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Detection of Intimin Gene and Its Variants, In *E. coli* Isolates from Calf Diarrhoea Cases

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A B S T R A C T

The present study was designed to study the presence of intimin gene and its variants in 91 *E. coli* isolates. All 91 *E. coli* isolates were screened for the presence of intimin gene by PCR, among which only 15 isolate (16.8 percent) were positive for eaeA gene. Out of 15 isolates positive for eae (intimin) were assayed for intimin variants *viz*., eaeβ1(811bp), eaeγ1(804bp), eaeγ2(808bp), eaeδ (833bp), eaeζ (206bp), and eaeξ (468bp) by PCR. Eight isolates could be typed using 6 intimin variant primer set and 2 isolate (13.3percent) were positive for eaeβ1 variant, one isolate (6.67percent) was positive for eaeγ1 and five (33.3percent) were positive for eaeγ2. Intimin gene codes for different types of protein which are immunogenic in nature and thus it can represent an important tool for *E. coli* typing in routine diagnostic as well as epidemiological and clonal studies. Different intimin variants are responsible for different host tissue cell tropism which could be taken into consideration to identify the specific intimin variant types responsible for attachment to specific tissue tropism.

Keywords

E. coli, Intimin, Intimin gene variants.

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Introduction

Enteropathogenic *E. coli* (EPEC) is an important category of diarrheagenic *E. coli* which has been linked to infant diarrhea in the developing world. EPEC harbors a pathogenicity island that encodes a series of proteins involved in the attaching and effacement lesions of the intestinal microvilli of the host cell (Jerse *et al.,* 1990) and the presence of the large EPEC adherence factor (EAF) plasmid, on which also the cluster of genes encoding bundle-forming pili (bfp) is present (Kaper, 1996). Based on these, EPEC strains are classified as typical when they possess the EAF plasmid, whereas atypical EPEC strains do not possess the EAF plasmid (Trabulsi *et al.*, 2002).

Like all diarrheagenic *E. coli* strains, EHEC and EPEC must first colonize the intestinal mucosa. Both pathovars produce a

characteristic histopathological feature, known as the "attaching-and-effacing" (A/E) lesion, by subverting the intestinal epithelial cell function (Frankel *et al.,* 1998). This lesion is characterized by the effacement of microvilli and by intimate adherence between the bacteria and the epithelial cell membrane. Marked cytoskeletal changes, including accumulation of polymerized actin, occur directly beneath the adherent bacteria. The formation of A/E lesions is governed by a pathogenicity island known as the locus of enterocyte effacement (LEE), which was first described in the EPEC O127 strain E2348/69 (MacDaniel *et al.,* 1995).

The LEE central region contains the eae (for *E. coli* attachment effacement) gene encoding a 94- to 97-kDa outer membrane protein known as intimin (Jerse *et al.,* 1990). This protein mediates close contact between the bacteria and the target cell, upon interaction with its translocated receptor Tir (for translocated intimin receptor), encoded by a gene located upstream eae (Kenny *et al.,* 1997; Deibel *et al.,* 1998).

Tir had been initially identified as a 90-kDa tyrosine phosphorylated protein from the target cell membrane and was called Hp90 (Rosenshine *et al.,* 1992). In animal models, intimin has been shown to be necessary for EHEC O157:H7 to intensively colonize the intestines and cause diarrhea and A/E lesions in calves and colonic edema and A/E lesions in piglets (Donnenberg *et al.,* 1993; McKee *et al.,* 1995; Dean-Nystrom *et al.,* 1998). *E. coli* eae gene have been cloned and sequenced from different EPEC and EHEC strains from different sources.

The overall pattern for these sequences shows high conservation in the N-terminal region and variability in the last C-terminal 280 amino acids of the intimin, where binding to the enterocytes (Frankel *et al.,* 1994) and Tir (Hartland *et al.,* 1999) occurs. Various studies have investigated the heterogeneity of eae among *E. coli* strains by amplification of the variable 39 region by PCR and restriction fragment length polymorphism (RFLP) analysis of PCR products. Schmidt *et al.,* (1993) designed two primer pairs capable of differentiating the *eae* genes of EPEC and EHEC strains of serogroup O157. Agin and Wolf (1997) provided the existence of atleast 3 immunologically distinct intimin types called α, β, and γ. A multiplex PCR was designed to detect eae and simultaneously identify the specific alleles encoding these three intimin variants (Reid *et al.,* 1999). In another study, Adu-Bobie *et al.,* (1998) used antisera to the C-terminal region and PCR to investigate antigenic variation and classify the cell-binding domain of intimin expressed by A/E lesion-forming bacterial pathogens. By these means, these authors identified four distinct intimin types: intimin α, intimin β, intimin γ, and intimin δ. Detection of Intimin alleles/variants represent an important tool for *E. coli* typing in routine diagnostic as well as epidemiological and clonal studies. The C-terminal end of intimin is responsible for receptor binding, and it has been suggested that different intimins may be responsible for different host tissue cell tropism (Zang *et al.,* 2002).

Intimin type specific PCR assays identified 14 variants of the eae gene that encodes 14 different intimin types and subtypes (α1, α2, β1, β2, γ1, γ2/θ, δ /κ,ξ,η,ι,λ,μ,ν), (Blanco *et al.,* 2004) Nevertheless, some EHEC and EPEC strains still express non typeable intimins (China *et al.,* 1998; Pelayo *et al.,* 1999; Reid *et al.,* 1999).

Materials and Methods

The present study was carried out for PCR based detection of intimin gene variants of *E. coli* isolates obtained from fecal samples of

diarrhoeic calves. The study was conducted on ninety one field isolates of *E. coli*, which were maintained at the Department of Veterinary Microbiology, Veterinary College, Anand.

DNA extraction

The genomic DNA of *E. coli* isolates was isolated according to Wilson (1987) with minor modifications. These DNA samples were used for PCR reactions.

Preparation of broth culture of *E. coli*

The culture was prepared by inoculating the isolate in Luria broth (HiMedia, Mumbai) and incubating at 37°C for 24 h in a shaker water bath.

Preparation of material for nucleic acid extraction

About 50 ml of broth culture was centrifuged at 10,000 rpm for 10 min at 5°C. The supernatant was discarded and the pellet was used for extraction of nucleic acid extraction.

Following solutions were used for extraction

Tris-EDTA (pH 8.0)

10 mM Tris-HCl

1 mM EDTA

SDS (10% w/v)

Proteinase K solution (20mg/ml, w/v)

5 M Sodium chloride

CTAB (Hexadecyl trimethyl ammonium bromide, 10% solution in 0.7M NaCl)

Saturated phenol (pH 8.0)

Chloroform

Isoamyl alcohol

7.5 M Ammonium acetate

Chilled absolute ethanol

Isolation of genomic DNA by Proteinase-K-SDS method

Pellet containing bacterial cells was suspended in 2 ml Tris-EDTA (pH-8.0), 250 µl SDS (10% w/v) and10µl of proteinase K solution (20mg/ml, w/v) and incubated for 1 h at 37°C.

Subsequently, 500 µl of 5M NaCl followed by 100 µl CTAB (10% solution in 0.7 M NaCl) was added and incubated in water bath for 10 min at 65°C.

The solution was spun at 8,000 rpm for 10 min after mixing with equal volume of chloroform: isoamyl alcohol (24:1) and upper phase was transferred to clean microfuge tube.

Equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) was added, mixed well by inverting, spun for 10 min at 10,000 rpm and upper aqueous phase was transferred again to a clean microfuge tube.

In the collected supernant, the DNA was precipitated with one-tenth volumes of ammonium acetate (7.5M) and double the volume with chilled absolute ethanol.

Tube was centrifuged for 10 min at 11,000 rpm and ethanol was discarded.

The pellet was washed in 70% ethanol and again spun for 5 min at 11,000 rpm.

Ethanol was discarded and pellet was dried.

DNA was resuspended in 200 µl sterile distilled water and kept in water bath at 65°C for one hour and stored at -20°C till use.

Quality checking and quantitation of DNA

Quality and purity of DNA were checked by agarose gel electrophoresis. 0.8 percent agarose in 0.5X TBE (PH 8.0) buffer (Sambrook *et al.,* 1989) was used for submarine gel electrophoresis. Ethidium bromide (1 %) was added @ 5µl /100ml.

The wells were charged with 5µl of DNA preparations mixed with 1X BPB dye.

Electrophoresis was carried out at voltage 5V/cm for 60 min at room temperature. DNA was visualized under UV transilluminator.

Quantity of DNA was calculated by spectrophotometric method. OD at 260 and 280 were taken in UV spectrophotometer with distilled water as reference.

Concentration (µg/ml) = OD at 260 x dilution factor x 50

Where 50 is concentration of dsDNA expressed in 1 µg/ ml at OD of 1.

PCR reaction for Intimin (eae) gene variants

Out of the 91 isolates, fifteen isolates which were positive for intimin gene (eae) were used for detection of intimin variants. Primer details (Table 1)

The following components were used in PCR mixture.

2X PCR Mastermix (Fermentas, Life Sciences):

4mM $MgCl_2$

0.4mM of each dNTPs (dATP, dCTP, dGTP, dTTP)

0.05 units/ml of *Taq* DNA polymerase

150 mM tris-HCl PCR buffer

DNA samples were diluted to a final concentration of 30ng/µl and 3µl of this preparation was used as template for PCR.

PCR conditions

PCR was carried out in a final reaction volume of 25µl using 0.2 ml thin wall PCR tube. A master mix for 15 samples was prepared and aliquoted in 22µl quantities in each PCR tube. Three µl sample of DNA was added in each tube to make the final volume of 25µl as in Table 2.

PCR tubes containing the mixture were tapped gently and quickly spun at 10,000 rpm for few seconds. The PCR tubes with all the components were transferred to thermal cycler (Eppendorf, Germany).

PCR protocol

The PCR protocol was as described in Table 3 for the primers used.

Agarose gel electrophoresis

The following reagents were used for agarose gel electrophoresis

Agarose

Agarose	2.00 g
TBE (0.5X)	100 ml
Ethidium bromide (1%)	5 µl

Tris Borate EDTA (TBE) buffer, pH 8.3 (5X)

Tris HCI	0.445 M
Boric Acid	0.445 M
EDTA	10 mM

Ethidium bromide (1%)

Ethidium bromide	10 mg
Distilled water	1.0 ml

To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1µl of 6X gel loading buffer from each tube and electrophoresed on 2.0 per cent agarose gel along with 100bp DNA Ladder (GeneRuler- Fermentas) and stained with ethidium bromide (1 per cent solution at the rate of 5 µl/100 ml) at constant 80 V for 30 minutes in 0.5X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

Results and Discussion

Detection of Intimin alleles/variants represent an important tool for *E. coli* typing in routine diagnostic as well as epidemiological and clonal studies. The C-terminal end of intimin is responsible for receptor binding, and it has been suggested that different intimins may be responsible for different host tissue cell tropism (Zang *et al.*, 2002). Intimin type specific PCR assays identified 14 variants of the eae gene that encodes 14 different intimin types and subtypes (α1, α2, β1, β2, γ1, γ2/θ, δ /κ, ξ, η, ι, λ, µ, ν), (Blanco *et al.*, 2004)

Out of 91 isolates, 15 isolates which were positive for intimin gene were, typed for 6 intimin variant types by PCR amplification using the total 6 sets of primer *viz*., eaeβ1(811bp), eaeγ1(804bp), eaeγ2(808bp), eaeδ (833bp), eaeζ (206bp), and eaeξ (468bp).

Eight out of 15 isolates could be typed for intimin variant types, however in seven isolates no intimin variant type was detected. Two out of 15 (13.3%) were positive for eaeβ1 (811bp) primer. One isolate (6.67%) was positive for eaeγ1 primer (804bp). Five isolates (33.3%) were positive for eaeγ2 (808bp) and no isolate was positive for other three primer *viz.*, eaeδ (833bp), eaeζ (206bp), and eaeξ (468bp). (Table 4)

Intimin mediates the intimate attachment of the bacteria to the host cell surface and is required for the formation of the characteristic A/E lesion associated with EPEC and EHEC infections. Several studies have shown that a considerable heterogeneity exists within the DNA sequences of the eae genes of different *E. coli* strains and that the corresponding changes in the amino acid sequence also represent antigenic variations. Using immunological and genetic approaches, Agin and Wolf (1997) and Adu- Bobie *et al.*, (1998) revealed the existence of four distinct intimin types: intimin α, intiminβ, intiminγ, intimin δ.

Detection of Intimin alleles/variants represent an important tool for *E. coli* typing in routine diagnostic as well as epidemiological and clonal studies. The C-terminal end of intimin is responsible for receptor binding, and it has been suggested that different intimins may be responsible for different host tissue cell tropism (Zang *et al.*, 2002).

Out of 91 isolates, 15 isolates were typed for 6 intimin variants *viz*., eaeβ1(811bp), eaeγ1 (804bp), eaeγ2(808bp), eaeδ (833bp), eaeζ (206bp), and eaeξ (468bp) by PCR. Intimin of 8 out of 15 isolates could be typed for intimin variants, while seven isolates could not be typed for any of the variants. Two isolates out of 15 (13.3%) were positive for eaeβ1 primer. One isolate (6.67%) was positive for eaeγ1 primer. Five isolates (33.3%) were positive for eaeγ2 and no isolate was positive for other three primer *viz*, eaeδ (833bp), eaeζ (206bp), and eaeξ (468bp).

Table.1 Details of primers used for PCR for intimin gene variants

Primers	Sequences (5'- 3')	Target Gene	Expected Size of amplified product(bp)	Reference
eae-β1 (F) eae-β1 (R)	CGC CAC TTA ATG CCA GCG CTT GAT ACA CCT GAT GAC TGT	eae-β1	811	Blanco *et al.*, (2004)
eae-γ1(F) eae-γ1 (R)	AAA ACC GCG GAG ATG ACT TC AGA ACG CTG CTC ACT AGA TGT C	Eae-γ1	804	Blanco *et al.*, (2004)
eae-γ2 (F) eae-γ2 (R)	AAA ACC GCG GAG ATG ACT TC CTG ATA TTT TAT CAG CTT CA	eae-γ2	808	Blanco *et al.*, (2004)
eae-δ (F) eae-δ (R)	AAA ACC GCG GAG ATG ACT TC CTT GAT ACA CCC GAT GGT AAC	eae-δ	833	Blanco *et al.*, (2004)
eae- ζ (F) eae- ζ (R)	GGT AAG CCG TTA TCT GCC ATA GCA AGT GGG GTG AAG	eae- ζ	206	Blanco *et al.*, (2004)
eae- ξ (F) eae- ξ (R)	AAA ACC GCG GAG ATG ACT TC ACC ACC TTT AGC AGT CAA TTT G	eae- ξ	468	Blanco *et al.*, (2004)

Table.2 Composition for PCR reaction for Intimin gene variants

Components	Quantity	Final Concentration
2X PCR Mastermix	12.50 µl	1X
DNase free water	7.50 µl	--
Primers Forward	1 µl	10 pmol/µl
Reverse	1 µl	10 pmol/µl
DNA template	3.0 µl	--
Total	25.0 µl	--

Table.3 Steps and conditions of thermocycling for PCR for Intimin gene variants

S. No.	Primers	Initial denaturation		Denaturation		Annealing		Extension		Final extension		Cycles
		Temp	Time	Temp	Time	Temp	Time	Temp	Time	Temp	Time	
1	eae β1	94°C	2 min	94°C	1 min	60°C	1 min	72°C	1 min	72°C	10 min	35
2	eae γ1	94°C	2 min	94°C	1 min	60°C	1 min	72°C	1 min	72°C	10 min	35
3	eae γ2	94°C	2 min	94°C	1 min	58°C	1 min	72°C	1 min	72°C	10 min	35
4	eae δ	94°C	2 min	94°C	1 min	60°C	1 min	72°C	1 min	72°C	10 min	35
5	eae ζ	94°C	2 min	94°C	1 min	62°C	1 min	72°C	1 min	72°C	10 min	35
6	eae ξ	94°C	2 min	94°C	1 min	66°C	1 min	72°C	1 min	72°C	10 min	35

Table.4 Results of Intimin gene and PCR based detection of intimin variant

S. No.	Isolate No.	Intimin (eae) gene (815bp)	Intimin Variants (eae)					
			Gene detected (amplified product size)					
			Beta1 β1 (811bp)	Gamma1 γ1 (804bp)	Gamma2 γ2 (808bp)	Deltaδ (833bp)	Zeta ζ (-206-bp)	Xi ξ (468bp)
1	AU36	-	-	-	-	-	-	-
2	AU37	-	-	-	-	-	-	-
3	AU25	-	-	-	-	-	-	-
4	AU15	-	-	-	-	-	-	-
5	AU16	-	-	-	-	-	-	-
6	AU5	-	-	-	-	-	-	-
7	AU17	-	-	-	-	-	-	-
8	AU18	-	-	-	-	-	-	-
9	AU29	-	-	-	-	-	-	-
10	AU2	+	+	-	-	-	-	-
11	AU24	+	-	-	-	-	-	-
12	AU22	-	-	-	-	-	-	-
13	AU26	+	-	-	+	-	-	-
14	AU27	-	-	-	-	-	-	-
15	AU19	-	-	-	-	-	-	-
16	AU20	-	-	-	-	-	-	-
17	AU6	+	-	-	-	-	-	-
18	AU7	+	-	-	-	-	-	-
19	AU3	-	-	-	-	-	-	-
20	AU4	-	-	-	-	-	-	-
21	AU23	-	-	-	-	-	-	-
22	AU12	-	-	-	-	-	-	-
23	AU38	-	-	-	-	-	-	-
24	AU39	-	-	-	-	-	-	-
25	AU21	-	-	-	-	-	-	-
26	AU1	-	-	-	-	-	-	-
27	AU8	-	-	-	-	-	-	-
28	AU9	-	-	-	-	-	-	-
29	AU40	-	-	-	-	-	-	-
30	AU28	-	-	-	-	-	-	-
31	AU32	+	-	-	-	-	-	-
32	AU33	+	-	-	+	-	-	-
33	AU13	+	+	+	-	-	-	-
34	AU14	-	-	-	-	-	-	-
35	AU30	-	-	-	-	-	-	-
36	AU31	-	-	-	-	-	-	-
37	VC4	-	-	-	-	-	-	-
38	VC3	-	-	-	-	-	-	-
39	OF17	-	-	-	-	-	-	-
40	OF11	-	-	-	-	-	-	-
41	OF10	-	-	-	-	-	-	-
42	OF16	-	-	-	-	-	-	-
43	OF12	-	-	-	-	-	-	-

44	OF4	-	-	-	-	-	-	-
45	OF19	-	-	-	-	-	-	-
46	OF21	-	-	-	-	-	-	-
47	OF14	-	-	-	-	-	-	-
48	OF15	-	-	-	-	-	-	-
49	OF24	+	-	-	-	-	-	-
50	OF25	+	-	-	-	-	-	-
51	OF26	-	-	-	-	-	-	-
52	OF7	-	-	-	-	-	-	-
53	OF1	-	-	-	-	-	-	-
54	OF27	-	-	-	-	-	-	-
55	OF22	-	-	-	-	-	-	-
56	OF23	-	-	-	-	-	-	-
57	OF18	-	-	-	-	-	-	-
58	OF13	-	-	-	-	-	-	-
59	OF552	-	-	-	-	-	-	-
60	VC5	-	-	-	-	-	-	-
61	VC6	-	-	-	-	-	-	-
62	OF8	-	-	-	-	-	-	-
63	OF9	-	-	-	-	-	-	-
64	JU17	+	-	-	+	-	-	-
65	JU18	+	-	-	+	-	-	-
66	JU9	-	-	-	-	-	-	-
67	JU3	-	-	-	-	-	-	-
68	JU4	+	-	-	+	-	-	-
69	JU5	-	-	-	-	-	-	-
70	JU11	-	-	-	-	-	-	-
71	JU12	-	-	-	-	-	-	-
72	JU13	+	-	-	-	-	-	-
73	JU16	-	-	-	-	-	-	-
74	JU10	+	-	-	-	-	-	-
75	JU8	-	-	-	-	-	-	-
76	JU14	-	-	-	-	-	-	-
77	JU1	-	-	-	-	-	-	-
78	JU6	-	-	-	-	-	-	-
79	JU7	-	-	-	-	-	-	-
80	JU2	-	-	-	-	-	-	-
81	JU15	-	-	-	-	-	-	-
82	VC1	-	-	-	-	-	-	-
83	VC2	-	-	-	-	-	-	-
84	AU10	-	-	-	-	-	-	-
85	AU11	-	-	-	-	-	-	-
86	AU34	-	-	-	-	-	-	-
87	AU35	-	-	-	-	-	-	-
88	OF6	-	-	-	-	-	-	-
89	OF2	-	-	-	-	-	-	-
90	OF3	-	-	-	-	-	-	-
91	OF20	-	-	-	-	-	-	-

+: amplification of desired product size, -: no amplification

Our findings were in accordance with that of finding of Reid *et al.,* (1999) who detected the eae gene and simultaneously identified specific alleles in 87 pathogenic *E. coli* strains encoding for α, β and γ intimin variants. Blanco *et al.,* (2004) who also identified eaeβ1 variant in 41 isolates, eaeγ1 in 82 isolates, three isolates had eaeγ2, one isolate showed eaeδ, 13 isolates had eae ε, and 8 isolates had eaeζ among 514 samples of *E. coli* from diarrheic calf. Oswald *et al.,* (2000) also studied the distribution of different types of intimin variants, among the EPEC and EHEC and identified new intimin variant "ε" which is very much similar to intiminβ.

Blanco *et al.,* (2005) studied intimin variants among 51 eae positive *E. coli* strains and found that EPEC strain harbored variety of intimin variants with eae- β1 being most frequent and also identified new intimin variant gene (eae-η2).

There is paucity of literature regarding the intimin gene variants.

Different intimin types and subtypes are closely associated with different pathogenic *E. coli* clones could contribute to our understanding of the evolution of intimin genes. Since intimin is found to be a 94-97kda protein and is immunogenic in nature it can targeted for vaccine designing against pathogenic *E. coli* strains.

Intimin gene codes for different types of protein which are immunogenic in nature and thus it can represent an important tool for *E. coli* typing in routine diagnostic as well as epidemiological and clonal studies. Different intimin variants are responsible for different host tissue cell tropism which could be taken into consideration and further work could be done to identify the specific intimin variant types responsible for attachment to specific tissue tropism. Intimin interaction with the host cell unfolds a novel mechanism of pathogen host cell interaction in the form of bacterial secreted protein translocated on host cell membrane acting as a receptor for bacterial protein and this pathogenic mechanism needs to be studied further for understanding tissue tropism of pathogen. Intimin protein being immunogenic in nature can be target for vaccine design for production of vaccine against pathogenic *E. coli* strains.

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