# Regulation of *eae*A Gene in Relation to *ton*B Gene in Diarrheagenic *Escherichia coli* by Changes in Iron Availability

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## ABSTRACT

The *eae*A gene encodes the intimin or attachment protein in diarrheagenic *E coli*. Its role in establishing an infection is important. In this study, we attempted to assess the role of iron in regulating *eae*A gene in EPEC, in relation to *ton*B gene, which encodes the TonB protein required for uptake of iron and as a regulator of expression of iron dependent genes. Possible regulation of *eae*A gene by changes in iron availability through TonB protein is important for inhibition of bacterial adhesion and the establishment of infection. To this purpose, we determined the transcriptional levels of *eae*A and *ton*B genes in standard EPEC strain harboring both genes and *eae*A in a mutant strain for the *ton*B gene to determine the role of *ton*B gene in regulating the *eae*A gene by changes in iron availability. RNA extracts of both strains were subjected to a semi-quantitative RT-PCR to measure the transcriptional levels of *eae*A and *ton*B genes in the non-mutant strain and the *eae*A gene in the *ton*B mutant strain in an iron supplemented and an iron unsupplemented growth medium. Our data have shown that the transcriptional levels of the *eae*A and the *ton*B genes in the non-mutant strain were significantly depressed in the presence of iron, in comparison to higher transcriptional levels in its absence. On the other hand the transcriptional level of the *eae*A gene was similar under iron supplemented and unsupplemented conditions in the mutant strain. In conclusion our findings are novel for *eae*A gene encoding the intimin protein and indicate that *eae*A appears to be regulated by iron through its uptake by the TonB protein encoded by the *ton*B gene. This will classify the *eae*A gene as an iron dependent gene and its potential regulation by iron availability is possible. Additional studies are underway to determine the regulation of *eae*A gene by iron availability in cell cultures.

## INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) are the major cause of infantile watery diarrhea in developing countries.[1-3] EPEC affect infants and toddlers in the first 3 years of life specially in nurseries and day-care centers[2,4] causing severe, prolonged and non-bloody diarrhea, vomiting and

fever.[2] The histopathological hallmark of EPEC infection is a phenomenon called "attaching and effacing" (A/E) lesion of cells. A/E lesion formation is mediated by a chromosomal gene *eae*A coding for a 94-KDa outer-membrane protein now designated intimin.[1,3] Intimin allows the bacterium to intimately attach to the epithelial cell membrane.[5] The loss of microvilli, known as effacement, will dramatically reduce the brush border absorbance. The net result will be activation of intestinal secretion, which will lead to watery diarrhea.[4] In order for EPEC to establish an infection in the host intestines, they require the uptake of nutrients to survive. Iron is one of the essential nutrients because it is used as a cofactor for enzymes in the cellular metabolism. To obtain iron, microorganisms secrete powerful iron chelators called siderophores that remove iron from the host proteins, forming iron-siderophore complexes, which are then actively transported across the outer membrane through the TonB protein. Iron availability and the *fur* (Fe uptake regulation) gene product regulate the transcriptional level of all genes involved in *E coli* siderophore-mediated iron transport systems.[6,7] In the presence of excess iron, the Fur protein binds to $Fe^{2+}$(reduced to this form intracellularly).[8] This complex formed will then bind to the promoter region on the DNA sequence thus inhibiting the expression of all the genes involved in the iron uptake[8]. However in iron starvation condition the opposite will happen. Fur protein and iron will not form a complex, therefore no binding will occur to the promoter region leaving the promoter region free for RNA polymerase access and the genes involved in ferric iron transport systems are expressed.[8] Given the crucial role that the *ton*B plays in iron transport and its potential ability to regulate other iron dependent genes that encode virulence factors, we aimed at assessing the role of iron in regulating *eae*A gene in relation to *ton*B gene in EPEC strains. This was done by measuring the transcriptional levels of both of these genes at different iron concentrations in the growth media and attempting to establish a possible regulatory link between the *eae*A gene and *ton*B gene, since control of iron concentration by the *ton*B may lead to possible control of expression of iron-dependent virulence genes.

## MATERIALS AND METHODS

### Source of Isolates

In this study two strains of *E coli* belonging to EPEC were used. One EPEC was isolated from a diarrheal stool sample in a previous study[9] and the other EPEC (*ton*B mutant) was obtained from the Centers for Disease Control and Prevention (CDC; Atlanta, Ga).

### Culture on Special Media

*Mac Conkey agar*

Mac Conkey agar was used to isolate and identify lactose positive *E coli* colonies. Mac Conkey agar was either supplemented with 100 µM of iron in the form of ferric chloride $FeCl_3$ (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) or it was left unsupplemented.

*Trypticase Soy broth (TSB)*

Trypticase Soy broth (Difco) was used to subculture one to two colonies of *E coli* from Mac Conkey agar plate in order to propagate the cells. To vary the iron availability of the medium, TSB was either supplemented with 100 µM of iron $FeCl_3$ (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) or with 100 µM of iron and 2mM EDDA chelator (Ethylenediamine-N,N'-diacetic acid) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) or it was left unsupplemented. Selection of iron concentrations was done according to

Postle.[6] Iron chelator, EDDA, makes the iron unavailable for transport into cells. Tubes containing EDDA were used no sooner than 3 days after preparation to allow time for iron chelation.[6]

**DNA Extraction and PCR**

Total DNA was extracted from the enteropathogenic *E coli* strains using the GFX™ Genomic Blood DNA Purification kit (AmershamPharmacia Biotech, Piscataway, NJ). PCR was performed on DNA extracts to amplify the *ton*B gene, using primers that were designed in our laboratory and manufactured by HVD Biotech (Vienna, Austria). The primers were derived from the *E coli ton*B gene sequence.[10] PCR amplification was performed in 100 µL reaction mixtures consisting of 10 µL DNA (2 µg/µL) and 90 µL of the amplification mix containing 16 pmol of each primer, 200 µM concentrations of each deoxynucleoside triphosphate, 10 µL of PCR buffer (Amersham Pharmacia Biotech, Piscataway, NJ) and 2.5 U of Taq DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ). A thermal cycler (PTC-100, MJ Research Inc. Watertown, Mass) was used for amplification for 30 cycles. Each cycle consisted of denaturation at 94˚C for 1 minute, primer annealing at 55˚C for 1 minute and extension at 72˚C for 1 minute. The cycles were followed by a final extension step at 72˚C for 10 minutes.

**RNA Extraction and RT-PCR**

RNA was extracted using the RNeasy Mini Kit (Qiagen, Maryland, USA) according to manufacturer's specifications. RT-PCR was performed using The Ready-To-Go You-Prime-First-Strand Beads kit (Amersham Pharmacia Biotech) according to manufacturer's specifications. RT-PCR was performed on *ton*B and *eae*A genes. The concentration of extracted RNA was determined by using a spectrophotometer (Utrospec 1000E, Pharmacia Biotech, Uppsala, Sweden) at 260 nm.

*First-Strand cDNA synthesis*

Twenty five µL corresponding to 1 µg of RNA was pipetted from the extracted RNA. The RNA sample was brought to a volume of 30 µL in an RNase-free microcentrifuge tube using DEPC-treated water. The sample was heated at 65˚C for 10 minutes then chilled on ice for 2 minutes. The RNA solution was then transferred to the tube of First-Strand Mix Beads (provided with the kit) without mixing. The beads contain the Maloney Murine Leukemia virus (M-MuL V) reverse transcriptase enzyme and dNTPs required for cDNA synthesis. Three µL of sense primer (same for all the genes) were added to bring the volume to 33 µL (as specified by the manufacturer). The amount of primer for all the genes added was 40 pmol. The sample was incubated at room temperature for 1 minute, then the contents of the tube were mixed gently by vortexing or pipetting. The contents were then collected at the bottom of the tube by brief centrifugation and incubation at 37˚C for 60 minutes.

*Semi-Quantitative RT-PCR[11]*

In order to semi-quantitate the mRNA transcripts of the *ton*B, and *eae*A genes, the cDNA synthesized in the first reaction was subjected to ten fold serial dilutions.

<u>(Semi-quantitative) RT-PCR for detection of the *ton*B gene</u>

CT1: 5' gaa atg att atg act tca atg acc3'
CT2: 5' aat ttc ggt ggt gcc gtt aat ttt 3'

RT-PCR was done on each dilution by adding l0X PCR buffer (500 mMKCl, 15 mM $MgC1_2$, 100 mM TrisHCl) (Pharmacia Biotech), *Taq* DNA polymerase (5 U/µL in a storage buffer containing 50 mM TrisHCl, 0.l mM EDTA, 5

mM dithiothreitol stabilizers and 50% glycerol) (Pharmacia Biotech), primers CT1 and CT2, dNTPs, and water as specified by the manufacturers. The concentration of primers needed was 40 pmol, which is a volume of 0.4 µL of primer CT1 (30.0 nmol/300 µL) brought with DEPC-treated water to a volume of 3 µL and a volume of 0.39 µL of primer CT2 (30.9 nmol/309 µL) brought with DEPC-treated water to a volume of 3 µL. For all the dilutions except the undiluted cDNA, the 100 µL mix contained in addition to cDNA (10 µL), 3 µL of each primer, 10 µL of 10X PCR-buffer, 1 µL of dNTPs (20 mM), 0.5 µL *Taq* DNA polymerase, and 72.5 µL of distilled water. The undiluted cDNA contained 3 µL of each primer, 10 µL of l0X PCR-buffer, 0.5 µL *Taq* DNA polymerase and 73.5 µL of distilled water. These mixtures were overlaid with a drop of mineral oil. The target gene was amplified using the PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, Mass) according to the following conditions: a denaturation step at 94˚C for 1 minute, an annealing step at 55˚C for 1 minute, followed by an extension step at 72˚C for 1 minute. This cycle was repeated for 30 times and a final extension step was performed at 72˚C for 10 minutes.

(Semi-quantitative) RT-PCR for detection of the *eae*A gene

Previously published primers[12] and manufactured by HVD Biotech (Vienna, Austria) were utilized in the protocol.

Cl: 5' tcg tca cag ttg cag gcc tgg 3'
C2: 5' cga agt ctt atc cgc cgt aaa gt 3'

RT-PCR was performed similarly to that of the *ton* B gene except for the primers, which are listed above. The concentration of the primers needed was 40 pmol, which is a volume of 0.4 µL of primer C1 (96.6 nmol/966 µL)brought with DEPC-treated water to a volume of 3 µL and a volume of 0.4 µL of primer C2 (80.0 nmol/880 µL) brought with DEPC-treated water to a volume of 3 µL. To amplify this gene thirty cycles were performed in a thermal cycler. Each cycle consisted of a denaturation at 94˚C for 20 seconds, a primer annealing at 53˚C for 45 seconds, followed by an extension at 72˚C for 45 seconds. The cycles were followed by a final extension at 72˚C for 10 minutes.

Amplicons of the *ton*B (732 bp) and *eae*A (1110 bp) genes were electrophoresed on 1% agarose (Sigma. St. Louis, Mo) gel in 1x Tris-borate-EDTA buffer at 117 V for 45 minutes. Ethidium bromide (Sigma. St. Louis, Mo) of 1 µg/mL was incorporated into the gel for staining. Amplicons were detected under UV light and photographed with type 667 Polaroid film.

## RESULTS AND DISCUSSION

### Polymerase Chain Reaction

Upon testing the transcriptional level of the *ton*B gene by RT-PCR in both EPEC strains, we found that EPEC strain (CDC) did not transcribe the *ton*B gene. A PCR performed on the genomic DNA of this strain confirmed the absence of the *ton*B (data not shown) This strain served as a negative control for the *ton*B gene to demonstrate its role in uptake of iron in the bacterial cell.

### Semi-Quantitative RT-PCR

The transcriptional level of each gene was assessed by the titer of the mRNA transcripts, determined as the highest dilution at which RT-PCR would give a positive result.[11] This is manifested as a band of a size of 732 bp for *ton*B, and 1110 bp for *eae*A gene along with the intensity of the bands.

The transcriptional level of *eae*A gene in the EPEC strain (isolated from a stool specimen in a previous study)[9] varied at different iron concentrations

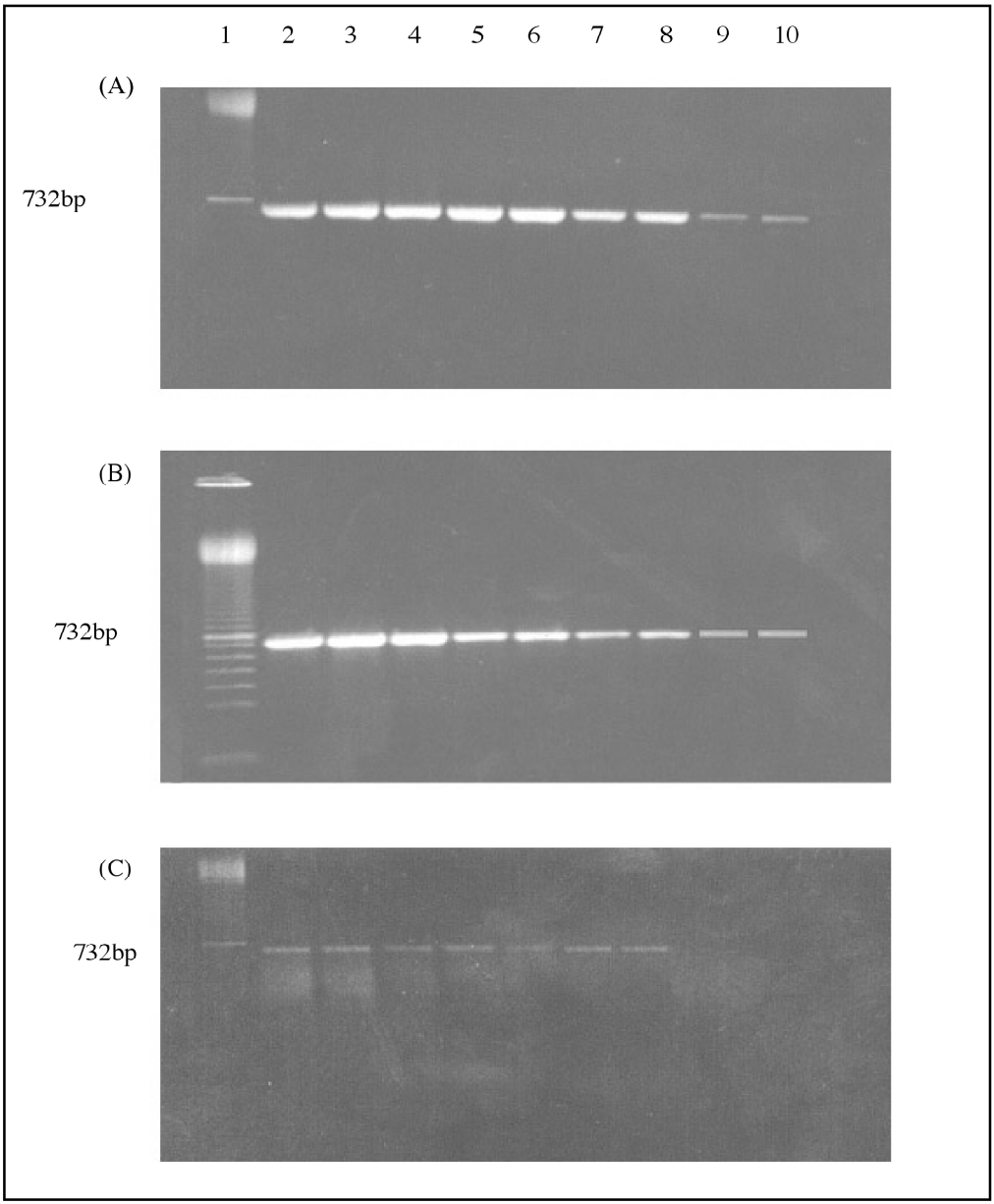


**Figure 1.** Transcriptional level of *ton*B gene in entropathogenic *E coli.* Lane 1, ladder; lanes 2-10, RT-PCR amplicons of serially diluted mRNA transcripts. (A) Trypticase soy broth without iron supplement (B) Trypticase soy broth supplemented with $FeCl_3$ (100 µM)+EDDA (2 µM) (C) Trypticase soy broth supplemented with $FeCl_3$ (100 µM).

and this same variation was seen with its *ton*B gene transcriptional level. In the TSB unsupplemented with iron, the transcriptional levels of both genes were not repressed and showed a titer of 1:160 with high band intensity. In TSB supplemented with iron, their transcriptional levels were repressed; the *ton*B had a titer of 1:40 with low band intensity while the *eae*A had a titer of 1:160 with low band intensity. In the TSB where the chelator was added, the transcriptional levels of both genes were observed to be the same as in TSB unsupplemented with iron (titer 1:160 with high band intensity) (Figures 1 and

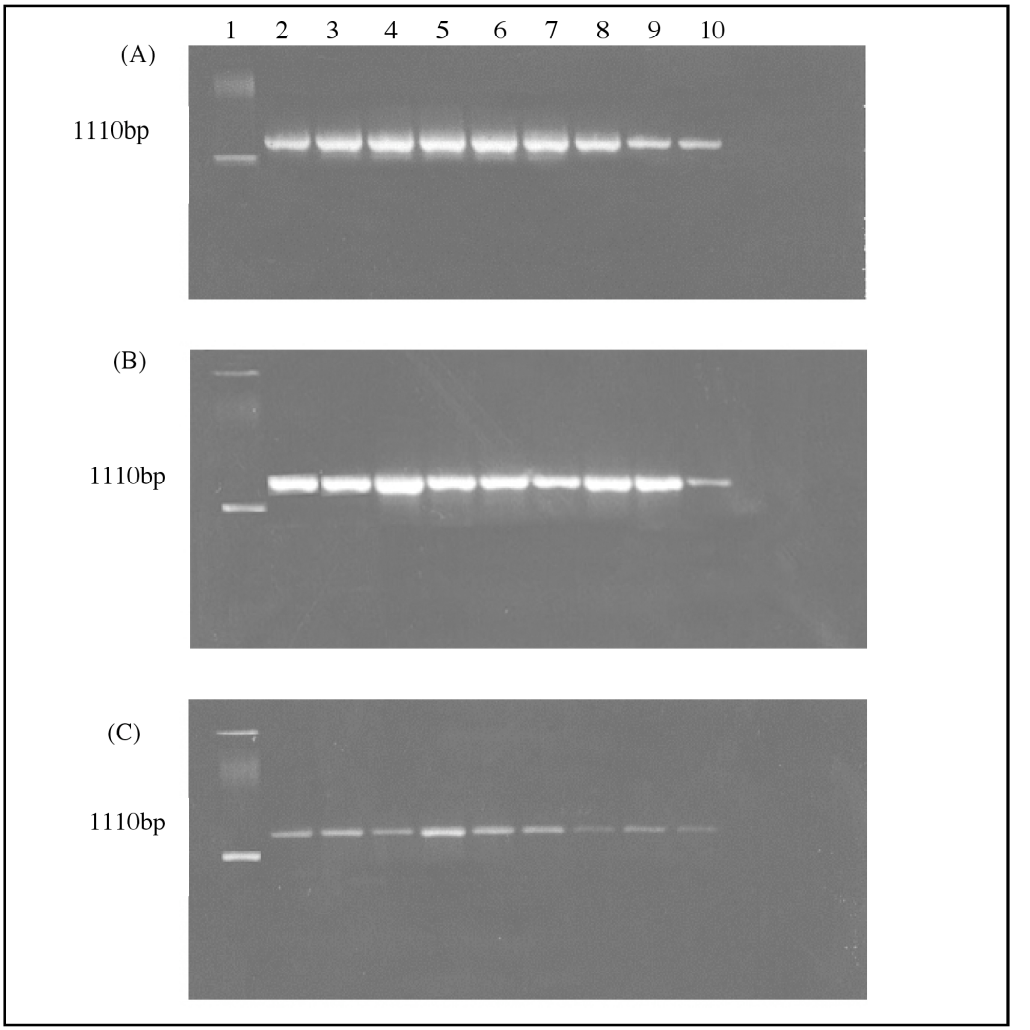


**Figure 2.** Transcriptional level of *eae*A gene in entropathogenic *E coli.* Lane 1, ladder; lanes 2-10, RT-PCR amplicons of serially diluted mRNA transcripts (A) Trypticase soy broth without iron supplement (B)Trypticase soy broth supplemented with $FeCl_3$ (100 µM)+EDDA (2 µM) (C) Trypticase soy broth supplemented with $FeCl_3$ (100 µM) .

2).

Based on this data, the *eae*A gene seems to be regulated by changes in iron availability in a similar manner than the *ton*B gene. The iron uptake appears to be mediated by the TonB protein. The *eae*A gene being regulated by changes in iron concentrations is a novel finding, since it was not previously reported in the literature. However, whether the Fur protein is involved in this regulation or not requires additional testing.

Confirmation of data on the role of *ton*B in the uptake of iron and its relation to *eae*A transcription under different conditions of iron availability is done on the *ton*B mutant strain belonging to EPEC by assessing the effect of iron on the transcriptional level of *eae*A gene. Data have shown that transcriptional level of the *eae*A gene in the *ton*B mutant EPEC (CDC) was not repressed and had the same titer of 1:40 with low band intensity regardless of the iron availability (Figure 3). This can be explained by the following assumption: since this strain is *ton*B mutant, then, there was no active transport of iron

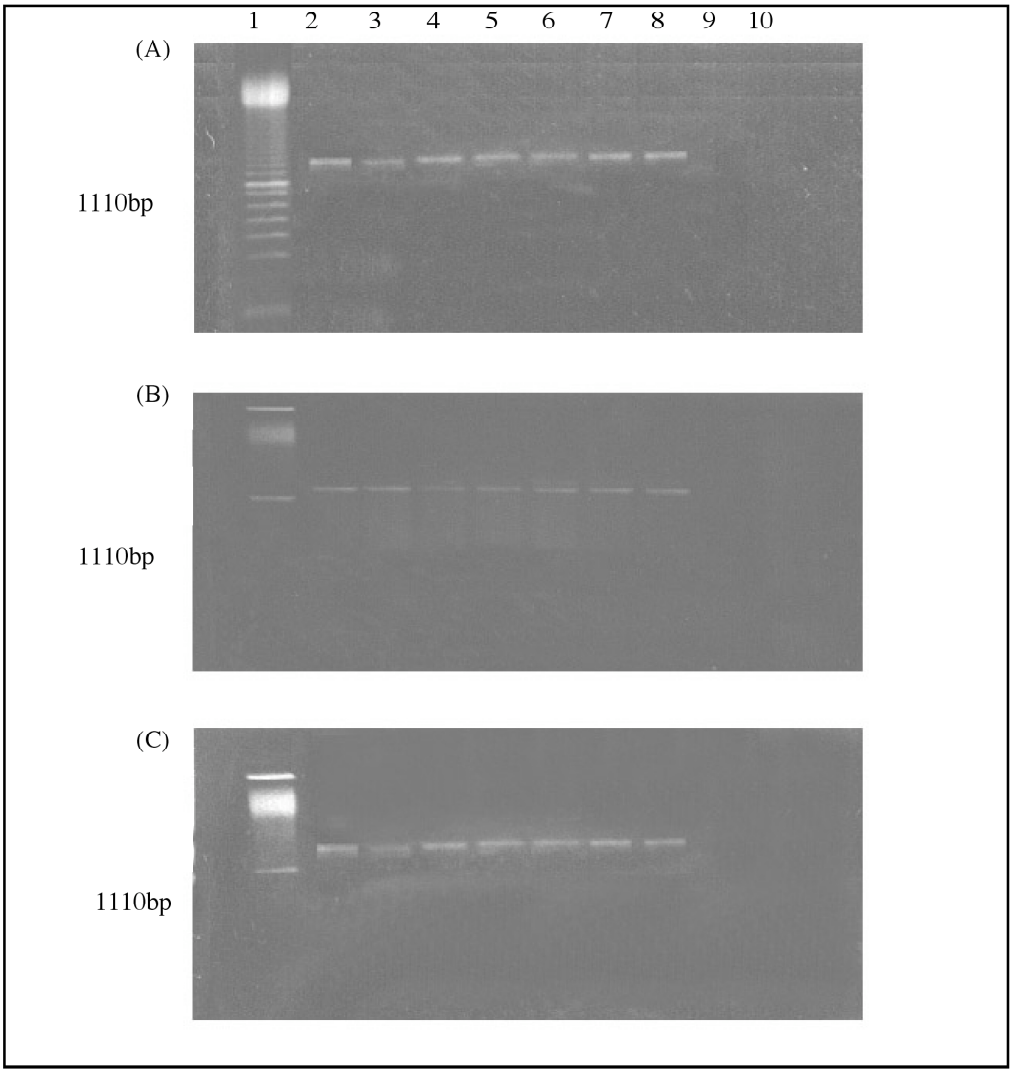


**Figure 3.** Transcriptional level of *eae*A gene in the *ton*B mutant entropathogenic *E coli* strain (CDC). Lane 1, ladder; lanes 2-10, RT-PCR amplicons of serially diluted mRNA transcripts. (A) Trypticase soy broth without iron supplement (B) Trypticase soy broth supplemented with $FeCl_3$ (100 μM)+EDDA (2 μM) (C) Trypticase soy broth supplemented with $FeCl_3$ (100 μM) .

across the outer membrane and hence no change in the transcriptional level of *eae*A gene was observed. Our findings are preliminary at this point since only two *E coli* strains were considered in this study. Additional testing on more *E coli* strains belonging to EPEC obtained from clinical isolates would be required to draw definitive conclusions. Cell lines infected with EPEC at different iron concentrations may serve as an efficient assay to determine the severity of A/E lesion formation by the intimin protein encoded by the *eae*A gene.

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