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New approach for detection of *Escherichia coli* invasion to HeLa cells

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ABSTRACT

To establish a successful infection, microorganisms have developed strategies to invade host cells. One of the most important human pathogens and the greatest cause of urinary tract infections, *Escherichia coli*, still do not have its invasion mechanisms fully understood. This work aims to present a new approach for detecting bacterial invasion of lineage cells, based on an enzymatic-fluorogenic method. The focus of this technique is the detection of *E. coli* invasion of HeLa cells, exploring β-glucuronidase, a specific constitutive enzyme of this bacterium. This enzyme hydrolyses the key substrate of this work, 4-methylumbelliferyl-β-D-glucuronide (MUG), resulting in a fluorogenic molecule, 4-methylumbelliferone. The fluorescence curve created by this method, analyzed by Tukey statistical test, demonstrated that this detection can be efficiently performed after 5 h incubation with MUG. When testing uropathogenic *E. coli* and *E. coli* isolated from human gastrointestinal microbiota, the proposed method presented similar results to those exhibited by plate counting invasion detection. Data examination by Duncan statistical test allowed the creation of an intensity range of bacterial invasion, which is part of the process of results interpretation. Detection by this enzymatic-fluorogenic method, compared to other existing bacterial invasion detection techniques, is less burdensome, more sensitive and allows fast achievement of reliable results.

1. Introduction

The ability of invading epithelial and endothelial cells is a pathogenic mechanism of several bacteria. Although the intracellular environment is generally limited in resources and nutrients, host cell invasion represents an important step for microbial pathogenicity, ensuring a privileged habitat for multiplication and protection against host defenses, facilitating microbial dissemination into adjacent tissues (Lewis et al., 2016). The invasion mechanisms of some bacteria such as *Yersinia, Listeria*, *Salmonella* and *Shigella* are well described (Drolia et al., 2018; Hume et al., 2017; Ke et al., 2013; Mellouk and Enninga, 2016; Cossart and Sansonetti, 2004), however, for *Escherichia coli*, one of the most important human pathogens and the major cause of urinary tract infections (UTI) (Vila et al., 2016), these mechanisms are not fully understood.

Considered for a long time as an extracellular pathogen, now it is proven by *in vitro* and *in vivo* experiments that uropathogenic *Escherichia coli* (UPEC) is capable of invading urinary tract cells (Bower et al., 2005). After adherence, UPEC can invade epithelial cells through many processes and different pathways, controlled by a variety of virulence genes (Lewis et al., 2016). Once internalized, UPEC is able to multiply and form intracellular communities or enter into a quiescent state, which can lead to recurrent or chronic infections, significantly contributing for its pathogenesis in UTI (Lewis et al., 2016; Dhakal et al., 2008). Thus, the investigation of this host-parasite interaction should result in a better bacterial pathogenicity comprehension, crucial for research on prevention and treatment alternatives for UTI. With this in mind, the first step is to detect whether or not the bacterium is capable of invading the host cell.

The techniques for bacterial invasion detection usually involve methods that distinguish extracellular from intracellular bacteria (Nizet et al., 1998). Electronic microcopy, for example, is one of the most used techniques for this type of detection, however, it is a laborious and time consuming method. Other approaches generally depend at some point,

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of quantification of viable microorganisms, that limits testing a great number of samples and conditions in which they can be tested. Consequently, these techniques become extremely laborious when investigating a great number of samples, and results frequently show great differences between repetitions due to variables added by the dilution and plating steps, required for quantification (Nizet et al., 1998). Therefore, there is a lack of suitable tests to provide reliable results in such a short period of time, without requiring burdensome work. In this manner, our work presents a new approach to detect *E. coli* invasion into HeLa cells, based on enzymatic-fluorogenic activity.

2. Materials and methods

2.1. Bacterial lineages

Samples of *E. coli*, isolated from stool of healthy patients (intestinal microbiota), and UPEC used in this work were previously characterized (Koga et al., 2014; Cyoia et al., 2015). We also tested UPEC V27, an isolate from human urosepsis (Johnson and Stell, 2000), enteroinvasive *E. coli* O124 (EIEC O124), and two others diarrheagenic *E. coli*, EIEC 1 and EIEC 2, identified as EIEC by agglutination with anti B (*E. coli* O112Ac, O124, O143, O164, O167), polyvalent sera (according to the manufacturer's recommendations – Probac do Brasil). *Escherichia coli* K12 HB101 (Boyer and Roulland-Dussoix, 1969) and EIEC O152 (Beutin et al., 1997) were used as negative and positive controls for invasion, respectively. These bacterial strains were stocked in Brain-Heart infusion broth (Neogen®) supplemented with 25% of glycerol (Sigma-Aldrich®) at −20 °C and −80 °C. For bacterial invasion tests, samples were inoculated in Luria Bertani broth (KASVI®) for approximately 18 h, at 37 °C under constant agitation. All bacteria utilized in this work are susceptible to gentamicin and have β-D-glucuronidase activity. To test β-D-glucuronidase activity, strains were incubated in nutrient broth with 4-methylumbelliferyl-β-D-glucuronide (MUG, 50 μg/mL, ≥ 98% purity, SIGMA®), overnight at 37 °C, and the fluorescence read under UV light after incubation. Fluorescence under UV light represents MUG cleavage (explained below), consequently, positive β-D-glucuronidase activity.

2.2. Cell culture and 96 well plate preparation

HeLa cells were cultivated in 96 well plates with Dulbecco modified eagle medium (DMEM Gibco®, 10^4 cells/well), supplemented with 10% fetal bovine serum (FBS- Gibco®) and 1% of antimicrobial solution (penicillin 100 IU/mL, streptomycin 100 μg/mL and amphotericin B 2,5 μg/mL - Gibco®). Cells were maintained at 37 °C under atmosphere with 5% CO_2, until cell monolayer total confluence was reached for bacterial invasion tests.

2.3. Bacterial invasion test with detection by counting of colony-forming units

The gentamicin protection assay, proposed by Sansonetti et al. (1986) was performed with controls and samples. Bacterial cultures were washed by centrifugation at 5000*g* for 5 min to withdraw any potential toxins produced over incubation that could disturb the cell monolayer (Edwards and Massey, 2011). The 96 well plate with HeLa cells was washed with phosphate saline buffer 0,01 M (PBS) and 95 μL of DMEM were added to each well. Five microliters from bacterial cultures (approximately 10^8 CFU/mL) were seeded in each well, reaching a multiplicity of infection of 50 bacteria per cell. Bacterial cultures were seeded in triplicates in each experiment. Next, the plate was incubated for 3 h at 37 °C under atmosphere with 5% CO_2. After incubation, 100 μL of DMEM supplemented with gentamicin were added, with gentamicin final concentration of 200 μg/mL per well. The plate was incubated for 1 h at 37 °C for antibiotic action. After incubation, the plate content was removed and wells were washed with PBS, and 50 μL of Triton™ X100 1% were added. After five minutes, 10 μL from each well and dilutions thereof were plated on MacConkey agar, in triplicates, for invasion detection by counting of colony-forming units after incubation overnight.

2.4. Bacterial invasion test with detection by enzymatic-fluorogenic approach

The invasion detection by enzymatic-fluorogenic method is based on the action of an *E. coli* constitutive enzyme, the β-D-glucuronidase, widely used for *E. coli* identification (Manafi et al., 1991). Approximately 97% of *E. coli* are positive for this enzyme, while the same enzyme is not as prevalent in others microorganisms from the *Enterobacteriaceae* family (Feng and Hartman, 1982). A substrate commonly utilized in enzymatic tests for *E. coli* detection is the 4-methylumbelliferyl-β-D-glucuronide (MUG), which when hydrolyzed by β-D-glucuronidase releases 4-methylumbelliferone (4-MU), a fluorogenic molecule that emits fluorescence when irradiated with ultraviolet light wavelength (Manafi et al., 1991). Gentamicin protection assay was carried out as described above, but after Triton addition, 100 μL of nutrient broth supplemented with 100 μg/mL of MUG were added to each well. The minimal concentration of MUG for an efficient distinction of fluorescence after its cleavage is 50 μg/mL in culture medium (Manafi et al., 1991). The plate was then incubated at 37 °C and the fluorescence readings were recorded at each one hour interval for a period of 8 h, with both excitation and emission wavelengths of 365 nm, and fluorescence expressed in relative fluorescence units (RFU) (Perry et al., 2006). For fluorescence reading it is necessary to alkalinize the content in each well (minimum of pH = 10), as 4-MU fluorescence is pH dependent (Manafi et al., 1991). Therefore, 50 μL of NaOH 1 N were added to each well, followed by the transference of 100 μL from each well to a black 96 well plate. The fluorescence reading was executed with the *Synergy HT* - BioTek® spectrophotometer and the Gen5™ software. Thus, a fluorescence curve of the bacterial controls invasion was constructed. The curve enabled the determination of the best period of incubation of bacterial controls with MUG, for an efficient detection of bacterial invasion. With this result, *E. coli* isolated from intestinal microbiota and UPEC samples were tested for invasion, and these results compared with those obtained by the invasion detection by counting of colony-forming units. For comparison purposes, both invasion tests, with detection by CFU counting and detection by fluorescence, were always performed at the same day, using the same conditions for lineage cells and bacteria.

2.5. Statistical analysis

The determination of the best period of incubation of bacterial controls with MUG to detect invasion was calculated using the Tukey test. The answer for bacterial invasion to HeLa cells was given by statistical comparisons between values of samples and controls, using the Duncan statistical test in both types of detections. Differences were considered statistically significant when $p < 0.05$ (confidence level of 95%, $\alpha = 0.05$). Statistical analysis were performed with the R software, version 3.4.2. Statistical results are represented in figures and tables below as letters (a, b, c, d, e and f), where different letters between results represent statistically significant differences, and equal letters show that results do not differ significantly between them. All data are expressed as mean values based on triplicate assays, repeated three times (Table 1).

3. Results and discussion

3.1. Fluorescence curve and determination of the best incubation period of bacterial controls with MUG

For bacterial invasion detection by the enzymatic-fluorogenic