Identification of Verotoxigenic *E. coli* (VTEC) Isolated from Diarrhoeic Calves in Beni Suef Governorate by PCR Assay

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Abstract

In order to determine the occurrence, serotypes and some virulence markers of verotoxigenic *E.coli* (VTEC), 75 faecal swabs of diarrhoeic calves were randomly taken from different villages in Beni Suef Governorate and examined bacteriologicaly and serologicaly. Bacteriological examination of these swabs revealed isolation of 21strains (28%) of *E.coli* with different serogroups as follows, O111 (5 strains) O55 and O8 (3 strains for each), O26, O127 and O6 (2 strains for each), O29 and O125 (one strain for each) and two untypable isolates. Out of 21 strains of *E.coli* 12 (57.14%) strains were verocytotoxic in Vero cell assay and were belonged to serogroups O111 (5 strains), O26, O8 and O6 (2 strains for each) and one untypable isolate. Some strains presenting verocytotoxic activities were submitted to PCR assay with specific primers for VT-1 and VT-2 genes. Detection of VT genes by PCR assay on 4 different serogroups of *E.coli* which were positive in Vero cell assay and one negative strain revealed that *E.coli* O111 possessed VT-1 & VT-2 genes, *E.coli* O8 and O6 possessed VT-2 gene, O26 possessed VT-1 gene and *E.coli* O55 was negative. In conclusion PCR assay provide a rapid, sensitive and specific method for identification of VTEC isolated from diarrhoeic calves.

Introduction

E.coli is one of the most common bacterial enteric pathogens capable of causing diarrhoea in calves. Several classes of *E.coli* are now recognized on the bases of production of virulence factors, these bacteria include strains of enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC) and enteroadherant (EAEC) *E.coli* (**16 and 6**). Strains of an additional *E.coli* group termed verotoxin producing *E.coli* (VTEC) or shiga-like toxin (SLT) producing *E.coli* have been shown to produce cytotoxins active on Vero and Hela cells and often include representative of both (EPEC) and (EHEC), (**22**). Verocytotoxin producing *E.coli* (VTEC) of different serotypes have become a major concern in animal disease in different countries in the last few years (**11 and 32**), many

authors have concluded that bovines are the most important reservoir of (VTEC) (**12 and 13**) and have been also described as agents of calve diarrhoea. The main virulence factor of (VTEC) is the verotoxins (VTs) of which 2 major types, VT-1 and VT-2,were detected (**21**). Since (VTEC) strains have biochemical properties similar to those of *E.coli* from normal enteric flora, the detection of VTs, using the cytotoxicity assay in Vero cell and PCR amplification of VT gene segments are required to identify such strains (**15 and 4**). More than 400 serotypes of (VTEC) have been described and over 150 implicated in human disease (**31 and 33**). This work was aimed to, isolation and identification of *E.coli* from diarrhoeic calves, by conventional methods, determination of verocytotoxin producing *E.coli* using Vero cell assay, and detection of VT1&VT2 genes in *E.coli* by PCR.

Material and Methods

Samples: -

A total of 75 faecal swabs were collected in plastic bags from diarrhoeic calves aged one week up to six months in different villages in Beni Suef Governorate and delivered directly to Beni Suef laboratory of animal health researches.

Bacterial isolation and identification: -

E.coli were isolated and identified biochemicaly according to (**6**)

Serological typing of *E. coli*: - According to (**9**) Agar slants containing generous growth of the isolates of *E. coli* were submitted to agglutination test using polyvalent and monovalent O, E. coli antisera obtained from Denka Seiken Co., LTD. Japan.

Detection of verocytotoxins produced by *E.coli* in Vero cell: -

According to (**29**) and (**3**).

Specific activities of the extracellular verotoxins were determined for all isolated strains of *E.coli* by Vero cell assay to establish any correlation between genotypic and phenotypic expression.

Five *E.coli* strains (4 were positive in Vero cell assay and one negative strain) were used in PCR assay as follows: -

Detection of virocytotoxin genes by PCR:-

According to (**25**), (**22**), (**23**) and (**3**).

Processing of samples for PCR: -

E.coli strains were harvested from tryptone soya agar slants and mixed with 0.9 ml of PBS and centrifuged at 12.000 rpm (Eppendrorf 5415C microcentrifuge) for 5 min. The pellets were washed twice with 1.0 ml of PBS and were resuspended in 0.5 ml water-0.01 ml of 10% sodium dedocyl sulfate (SDS). This cell suspension was boiled in water bath for 10 min, cooled, and then extracted twice with phenol-chloroform. The upper aqueous layer was recovered, and the DNA was precipitated in isopropanol and resuspended in 0.03 ml of water. One microliter of this DNA was used in the PCR.

The primers used were designed on published sequence for the VT-1 (**5**) and VT-2 (**7 and 14**) as shown in table (1)

Table (1) Base sequences, locations, and predicted sizes of amplified products for the VT-specific oligonucleotide primers

Primer	Oligonucleotide Sequence (5'-3')	Location within gene	Size of amplified Product (bp)
VT1a	GAAGAGTCCGTGGGATTACG	1191-1210	130
VT1b	AGCGATGCAGCTATTAATAA	1301-1320	
VT2a	TTAACCACACCCACGGCAGT	426-445	346
VT2b	GCTCTGGATGCATCTCTGGT	752-771	

PCR protocol:

The PCR mixture consisted of 5µl of 10x PCR buffer (final concentration, 50 mM KCL, 0.01% gelatin, 10mM Tris HCL {PH 8.3}), 2.5 mM $MgCL_2$, 0.2mM (each) dATP, dCTP, dGTP, and dTTP, 0.001 mM (each) VT- specific primer pair, and 1µl of DNA in a final volume of 50 µl. PCR assays were set up as an individual primer set (i.e, DNA was amplified by VT-1 or VT-2 primers in separate testes). The PCR was started with 2.5 U of Taq polymerase (Perkin-Elmer Cetus), and the PCR mixture was overlaid with mineral oil and processed in a DNA thermal cycler (Perkin-Elmer Cetus). The cycler conditions consisted of initial denaturation at 95 ^{o}C for 5 min and then 40 cycles of 94 ^{o}C for 1 min, 55 ^{o}C for 1 min, and 72 ^{o}C for 1 min. In the final cycle, extension was carried out at 72 ^{o}C for 7 min. A reagent blank which containing all the components of the reaction

mixture with sterile distilled water instead of template DNA was included in every PCR procedure.

Specificity of the primers:

VT-1 and VT-2 primers specificities were tested with the following gram negative micro- organisms in mixed cultures: -

(i) known VT-negative isolates Shigella sonnei ATCC 25931, Salmonella typhimurium ATCC 14028 and Klebisella oxytoca (clinical isolate)

(ii) Known VT-positive *E.coli* C600 (H19B) (VT-1) and *E.coli* C600 (933W) (VT-2)

Sensitivity of the primers:

A single colony of each *E.coli* C600 (H19B) (VT-1) and *E.coli* C600 (933W) (VT-2) and two additional clinical strains each of VT-1 and VT-2-producing *E.coli* were incubated in 5 ml of BHI broth for 4h in a shaking water bath at 37 ^{o}C and were adjusted to 0.5 McFarland units (10^{8} CFU/ml). Serial 10 fold-dilutions of this culture were made in PBS; DNA was extracted from 10^{1} to 10^{7} CFU/ml containing tubes and was used in PCR assay.

Amplified products from the PCR were electrophoresed on 2% agarose gels and were stained with ethediume bromide. A 100 bp molecular size ladder (Gibco BRL, Gaithersburg, Md.) was used.

Results

Bacteriological examination of 75 faecal swabs collected from diarrhoeic calves revealed isolation of 21 *E.coli* isolates (28%), serological identification of these isolates were shown in table (2)

Table (2) Serological identification of *E.coli* isolated from diarrhoeic calves

Serogroup	No	%
O111	5	23.810
O55	3	14.285
O8	3	14.285
O26	2	9.524
O127	2	9.524
O6	2	9.524
O29	1	4.762
O125	1	4.762
Untypable	2	9.524
Total	21	100

Examination of 21 strains of *E.coli* isolated from diarrhoeic cavles in Vero cell assay cleared that 12 strains (57.14) were VTEC as shown in table (3).

Table (3) Verocytoxin activities of E.coi strains isolated from diarrhoeic calves

Serogroup	No	Vero-cell assay	
		+	%
O111	5	5	100
O55	3	0	0
O8	3	2	66.67
O26	2	2	100
O127	2	0	0
O6	2	2	100
O29	1	0	0
O125	1	0	0
Untypable	2	1	50
Total	21	12	57.14

Detection of VTs genes by PCR assay on 4 different serogroups of *E.coli* which were positive in Vero cell assay and one negative strain, revealed that *E.coli* O111 possessed VT-1 & VT-2 genes, *E.coli* O55 was negative and *E.coli* strains, O26, O8, O6 possessed one gene either VT-1 or VT-2 as shown in table (4) and Fig 1.

Table (4) Occurrence of VT-1 & VT-2 genes in some *E.coli* strains isolated from diarrhoeic calves

Serogroup	VT-1	VT-2
O111	+	+
O26	+	-
O8	-	+
O6	-	+
O55	-	-

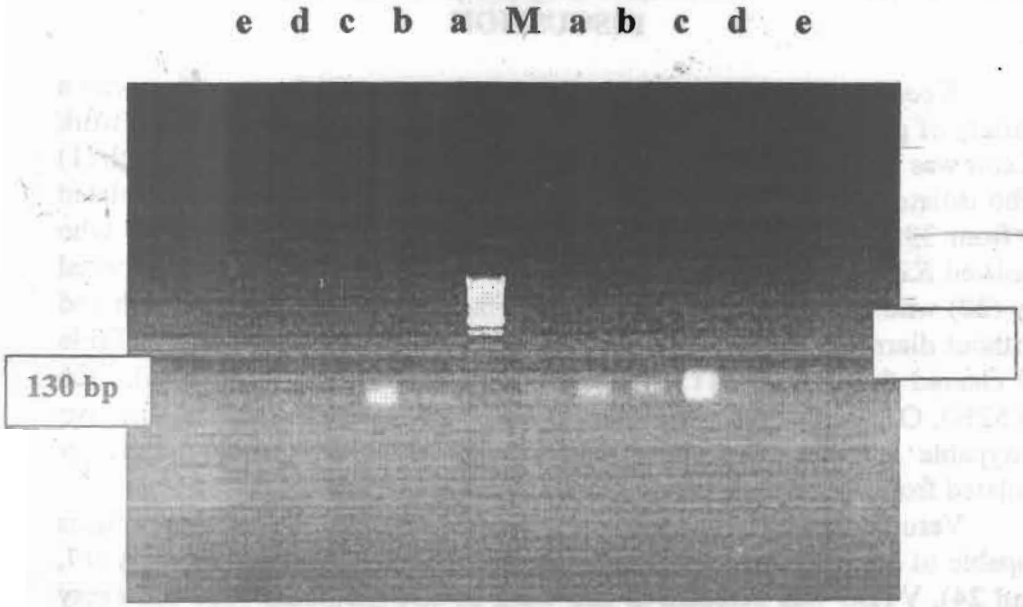

Fig (1) Electrophoretic pattern of PCR amplification products (130 bp and 346 bp specific for VT-1 and VT-2 genes of verotoxigenic strain of E. coli respectively) in 2% agarose gel stained with ethidium bromide. M- 100 bp Ladder, Lane (a) E.oli O6, Lane (b) *E.coli* O8, Lane (c) *E.coli* O111, Lane (d) *E.coli* O26 and Lane (e) *E.coli* O55.

Specificity of the PCR assay: -

It was clear from table (4) and Fig1 that the size of amplified products of the examined *E.coli* strains (O111&O26) was 130 bp and the size of amplified products of the examined *E.coli* strains (O111, O8 and O6) was 346 bp. *E.coli* O55 did not show amplification of the requisite fragments for both VT-1or VT-2 genes.

Sensitivity of the PCR assay: -

PCR assay detected VT-1 gene in *E.coli* strains at 10^4 CFU/ml and VT-2 gene in *E.coli* at 10^7 CFU/ml.

DISCUSSION

E.coli is one of the most important pathogens which associated with a variety of pathological conditions in calves specially diarrhoea. In this work *E.coli* was isolated from 28% of diarrhoeic calves, this result agreed with (1) who isolated *E.coli* from 25.83% of diarrhoeic calves and (18) who isolated it from 39.29% of diarrhoeic calves, low rate was recorded by (10) who isolated *E.coli* from 11.18% of diarrhoeic calves and high rate was recorded by (20) who isolated *E.coli* from 94% of faecal samples of calves with and without diarrhoea. Serological identification of 21 isolates of *E.coli* (Table 2) cleared that *E.coli* O111 (23.81%), O55 (14.28%), O8 (14.28%), O26 (9.52%), O127 (9.52%), O6 (9.52%), O29 (4.76%), O125 (4.76%) and two untypable isolates were found, such *E.coli* serogroups most frequently isolated from faecal samples of diarrhoeic calves (**2 and 27**).

Verotoxigenic *E.coli* (VTEC) comprises a diverse group of organisms capable of causing sever gastrointestinal disease in human and animals (**17, and 24**), VTEC was detected in this work by two methods, Vero cell assay and PCR. The results of Vero cell assay for detection of VTEC showed that 12 (16%) strains of VTEC were recovered from 75 faecal swabs of diarrhoeic calves, this result confirmed the results recorded by (19) who isolated VTEC from 9.0% of diarrhoeic calves and (12) who explained that the isolation rate of VTEC from faecal samples of dairy cattle ranged from 3.8% to 84.6%.

As shown in Table (3) the most frequent strains of *E.coli* which were VT positive in Vero Cell assay were belonged to serogroups, O111 (5 strains), O26, O8 and O6, (2 for each) and one untypable isolate, this results achieved the results recorded by (26) who isolated 26 different serogroups of verocytotoxins producing *E.coli*, (24) who showed that 45 different serogroups of *E.coli* isolated from faecal swabs of cows, calves, human and dogs and milk samples were positive in Vero cell assay and (21) who recorded that from 7 to 10% of untypable isolates of *E.coli* isolated from faecal samples of cattle were positive in Vero cell assay.

The results of PCR assay of some strains of *E.coli* which gave positive results in Vero cell assay (Fig1 and Table 4) showed that, the size of the

amplification products identifying VT-1 and VT-2 genes sequences in DNA extracted from VTEC were 130 bp and 346 bp respectively. Positive strains were belonged to serogroups O111 (VT-1 and VT-2), O26 (VT-1), O8 and O6 (VT-2) and O55 was negative in Vero cell and PCR assays, similar results were recorded by **(30)**, **(23)** and **(3)** who used PCR assay in identification of different strains of VTEC particularly including serogroups O111, O26, O8 and O6. PCR assay demonstrated a high specificity in that all VT producing *E.coli* either clinical samples which were positive in Vero cell assay or control positive strains were detected and the negative control isolates and *E.coli* strain which gave negative result in Vero cell assay did not show amplification of the requisite fragments for VT-1 orVT-2 genes in the used PCR protocol. Furthermore PCR assay demonstrated high level of sensitivity in that the amplified fragment specific for VT-1 andVT-2 was clearly detected when the DNA used in PCR was extracted from 10^4 and 10^7 C.F.U/ml respectively, the results confirm the results recorded by **(27)** and **(28)**. In conclusion PCR assay is a rapid, sensitive and specific method for identification of VTEC isolated from diarrhoeic calves.

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تصنيف ميكروب الأشيريشيا القولونى المفرزللسموم والمعزول من العجول المصابه بالأسهال فى محافظة بنى سويف بأستخدام تفاعلات أنزيم البلمره المتسلسل

سامیه أبراهيم عفيفي

فى محاولـه لتصنيف وتحديد مدى تواجد ميكروب الأشيريشيا القولونى المفرزللسموم فى العجول المصابه بالأسهال فى محافظة بنى سويف تم أخذ ٧٥ عينة براز حيث تبين أن (٢٨%) منها تحتوى على عترات ميكروب الأشيريشيا القولونى
١١١ Oو ٥٥ Oو ٨ Oو ٢٦ Oو ١٢٧ Oو ٦ Oو ٢٩ O، ١٢٥ O بالأعداد الأتيه على التوالى ٥ و ٣ و ٣ و ٢ و ٢ و ٢ و ١ و١. ولم يتحدد نوع عترتين وبأختبار هذه االعترات بأستخدام بيئة الخلايا كانت العترات الاتيه ١١١ O و ٨ O و ٢٦ O و ٦ O وعتره أخرى غير محدده مفرزين للسموم وعند فحص بعض هذه العترات بأستخدام تفاعلات أنزيم البلمره المتسلسل كانت نتائجه مؤيده لأختبار الخلايا غير أنها كانت أكثر دقه و أسرع كما أنها حددت نوع العامل الجينى المفرز للسموم.