



Effect of oxidative stress on glutathione reductase activity of *Escherichia coli* clinical isolates from patients with urinary tract infection

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Abstract

Background: Urinary tract infection (UTI) is frequently encountered by female population where the episode of occurrence increases with advancing age. *Escherichia coli*, a common UTI causing organism, retains glutathione defense mechanisms that may allow the organism to withstand host oxidative immune response as well as the harsh physiological environment of urinary tract. The aim of this study was to analyze glutathione reductase (GR) activity of UTI causing *E. coli* under stressful condition. **Material and Methods:** *E. coli* isolates from urine samples of UTI female patients of different ages were sampled. Samples of isolated *E. coli* were grown in conditions with and without oxidative stress where the stressful condition was induced by hydrogen peroxide (H₂O₂). In both conditions measurement of reduced glutathione level of the isolates was performed calorimetrically using microplate reader assay. **Results:** Samples from elderly patients showed that GSH level significantly altered, the value of GSH (mM/mg) are 5.01±0.48 and 5.20±0.64 in normal and under stressful condition respectively, which showed increase of GSH level by 3.83% (p = 0.024) under oxidative stress condition. On the other hand, in case of adult patients, GSH (mM/mg) level found to be decreased by 5.11% (p = 0.011) with the values of 5.08±0.1 and 4.82±0.18 respectively for normal and stressed condition. **Conclusion:** Our data suggesting an association of GR activity with patient's age, may signify that *E. coli* isolates respond differently to oxidative stressful environment in different age-related physiological changes and could help to uncover ways to gain better insight into *E. coli* pathogenesis of UTI predominance in aged population.

Key words: Aged, *Escherichia coli*, glutathione reductase, oxidative stress, reduced glutathione (GSH), Urinary tract infections (UTI).

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Introduction

Urinary Tract Infection (UTI) is a commonly encountered infectious disease and a major cause of morbidity in most of the developing countries. *Escherichia coli* has been known to be the most prevalent causative agent of UTI that is responsible for more than 85% of all UTIs (1). The incidence of UTI is higher in female and with advancing age of the patient increases the prevalence of the disease. About 60% of all women experience at least one UTI within their lifetime and at the age of 70 about 10% women have UTI where *E. coli* is the most frequent organism isolated (2).

E. coli also possesses several defense mechanisms for survival and has the ability to bypass stressful environment created by the host physiology and immune system (3). This ability may contribute to the pathogenicity of the organism and episode of infectious diseases of variable degrees. Human urine is allegedly a challenging environment for *E. coli* growth that struggles with the averagely oxygenated setting of limited nutrient and high-osmolality. There are also innate and adaptive immune systems that protect the host against a multitude of pathogens and infections (4). Oxidative stress is one of the host cellular responses that gets activated in response to stressful stimuli, in this case uropathogenic *E. coli* (UPEC), in an effort to eradicate the pathogen by producing reactive oxygen and nitrogen species (ROS/RNS) (5). On the other hand, different inducible defense responses of *E. coli* allow the organism to endure the host oxidative immune response. Glutathione-glutaredoxin is a major redox system involved in maintaining a reduced environment in the *E. coli* cytosol. Reduced form glutathione (GSH) is an abundant non-protein low molecular weight thiol in *E. coli*, where the intracellular concentration is approximately 5 mM and regulated by the transcriptional activator OxyR (6). The antioxidant properties of GSH play significant role in UPEC to persist the hostile environment of the urinary tract.

In this study, we observed GSH level alteration of *E. coli* strains under in vitro oxidative stress that were isolated from urine samples of UTI female patients of different ages.

Material and methods

Subjects

The study population was considered to be inpatients and outpatients at Uttara Adhunik Medical College Hospital, Uttara, Dhaka (Ethical reference no. UAMC/ERC(Re)/Recommended-47/2016), with clinically diagnosed uncomplicated symptomatic UTI. The clinical diagnosis of UTI was suggested based on the microscopic examinations of more than 5 white blood cells per high power field (1000x for high power) and a colony count of 10^5 CFU/mL of a single pathogen. UTI patients were selected between age of one month to 80 years over a period from August to September 2014, excluding the patients who had the history of taking antibiotic during the study or near past of study period. Clean catch of midstream urine was sampled from 58 patients with diagnosed UTI.

Methods

Sampled urine was transported to the laboratory within one hour of collection and carried out the procedure within 2-4 hours. Isolation and identification were done according to previous reference method (7) using suitable biochemical tests. *E. coli* was isolated from 88% of the samples as UTI causing organism among which 9 isolates were randomly selected for this investigation, obtained from the urine samples of female patients.

The isolates suspended in Luria broth (Sisco Research Laboratories Pvt. Ltd., India), were cultured overnight in a shaking incubator at 37°C at 120 rpm. The culture was centrifuged and cells were resuspended in fresh medium (OD600= 0.05), with shaking grown aerobically at 37°C until OD600 = 0.6 was obtained. To provide oxidative stress, 8 mM hydrogen peroxide (H₂O₂) (Merck, Germany) was added to the fresh medium during resuspension of the centrifuged cells following all the steps carried out in the normal growth condition. OD600 values in both normal and stressful conditions were monitored spectrophotometrically every 30 minutes of interval until OD600 value of 0.6 was reached.

Sample of bacterial cell culture was harvested by centrifugation, suspended in cold 20 mM EDTA (Sigma-Aldrich, Germany) and lysed by using six sonication cycles of 30 s pulse at 0°C temperature. A final concentration of 0.5 mM perchloric acid (Sisco Research Laboratories Pvt. Ltd., India) was added to the lysate to precipitate proteins. After 30 min, the suspension was centrifuged; supernatant was adjusted to pH 7.5 with KOH (Merck, Germany), frozen, and centrifuged to eliminate the potassium perchlorate. The supernatant was collected carefully, which was undertaken for protein concentration measurement using the method described by Lowry et al., (8). Unknown protein concentration was estimated comparing with the standard curve plotted by using Bovine serum albumin (BSA). Using the values of protein estimation sample was drawn accordingly and added 10 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) (Sisco Research Laboratories Pvt. Ltd., India) with 10 minutes of incubation at room temperature.

GSH concentration was determined colorimetrically in a microplate spectrophotometer (Thermo Fisher Scientific, USA) by measuring the absorbance of GSH and DTNB reaction product at a wavelength of 560 nm. An overwhelming fraction of acid-soluble thiols in *E. coli* is comprised by GSH, making this technique functional regardless the inadequacy to differentiate between GSH and other low-molecular-weight thiol-containing compounds. GSH standard equation was used to estimate mM amount of GSH per mg of protein.

Statistical analysis

The statistical analysis was performed by using SPSS V22.0 program. The test distribution was analyzed with One-sample Kolmogorov-Smirnov test and p value was calculated using paired t-test, where p < 0.05 was considered to be statistically significant. Partial correlation analysis was also performed.

Results

In normal condition the growth of the *E. coli* isolates reached OD600= 0.6 in 3~3.5 hours where in presence of H₂O₂ the growth time got extended to 7~8 hours (Figure

1) In normal condition the isolates 1,3,4,7,9 and 5,6,8 exhibited similar growth profile (Figure 1A). On the contrary, isolates 1,7; 3,4,9 and 5,8 behaved similarly to each other with the growth pattern under stress (Figure 1B). The expression level of soluble proteins of *E. coli* is the highest at the mid-log phase. Thus, in this study the isolated *E. coli* cell growth was carried out till the mid-log phase (OD600=0.6) instead of the entire growth timeline in order to ensure the optimum expression level of the soluble protein (Figure 1 A and B).

The glutathione content of isolated *E. coli* was expressed as mM/mg protein, respective to the patient's age and growth conditions (Table 2). The amount of GSH production changed in all samples due to H₂O₂ treatment. Compared to normal condition, the level of GSH production of isolated *E. coli* sampled from adult patients, below 40 years of age, found to be decreased under stressful condition. On the contrary, GSH production demonstrated increased level in samples that were collected from aged patients, age above 40 years. Such change in GSH level was found to be statistically significant ($p < 0.05$). A significant partial correlation was found between age and change of GSH level as well ($p = 0.007$).

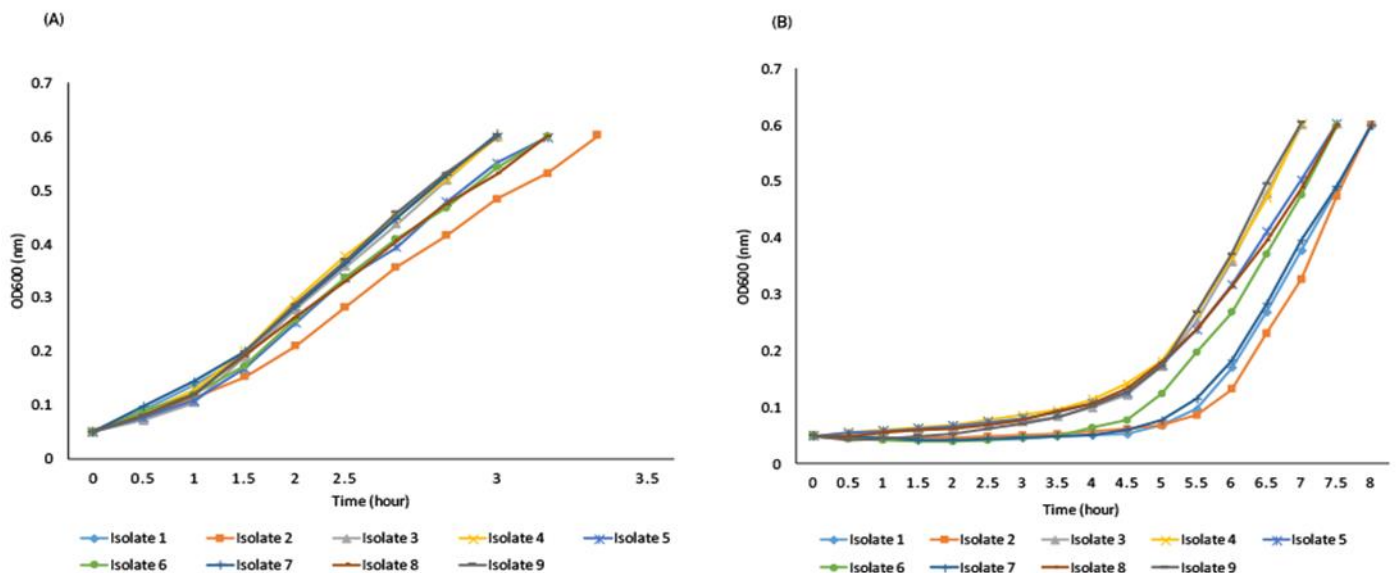


Figure 1. Growth of *E. coli* strains isolated from urine sample of UTI patients. (A) Isolated *E. coli* cell growth in normal condition and (B) and isolated *E. coli* cell growth under stressful condition induced by 8 mM H₂O₂. Cells were cultured in LB medium at 37°C with shaking at 120 rpm until OD600= 0.6 was obtained. The OD600 readings were carried out every 30 minutes of interval. Each data point is the mean of three replicates.

Table 1. Profile of selected patients with UTI.

| Sample | Patient's Age (years) | Gender | Complaints |
|--------|-----------------------|--------|--|
| 1 | 32 | Female | Burning sensation during micturition, fever, pain in lower abdomen. |
| 2 | 51 | Female | Burning sensation during micturition, fever, pain in lower abdomen. |
| 3 | 50 | Female | Burning sensation during micturition, fever, nausea, vomiting, pain in lower abdomen, red color urine, history of diabetes |
| 4 | 55 | Female | Burning sensation during micturition, fever, nausea, vomiting, pain in lower abdomen |
| 5 | 59 | Female | Burning sensation during micturition, fever, reddish color urine, pain in lower abdomen |
| 6 | 24 | Female | Burning sensation during micturition, fever, pain in lower abdomen. |
| 7 | 29 | Female | Burning sensation during micturition, fever, pain in lower abdomen. |
| 8 | 42 | Female | Burning sensation during micturition, fever, Dysuria |
| 9 | 36 | Female | Burning sensation during micturition, fever, pain in lower abdomen. |

Table 2. Change of GSH level of different strains of *E. coli* under stress compared to normal conditions.

| Age Grp. | Sample | GSH (mM/mg) level | | | | Diff. | SEM | Change (%) | p value |
|----------|-----------|-------------------|-------------------------|---------------------|----------------------------|-------|-------|------------|---------|
| | | Normal condition | Normal condition (Avg.) | Stressful condition | Stressful condition (Avg.) | | | | |
| <40 yrs. | 1 | 5.46 ±0.11 | | 5.28±0.26 | | -0.18 | | | |
| | 6 | 4.71±0.02 | 5.08±0.1 | 4.34±0.39 | 4.82±0.18 | -0.37 | 0.396 | -5.11 | 0.011 |
| | 7 | 4.91±0.11 | | 4.67±0.06 | | -0.24 | | | |
| | 9 | 5.23±0.17 | | 5.04±0.02 | | -0.19 | | | |
| 8 | 4.97±0.13 | 5.11±1.42 | | 0.14 | | | | | |
| >40 yrs. | 2 | 5.06±1.11 | 5.01±0.48 | 5.43±1.04 | 5.20±0.64 | 0.37 | 0.325 | 3.83 | 0.024 |
| | 3 | 5.00±0.09 | | 5.19±0.13 | | 0.19 | | | |
| | 4 | 4.82±1.02 | | 5.02±0.48 | | 0.20 | | | |
| | 5 | 5.21±0.08 | | 5.26±0.13 | | 0.05 | | | |

"±" = Standard deviation from the mean; Grp. = Group; yrs. = years; Avg. = Average; Diff. = Difference; SEM = Standard error of the mean.

Discussion

Studies have shown that there is a relation between the cellular redox state, stress proteins and immune activation (5). In bacteria, the adaptive response to oxidative stress is regulated by specific transcription factors, which produces the ion of antioxidant activities. In *E. coli* glutathione reductase activity is upregulated upon H₂O₂ oxidative stress via the induction of OxyR, a transcriptional activator to encode glutathione biosynthetic enzymes, glutathione reductases, glutaredoxins, as well as glutathione-dependent peroxidases (4). Cells retain glutathione reductase enzyme activity that uses glutathione to convert the dimeric oxidized form (GSSG) to the reduced form (GSH) of the tripeptide in recycled manner. The concentration of GSH in *E. coli* has been found to increase during oxidative stress and a mutant with malfunctioned GSH synthesis system demonstrated defective growth in media with elevated ROS (9). Thus, higher GSH concentrations increase the chances of UPEC to endure oxidative damage provoked by stressful and harsh environment of the urinary tract.

OxyR is inactivated in “unstressed” cells. Studies have shown that OxyR is reversibly triggered by the intramolecular disulfide bond formation due to altered cytosolic redox state and activation is reversed by cellular disulfide-reducing machinery (10). The easiness of formation of such disulfide bond depends on pH of the environment as well. In elderly women, due to deficiency of estrogen the vaginal pH results into more basic environment that may promote the formation of disulfide bond in *E. coli* cell (11, 12).

Ageing associating enhanced inflammation, pathogen-dependent tissue destruction, or accelerated cellular ageing may be contributing factors for the occurrence of UTI in the elderly population where *E. coli* manages to be the most common uropathogen. Studies have been reported that ROS levels increase with advancing age. Moreover, phagocyte ROS production in elderly patients is markedly elevated which suggests that this cytotoxic ROS overproduction could contribute to raised GSH level in UPEC (5). Aubron et al. showed that in asymptomatic bacteriuria (ABU) strains, increased level of endogenous ROS might be accountable for adaptive mutations as well as more effective antioxidant mechanism that is reportedly associated to enhanced growth of *E. coli* in urine, low abundance of fimbriae, and possible biofilm formation, impelling the capacity to colonize the bladder (4). Hence there could be a possibility that in case of elderly patients OxyR is more induced than that in adult patients allowing *E. coli* to produce higher level of GSH, subsequently high level of antioxidant defenses. Due to possession of specialized virulence genes contributing to pathogenicity, UPEC isolates are capable of exhibiting high degree of genetic diversity (13). Moreover, age or sex variation has influence on the distribution of *E. coli* genotypes (14). Studies suggest that differences in population, age group, diagnostic criteria and methods used for diagnosis are associated factors for the variability of prevalence of enteropathogenic *E. coli* in humans (15). Uropathogenic *E. coli* has found to exhibit different lipopolysaccharide characteristics respective to patient’s age (16). Study showed that pregnant women with UTI caused by resistant microorganisms were older than those with UTI caused by microorganisms sensitive to fosfomycin (17).

Conclusion

According to the finding of this study, it is most likely that the elevation of GSH concentration in UPEC increases the organism survival capacity as well as the chances of UTI predominance in female elderly patients. We hope that findings obtained from the present study will encourage larger experimental and clinical studies to infer the connection between patient's age and *E. coli* glutathione reductase activity contributing to UTI prevalence in elderly patients.

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