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Characterization of killed but metabolically active uropathogenic *Escherichia coli* strain as possible vaccine candidate for urinary tract infection

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ABSTRACT

Background and aim: Urinary tract infection (UTI) is the second most frequent infection in human, and uropathogenic *Escherichia coli* is its most common cause. Although antibiotics are the standard treatment for UTI, they can cause harmful effects on gut microbiome and increase the rate of existing drug-resistant bacteria, which make the vaccine research reasonable.

This study was conducted to construct a Killed but Metabolically Active (KBMA) *E. coli* strain, and to determine its characteristics as a possible vaccine candidate for UTI, which will be evaluated in further investigations.

Methods: The *uvrB* gene of *uvrABC* excision repair system of *E.coli* was deleted to construct a *ΔuvrB* mutant, lacking repairing system of intercross linkages between DNA strands. To construct KBMA strain, the *ΔuvrB* mutant was PUVA-treated, using different doses of 8-methoxypsoralen (8-MOP) followed by different doses of ultraviolet A (UVA) irradiation (365 nm), until the optimal doses of each were achieved. Then, different characteristics of the PUVA-treated *E. coli* (with the optimal doses) were assessed, using cell counting, colony formation assay, MTT and XTT assays, fluorescent staining, and flow cytometry.

Results: PUVA treatment's optimal dose for *E. coli* isolates was 150 ng/ml 8-MOP plus 1000 mj/cm^2 UVA. While the PUVA-treated isolates had a significant decrease in cell counting, the fluorescent dying of the un-grown parts of the culture plates revealed living bacteria with bizarre shapes. Meanwhile, MTT and XTT assays demonstrated the metabolic activity of these bacteria and flow cytometry confirmed their aliveness.

Conclusion: These PUVA-treated bacteria, with metabolic activity and proliferation inability, seem to be good enough to be tested *in vitro* and *in vivo* as a candidate for vaccine against UTI. Therefore it seems the first step toward development of a vaccine candidate is successfully done. The immunogenicity and protectivity of these treated bacteria is under evaluation.

1. Introduction

The “Killed but metabolically active (KBMA) bacteria” phrase was first introduced in 2005 [1]. KBMA bacteria have been mainly achieved by PUVA treatment which utilizes the combination of a natural or synthetic psoralen metabolite following by ultraviolet A (UVA, 320–400 nm wavelengths) irradiation. The planar tricyclic structure of photosensitive psoralen molecule facilitates its intercalation between DNA base pairs; preferentially at the 5′-TA-3′ site [2]. Psoralen molecules form hydrogen bonds with pyrimidine bases (especially thymidine residues). UVA irradiation converts these hydrogen bonds to the stable covalent bonds to make DNA interstrand cross-links (ICLs), which inhibit DNA replication and thereby duplication and growth, while the microorganism is alive. Here, while the structure of bacterial cell is intact, expression of genes in which ICLs have been formed is interrupted [3,4]. Therefore, depending on the amount of formed ICLs, some of the bacterial functions are interfered and some not.

The lower concentration of psoralen means the lower number of the inactivated genes. Therefore, it is vital to achieve the lowest concentration of psoralen [5]. To protect changes made by UV irradiation, bacteria such as *E. coli* have a nucleotide excision repair mechanism using a specific nuclease named ABC exonuclease [6]. So, it is also important to mutate all or a part of its gene: *uvrABC*, to prevent the breakdown of the generated ICLs [1]. The *uvrB* deleted strains are more sensitive to PUVA treatment in comparison to the *uvrA* and *uvrC* deleted ones [7].

Up to now, several studies have evaluated KBMA strain of bacteria such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Bacillus anthracis*

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and *Leishmania infantum* either as vaccine candidates [1,5,8,9] for the diseases they are responsible for or as safe vectors [10,11]. As vaccine candidates, KBMA bacteria have been promised to possess the safety (reducing the risk of post-vaccination infection, especially in immune-deficient patients) of killed vaccines, and the potency (generating strong, extensive, and long-term immune responses) of live attenuated ones.

Urinary tract infection (UTI) is the second most frequent infectious disease in human [12]. It is estimated that 40–50% of women suffer from symptomatic UTI through their life, while 25% of them experience recurrent UTI6 to 12 month later [12,13]. Uropathogenic *Escherichia coli* (UPEC) cause 90% of all UTI cases [14]. Although some antibiotics still are effective against uropathogenes such as UPECs, pervasive effects on human microbiota [15] as well as increasing rate of drug-resistance bacteria [16–18] have been made great concerns about their usage. Therefore, great efforts have been done to develop an effective vaccine for UTI up to now, mostly for those who suffer from or have the high possibility of recurrent infections [19]. Diverse targets such as different virulence factors have been issued as vaccine candidates [20,21]; yet, none of them have been approved. Therefore, it seems that seeking a new probable vaccine candidate for UTI would be logical.

The purpose of this study is to construct a KBMA *E. coli* strain, and to evaluate its viability and metabolic activity as well as lack of its dividing ability.

2. Material and methods

2.1. Construction of uvrB deletion mutant (ΔuvrB mutant)

The standard *E. coli* strain, ATCC 25922was used and the its *uvrA, uvrB* and *uvrC* genes of ABC excinuclease [6] was detected by PCR amplification of the extracted chromosomal gene using the previously described primers [22]. The *ΔuvrB* mutant was constructed using established published techniques [23,24]. Briefly, 50–100 base pairs flanking regions of *uvrB* gene were amplified by PCR using the following primers: uvrB1f (5′-GGGATTAATCACGCGATGTTGAC-3′), uvrB1r (5′-GGGGAATTCTTCTTCGCTATCCTG-3′), targeting the upstream flanking region of the gene with VspI and EcoRI sites respectively, and uvrB2f (5′-GGGGAATTCCAGGATAGCGAAGAAGA-3′), uvrB2r (5′-GGGGTTAACGGAAATCCGAAGGA-3′), targeting the downstream flanking region of the gene with EcoRI and HpaI sites respectively. After purification, the PCR fragments were digested with the mentioned enzymes and ligated with pBHA vector (Bioneer, South Korea) which carries the gene for ampicillin selection in *E. coli* and was digested with VspI and HpaI. The ligation mixtures were transformed into competent *E. coli* BL21 (DE3) cells and the transformed bacteria were selected by plating on Luria-Bertani (LB) agar containing 100 μg/ml ampicillin. The ampicillin resistant colonies were picked and cultured, and plasmids were purified and digested with VspI and HpaI to check for appropriate inserts. Finally, selected plasmids were transformed into competent *E. coli* to construct the *ΔuvrB* mutant which was used in the next experiments.

2.2. Inactivation of UPEC using 8-MOP plus UVA

E.coli cultures incubated in LB broth at 37 °C overnight in shaking incubator until OD_{600} was reached to around 1.25. Then, 1×10^7 cells/ml were transferred to each well of the sterile 6-well plates. Next, in different stages, varying concentrations of 8-MOP (0, 25, 50, 75, 100, 125, 150, 200 and 250 ng/ml) were added to each well. The plates were covered with aluminum foils, and were incubated and shacked for 20 min at 37 °C. Eventually, the plates were placed on ice (to overcome the heat due to irradiation) and irradiated or not by UVA light (365 nm) at various energy level (0.5, 1, 1.5, 2, 2.5, 3.5, 5, 10 J/cm^2) using Ultraviolet Crosslinker (Hoefer, USA).

2.3. Confirmation of inactivation of UPEC

2.3.1. OD measurement

The OD_{600} of each well was measured by spectrophotometry (Eppendorf BioPhotometer, Germany) 20 min, 1 h and 2 h after PUVA treatment.

2.3.2. Colony formation measurement

Ten-fold serial dilutions of PUVA-treated/-untreated bacteria from each well were prepared (10^0-10^{-5}). Next, two different culture methods were used: (1) 2 μl of each dilution (equal to a dot) transferred on LB agar; (2) the 10^{-3} dilutions of each well were spread on separate LB agar media using pour plate method. All plates were grown at 37 °C overnight. Then, in both methods, colony formations of different dilutions were compared with each other and the plates were scanned.

2.3.3. Fluorescence microscopy

The procedure of staining was performed based on what was explained before by Matsuyama T [25]. The areas of the pour plate cultures where no colony was formed were harvested and suspended in 1 ml PBS. They were stained with 5 μl of rhodamine 123 (Sigma-Aldrich, USA, 5 μg/ml) and incubated at 37 °C for 30 min. Then, they were centrifuged in 9000 rpm for 3 min and washed 2 times. Using the chamber slides, they were directly observed under fluorescent microscope.

2.3.4. Metabolic assay (MTT assay)

Around 10^5 L929 mouse fibroblast cells (obtained from National Cell Bank of Iran, Pasteur institute of Iran) were cultivated in each well of the 96-well flat-bottomed tissue culture plates (3 repeats). Medium was removed and the cells were washed with Phosphate Buffer Saline (PBS). Three Prepared serial dilutions (10^0, 10^{-3} and 10^{-5}) of 3 PUVA-treated bacteria with different doses of 8-MOP (0, 150 and 250 ng/ml) were added to each well. Plates incubated in CO_2 incubator for 24 h. Then, 100 μl of MTT solution (5 mg/ml) was added to each well; plates were wrapped with foil, and were incubated again in 37 °C. Four and 24 h after treatment, the supernatants were removed, the solubilization solution was added, and the reaction was quantified by measuring the absorbance at 570 nm, using a multi-well spectrophotometer (BioTek Synergy 4, USA).

2.3.5. XTT assay

Using the XTT Cell Proliferation Assay Kit (Abnova, Taiwan), equal volumes of the Electron Mediator solution with XTT Developer reagent were mixed. Like MTT assay, 100 μl of serial dilutions were added in each well of the 96-well plates (3 repeats). Then, 10 μl of the XTT mixture was added to each well. After 5 min orbital shaking, plates were incubated in 37 °C. Four and 8 h after treatment, absorbance of each sample were measured at a wavelength of 475 nm, using the multi-well spectrophotometer (BioTek Synergy 4, USA).

2.3.6. Flow cytometry

Flow cytometry was done based on the protocol previously described by Lopez-Amaros et al. [26]. Briefly, 2 h after PUVA treatment, Ethylene glycol-bis(b-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA, Sigma-Aldrich, USA) was added to each sample to a final concentration of 1 mM to permeabilize the bacterial outer membrane. Sample and control cells (10^6 cells/ml) were placed into suitable tubes for flow cytometry analysis. Rhodamine 123 and propidium iodide were added to separate tubes to a final concentration 2 and 10 μg/ml respectively, and mixed gently. The tubes were incubated for 2 min at 37 °C. A 1:10 dilution was prepared using LB agar. Flow cytometric analysis was performed using the CyFlow SL flow cytometry (Sysmex Partec, Germany) equipped with a 488 nm laser. Two filters with the ranges of 520–530 nm and 670–680 nm were used to collect the emission of rhodamine 123 and propidium iodide respectively. Non-stained

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