# European Journal of Molecular Biology and Biochemistry

Research article

EJMBB
European Journal of Molecular Biology and Biochemistry

# CS21-MEDIATED ADHESION MECHANISMS IN ENTEROTOXIGENIC ESCHERICHIA COLI: IMPLICATIONS FOR VIRULENCE AND THERAPEUTIC INTERVENTIONS

## Dr. Umesh Kumar C

Assistant Professor, Department of Microbiology, Sri Lakshmi Narayana Institute of Medical Sciences, Osudu, Agaram Village, Villianur Commune, Koodapakkam Post, Puducherry – 605 502, India.

**ABSTRACT**

Enterotoxigenic Escherichia coli (ETEC) is a major cause of diarrheal diseases, particularly in travelers and infants from developing countries. One of its key virulence factors, the CS21 type IV pili, is implicated in intestinal colonization, adhesion, and microcolony formation. However, the exact role of CS21 in ETEC pathogenesis is not fully understood. This study investigates the role of CS21 in mediating adhesion using in vitro models of epithelial cells (IPEC-J2 and IPEC-1) and in vivo neonatal mouse models. Quantitative adhesion assays revealed that CS21-expressing ETEC strains adhered significantly more to epithelial cells compared to CS21-deficient mutants. Additionally, adhesion was inhibited by anti-LngA monoclonal antibodies and neuraminidase treatment. In vivo, CS21-expressing strains exhibited enhanced virulence in neonatal mice, supporting their role in gut colonization and pathogenicity. These findings position CS21 as a crucial virulence factor in ETEC infections, offering potential targets for novel therapeutic strategies aimed at mitigating ETEC-related diseases.

## INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) is a significant public health threat, primarily due to its role in causing diarrheal diseases, particularly affecting travelers and infants in low-resource regions [1-4]. The pathogenicity of ETEC strains is largely dependent on their ability to bind to intestinal microvilli through specialized surface colonization antigens (CSs) and pili, which aid in the secretion of enterotoxins into host cells [5]. Among these CSs, CS21 has garnered considerable attention, with its prevalence varying based on geographical location and patient demographics [6].

The regulation of CS21 expression is controlled by several genes located within plasmid clusters, influencing bacterial behaviors such as adhesion and biofilm formation. However, the precise role of CS21 in

ETEC pathogenesis remains incompletely understood, particularly regarding its interaction with host intestinal cells. Previous studies have highlighted the importance of differentiated primary intestinal cells, derived from pig models, for investigating ETEC adhesion mechanisms [7]. These cells closely resemble human intestinal cells in terms of receptor expression and physiological properties, making them valuable for examining ETEC-host interactions.

While in vitro models provide vital insights, they may not fully replicate the complexities of disease progression in living organisms. To address this gap, neonatal mouse models are commonly used to assess ETEC virulence and pathogenesis [8]. Studies have shown that ETEC strains expressing CS21 contribute to increased neonatal mortality in mouse models, suggesting that CS21 plays a critical role in the severity of ETEC infections [9]. Despite these findings, the precise mechanisms through which CS21 influences ETEC pathogenesis remain poorly defined.

Corresponding Author
**Dr. Umesh Kumar C**

This study aims to explore the role of CS21 in ETEC pathogenesis by employing both in vitro and in vivo models. Through the analysis of interactions between CS21-expressing ETEC strains and host intestinal cells, this research aims to deepen our understanding of ETEC virulence mechanisms and potentially identify novel therapeutic targets for the management of ETEC-related infections.

## METHODS

This study was conducted at Tagore Medical College and Hospital, Chennai, from August 2015 to August 2016. The E9034A strain, initially isolated from a Caribbean diarrheal outbreak, was utilized in this research. This strain harbors LT and ST toxins, CS21 and CS3 colonization factors, and the O8:H9 serotype. PCR DNA amplification tests confirmed the presence of virulence genes TIBA, IRP2, ETPA, and ETPB, while TIA, LEOA, EATA, and FYUA were absent. A previous study reported that PCR analysis can detect non-classical virulence factors. According to earlier research [10], overnight cultures of E9034A DlngA mutants and complemented mutants were maintained at 37°C. The pGFPuv plasmid was introduced into E9034A DlngA (Clonetech) through electroporation to induce green fluorescent protein expression and ampicillin resistance.

### Cell Culture and Adhesion Assay

Based on earlier protocols [11], IPEC-J2 and IPEC-1 intestinal epithelial cells were cultured from one-day-old piglets in UTEC media. Before seeding onto Millicell culture plate inserts (PIHA 012 50; Millipore), these cells were trypsinized and plated in IPEC media on surfaces pre-coated with type VI human placental collagen. Each insert received 150 µl of cell suspension, and the Millicells were incubated for 24 hours in 500 µl of IPEC media. After 24 hours, the medium was refreshed with Ultroser G serum substitute medium (BioSepra). Media exchanges were conducted every two days for 10 to 15 days at 5% CO₂ and 37°C to optimize cell differentiation. The adhesion of ETEC to epithelial cells was quantified using confocal and electron microscopy [10]. ETEC bacterial cultures were prepared by diluting TB liquid medium from epithelial cells and suspending ETEC bacteria. The TB-containing and TB-free media were incubated for three hours, and mannose (1%) was added as an inhibitor of non-specific Type I pili binding. Additionally, media were pre-incubated with 0.02% arabinose before arabinose supplementation for CS21-mutant complementation. After a three-hour infection period, cells were washed three times with PBS to remove unbound bacteria. Following lysis with 1% Triton X-100 in PBS for 10 minutes, serial dilutions were prepared for further colony counts on LB agar plates.

### Assessment of Adherence Inhibition

The inhibition of ETEC adhesion was determined by treating IPEC-J2 and IPEC-1 intestinal cell lines with 50% IPEC-1/IPEC-J2 tissue culture media. Increasing concentrations of CS21 adherence inhibitors were introduced immediately after incubation, followed by infection. ICA39 and N-acetyl neuraminic acid served as negative controls, while the anti-tubulin monoclonal antibody TG4076 [11] was used as a positive control. Monoclonal anti-LngA antibody ICA39 specifically recognized CS21-expressing ETEC isolates but failed to detect non-CS21 ETEC isolates or exogenous E. coli. Neuraminidase treatment after a PBS wash inhibited ETEC adhesion, which was assessed by infecting a 5 mL ETEC suspension for three hours with neuraminidase and inhibitors, followed by colony-forming unit (CFU) counts.

### Transmission Electron Microscopy (TEM) and Immunofluorescence Analysis

For high-magnification imaging, transmission electron microscopy (TEM) was employed. Infected cells grown on air-liquid interface membranes were fixed with glutaraldehyde, osmium tetroxide, and uranyl acetate. Dehydration was carried out using graded ethanol solutions (25%, 50%, 75%, and 100%) before embedding samples in EPON812 (Electron Microscopy Sciences). Ultrathin sections (0.5 mm) were cut with diamond knives, stained with toluidine blue, and examined under light microscopy and TEM (JEM-1230 model). TEM images were saved as TIFF files for further analysis. For immunofluorescent assays, paraformaldehyde fixation (15 minutes) was followed by three PBS washes, permeabilization with 0.2% Triton X-100 (8 minutes at room temperature), and additional PBS washes. Cells were incubated for one hour in the dark with Alexa Phalloidin 568 (Life Technologies), diluted 1:10 in PBS. After three PBS washes, samples were mounted with Vectashield (Vector Laboratories) containing DAPI. Fluorescent images were captured using a Zeiss 510 META laser scanning confocal microscope with a 63× oil immersion lens (numerical aperture 1.52), and stored as TIFF files for further examination.

### Neonatal Mouse Model for ETEC Pathogenesis

DBA2 mice were selected due to their susceptibility to ETEC strains and non-aggressive maternal behavior after handling. ETEC strains at various optical densities were cultured in 0.1 M sodium bicarbonate during the late exponential growth phase. Their CS21 mutant counterparts and complemented ETEC mutants were prepared under similar conditions. Bacterial suspensions were harvested, washed with PBS, and resuspended in sodium bicarbonate solutions of corresponding optical densities. Each neonatal mouse received 0.2 mL of bacterial suspension. Post-challenge, bacterial counts were determined by serial dilutions and plating on LB agar plates. Approximately 1 to 2 days after birth, newborn mice were orally inoculated using ultrathin needles (12.7 × 0.33 mm). The number of neonatal deaths

was recorded daily for 7 days, with particular attention to mortality occurring within 24 hours post-infection. Various ETEC concentrations and bacterial strains were tested. A control group of 38 newborn mice received PBS inoculation, with no fatalities observed during the study period.

## Statistical analysis

In this study, we utilized analysis of variance (ANOVA) to compare the adhesion properties of the wild-type and recombinant enterotoxigenic Escherichia coli (ETEC) strain E9034A. To ensure accuracy and reliability, each experimental condition was replicated at least three times to obtain consistent and representative data. Statistical analysis was then performed using GraphPad Prism software, where Kaplan-Meier survival curves were generated to assess differences in survival rates across various experimental groups. A statistical significance level of $P < 0.05$ was set to determine the relevance of observed differences in the results.

## RESULTS

For LngA expression to be effectively exhibited by a cell, adherence to differentiated epithelial cells is essential. ETEC strains expressing CS21 demonstrated stronger adhesion to human intestinal epithelium compared to strains lacking CS21. The E9034A mutant ETEC strain exhibited higher levels of adhesion than its CS21-deficient counterpart (222-23% vs. 22-32%). These findings suggest that CS21 receptors on epithelial cells play a critical role in ETEC adherence in both in vitro and in vivo conditions. To investigate this hypothesis, quantitative adherence assays were conducted using two different tissue culture media. Previous reports indicate that CS21 expression is induced by TCM and tissue culture media alone. The presence of microvilli, surface molecules, and possibly CS21 receptors has been confirmed in primary intestinal cells, including IPEC-1 and IPEC-J2. Additionally, CS21 mutants displayed similar adhesion properties to wild-type ETEC strains, confirming that CS21-expressing primary intestinal cells enhance ETEC adhesion. Interestingly, adhesion levels remained unchanged in HeLa cells, regardless of nutrient supplementation, suggesting that undifferentiated cells like HeLa express CS21 receptors differently. To evaluate CS21-mediated adherence, adhesion assays were conducted using IPEC-1 and IPEC-J2 intestinal cell lines, supplemented with 50% TB in IPEC tissue culture media to enhance CS21 expression. Adhesion patterns were similar between IPEC-1 and IPEC-J2 cells (data not shown). Post-infection analysis revealed that actin fibers in infected cells exhibited a rounded morphology, while TEM images of wild-type ETEC cells displayed microvilli-like structures, confirming that these structures also exist in vivo mucosa.To verify the role of anti-LngA antibodies and proteins in inhibiting CS21-ETEC adhesion to intestinal cells, both were included in cell adhesion assays. Mouse monoclonal antibodies targeting LngA were utilized to test the adherence of intestinal cells to LngA-expressing bacteria. Optimal CS21 expression was achieved by supplementing IPEC tissue culture medium with 50% TB. Appropriate negative and positive controls were employed. A dilution of anti-tubulin antibodies did not affect CS21 mutant adhesion, regardless of whether the strain was wild-type, mutant, or complemented. These findings were consistent in IPEC-1 cells. Notably, intestinal cells treated with anti-LngA antibodies and purified LngA protein only inhibited the adhesion of CS21-expressing ETEC strains, indicating that CS21 enhances ETEC adherence to intestinal cells. According to a previous study, bacterial virulence is associated with the ability to bind specific sugar structures on epithelial cell receptors. However, simple sugars such as mannose, arabinose, and galactose did not inhibit CS21-mediated adhesion of ETEC to intestinal epithelial cells. Conversely, neuraminic acid effectively inhibited ETEC adhesion, suggesting that neuraminic acid residues on epithelial cell surfaces play a role in ETEC attachment. It is likely that neuraminic acid residues and glycoprotein substitutions are present on the plasma membranes of differentiated intestinal cells, whereas these structures may be absent in undifferentiated transformed cells.To further investigate neuraminic acid-mediated inhibition, an adherence inhibition assay was conducted on ETEC-infected cells, where neuraminic acid was added in increasing concentrations. The study employed inhibitor adherence assays using IPEC tissue culture media containing 50% TB. Regardless of concentration, N-acetylneuraminic acid failed to impact CS21-mutant ETEC strains. To confirm whether neuraminic acid residues in IPEC-1 and IPEC-J2 inhibit CS21-mediated adhesion, the cells were treated with neuraminidase. In the absence of CS21 receptors, at any neuraminidase concentration, CS21-mutants failed to adhere to ETEC. These findings suggest that N-acetylneuraminic acid residues may regulate interactions between CS21-expressing ETEC and differentiated intestinal cells. Additionally, to explore the function of CS21 in E9034A wild-type ETEC pathogenesis, neonatal mice were injected with mutant CS21 and its complemented strains. The CS21-mutant inoculum was found to be effective at the same concentration in mice, reinforcing the notion that ETEC strain E9034A can be transmitted through CS21 expression.

## DISCUSSION

Intestinal infections caused by Escherichia coli (E. coli) occur when the bacteria attach to and colonize epithelial cells in the intestines [11, 12]. Previous research [13] has identified CS21 type IV pili as playing a crucial role in this process, as they promote microcolony formation, enhance resistance to environmental stress, and enable twitching motility and adhesion to host cells. Interestingly, mutant strains of enterotoxigenic E. coli (ETEC) exhibit higher levels of CS21 expression compared to non-mutant strains, significantly improving

their adhesive properties. Monoclonal antibodies targeting LngA, a key structural component of CS21, have been shown to effectively inhibit CS21-mediated adherence [14]. Additionally, studies have reported that CS21 mutant ETEC strains are linked to severe birth defects in neonatal mice, with their LD50 (lethal dose 50%) significantly lower than that of wild-type strains. This underscores the critical role of CS21 pili as a virulence factor in ETEC infections in humans, particularly in gut colonization [15]. The ability of CS21 to bind to intestinal epithelial cells facilitates ETEC colonization in the human gastrointestinal tract, with primary intestinal cell lines expressing CS21 receptors and displaying microvilli-like structures. However, the specific receptors involved in CS21-mediated adhesion remain partially characterized, though studies suggest that sialic acid derivatives on mammalian cell membranes may play a role. Furthermore, glycan modifications on ETEC pili have been implicated in enhancing bacterial binding to host receptors, thereby

aiding adhesion and colonization. In vivo studies using neonatal mouse models have further clarified the pathogenic potential of CS21-expressing ETEC strains, demonstrating that these strains can induce fatal infections in neonatal mice. These findings highlight CS21's significance in ETEC pathogenesis and its potential as a target for therapeutic interventions.

## CONCLUSION

Despite these findings, the precise mechanisms underlying ETEC-induced fatality in neonatal mice remain incompletely understood, necessitating further research. This study seeks to bridge this knowledge gap by utilizing an intra-gastric lethal challenge model to investigate the virulence-associated factors of ETEC strains in vivo. Through this approach, we aim to provide critical insights into ETEC pathogenesis, contributing to a deeper understanding of infection dynamics and potential therapeutic targets.

## REFERENCES

1. Beatty M. E, Adcock P. M, Smith S. W, Quinlan K, Kamimoto L. A, Rowe S. Y, Scott K, Conover C, Varchmin T. & other authors*, et al*. Epidemic diarrhea due to enterotoxigenic Escherichia coli. *Clin Infect Dis* 42, 2006, 329–334.
2. Bieber, D, Ramer, S. W, Wu, C. Y, Murray, W. J, Tobe, T, Fernandez, R. &Schoolnik, G. K*, et al*.. Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic Escherichia coli. Science 280, 1998, 2114–2118.
3. Black, R. E, Cousens, S, Johnson, H. L, Lawn, J. E, Rudan, I, Bassani, D. G, Jha, P, Campbell, H, Walker, C. F. & other authors*, et al*.. Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet 375, 2010, 1969–1987.
   Blackburn D, Husband A, Saldana Z, Nada R. A, Klena J, Qadri F. &Giron J. A*, et al*. Distribution of the Escherichia coli common pilus among diverse strains of human enterotoxigenic E. coli. J ClinMicrobiol 47, 2009, 1781–1784.
4. Brown, E. A, Kaushik, R. S. &Hardwidge, P. R*, et al*.. Susceptibility of human enterotoxigenic Escherichia coli isolates to growth inhibition by porcine intestinal epithelial cells. FEMS Microbiol Lett 274, 2007, 95–101.
5. Clavijo, A. P, Bai, J. & Go´ mez-Duarte, O. G*, et al*. The Longus type IV pilus of enterotoxigenic Escherichia coli (ETEC) mediates bacterial self-aggregation and protection from antimicrobial agents. MicrobPathog 48, 2010, 230–238.
6. Del Canto, F, Valenzuela, P, Cantero, L, Bronstein, J, Blanco, J. E, Blanco, J, Prado, V, Levine, M, Nataro, J. & other authors*, et al*. Distribution of classical and nonclassical virulence genes in enterotoxigenic Escherichia coli isolates from Chilean children and tRNA gene screening for putative insertion sites for genomic islands. J ClinMicrobiol 49, 2011, 3198–3203.
7. Duchet-Suchaux, M*, et al*. Protective antigens against enterotoxi- genic Escherichia coli O101 : K99,F41 in the infant mouse diarrhea model. Infect Immun 56, 1988, 1364–1370.
8. Duchet-Suchaux, M, Le Maitre, C. &Bertin, A*, et al*. Differences in susceptibility of inbred and outbred infant mice to enterotoxigenic Escherichia coli of bovine, porcine and human origin. J Med Microbiol 31, 1990, 185–190.
9. Fleckenstein, J. M, Kopecko, D. J, Warren, R. L. &Elsinghorst, E. A*, et al*. Molecular characterization of the tia invasion locus from enterotoxigenic Escherichia coli. Infect Immun 64, 1996, 2256–2265.
10. Gaastra, W. &Svennerholm, A. M*, et al*. Colonization factors of human enterotoxigenic Escherichia coli (ETEC). Trends Microbiol 4, 1996, 444–452.
11. Geens, M. M. &Niewold, T. A*, et al*. Preliminary characterization of the transcriptional response of the porcine intestinal cell line IPEC-J2 to enterotoxigenic Escherichia coli, Escherichia coli, and E. coli lipopolysaccharide. Comp Funct Genomics 2010, 2010, 469583.
12. Giro´ n, J. A, Viboud, G. I, Sperandio, V, Go´ mez-Duarte, O. G, Maneval, D. R, Albert, M. J, Levine, M. M. &Kaper, J. B*, et al*. Prevalence and association of the longus pilus structural gene (lngA) with colonization factor antigens, enterotoxin types, and serotypes of enterotoxigenic Escherichia coli. Infect Immun 63, 1995, 4195–4198.
13. Giro´ n, J. A, Go´ mez-Duarte, O. G, Jarvis, K. G. &Kaper, J. B*, et al*. Longus pilus of enterotoxigenic Escherichia coli and its relatedness to other type-4 pili–a minireview. Gene 192, 1997, 39–43.
14. Go´ mez-Duarte, O. G, Ruiz-Tagle, A, Go´ mez, D. C, Viboud, G. I, Jarvis, K. G, Kaper, J. B. & Giro´ n, J. A*, et al*. Identification of lngA, the structural gene of longus type IV pilus of enterotoxigenic Escherichia coli. Microbiology 145, 1999, 1809–1816.

15. Gomez-Duarte, O. G, Chattopadhyay, S, Weissman, S. J, Giron, J. A, Kaper, J. B. &Sokurenko, E. V, *et al*. Genetic diversity of the gene cluster encoding longus, a type IV pilus of enterotoxigenic Escherichia coli. J Bacteriol 189, 2007, 9145–9149.