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Research Article

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Effect of acid stress on adherence of *Escherichia coli* to intestinal epithelial cells and ability to grow in caecal mucus and colonize mouse intestine

Sanjay Chhibber [1], Sonia B Bhardwaj[2]*and Preeti Aghi [3]

[1]Professor, Department of Microbiology, Panjab University, Chandigarh.
[3]Department of Microbiology, Panjab University, Chandigarh.
[2]Assistant Professor, Department of Microbiology, Dr Harvansh Singh Judge Institute of Dental Sciences and Hospital, Panjab University, Chandigarh.
*Corresponding Author E-mail: sbbhardwaj2002@yahoo.com

ABSTRACT

Enterohaemorrhagic Escherichia Coli (EHEC) 0157:H7 is exposed to a wide variety of stresses including gastric acid shock, yet little is known about the impact of stress on EHEC – host cell adhesion. In this study the attachment to chicken epithelial cells at different pH range, showed that bacteria were able to attach to intestinal cells in vitro. In addition bacteria subjected to acid shock after growth at an acidic pH (pre shock-pH 5.0) were found to retain the ability to adhere to chicken epithelial cells, which was absent in the bacterial cells subjected to acid shock after growth at pH 7. Acid adapted enterohemorrhagic E. coli could grow well in mucus of streptomycin treated mice after acidic shock, while non- adapted strain in acid shock was unable to grow in the mucus. Acid adapted, streptomycin resistant strain of EHEC E.coli was able to colonize and establish infection in the intestine of the streptomycin treated mice 24 hours after oral feeding. The animals were found to excrete E. coli 0157:H7 in their faeces till 2 weeks period. These data indicate that acid stress of EHEC significantly enhances adhesion to host intestinal cells both in vitro and in vivo.

Key- Words: *Enterohemorrhagic E. Coli, acid stress, intestinal cells.*

INTRODUCTION

Enterohaemorrhagic *E. Coli* (EHEC) can cause severe clinical manifestations such as hemorrhagic colitis (HC), haemolytic uraemic syndrome [1]. Of the EHEC serotypes 0157-H7 has the highest correlation worldwide with HUS [2].

Stress has a major impact on gut physiology and may affect the clinical course of gastrointestinal diseases. All kinds of stress mechanisms including heat stress [3], alkaline stress[4] and acid stress [5,6] have been found to play an important role in the survival of bacteria which may not be conducive for their survival.

Enterohemorrhagic *E. coli* due to their resistance to acidic pH are able to colonize the intestine at lower infective dose [7], acid tolerance and resistance are critical virulence traits. Both non pathogenic and pathogenic *E. coli* encode four different acid resistance systems that provide protection against exposure to pH as low as 2-2.5 [6]. *E. coli* 0157:H7 demonstrates intimate adherence to enterocytes in animal models of human disease [8] showing characteristic lesions in the intestine of gnotobiotic piglets, rabbits , chickens and in selected cell lines in vitro. The enterohemorrhagic *E. coli* has been shown to colonize the terminal ileum, caecum and colon of experimental animals. The ability of *E. coli* to grow in mucus which is abundantly present in large intestine may be an important prerequisite for this pathogen to colonize the intestine. There is much information on the adherence of EHEC 0157:H7 in vitro [9, 10] and much less about

adherence in vivo. One of the in vivo systems that has been used to study EHEC 0157:H7 adherence in the ligated pig intestine [11].

In natural infection, EHEC 0157:H7 is thought to pass through the acid environment of the stomach before expressing virulence factors in the upper intestine, but there is no information on the effect of this acid exposure upon adherence in lower intestine. Although murine models do not fully recapitulate human infection, they are commonly used to evaluate EHEC vaccines and immune protective responses elicited in the host [12]. The objective of the study was to assess ,The effect of acid stress on adherence of *E coli* to intestinal epithelial cells and its ability to grow in the caecal mucus and colonize mouse intestine."

MATERIALS AND METHODS

Bacterial strain- A clinical isolate of EHEC 0157:H7 was used in this study.

Animals: LACA male mice weighing 15-20 g were procured from the animal house.

Cell Adhesion Assay- Adhesion of EHEC 0157:H7 to chicken intestinal epithelial cells at four different pH's(7,5,5→3.5 and 7→3.5) was checked by mixing 0.5 ml of bacterial cell suspension (10^9 cell/ml) to 0.5 ml of intestinal cells suspension (10^9 cells/ml) in Krebs Henslet buffer. The contents were incubated at 37^0C for 1 hr. The tube was centrifuged at 1800 rpm for 15 min. Pellet was washed thrice in 1 ml Krebs Henslet buffer . Smear was made on the slide fixed with methanol and stained with CV for 1 min. The slides were observed under microscope at 100X. The number of bacteria adhering to 30 different intestinal cells were counted and mean number of adhering cells calculated at each pH.

Growth of Bacterial strains in Mouse Caecal Mucus.

The method of Cohen *et al* [13] was used for extracting mucus from the caecum of mice. The mice were fed with normal feed and water containing streptomycin sulphate (5g/l). The mice were sacrificed and their caecal contents washed with sterile N-2 hydroxy ethyl piperazine –N-2 ethane sulphonate (HEPES). The caecal mucus obtained from different mice was pooled and the final product was homogenized. Enterohemorrhagic *E. coli* was grown at different pH (7, 5, 7→3.5, 5→3.5). 250 ul of mucus sample was put in a sterile eppendorf tube. To this 50 ul of inoculum was added to each tube, incubated at 37^o C. Samples were withdrawn at 0, 2, 4 and 6 hr time interval and serial dilutions made in normal saline blanks. These were spread plated on MacConkey's agar plates containing streptomycin (100 ug/ml). All the plates were incubated at 37^oC overnight and their viable counts were determined.

Mice Colonization Experiment

The ability of enterohemorrhagic *Escherichia coli* to colonize mouse intestine was studied as described by Wadolowski *et al* [14]. Bacterial strains were grown overnight in Luria broth, washed twice with PBS and finally suspended in PBS. Animals were divided in four sets.

Set –I Animals received organisms grown at pH 7.0.

Set-II Animals received organisms at pH 5.0.

Set-III Animals were grown at pH 7.0 and brought to pH 3.5 before feeding.

Set –IV Animals were grown at pH 5.0 and brought to 3.5 before feeding.

50 mg of pooled faeces were collected from each group after 5 hr, 24 hr and 2 week interval. Faecal samples were homogenized by adding 1 ml PBS. Serial dilutions prepared in PBS were spread plated on MacConkey's agar plates, containing streptomycin (100ug/ml). Plates were incubated overnight at 37 ^{0}C and bacterial count noted.

RESULTS

The adherence of EHEC strains of *E.coli* to chicken epithelial cells on incubation at 37^0C at different pH was seen. The results of adhesion experiment are presented (Table-I) . The adhesion index was maximum in case of EHEC *E.coli* at neutral pH(7.0) as well as at acidic pH (5.0). When acidic shock (pH 3.5) was given after growth at pH 5.0 the adhesion index was high with E coli 0157:H7. Enterohemorrhagic *E. coli* could take the acid shock after growth at pH 7.0 as adhesion index of 0.375 was obtained .

Table I: Adherence of E coli strains grown at different pH to chicken epithelial cells

pH	Shock pH	Enterohemorrhagic E. Coli(mean adhesion index)
7	-	4.62±7.65
5	-	3.40±7.47
5	3.5	2.60±6.17
7	3.5	0.375±0.99

Mucus was extracted from mice caecum and inoculated with overnight grown EHEC (10^5 CFU/ml) grown at pH 7.0. The inoculated caecal contents were then incubated at 37^0C and samples were withdrawn at 0, 2, 4 and 6 hr. The results showed that the strain was able to grow in mouse caecal mucus(Table II). The EHEC showed an upward trend and at 6 hrs the log (CFU/ml) was 6.38.

Table II: Growth profile of enterohemorrhagic E.Coli after acid shock in pooled mice mucus obtained from streptomycin treated animals

pH	Shock pH	Length of exposure(hrs)	EHEC E coli (log CFU/ml)
7	-	0	5.38
		2	5.47
		4	5.62
		6	6.32
5	3.5	0	5.30
		2	5.17
		4	5.04
		6	5.36
7	3.5	0	5.44
		2	2.09
		4	-
		6	-

The growth of overnight grown enterohemorrhagic *E. coli* in nutrient broth at pH 5.0 and pH 7.0 followed by acid shock (pH 3.5) was also monitored in the mouse caecal mucus. The strain initially grown at pH 7.0 followed by acid shock showed growth till 2 hrs of incubation. The survival of pH 5.0 growth organisms followed by acid shock was compared with that of pH 7.0 grown organisms which were given no acid shock. The results showed that though initially the bacterial counts obtained were similar with strain receiving acid shock or no shock but with the increasing time interval a marginal decrease in the bacterial count of acid shock subjected bacterial strain was seen.

The effect of pH on the intestinal colonization of mice with EHEC *E. coli* was studied. An increase in bacterial number was seen with EHEC *E. coli* and the counts were 3.78 log cfu/ml at 5 hrs after challenge were 1.32 log cfu/ml at 2 weeks interval (Table III).

Table III: Survival and implantation of enterohemorrhagic E coli in mouse intestine after acid shock

Sampling Time	pH	Shock pH	EHEC E Coli Log CFU/ml
At 5 hrs	7	-	3.78
	5	3.5	3.07
	7	3.5	1.32
At 24 hrs	7	-	6.47
	5	3.5	5.30
	7	3.5	0.07
1 week	7	-	8.60
	5	3.5	8.77
	7	3.5	-
2 week	7	-	8.60
	5	3.5	8.57
	7	3.5	-

DISCUSSION

Enterohemorrhagic *E. coli* (Luria Broth + Glucose) grown organisms showed resistance as only 0.5 to 1.0 log loss of viability at pH 3.5 was observed. The results indicate that acid tolerance was dependent on the pH of the growth medium [15]. There is evidence that environmental stress increases adhesion of other pathogens, including *Helicobacter pylori*, *Legionella pneumophila* and *Clostridium difficile* [16, 17, 18].

In one study, adherence of EHEC clinical isolates was compared with that of EHEC isolates that were from a contaminated food source but not found in patients [19]. The clinical isolates adhered significantly better to human colonic cells than did the food isolates, implying that ingestion stress may have enhanced host cell adhesion.

The colonization of mucous membrane of gastrointestinal tract is important as bacteria must adhere to mucosal cells because surfaces of these membranes are recurrently washed with fluids that otherwise sweep away unattached organism [20]. The results show that *E. coli* 0157:H7 could tolerate pH 3.5 exposure once the strain was preadapted at acidic pH before acid shock. Initially the growth declined upto4 hrs in mucus. On further incubation however, an increase in bacterial counts was observed indicating that probably during early initial incubation there is a lag period following which bacterial growth takes place. However, bacteria grown at pH 7.0 when subjected to low acidic pH (3.5) showed decline in bacterial numbers within 4 hrs of incubation. This observation suggests that prior adaptation to acidic pH has a significant effect on subsequent survival in mucus in vivo as well. The subsequent mouse colonization experiment also showed similar trend in the acid adapted *E. coli* 0157:H7 in mouse intestine as it was subsequently able to establish itself in the intestine of streptomycin treated mice within a weeks time. However, the organisms took some time to adapt itself as in the initial few days the increase in the count was not so marked as compared to animals who were fed with *E. coli* grown at normal pH. No colonization was observed with *E. coli* strain which was grown at pH 7 and subsequently subjected to pH 3.5 before oral challenge to animals.

The environment of the intestinal tract is characterized by variable oxygen levels. While the lumen of the intestinal tract is relatively anaerobic, there is a zone of relative oxygenation adjacent to the mucosal surface [21, 22, 23]. It has been shown that *E. coli* can sense changes in oxygen availability and switch from aerobic to either anaerobic or microaerobic. Despite the understanding of this respiratory flexibility in *E coli*, there is a little known about varying oxygen concentrations modulate EHEC virulence. Flagella have also been reported to function as EHEC adhesions, promoting adherence to mucins, the major component of the mucus that lines the gastrointestinal tract [24].

The results of this in vitro and in vivo study on the adherence and colonization of enterohemorrhagic *Esherichia Coli* 0157:H7 indicate that this process is independent of the acidic stress which this bacterium might encounter in the environment outside or inside the body.

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