernery and Kaaden 2002).

In our study the bacteriological examination of internal organ specimens (45) and the faecal samples (77) collected from camel-calves with a history of diarrhoea revealed positive isolation of *E. coli* from 33/45 (73.3%) of organ specimens and 58/77 (75.3%) of the faecal samples. Eleven different E. coli serotypes were demonstrated by the slide agglutination test (Table 2) with high prevalence of O3 (18/91, 19.8%), O11 (16/91, 17.6%), O80 (15/91, 16.5%). Two enterohemorrhagic enterotoxigenic O157 isolates (2/91, 2.2%) were also detected. Other serogroups were recorded as O4, O2, O136 and O26 which were isolated from both groups of samples. Some *E. coli* serotypes were isolated only from faecal specimens as O91, O125 and O29 (Table 2).

Many other authors reported similar isolation pattern from camel-calves with similar serogroups and with wide virulence markers (Chauhan et al., 1987; Salih 1997, 1998; and Wernery and Kaaden, 2002). They detected haemolytic, toxin producing and attaching surface proteins. Though similar *E. coli* serotypes were revealed from bovine, ovine and caprine species, that was considered understandable due to the mixed rearing system (Blanco et al., 1996; Asakura et al.,1998; Mohamed, 1998 and Cid et al., 2001) .

Investigating some virulence markers of the isolates reveled that the hemolytic activity was expressed significantly higher ($p<0.01$) higher than the other investigated markers as 43/91 (47.3%) were hemolysin producers. High hemolytic activity was expressed by O157, O26 and O29 as all isolates (100%) were hemolytic to sheep erythrocytes as expresed with wide zone of hemolysis on the blood agar plates. This virulence phenomena was supported by Ibrahim et al., (1998) and Salih (1998). It was also observed that, all isolates of the serotype O26 (4) and O157 (2) beside being hemolytic were 100% positive for the possession of the VT2 genes as revealed by the production of 255bp band in the VT2-PCR assay (Figure 1). VT2 gene which is encoding the major subtype of Shiga-like toxins is not perminant in the isolate toxin-profile which can change overtime, since this gene was encoded by phages (Gyles 1994 and Lee et al., 1996). Other serotypes in our results as O91, O125, and O29 were lacking the VT2 gene. The expression of the VT2 gene and its effect was demonstrated in vitro by the cytotoxic effect on the vero cell cultures. Our results revealed that the frequency of

the phynotypic demonstration of (CPE) on Vero cells was significantly ($p<0.01$) lower than the genotypic expression, as only 26/91 (28.6%) of the isolates were inducing CPE on the Vero cells against 34/91 (37.4%) which actually possess the gene. These results were supported and explained as the VT2 gene might be switched off in some islates or that the expression of the toxins were below the detectable levels (Lee et al., 1996 Bonardi et al 1999) In our results there was one isolate of the O91 had CPE and did not possess the VT2 sequence which may be attributed to other subtype Shiga-like toxins.

Investigating the Congo red binding (Crb) affinity, showed that 31/91 (34.1%) were Crb-positive (Crb+). Relatively high hydrophobic activity was recorded in most of the isolates, only low affinity was recorded in serotype O80 (3/15, 20%) as well as the untypable isolates (1/5, 20%). The highest percentage of hydrophobic activity of *E. coli* isolates was (81.2 ± 5.7) by the O163 isolates, whereas the lowest hydrophobic activity (40.2 ± 3.2) was recorded by the O125 isolates (table 3). Hydrophobicity and Crb affinity were reported to relate to *E. coli* attachment to host cells which is considered as thefundamental step in pathogenesis (Corbett et al., 1987, Pencu et al., 1991; Gyles et al., 1994 and Silveira et al., 2001). Our results showed, that the highest expression of virulence markers was among the *E. coli* isolates of O157 (2 isolates), O26 (3 isolates) and O163 (2 isolates), as these isolates expressed full range of investigated virulence markers (100% positive for each of hemolysine production, possession of VT2, detection of CPE on Vero cells and Crb+). Also the three serotype groups demonstrated relatively high percentage of hydrophobic activity (63.1 ± 7.5), (60.8 ± 3.5) and (81.2 ± 5.7) respectively. Most of the rest of *E. coli* isolates from other serogroups revealed either one or two virulence criteria (Table 3). These findings were also supported by Gyles (1994), Lee et al., (1996); Bonardi et al., (1999) and Pabst et al., (2003).

Antibiotic sensitivity testing of *E. coli* isolates revealed that the highest sensitivity was against gentamycin (94.4%), trimethoprim sulphamethazole (91.2%), streptomycin (87.9%) and cephalosporine (84.6%) (table 4). These results agreed with the findings of Schwartz and Dioli (1992) and Wernery and Kaaden, (2002) who investigated *E. coli* in camels. These antibiotics when recommended for therapy the episode of diarrhoea in diseased camel-calves ceased in few days and most animals recovered completely.

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