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Differential Gene Expression of Peroxide Resistant Protein (AhpC) in Planktonic and Biofilm State of Uropathogenic *Escherichia coli* Cells

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Abstract: The gastrointestinal tract seems to be the primary reservoir of Uropathogenic *Escherichia coli* (UPEC) in humans. UPEC strains harbor the Urinary Tract (UT) and cause Urinary Tract Infections (UTI) which might represent a serious threat to human life. To counteract with the damage caused by the Reactive Oxygen Species (ROS), bacterial strains produce various enzymes and proteins like Alkyl hydroperoxide reductase (AhpC*)* to scavenge the toxic oxygen molecules. The present study was designed to find the relation between the growth and biofilm formation conditions in the natural and artificial media along with the increasing resistance to the oxidative stress conditions. We studied antibacterial activity by broth dilution, antibiofilm assay, and primary adherence assay on *E. coli* (UPEC) (MTCC 729). Oxidative stress was studied by hydrogen peroxide assay and Lipid peroxidation assay. We further evaluated the oxidative stress by real-time PCR using alkyl hydroperoxide reductase AhpC as the gene member. Throughout the study, bacterial growth and biofilm formation were found to be more in synthetic urine. Biofilms in synthetic urine showed increased accumulation of total ROS and LPO compared to the media. From the qPCR study, we found that, when grown in the presence of favorable media, the cells showed increased gene expression. Further studies that clarify the susceptibility of strains to stress conditions and treatments need to be confirmed at the protein level.

Keywords: Biofilms, H_2O_2 Challenge Assay, Alkyl Hydroperoxide Reductase, Real-Time PCR

Introduction

Oxidative stress is a common threat to living organisms. It not only influences the number of biomolecules but also leads to the disruption of many critical and important cellular functions (Bisht and Dada, 2017). Oxidative stress is caused by free radicals such as hydrogen peroxide (H_2O_2), superoxide (•O_2 -) and hydroxyl radical (•OH). The most frequent is •OH, which is formed within the cells due to the presence of redox-cycling transition metals such as iron and copper (Kim *et al*., 2019).

Recent studies confirmed that bacterial species acclimatization is tightly related to ROS by various mechanisms (Lastochkina *et al*., 2020). In order to survive in harsh conditions, they do not only adapt, but bacterial species also modify the nearby environment. Some of the mechanisms depend on the production of proteins like DNA-binding proteins from starved cells (Dps) (Babele *et al*., 2019; Somayaji *et al*., 2022). These proteins protect the cells by binding and oxidizing Fe^{2+}, thus greatly reducing the production of •OH (Zhen *et al*., 2018).

The oxidized iron is only stored inside the iron core. Moreover, DPS proteins bind to DNA to form a protective coating that shields the DNA from harmful agents. Therefore, the Dps proteins elicit protective functions in cells and render the bacteria resistant to harsh conditions like ROS (Panwar *et al*., 2021; Edwardson *et al*., 2022). Recent studies suggest the possible and important role of the Dps proteins in antibacterial resistance. Bacteria express Dps-related resistance molecules such as peroxide resistance proteins (Dpr proteins). It seems that Dpr proteins are a promising novel target for drug design, as they might be involved in bacterial virulence (Guerra *et al*., 2021; Sevilla *et al*., 2021).

Science Publications

Antibiotic resistance is the capability of the strains to resist or to protect against the effects of an antibiotic. Antibiotic resistance occurs when the bacteria changes to reduce the action of the drugs or other agents on their cell membrane or other components (Walsh, 2000; Abushaheen *et al*., 2020). Also, non-pathogenic strains can naturally transform into pathogenic strains. Antibiotic resistance not only causes severe symptoms but also makes the strain more aggressive. These strains spread dangerous infections and prolong the suffering in children and adults (Minasyan, 2019). Such antibiotic-resistant bacteria can spread very fast to other family members and other people and in favorable conditions may lead to epidemics. Epidemics can lead to endemic and endemic pandemics. Such resistant bacteria are more difficult to handle and cannot be destroyed. In such cases, antibiotic-resistant infections can lead to more serious disability or might even become fatal to human organisms (Sadik *et al*., 2017; Gómez-Núñez *et al*., 2020).

Nowadays, Urinary Tract Infections (UTIs) are worldwide spread, affecting a large percentage of the human population, with more than 150 million people developing UTI infections. It is also estimated that more than 50% of women become infected with at least one UTI during their lifetime. With more than 11 million cases reported around the world each year, the cost of treatment and therapy is estimated to exceed 25 billion dollars annually (Sihra *et al*., 2018; Zeng *et al*., 2022). The gastrointestinal tract seems to be the primary reservoir of UPEC in humans. UPEC strains harbor the UT and cause UTI. Recent reports suggest that UPEC strains can be a serious threat to human life. UPEC not only colonizes the Urinary tract, but it also can generate local and systemic symptoms (Neugent *et al*., 2020). For successful colonization, *E. coli* needs to survive during the gastrointestinal passage and resist the extreme pH conditions of the stomach and intestine. Further, it penetrates the mucus layer of the colon and survives against other host defense mechanisms (Neugent *et al*., 2020; Hu *et al*., 2021). Here, they compete with other strains to acquire nutrients and other factors. Therefore, they become more pathogenic and resistant to other infections (Pickard *et al*., 2017). To counteract the damage caused by the ROS, bacterial strains produce various enzymes and proteins to scavenge the toxic oxygen molecules. Especially for peroxides, bacteria produce various catalases and peroxidases to scavenge H_2O_2 and organic peroxide molecules (Vojnar, 2020). Catalases, often heme-containing enzymes, catalyze the dismutation of two H_2O_2 molecules into molecular oxygen and water. In contrast, peroxidases catalyze the reduction of H_2O_2 and organic peroxides into their respective water and alcohol molecules, oxidizing an electron donor in the reaction (Patlevič *et al*., 2016; Asada, 1994).

Alkyl hydroperoxide reductase (AhpC) represents a class of peroxidases found in most bacterial species, with AhpC representing the classical form of this enzyme. AhpC, as well as all peroxidases from the peroxiredoxin family, uses redox-active cysteine residues to reduce its peroxides target (Shrivastava *et al*., 2020). In *Escherichia coli*, AhpC scavenges the low concentrations of H_2O_2 produced during normal cellular metabolism, these proteins were found to be responsible for defending the cells against peroxide stress (Carriel, 2017; Gu *et al*., 2020).

The present study aimed to determine the extent to which biofilm production protects the UPEC strains against oxidative damage or stress. For this, we evaluated the growth and biofilm formation with regard to antibiotic stress in both chemical media and artificial urine (mimicking the natural media of UPEC). Furthermore, we also quantitatively measured the expression of the AhpC gene in relation to the ROS damage that was induced.

Materials and Methods

Bacterial Strains

The strains of *E. coli* (UPEC) were procured from the MTCC repository. The strain (MTCC 729) was found to contain fimbriae and it is uropathogenic in nature. All these cultures were maintained on nutrient agar plates at 4°C.

Chemicals and Reagents

All the chemicals and the reagents commonly used in the tissue culture processes were obtained from HiMedia. The RNeasy Mini Kit (Cat No.74104), used for the total RNA extraction from cells and tissues was purchased from Qiagen. The components: Random primers, Taq polymerase, and the 100 bp DNA reference ladder were all purchased from Sigma Aldrich, Bangalore. All the set of primers used in this project was designed using the primer3 software cross-checked for specificity, using the Clustal W software, and were purchased from Sigma Aldrich through Eurofins, Bangalore. Superscript II reverse transcriptase and SYBR green Supermix were bought from HiMedia.

Preparation of Synthetic Urine

Synthetic urine was prepared according to the protocol prescribed by Barredo and Barredo (2005). All of the components ($CaCl_2 \times 2H_2O$, 0.651 g; $MgCl_2 \times 6H_2O$, 0.651 g; NaCl, 4.6 g; Na_2SO_4, 2.3 g; sodium citrate, 0.65 g; sodium oxalate, 0.02 g; KH_2PO_4, 2.8 g; KCl, 1.6 g; NH_4Cl, 1.0 g; urea, 25.0 g; creatine, 1.1 g) were dissolved properly in 200 mL of distilled water and made up to 1 L with distilled water. The solution was then supplemented with 0.2% glucose and pH was adjusted to 5.8.

Antibacterial Activity Using the Broth Dilution Method

The antibacterial activity was determined according to the protocol described by Mohammed and Malla (2015). In brief, antibiotic solutions (Nitrofurantoin CAS; No: 67-20-9) in various concentrations (50, 100, 200, 300, 400, and 500 µg/mL) were prepared and used for the antibacterial assay. A negative control without treatment was used in the study. 10 µL UPEC was used as inoculum. The experiment was performed with both Luria Broth (LB) and synthetic urine and carried out in triplicates. The percentage inhibition was calculated by using the formula:

$$Percentage\ Inhibition\ (\%) = [(dc - dt)/dc]\ x\ 100$$

where, *dc* and *dt* represent OD600 of control and treated sample strains, respectively.

Biofilm Assay

The biofilm study was carried out according to the protocol (Bao *et al*., 2017; Mohammed and Malla, 2015). Briefly, overnight cultures grown in LB were diluted (1:200) using LB + Glucose solution, and 200 µL was added to each well of the microtitre plate. UPEC was used as inoculum. Inoculum was added to each well, along with DNaseI (0.02 mg/1) at different time points (24, 48, 72 and 96 h). The same set of experiments was done with synthetic urine and carried out in triplicates. The plates were then incubated at 37°C for 24 h. Following incubation, the wells were washed thrice with 200 µL PBS and stained with 2% crystal violet for 15 min after drying at room temperature. The plates were then rinsed with 200 µL of ethanol: Acetone 80:20 to solubilize the stain. Absorbance was read at $\lambda = 590$ nm.

Primary Adherence Assay

The assay was performed according to the protocol (Gomroki *et al*., 2015). Briefly, 200 µL of the broth with culture was diluted with sterile Luria Broth in a boiling tube, up to an absorbance of 0.1 at $\lambda = 578$ nm. UPEC was used as inoculum and incubated at 37°C overnight. The same set of experiments was done but the synthetic urine and carried out in triplicates. Following incubation, 10 mL of the suspension was added to Petri dishes and incubated for 2 h at 37°C. Following incubation, the Petri dishes were washed thrice with PBS and the cells were stained with Gram's iodine followed by glycerine fixing. Adherent bacterial cells were observed under 40X magnification and the mean count was recorded with 5 microscopic fields.

H_2O_2 Challenge Assays

Hydrogen peroxide assay was performed using the method described by Uhlich *et al*. (2006). This assay is based on the capacity of the cells to scavenge free radicals generated by H_2O_2 in the medium. A sterile glass slide was added into the 20 mL of LB in a 50 mL conical flask and incubated at 35°C. The slides were transferred to a tube containing 25 mL H_2O_2 (20%) and incubated for 10 min at 25°C. Further, they were quenched in approximately 25 mL of 1% sodium pyruvate. Following quenching, the slides were rinsed with sterile distilled water and transferred to the sterile tubes containing 25 mL 0.1% Peptone Water (PW). The formed biofilms were scraped using a sterile spatula and the contents were vortexed for 30 sec. The dislodged planktonic forms were further diluted and plated onto brain heart infusion agar plates. Cells treated with sterile distilled water served as control. The same set of experiments was done with synthetic urine and carried out in triplicates. Following vertexing, the amount of hydrogen peroxide remaining within the solution was estimated using 50 mm hydrogen peroxide.

Lipid Hydroperoxide (LPO) Assay

Lipid peroxidation plays an important role in estimating the role of oxidative injury and can be estimated by measuring the Malondialdehyde (MDA) values. LPO levels were estimated using the procedure implemented by Akalın *et al*. (2007). The vortexed contents from the previous experiment were used in the assay, for the extraction step.

50 µL of sample collected from each tube was put into a sterile test tube and mixed with an equal volume of chloroform saturated with methanol. The contents were vortexed for 2 min and 1 mL of ice-cold chloroform was added. The contents were mixed thoroughly and centrifuged at 7000 rpm for 5 min at 4°C. The bottom chloroform layer was removed carefully with a Pasteur pipette and used for the LPO assay. An equal volume of chloroform-methanol mixture was added to each 500 µL of the above chloroform extract. 50 µL of freshly prepared chromogen reagent (4.5 mm ferrous sulfate in 0.1 m HCl and 3% methanolic solution of ammonium thiocyanate) was added to each tube and mixed thoroughly. The absorbance values were recorded at $\lambda = 500$ nm following incubation at dark. Chloroform-methanol mixture was used as blank and a standard curve was prepared using 13-hydroperoxy-octadecadienoic acid. The mean values of the absorbance were recorded for each standard and sample.

Gene Expression Studies

As Alkyl hydroperoxide reductase AhpC was found to be involved in the scavenging activity, our experiments were designed to confirm the role of the gene in the scavenging process and antimicrobial resistance mechanism. The gene expression studies were designed to study the significance of the genes in the biofilm formation and to assess the significance of ROS scavenging, comparatively among the planktonic cells and the biofilm.

RNA Extraction from the Biofilm Assay

The planktonic cells from the biofilm assay which were not linked to the biofilm were washed with sterile distilled water and collected in centrifuge tubes. The cells that were attached to the wells and involved in the biofilm formation were scraped out and collected in separate centrifuge tubes. The cells from both tubes were pelleted down and used for the RNA extraction process. The samples collected from both the biofilm assay and the planktonic assay were used for the RNA extraction. The RNeasy mini kit was used for the procedure and the experiment was done according to manual instructions. The obtained RNA was quantified using UV-spectrophotometry and 2 µg of the RNA sample was used for cDNA synthesis.

Expression of AhpC Members

The genomic DNA obtained by the Real-Time Polymerase Chain Reaction (RT PCR) was studied for the expression of the AhpC gene (FW: TGCGTGAAGATGAAGGTCTG; RV: CGACCAGGTCTAAGGATGGA). The cDNA was amplified for the AhpC gene. Amplification was performed for 30 cycles at 93°C for 45 sec, 62°C for 50 sec, and 72°C for 60 sec, using their respective primers. The amplified products were then isolated on 1.5% agarose gels and analyzed with a Gene Genius gel analyzer (Syngene, Bioimaging systems).

Real-Time Assay

2 µg of the total RNA was reverse transcribed with random primers, using reverse transcriptase. RT amplification was performed at 65°C for 5 min, 25°C for 2 min, and 42°C for 50 min, with a final inactivation at 70°C for 15 min. The real-time quantification was then done using the iQ^{TM} SYBR Green Supermix (HiMedia). The primers in a final concentration of 600 nm and 1 µL of the RT products were used for the amplification. The reaction was carried out in a total volume of 12.5 µL. All the reactions were performed in duplicates. All four samples were studied separately for gene expression. The product size was assumed to be 222 bp. ΔΔCt method of quantification was used to analyze the expression levels of the gene.

Results

Antibacterial Activity Using the Tube Method

The growth of the bacteria was significantly high in synthetic urine and so was the antibacterial activity, as can be clearly observed in the Fig. 1. Significant effect on antibacterial activity was seen in both the chemical and synthetic urine, but with higher values for synthetic urine. The antibacterial activity was found to be dose-dependent (Fig. 1).

Cultivation of Biofilms

We determined a significant effect in the synthetic urine batch (Fig 2). The biofilm formation was increased in the case of synthetic urine compared to the chemical-defined LB media. The results were in accordance with the bacterial inhibition studies. The biofilm formation capacity was found to be higher for the samples grown in synthetic urine than in the chemical media. This proves the affinity of the bacterial strain towards the host specificity. The biofilm formation was found to be 0.14±0.24, 0.23±0.11, 0.29±0.17, 0.41±0.08 and 0.63±0.04 for 0, 24, 48, 72 and 96 h respectively for the chemical media. Respectively, for the natural media (synthetic urine), the biofilm formation was found to be 0.32±0.63, 0.46±0.10, 0.59±0.21, 0.65±0.44 and 0.87±0.51 for 0, 24, 48, 72 and 96 h.

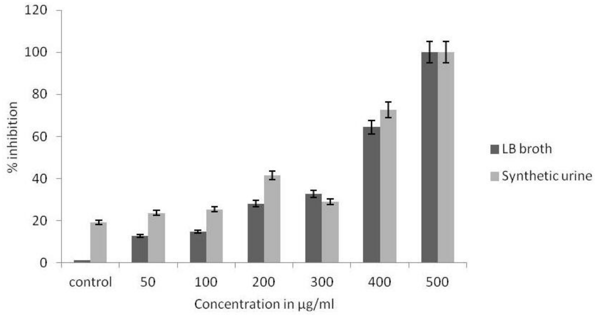

Fig. 1: The percentage of antibacterial activity inhibition. All the values were averages of triplicates. Values were represented as % inhibition ± s.e

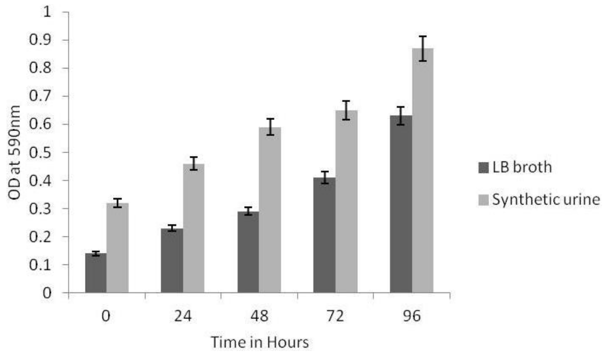

Fig. 2: The biofilm formation in the presence of LB media and synthetic urine. All the values were averages of triplicates. The values were represented as OD ± s.e

Primary Adherence Assay

The primary adherence values were in accordance with the biofilm formation assay. They were found to increase proportionally with the incubation time (Fig. 3). The results were found to be significant in the case of synthetic urine, compared to chemical-defined media. The primary adherence values were 0.08±0.14, 0.11±0.02, 0.25±0.06, 0.48±0.11 and 0.58±0.23 for 0, 24, 48, 72, and 96 h for the chemical media, respectively. The primary adherence values were 0.23±0.45, 0.46±0.34, 0.59±0.33, 0.72±0.12 and 0.94±0.22 for 0, 24, 48, 72, and 96 h for the synthetic urine, respectively. There was a significant effect determined in the case of synthetic urine, rather than when the chemical media was used.

H_2O_2 Challenge Assays

The resistance of the strains in the different media was analyzed using different concentrations of hydrogen peroxide. 20% of H_2O_2 was used as control and it corresponds to an assumption of 5%. The test samples in the urine and chemical media were calculated from the standard graph. The values were found to be 22.97±0.11 and 66.22±0.42 for chemical media and synthetic urine, respectively. The biofilms were found to be more effective in scavenging the hydrogen peroxide when synthetic urine was used, than in the case of chemical media. The values seem to be more promising for the biofilms grown more than 48 h (Fig. 4). This might suggest that as the biofilms mature, their peroxide resistance increases significantly.

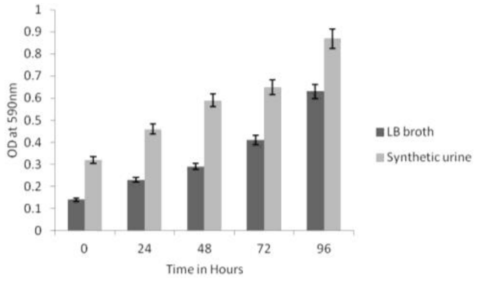

Fig. 3: The primary adherence values of the UPEC samples. All the values were averages of triplicates. The values were represented as OD ± s.e

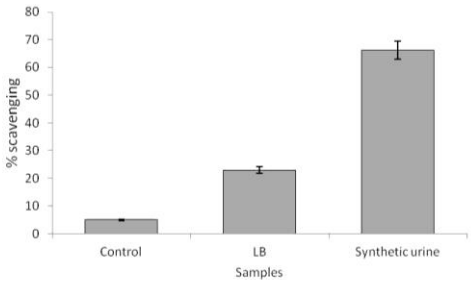

Fig. 4: The percentage of scavenging activity of the strains within the natural media and chemical media. All the values were averages of triplicates

Lipid Hydroperoxide (LPO) Assay

Consistent with the values, the biofilms in synthetic urine showed increased accumulation of total ROS and LPO compared to the media. In particular, the LPO level of the media was found to be more than double that of the synthetic urine sample. This proves the effective function of the UPEC strains in natural media settings. The positive control showed significant activity in Fig. 5. The results suggest that the strain in the natural setting might be involved in the detoxification of LPO. Different peroxide-resistant proteins might be involved in the overall mechanism of protection. Such an effect would be of great help to protect the strains from stress levels. The LPO levels were found to be 14.23±0.43, 70.42±0.23, and 35.21±0.11 for the positive control, media, and urine samples, respectively. This suggests that the strain functioned better in the synthetic urine sample with better protection from the ROS stress.

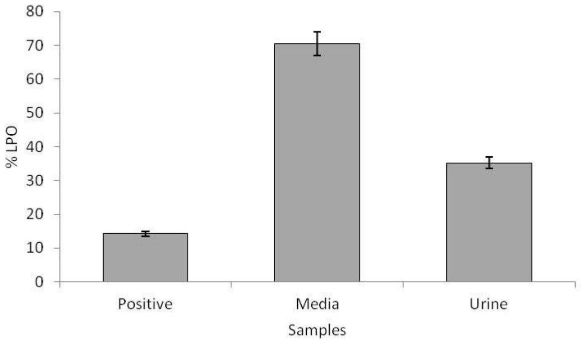

Fig. 5: The values of LPO assay. Values were obtained from the standard graph; (y = 0.0014x, R² = 0.9909). All the values are averages of triplicates

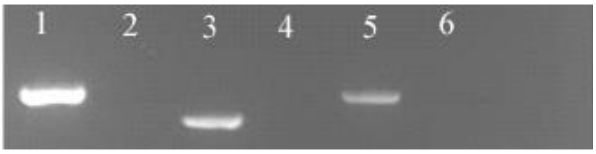

Fig. 6: Image of the agarose gel showing the amplified cDNA by RT PCR. Lane 1: cDNA of biofilm under synthetic urine; Lane 2: Negative control (urine sample); Lane 3: cDNA of Planktonic cells; Lane 4: Negative control; Lane 5: cDNA under media growth; Lane 6: negative control

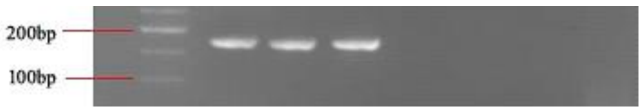

Fig. 7: The PCR amplification of cDNA for the gene AhpC. Lane M: Molecular marker, 100bp ladder; lane1: Biofilm in synthetic urine; Lane 2: Biofilm in media; Lane 3: Planktonic cells

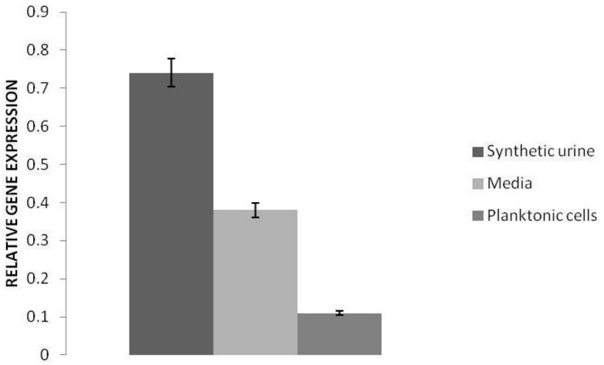

Fig. 8: The relative expression of the AhpC gene in synthetic urine, media, and planktonic cells. All the values are averages of duplicates

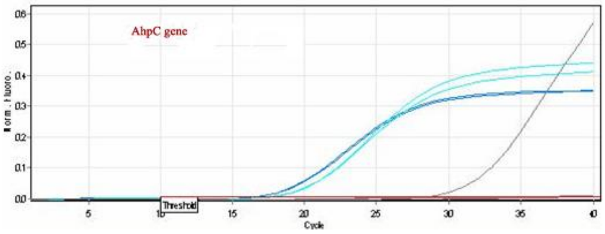

Fig. 9: Melting curve analysis of the samples. The samples were used in duplicates. The light blue line depicts the amplification curve of the biofilm in the synthetic urine sample; dark blue indicates the amplification curve of the biofilm in the media sample. There was no amplification for the planktonic cells

RNA Extraction and Expression of AhpC

The expression of the gene in the case of synthetic urine seems to be higher than in the other three samples. The amplified product was found to be approximately 222 bp. The PCR amplification of the gene showed a positive response. The strain was found positive and the expression level was also found to be more significant. The strains showed positive responses irrespective of the sample. Even the planktonic cells expressed the gene but in lower amounts. This shows the UPEC strains express more of this gene during the biofilm formation. It suggests that the biofilm is a strategy of protection by rendering increased protection towards harsh conditions.

Real-Time Assay

All four samples were studied separately for gene expression. The product size was assumed to be 222 bp. The level of expression seems to be significantly different for each sample. Samples grown in synthetic urine showed increased growth and biofilm formation and responded better to the ROS/ hydrogen peroxide treatment. When grown in the presence of favorable media, the cells showed increased gene expression. The AhpC gene was found to reduce the tension created by the free radicals. This scavenging capacity of the protein makes the bacteria more resistant when exposed to harsh conditions. This shows that AhpC is expressed more in order to scavenge the high level of free radicals as we see in Figs 6-7. The scavenging ability was found to be better for the strain grown in synthetic urine than in chemical media. The planktonic cells also showed a response in traditional PCR, but in RT PCR, the response was considered negligible. In an RT PCR, the ct values over 30 are considered negligible in terms of expression Figs. 8-9. The strains showed expression in synthetic urine 4 times more than those grown in chemical media.

Discussion

The growth of the bacteria was quite high in synthetic urine and so was the antibacterial activity (Sampath Kumar *et al*., 2021). A significant effect of antibacterial activity was seen in both chemical and synthetic urine samples (Sampath Kumar), but the effect was higher when synthetic urine was used (Sampath Kumar *et al*., 2021). The antibacterial activity was found to be dose-dependent. Previous studies also reported the same results with nitrofurantoin. It is used in many complicated urinary tract infections and it was found to exhibit low resistance prevalence (Munoz-Davila, 2014).

According to biofilms were found to be more in synthetic urine media than in chemical media. Our results are in accordance with previously reported data. Studies reported the possible role of H_2O_2 produced by *Streptococcus pneumoniae* to inhibit the growth of other inhabitants along the respiratory tract. *Streptococcus pneumoniae* was found to produce H_2O_2 with cytotoxic effects on the epithelial cells of the host. This was found to provide both resistance as well as virulence (Pericone *et al*., 2000).

As a novelty, our results reported the relation between the scavenging activity and the biofilm formation. The biofilms were found to be more effective in scavenging the hydrogen peroxide in synthetic urine than when using chemical media. The values seem to be more promising for the biofilms grown over 48 h. This might suggest that their peroxide resistance significantly increases as the biofilm matures. Consistent with the values, the biofilms in synthetic urine showed increased accumulation of total ROS and LPO compared to the media. In particular, the LPO level of the media was found to be more than double that of the synthetic urine sample. This proves the effective function of the UPEC strains in natural media settings.

The high gene expression in the case of synthetic urine may prove the possible role of the bacterial infection regarding the urinary tract. The PCR amplification was positive and the level of expression was also found to be significant. For the first time, our results report that UPEC strains express this gene more during the biofilm formation, suggesting the role of biofilms in preventing and protecting against harsh conditions. The strains showed 4 times higher expression in synthetic urine than in chemical media.

Conclusion

Very specific to human urinary tracts, UPEC strains are pathogenic in nature. They harbor the urinary tracts of humans and cause serious ailments that cannot be treated with antibiotics. These strains are gaining resistance to many of the antibiotics in use and are becoming real potential threats to human health. They not only cause serious and severe complications but also generate other serious infections. Various antibiotic treatments are now in use to treat bacterial infections, but these bacteria are rapidly acquiring antibiotic resistance and ROS conditions. Bacteria are capable of releasing enzymes and proteins protecting them in extreme conditions. Moreover, they are able to prevent the adverse conditions caused by ROS. We found the strains were more protective in the presence of oxidative stress/antibiotic therapy, as was evident by our growth assay, biofilm formation, and peroxidize assays. The real-time expression of the gene AhpC also confirmed the possible role of AhpC in deteriorating free radicals. The biofilms grown in synthetic urine are found to form more biofilms and express higher the AhpC gene. Further confirmation needs to be done at protein levels via blotting studies. These results could be of much help in predicting antibiotic susceptibility and deciding the dosing concentrations and dosing intervals of the correct antibiotics.

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Author’s Contributions

Omar Sadik Shalal: The development and publication of this manuscript.

Ani-Simona Sevastre: Preparation and lab techniques.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

Conflict of Interest

The authors declare no conflict of interest.

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Abbreviations

UPEC	Uropathogenic *E. coli*
AhpC	Alkyl hydroperoxide reductase
UTI	Urinary tract infections
ROS	Reactive oxygen species
MTCC	Microbial type cell culture repository
PBS	Phosphate buffered saline
LPO	Lipid hydroperoxide
RT	Reverse transcriptase