Inhibition of V. parahaemolyticus infection using the commensal E. coli HS in a tissue culture infection model

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Abstract. V. parahaemolyticus is a Gram-negative pathogenic bacterium. Its pathogenicity contributes to extracellular products (hemolysin) which considered as the most important virulence factor and controls of many biological activities like cytotoxicity, hemolytic activity, enterotoxicity. This bacterium is able to attach to different types of host cells and cause infection. This study was done to investigate the ability of the well-known commensal bacterium E. coli HS to inhibit the cytotoxic effect and the attachment of V. parahaemolyticus using Hela cell line. The commensal E. coli HS can strongly adhere to epithelial cells without any harm effects. The results revealed that the pre-incubation of Hela cells with the commensal E. coli HS leads to inhibit the cytotoxicity and the attachment of V. parahaemolyticus to Hela cells. Since, bacterial attachment to host cells is a critical stage towards pathogens infection, herein; we conclude that the inhibition of pathogens attachment to host cells may result in inhibition of infection.

Keywords: V. parahaemolyticus, cytotoxicity and attachment, Hela cells line

Introduction
V. parahaemolyticus is the major foodborne pathogen compared to other Vibrio isolates. Annually in the United States the V. parahaemolyticus causes about 34,664 episodes of locally acquired foodborne infection\[1,2\].

V. parahaemolyticus can contaminate undercooked shrimp, fish, oyster and the signs and symptoms are abdominal pain, diarrhea and acute gastroenteritis. V. parahaemolyticus is a Gram-negative halophilic bacterium that produces a capsule with different somatic (O) and capsular (K) antigens [3]. Infection by the O3: K6 strain, which is pandemic, lead to a massive number of human deaths in many countries [4,6]. In addition, V. parahaemolyticus pathogenicity contribute to extracellular products (hemolysin) which considered as the most important virulence factor and controls of many biological activities like cytotoxicity, hemolytic activity, enterotoxicity [7,9], wound infection and septicemia [1,2]. Also proteolytic enzymes, gelatinase and caseinase which damage and hydrolyze many protein substrates like hemoglobin and active peptides [10,11], Phospholipases [12] urease [13], DNase and amylase [14] all of these enzyme considered reasons for tissue distraction and harm.
Thermostable direct hemolysin (TDH) and thermostable direct-related hemolysin (TRH) remain the most separate virulence factors of *V. parahaemolyticus* which have hemolytic activity attributed to these two genes.[15,16,17]. The main targets for TDH activity are the epithelial and intestinal cells and TDH is a pore-forming toxin. It makes large pores on erythrocytes (RBCs) permit both water and ions to flow throughout membranes, this results in colloidal osmotic lysis[18,19]. TRH demonstrates hemolytic activity similar to that of TDH on blood cells[20].

Recent study noted that it may be important to determine the distribution of mutT gene in a broader range of clinical and environmental isolates of *V. parahaemolyticus* to found the role of mutT gene in disease as virulence factor and to investigate whether this gene is a useful marker of non-toxigenic strains of *V. parahaemolyticus* [21].

*V. parahaemolyticus* express adhesins that enable them to adhere to host cells and cause infections. Recently, it has been shown that *V. parahaemolyticus* express Multivalent adhesion molecules (MAM7) that enable the bacterium to attach to different types of host cells [22].

The commensal strains of *E. coli* can strongly adhere to epithelial cells without any harm effects mediated by flagella and fimbriae [23, 24]. These adhesive properties have been shown to be critical for commensal strains’ ability to compete with pathogenic bacteria such as *Salmonella* spp. and *E. coli*[24–26].

Antimicrobial susceptibility test of *V. parahaemolyticus* revealed that isolates were well sensitive to ampicillin sulbactam, meropenem, ceftazidime, and imipenem, but it is resist to penicillin G and ampicillin. In this study two isolates of *V. parahaemolyticus* were multidrug resistance (MDR) which appeared resistance to Seven antibiotics[28]. Another emerging challenge when a bacterial isolates become resistant toward multiple antibiotics. These strains can be achieved during several mechanisms like enzymatic inactivation, chromosomal DNA mutations, as conjugation as well as transformation [29]. This study aimed to investigate the effect of non harm *E. coli* on cytotoxicity and attachment of pathogenic *V. parahaemolyticus* on Hela cells line model.

**Materials and methods:**

**Cell line and growth conditions**

Hela cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% HIFBS, 5mM L-glutamine, 50µg/ml streptomycin and penicillin at 37 °C in an atmosphere containing 5% CO₂.

**Bacterial attachment to host cells**

Into a 24-well plate, Hela cells (15x10⁴ cells/ml) were seeded and incubated for 24 hours. Following washing with PBS, *V. parahaemolyticus* or *E. coli* strain HS were added (MOI of 10). After incubation for 1 and 4 h, the medium was removed and Hela cells were lysed with 0.5% Triton X-100. Serial dilutions were prepared and grew on LB agar at 37 °C for 24 hours followed by counting the number of colonies.

**Immunostaining for Fluorescence Microscopy**

The fixed Hela cells were treated with 0.1% Triton X- 100 for 5 minutes. Hoechst or rhodamine-phalloidin was added for 10 min. Followed by washing with PBS. Slides were visualized using a Nikon Ti Eclipse microscope.
Cytotoxicity assay

Lactate dehydrogenase release (LDH) assay was used to detect the cytotoxicity. Hela cells (15x10^4 cells/ml) were seeded into a 24 well plate. Bacteria was incubated with Hela cells. In triplicate, 200 µl of supernatant was removed into a 96 well plate and centrifuged for 1000 xg for 5 min. In a fresh 96 well plate, 100 µl of the reagents mix was added to 100 µl of the supernatant and incubated at room temperature. The absorbance was measured at 490 nm. Below the formula of cytotoxicity percentage:

\[
\% \text{ cytotoxicity} = 100 \times \frac{\text{OD}_{490 \text{ for experimental release}} - \text{OD}_{490 \text{ for spontaneous release}}}{\text{OD}_{490 \text{ for maximum release}} - \text{OD}_{490 \text{ for spontaneous release}}}.
\]

Competition experiments

*E. coli* strain HS in DMEM without antibiotics at an MOI of 100 µl was added to cells line and incubated for 1 h., followed by adding *V. parahaemolyticus* at an MOI 10. These plates were incubated at 37 ºC for 4 hours. For the attachment competition, Hela cells line were washed three times by PBS and added 0.5% Triton X-100. Serial dilutions were prepared and plated on TCBS agar plates for *V. parahaemolyticus*. The plates were incubated at 37ºC for 20 hours. Then the number of colonies was counted. For measuring the cytotoxicity, lactate dehydrogenase (LDH) release assay was used as mentioned above.

Results

In the current study, the results revealed the cytotoxic effects of *V. parahaemolyticus* after infection Hela cells for 1h and 4h (30 % and 60% ) respectively(Figure 1A). In addition, the results showed the attachment of the bacteria to Hela cells as illustrated in Figure 1B. The cytotoxicity and attachment properties of *V. parahaemolyticus* on Hela cells were detected by fluorescence imaging(Figure 1C,D).
Figure 1: The cytotoxic effects and the attachment of *V. parahaemolyticus* to the Hela cells.

The figure shows the cytotoxic effects (A) and the attachment (B) of *V. parahaemolyticus* (at MOI 10) on Hela cells. The fixed infected cells were stained with Hoechst for DNA (blue) and rhodamine-phalloidin for actin (red) (C & D). The values shown are means ± SD.

There were no cytotoxic effects caused by the incubation of Hela cells line with the commensal *E. coli* at the detected time (2A). In addition to the ability of the bacteria to attach to Hela cells (2B). The cytotoxicity and attachment of *E. coli* on Hela cells were detected by fluorescence imaging (Figure 2C, D).
Figure 2: A: The commensal E. coli attach to the host cells without cytotoxic effects.

The figure shows no cytotoxic effects (A) and the attachment (B) of E. coli (at MOI 10) on Hela cells. The fixed infected cells were stained with Hoechst for DNA (blue) and rhodamine-phalloidin for actin (red) (C & D). The values shown are means ± SD. In this study results, the pre incubation of Hela cells with the commensal E. coli strain HS prior the infection with V. parahaemolyticus leads to reduction the cytotoxicity to 5% (***P-value 0.01) (Figure 3A). In addition to inhibit the cytotoxic effects, the commensal E. coli strain HS reduced the attachment of V. parahaemolyticus to 35000000 CFU/ml comparing to adherance of V. parahaemolyticusalone (42000000 CFU/ml) with significant difference (*P<0.05), Figure 3B. The cytotoxicity and attachment properties of V. parahaemolyticus with commensal E. coli on Hela cells were detected by fluorescence imaging (Figure 3C, D).
Figure 3: The commensal *E. coli* HS inhibit *V. parahaemolyticus* cytotoxicity and attachment.

The figure shows the inhibition of cytotoxic effects (A) and the attachment (B) of *V. parahaemolyticus* via pre incubation of Hela cells with the commensal *E. coli* HS (at MOI 100). To visualize to treatment, the fixed treated cells were stained with Hoechst (blue) and rhodamine-phalloidin (red)(C & D).The values presented are means ± SD. Two tailed student’s t test was used to determine the significance (* p <0.05, ** p <0.01).

**Discussion**

In this study, inhibition the cytotoxicity and adherence of *V. parahaemolyticus* by commensal *E. coli* was done using Hela cells model .According to the Figure 1A & B the cytotoxicity of *V. parahaemolyticus* reached to 60% after the incubation with Hela cells for 4 h. In addition to, the results show the ability of this pathogen to adhere to host cells. Previously, study has attributed the ability of *V. parahaemolyticus* to cause infection in humans to the existence of many genes encoding the TDH , TRH haemolysins and T3SS2. The environmental strains of this bacteria carry these virulence genes but less frequent and without causing illness [30]. However; facts that strains missing these genes can also cause diseases. VPA1380 is the most newly identified T3SS2 as effector protein. It is a typical cysteine protease that catalyzes its substrates target[31] and possibly invasion of the host cell occur by VPA1380[32].

The most recent to be described is the T6SS (Gram-negative bacterial secretion systems), It is a molecular machine that utilizes a bacteriophage-like cell-puncturing tool to inject effector proteins into target cells[33]. During the infection of bacterial cells, T6SS play role in intracellular trafficking, actin cross-linking, secretion and vesicular transport[34]. *V. parahaemolyticus* holds two set sof T6SSs, one on each chromosome. Firstly, VPT6SS1s related just with clinical strains, secondly, VPT6SS2s encode all strains of *V. parahaemolyticus*. These two systems have different aspects of adherence to Caco-2 and/or Hela cells[35].

In this study, the competition between pathogenic *V. parahaemolyticus* and the commensal *E. coli* was carried out. The pre incubation of Host cells with the commensal *E. coli* result in reduction the cytotoxic effects from 60% to 5% (***P-value 0.01) (Figure 3A). In addition to inhibit *V. parahaemolyticus* adhesion to host cells (*P<0.05) Figure 3B. The inhibitory activity of the commensal *E. coli* HS was also confirmed via visualization of the treatment(Figure 3 C,D).
The inhibition of the cytotoxic effects caused by *V. parahaemolyticus* could be because the competition for the binding sites on the host cells. In literatures, it has been shown that the attachment of pathogens to host cells is an important step towards pathogens infection. The commensal and recombinant bacteria have been used to prevent pathogens infections. Recently, it has been revealed that the ability of an engineered bacteria express an dhesin known as MAM7 from the commensal *E. coli* HS or *V. parahaemolyticus* to displace variety of pathogenic bacteria [27,36,37]. Another study results showed the ability of the commensal *E. coli* Nissle 1917 to compete pathogenic bacteria causing intestinal infections [38]. Furthermore, it has been found that the commensal lactobacilli prevent the adhesion of *Escherichia coli* and *Salmonella* spp in a tissue culture model[39]. In addition to the competition for the binding sites, bacterial ability to inhibit pathogens infections may result via the interspecies antagonism as well as the competition for the nutrients[27,40,41]. Some of the food producers could include probiotics like *Streptomyces* and *Lactobacillus pentosus*, to suppress the growth of *V. parahaemolyticus* and reduce the production of pathogenic extracellular products[42, 43].

Five *V. parahaemolyticus* isolates were cultivated with *E. coli* in marine-like environment to exam their virulence effects to this bacteria. Three of these isolates were not virulent toward *E. coli* versus two isolates were virulent. Using PCR for genomic DNA, these two virulent isolates have the genetic locus called T6SS1, while other three isolates which were not virulent against *E. coli* did not include T6SS1, provided that association between the presence of T6SS1 and antibacterial virulence factors effect. Two isolates have T6SSs might not play an important action in mammalian virulence, possible the regulatory mechanisms of the T6SSs have evolved to advantage this pathogenic bacteria in the marine location[44]. In this assay, suggestion that T6SS2 does not play a role in inter bacterial competition under many conditions (surface, temperature and salinity). *V. parahaemolyticus* virulence effects against other bacteria was selective, for the reason that it was not suppressed *P. aeruginosa* and *A. tumefaciens*. This effect could be explained in many ways: *V. parahaemolyticus* targets a bacterial component that is not found or is not vital for some bacterial species; T6SS1 activity can suppress by a number of bacterial species and some bacterial isolates are immune toward T6SS1 toxicity [45].

**Conclusion:**

We conclude that the inhibition of *V. parahaemolyticus* infection may happen as a result of the competition for the binding sites which is critical for the pathogens to cause infection.

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