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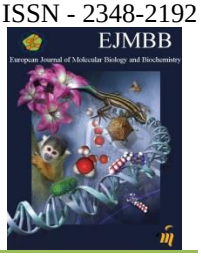

ROLE OF CS21 PILI IN ENTEROTOXIGENIC ESCHERICHIA COLI (ETEC) PATHOGENESIS: MECHANISMS OF ADHESION AND VIRULENCE IN IN VITRO AND IN VIVO MODELS

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ABSTRACT

Enterotoxigenic Escherichia coli (ETEC) is a significant cause of diarrheal disease, particularly affecting travelers and infants in developing regions. One of its key virulence factors, CS21 type IV pili, facilitates intestinal colonization, adhesion, and microcolony formation. However, the precise role of CS21 in ETEC pathogenesis remains unclear. This study explores CS21-mediated adhesion mechanisms using in vitro epithelial cell models (IPEC-J2 and IPEC-1) and in vivo neonatal mouse models. Quantitative adhesion assays demonstrated that CS21-expressing ETEC strains adhered significantly more than CS21-deficient mutants, with adhesion being inhibited by anti-LngA monoclonal antibodies and neuraminidase treatment. Furthermore, CS21-expressing ETEC strains exhibited increased virulence in neonatal mice, reinforcing their role in gut colonization and pathogenicity. These findings highlight CS21 as a critical virulence factor, offering potential targets for novel therapeutic interventions to mitigate ETEC-related infections.

INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) is a major public health concern, primarily due to its role in causing diarrheal diseases, especially among travelers and infants in developing countries [1-4]. The pathogenicity of ETEC strains depends on their ability to attach to intestinal microvilli using specialized colonization surface antigens (CSs) and pili, which facilitate the secretion of enterotoxins into enterocytes [5]. Among these CSs, CS21 has attracted significant attention, with its prevalence differing based on geographic location and patient population [6].

The genetic regulation of CS21 expression involves several genes located within plasmid clusters, influencing bacterial phenotypes such as adhesion and biofilm formation. However, the specific role of CS21 in ETEC pathogenesis is not yet fully understood, particularly in terms of its interaction with host intestinal cells.

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Previous studies have demonstrated the importance of differentiated primary intestinal cells, derived from pig models, in investigating ETEC adhesion mechanisms [7].

These cells exhibit similar receptor expression and physiological properties to human intestinal cells, making them valuable for studying ETEC-host interactions. Although in vitro models provide critical insights, they may not fully represent disease progression in living organisms. To bridge this gap, neonatal mouse models have been employed to assess ETEC virulence and pathogenesis [8]. Research has shown that ETEC strains expressing CS21 contribute to neonatal mortality in mouse models, suggesting that CS21 may play a significant role in ETEC infection and severity [9]. However, the precise mechanisms by which CS21 influences ETEC pathogenesis remain unclear. This study aims to investigate the role of CS21 in ETEC pathogenesis by utilizing both in vitro and in vivo models. By analyzing the interactions between CS21-expressing ETEC strains and host intestinal cells, this research seeks to enhance our understanding of

ETEC virulence mechanisms and potentially identify new therapeutic targets for managing 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% CO_2 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.

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