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DETECTION OF *eaeA* AND *hlyA* GENES IN ESCHERICHIA COLI-CAUSING DIARRHOEA IN YOUNG CALVES BY MULTIPLEX PCR

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SUMMARY

E.coli was isolated from the faeces of 28/31 (90.3%) diarrhoeic and 9/11 (81.8%) apparently healthy calves. *E. coli* O157 was the most prevalent serotype, detected from 5/31 diarrhoeic calves (16.1%). No O157 *E. coli* was revealed from the apparently healthy calves. Other serotypes were detected in diarrhoeic calves as O136, O111, O118, O103, O91, O121, O145, O87, O22 and O8 whereas O91, O113, O87, and O8 were revealed from the apparently healthy. There was also 3 untypable *E. coli* isolates. Screening virulence genes *eaeA* and *hlyA* in identified isolates or in faecal culture crude extract revealed clear PCR products, *eaeA* gene of 384bp and *hlyA* of 534bp. Isolates from diarrhoeic calves possessed 20/29 (69%) *eaeA* gene and 21/29 (72.4%) *hlyA* gene. Detection of the same genes in the faecal samples from apparently healthy calves was manifested in significantly ($p<0.01$) lower pattern as *eaeA* was detected only in 2/9 isolates (22.2%) and *hlyA* in 3/9 (33.3%). Use of 2-4h faecal culture crude extract in direct PCR assay for the detection of *eaeA* and *hlyA* genes demonstrated higher incidence of both genes than their detection from isolate lysates, as in diarrhoeic specimens, *eaeA* was detected in 23/31 (74.2%) and *hlyA* in 25/31 (80.6%), whereas in samples collected from apparently healthy *eaeA* was detected in 3/11 (27.3%) and *hlyA* in 4/11 (36.4%). All *E. coli* isolates from diarrhoeic calves, as well as the specimens from which they were isolated, were 100% positive for either single detection of one gene (*eaeA* or *hlyA*) or for double possession of both genes, as 12/29 (41.4%) isolates harbored both genes at the same time, whereas 17/29 (58.6%) possessed only one of either genes *eaeA* or *hlyA*. Faecal sampls from diarrhoeic calves showed signifiantly ($p<0.01$) higher detection of double possession of both genes *eaeA* and *hlyA*, 16/31 (51.6%), whereas single possession of one of either genes was 15/31 (48.4%). Isolates from apparently healthy calves, as well as faecal speci-

mens from which they were isolated, in all cases, possessed only one gene either *eaeA* or *hlyA* (5/9, 55.6% isolates, and 7/11, 63.6% faecal samples). There was no double possession of both genes in any case of samples from apparently healthy calves.

INTRODUCTION

Escherichia coli produces a wide range of illnesses in wide spectrum hosts. The infection in man can vary from mild diarrhoea to life threatening illnesses as haemorrhagic colitis (HC) and haemolytic uraemia syndrome (HUS). In animals as calves and sheep the infection takes the form of scour which could be watery or bloody. *E. coli* have been distinctively identified into six major pathogenic groups namely, enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC) and enteropathogenic *E. coli* (EPEC) and enteroadherent *E. coli* (Nataro and Kaper 1998; Pabst et al., 2003) according to the virulence factors contributing to the pathogenesis (Karmali 1989; Belanger et al., 2002). Among the virulence traits of *E. coli* are the *eaeA* gene encoding intimin, an outer membrane protein involved in the intimate attachment of the *E.coli* to the gut endothelial cells, there are five recognized types of intimins namely, α, γ, δ, (and (which can be a criteria of differentiation of isolates of the same serotype (Jenkins et al., 2002). *HlyA* gene which is the plasmid-borne enterohemolysin, encoded by EHEC and usually associated with serious-disease-causing strains, that included bloody diarrhoea.

Calves were considered a major reservoir of different groups of *E. coli* especially enterohemorrhagic *E.coli* which transmitted the infection to man through consumption of contaminated milk, meat and other animal products. Some species of the serotype O157, O145, O111, O103 and O26 which were associated with severe outbreaks in calves and were transmitted to man leading to cases of fatalities (Willshaw et al., 2001). Different techniques were employed for diagnosis of E. coli infection in diferent hosts including the isolation and seroidentification of the *E. coli* species, latex agglutination tests, ELISA and PCR assays. The aim of this work was to investigate the different types of *E.coli* involved in diarrhoea in calves and application of multiplex PCR assay in detection of putative virulence traits as well as diagnosis of infection directly from the faecal specimens withen 4h by direct PCR.

MATERIALS AND METHODS

Investigated animals and specimens:

In this study faecal samples were collected from 28 diarrhoeic calves aged from few days to 6 months with severe dirrhoea anorexia, fever that reached 41°C in some late progressive cases. Another 11 faecal samples were collected from apparently healthy calves not having any symptoms

of diarrhoea. All samples were collected during the period from August 2002 to February 2003 from governmental and private farms in Giza governorate.(buffaloe calves were not included in this study). All samples were transferred immediately to the laboratory in ice box with minimum delay and examined bacteriologically.

Isolation and Identification of *E coli*

Faecal specimens were cultured directly on blood agar containing 10% sheep blood, sorbitol Mac Conkey and Mac Conkey agar plates and incubated at 37°C for 24hrs. Suspected colonies were confirmed biochemically by API20 diagnostic strips (Biomereaux, France). The biochemically confirmed *E. coli* isolates were serologically identified by slide agglutination test using (Ewing, 1986) using polyvalent and monovalent somatic (O) and flagellar (H) antigens (Difco Laboratories USA).

Preparation of DNA template

Bacterial cultures 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 was transferred to microfuge tubes and used for PCR assays (Reischl et al., 2002).

Multiplex PCR assay for eaeA and hlyA:

Samples from each bacterial DNA extract (2(l) were amplified in 50(l reaction mixtures containing 200 µM of each deoxynucleoside triphosphates, 250nM of *eaeA* F and *eaeA* R primers as well as *hlyA* F and *hlyA* R 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 30 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 5 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).

Analysis of primary faecal cultures by direct multiplex PCR assay:

Crude extracts of all diarrhoeic faecal cultures 28 (2-4h Trypticase soy broth) as well as the 11 specimens which collected from apparently healthy calves were analyzed for the *eaeA* and *hlyA* using the forementioned primers and conditions in direct multiplex PCR assay (Paton and Paton 1998; Belanger et al., 2002)

Statistical analysis

Statistical analysis of the obtained results was carried out using the "t" test according to the method of Snedecor and Cochran (1967).

Table (1): Specific primers for *eaeA* and *hlyA* genes

Primer	Sequence	Specificity of targeted sequence	Targeted amplicon size
eae AF	5'GACCCGGCACAAGCATAAGC3'	From 27-410 nucleotide of the *eae A*	384bp
eae AR	5'CCACCTGCAGCAACAAGAGG3'		
hly AF	5'GCATCATCAAGCGTACGTTCC3'	From 70-603 nucleotide of the *hlyA*	534bp
*hlyA*R	5'AATGAGCCAAGCTGGTTAAGCT3'		

RESULTS

E. coli was the most prevalent bacteria isolated from the faeces of the investigated diarrhoeic or apparently healthy calves. No cases of Salmonella or other enteropathogenic bacteria were revealed from these examined samples. Out of the 28 diarrhoeic faecal samples 29 *E. coli* isolates were detected. Only one animal harbored two isolates of serotype O157 and O111 (Table 2). *E. coli O*157 was the most prevalent serotype, detected from 5/31 diarrhoeic calves (16.1%). No O157 *E.coli* was revealed from the apparently healthy calves. Other serotypes were detected in diarrhoeic calves as O136, O111, O118, O103, O91, O121, O145, O87, O22 and O8 whereas O91, O113, O87, and O8 were revealed from the apparently healthy (Table 3). Also 3 untypable *E. coli* isolates were detected, 2 from the diarrhoeic and 1 from apparently healthy calves. There were also 5 samples bacteriologically negative, 3 from diarrhoeic and 2 from apparently healthy animals (from which only Enterococci and other coliform were detected).

Screening the *E. coli* isolates for the the *eaeA* and *hlyA* genes by standard multiplex PCR assay or using faecal culture crude extract, revealed clear PCR products of the expected sizes. EaeA gene of 384bp and *hlyA* of 534bp (Figure 1). *E. coli* isolates showed that 20/29 (69%) isolates from diarrhoeic calves possessed the *eaeA* gene and 21/29 (72.4%) possessed the hlyA gene. Detection of the same genes in the faecal samples from apparently healthy calves was manifested in significantly lower pattern ($p<0.01$), as *eaeA* was detected only in 2/9 isolates (22.2%) and *hlyA* in 3/9 (33.3%) of the isolates (Table 3). The use of 2-4h faecal culture crude extract in direct multiplex PCR assay for the detection of *eaeA* and *hlyA* genes detected significantly ($p<0.05$) higher incidence of both genes than their detection from isolate lysates, as in diarrhoeic specimens, *eaeA* was detected in 23/31 (74.2%) and *hlyA* in 25/31

(80.6%), whereas in samples collected from apparently healthy *eaeA* was detected in 3/11 (27.3%) and *hlyA* in 4/11 (36.4%) (Table 2&3).

Analysis of results showed that all *E. coli* isolates from diarrhoeic calves, as well as the specimens from which they were isolated, when screened by PCR assays, were 100% positive for either single detection of one gene (*eaeA* or *hlyA*) or for double possession of both genes. There was 12/29 (41.4%) isolates harbored both genes, whereas 17/29 (58.6%) possessed only one gene either *eaeA* or *hlyA*. Faecal sampls from diarrhoeic calves showed significantly ($p<0.01$) higher detection of double possession of both genes *eaeA* and *hlyA*, 16/31 (51.6%), whereas single possession of one of either genes was 15/31 (48.4%). *E. coli* isolates from apparently healthy calves, as well as faecal specimens from which they were isolated possessed only one gene either *eaeA* or *hlyA* (5/9, 55.6% isolates, and 7/11, 63.6% faecal samples) There was no double possession of both genes in any case from the apparently healthy calves.

Table (2): Pattern of *eaeA* and *hlyA* genes distribution in different serotypes of *E. coli* from faecal samples of diarrhoeic calves

Animal condition	*E.coli* serotype	isolates/ animals	*E.coli* isolates PCR		*Faecal cultural PCR* isolates PCR	
			eaeA+ve	*hlyA*+ve	*eaeA*+ve	*hlyA*+ve
31 Diarrhoeic animals. 28 were culture +ve 3-ve (90.3%)	O157	5/5*	5/5	5/5	5/5	5/5
	O136	4/4	3/4	3/4	3/4	3/4
	O111	3/3*	2/3	2/3	2/3	2/3
	O118	3/3	1/3	2/3	2/3	2/3
	O103	3/3	1/3	2/3	2/3	2/3
	O91	2/2	1/2	2/2	1/2	2/2
	O121	2/2	1/2	1/2	1/2	2/2
	O145	2/2	2/2	1/2	2/2	1/2
	O87	1/1	1/1	0/1	1/1	1/1
	O22	1/1	1/1	1/1	1/1	1/1
	O8	1/1	1/1	1/1	1/1	1/1
	Un	2/2	1/2	1/2	1/2	1/2
	negative	0/3	0/0	0/0	1/3	2/3
Total		29/31*	20/29 (69%)	21/29 (72.4%)	23/31 (74.2%)	25/31 (80.6%)

*29 *E.coli* isolates from 28 culturally positive animals, 2 isolates were detected in one animal. (total no of animals 31). Un, untypable.

Table (3): Pattern of *eaeA* and *hlyA* genes distibution in different serotypes of *E. coli* from faecal samples of apparently healthy calves

Animal condition	*E.coli* serotype	isolates/ animals	*E.coli* isolates PCR		*E.coli* isolates PCR	
			cacA+ve	hlyA+ve	cacA+ve	hlyA+ve
11 apparently healthy (11 samples)	O91	3/3	1/3	1/3	1/3	1/3
	O113	2/2	0/2	1/2	0/2	1/2
	O87	2/2	1/2	0/2	1/2	0/2
	O8	1/1	0/1	1/1	0/1	1/1
	untypable	1/1	0/1	0/1	1/1	0/1
	negative	0/2	0/0	0/0	0/2	1/2
Total		9/11	2/9 (22.2%)	3/9 (33.3%)	3/11 (27.3%)	4/11 (36.4%)

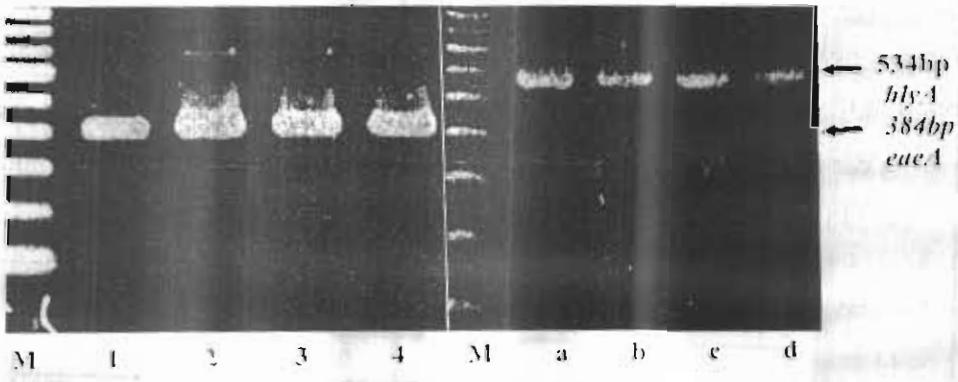

Figure (1): Electrophoretic analysis of the PCR products obtained from DNA extracts of *E. coli* isolates and primary faecal cultures, by amplification of highly conserved portion of *eaeA* 384 bp (lane1, 2, 3, and 4) and the plasmid-borne DNA sequences coding for the *hlyA* 534 bp (lane a, b, c, and d). Lane M is 1-kb DNA ladder (New England Biolabs, Inc.).

DISCUSSION

E. coli infection in calves is still one of the ubiquitous field problems that causes serious zoonotic health problems as it was considered one of the major health foodborne pathogens, that caused many deaths in many countries in the world, beside the great economic losses incured in animal industry (Lee et al., 1996; Hiramatsu et al., 2002). In our study, *E. coli* was the most prevalent bacteria isolated from the faeces of diarrhoeic or apparently healthy calves. No cases of Salmonella or other enteropathogenic bacteria were revealed from the examined samples that might interfered with our results. Out of the 28 culture-positive diarrhoeic faecal samples 29 *E. coli* isolates were detected, as only one animal harbored two isolates of serotype O157 and O111 (Table 2). Mixed infection with different serotypes of *E. coli* in a single animal is common in severe infections (Orden et al., 2002). *E. coli* O157 was the most prevalent serotype, detected from 5/31 diarrhoeic calves (16.1%). No O157 *E. coli* was revealed from the apparently healthy calves, which could incriminate this serotype of inistigating and establishing the pathogeneses. Many authors has confirmed the high virulence characteristics of the serotype O157 especially the motile strains O157:H7 which caused severe losses in humans and animals (Fratamico et al., 1995; Lee et al., 1996; Donkersgoed et al., 1999, Jenkins et al., 2002). Other serotypes were detected in diarrhoeic calves as O136, O111, O118, O103, O91, O121, O145, O87, O22 and O8 whereas O91, O113, O87, and O8 were revealed from the apparently healthy. Also 3 untypable *E. coli* isolates were detected, 2 from the diarrhoeic and 1 from apparently healthy calves. There were also 5 samples negative for *E. coli* isolation, 3 from diarrhoeic and 2 from apparently healthy animals (from which only Enterococci and other coliform organisms were detected) (Table 3). It was apparent from our results, that the wide diversity of serotypes revealed from clinical diarrhoeic cases were incomparable with the limited serotypes revealed from apparently healthy calves. This could be due to the complete absence of certain virulent strains in the apparently normal calves or due to the limited undetectable colonization of these virulent strains under the normal health status. Moreover some serotypes as O136, O111, O103, O145 which were detected in clinical cases were incriminated by other research groups as causing severe infections in humans and animals, due to involvement of different virulence factors as toxines and intimins (Bettelheim et al., 2001; Jenkins et al., 2002; Olsson et al., 2002 and Padola et al., 2002).

Among the major virulence genes of *E. coli* are the *eaeA* and *hlyA* gene, encoding intimin, 97KDa outer membrane protein involved in the intimate attachment of the E. coli to the gut mucosa and

the plasmid-borne enterohemolysin, encoded by EHEC which usually involved bloody diarrhoea. Both genes were reported as associated with serious infections (Fratamico et al., 1995; Jenkins et al., 2002). Screening the *E. coli* isolates for the the *eaeA* and *hlyA* genes by multiplex PCR assay, using faecal culture crude extract revealed clear PCR products of the expected sizes, *eaeA* gene of 384bp and *hlyA* of 534bp (Figure 1). *E. coli* isolates showed that 20/29 (69%) isolates from diarrhoeic calves possessed the *eaeA* gene and 21/29 (72.4%) possessed the *hlyA* gene. Detection of the same genes in the faecal samples from apparently healthy calves was manifested in significantly ($p<0.01$), lowed pattern, as *eaeA* was detected only in 2/9 isolate (22.2%) and *hlyA* in 3/9 (33.3%) of the isolates (Table 3) These results were expected as we mentioned earlier, due to the absence or the limited colonization of virulent strains in healthy status. These findings were also supported by other workers as Jenkins et al., (2002), Bettelheim et al., (2001), Fratamico et al., (1995), Olsson et al., (2002), Belanger et al., (2002).

The use of 2-4h faecal culture crude extract in direct multiplex PCR assay for the detection of *eaeA* and *hlyA* genes detected significantly ($p<0.05$) higher incidence of both genes than their detection levels from isolate lysates, as in diarrhoeic specimens, *eaeA* was detected in 23/31 (74.2%) and *hlyA* in 25/31 (80.6%), whereas in samples collected from apparently healthy eaeA was detected in 3/11 (27.3%) and *hlyA* in 4/11 (36.4%) (Table 2&3). These results confirmed that application of direct multiplex PCR of 2-4h enrichment cultures has been proven sensitive, specific and rapid method for clinical diagnosis than the conventional classical methods of culturing on patent media. Also it was reported of the negligable possibility of missing positive cases by the direct multiplex PCR due to the great sensitivity and specificity, which greatly supported our results (Belanger et al., 2002).

The PCR primers for the *eaeA* gene were selected to amplify a segment of a highly coserved region at the 5' of the *eaeA* gene that is conserved among ETEC and EPEC, which in turn would suggest that these primers would be efficient tool to detect both categories of diarrheagenic *E. coli*. Also the selection of the *hlyA* specific primers was targeted to detect the category of EHEC (Paton and Paton 1998; Reischel et al., 2002). It has been suggested that the protein product of the enterohemorrhagic *eaeA* gene is a 97-kDa intimin protein responsible for attaching and effacing, which is considered a major virulence character of the pathogenesis process. This intimin protein was similar to 94 kDa protein produced by the *eaeA* gene of enteropathogenic *E.coli* that showed 97% homology at the 5' and only 59% homology at the 3' when the AE19 and AE20 were used to flank the 1087bp eaeA fragment (Fratami-

co et al., 1995)

Analysis of results showed that all *E. coli* isolates from diarrhoeic calves, as well as the specimens from which they were isolated, when screened by PCR assays, were 100% positive for either single detection of one gene (*eaeA* or *hlyA*) or for double possession of both genes. There was 12/29 (41.4%) isolates harbored both genes, whereas 17/29 (58.6%) possessed only one gene either *eaeA* or *hlyA*. Faecal sampls from diarrhoeic calves showed signifiantly ($p<0.01$) higher detection of double possession of both genes *eaeA* and *hlyA*, 16/31 (51.6%), whereas single possession of one of either genes was 15/31 (48.4%). *E. coli* isolates from apparently healthy calves, as well as faecal specimens from which they were isolated possessed only one gene either *eaeA* or *hlyA* (5/9, 55.6% isolates, and 7/11, 63.6% faecal samples). There was no double possession of both genes in any case collected from apparentl healthy calf. Our resuls also demonstrated that, direct detection of the targeted sequences from bacterial isolates was not synonymous to the detection from broth enrichment, which serves two main purposes namely, dilution of the PCR inhibitors in the specimen and multiplication of the bacterial population which increase the copies of the targeted DNA sequebnces. This was also approved by Paton and Paton (1998).

In conclusion, our results demonstrated that direct multiplex PCR assay targeted *eaeA* and *hlyA* is very promising rapid, sensitive and specific for direct detection of attaching and effacing as well as enterohemorrhagic E. coli in few hours and can curb heavy economic losses due to false or delayed diagnosis.

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