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# ROLE OF HIGH-TEMPERATURE PROTEIN G IN THE VIRULENCE AND DEVELOPMENT OF ANTIMICROBIAL RESISTANCE IN SOME ORAL BACTERIAL ISOLATES

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## ABSTRACT

**A marked increase in antimicrobial resistance among common bacterial pathogens has made the search for new therapeutic targets highly imperative. The present study was aimed to analyse the potential of bacterial chaperone high-temperature protein G (HtpG) as a therapeutic target by determining its role in virulence and antibiotic resistance. Two bacterial isolates obtained from the oral cavity of healthy human subjects were characterized and tentatively identified as *Staphylococcus aureus* and *Streptococcus mutans*. Survival assays of these isolates showed induction of thermotolerance with around 24% increase in survival at lethal temperature after preconditioning. Protein profile studies revealed prominent expression of proteins of 99, 77 and 40 kDa in thermo-tolerance group as compared to the control. HtpG inhibition was achieved by using a pharmacological inhibitor of human Hsp90, geldanamycin (GA). In disc diffusion assays, there was no significant inhibitory impact of HtpG on antibiotic susceptibility to six antimicrobial compounds in both the isolates as compared to the control. An 18-19% reduction in biofilm-forming capacity was observed for both isolates after GA treatment. This study lays strong experimental evidence to the involvement of HtpG in the development of anti-microbial resistance through biofilm formation.**

**Keywords:** Antibiotic susceptibility, biofilm, geldanamycin, HtpG, oral isolates, thermotolerance

## INTRODUCTION

The unrestrained use of antimicrobial compounds over past several decades has led to a marked increase in antimicrobial resistance in common bacterial pathogens. The antibiotic resistance is one of the three most important public health threats of 21st century (Lowe, 1982). The antimicrobial resistance involves many different mechanisms; and it is presumed that the switch from planktonic lifestyle to sessile existence within the communities like biofilms is one of the most important factors conferring added protection to the bacteria from antimicrobial agents. It is an established fact that biofilms are much less susceptible to antimicrobial compounds than their genetically identical planktonic counterparts (Grudniak *et al*., 2018).

The formation of biofilms along gingival margins is one of the major pathogenic strategies exercised by the oral microorganisms involved in periodontal diseases (Chen *et al*., 2020). However, planktonic forms of oral bacterial strains also exhibit antibiotic resistance acquired through other molecular and biochemical mechanisms. As the advancement of disease is a combined effect of

multiple pathogenic microorganisms residing in the community, antibiotics designed against specific target microbes are not effective. Instead, the factors contributing to the community virulence and resistance would serve as effective therapeutic targets. Increasing understanding of genetic and biochemical basis of antimicrobial resistance has speculated the involvement of a group of protein-folding helper proteins or chaperones not only in virulence mechanisms but also in the development of antimicrobial resistance in bacterial pathogens (Chiappori *et al*., 2015).

Most pathogens encounter drastic changes in their environment on host entry. To cope with these changes, the pathogens elaborate the stress response that involves the induction/ overexpression of stress proteins or chaperones. The central role of chaperones is protein folding, thus prevents and clears the stress-induced aggregation (Neckers and Tatu, 2008). There are reports of different bacterial chaperones being involved in outer membrane protein biogenesis (Klein *et al.,* 2019), cell growth (Kumar and Balbach, 2017) stage transformation, and biofilm formation (Alam *et al.*, 2021) of pathogens. Bacterial chaperones can stabilize mutations in drug targets leading to the development of antimicrobial resistance (Fay *et al*., 2021). Inhibitors of bacterial Hsp70 chaperone reportedly reduce resistance to frontline antibiotics and disrupt the survival mechanisms (Hosfelt *et al*., 2021). Inhibition of chaperonin sensitize bacteria to aminoglycoside antibiotics (Goltermann *et al*., 2013).

Hsp90 or high-temperature protein G (HtpG) is the most commonly implicated chaperone in disease progression and pathogenesis of microbial infections. Hsp90 is involved in the development of drug resistance in *Leishmania* and fungi (Neckers and Tatu, 2008). Mutation in HtpG led to the compromises in motility, adhesion, biofilm formation, invasion, and inflammation induction of *S. typhimurium* (Dong *et al*., 2021). In *P. aeruginosa*, HtpG is reported to be involved in many physiological processes that are important for virulence like motility, biofilm formation, proteolytic activity, and pigment and biosurfactant production (Grudniak *et al*., 2018). HtpG reportedly is required for the production of genotoxin and siderophore in *E. coli* (Garcie *et al*., 2016). However, the literature related to the mechanisms of different molecular chaperones in various pathogenic bacteria is limited. More information needs to be generated to substantiate the recognition of molecular chaperones as promising antimicrobial targets. The present study was aimed to assess the involvement of HtpG in virulence-related activities of two bacterial isolates obtained from oral cavity. By causing functional inhibition of HtpG through use of eukaryotic hsp90 inhibitor, geldanamycin (GA), we analysed its role in biofilm formation. Also, to assess whether HtpG contributed to the development of drug resistance by other mechanisms, the effect of HtpG inhibition on antibiotic susceptibility was analysed.

## MATERIALS AND METHODS

All the chemicals used were of analytical or ultrapure grade. The media/reagents were purchased from HiMedia, India; biochemicals from SRL Pvt. Ltd. and geldanamycin from Sigma Aldrich. Molecular weight protein markers (35-205 kDa) were purchased from GeNei Laboratories Pvt. Ltd. The samples were collected from the oral cavity of healthy individuals by swabbing across the gingival and subgingival surfaces using sterile cotton swabs. The samples were initially inoculated in nutrient broth (NB) (HiMedia) and incubated for 48 h at 37°C. Diluted cultures were then plated on nutrient agar (NA). Visibly distinct colonies were selected and their pure cultures raised by frequent streak sub-culturing (Chen *et al*., 2020).

### *Screening for biofilm formation*

Luria Bertani (LB) broth (1 mL) was inoculated with 100 μL of overnight culture broth and incubated at 37°C for 72 h. The tubes were decanted and PBS (pH 7.3) was used to wash the test tubes before drying. Dried tubes were stained with 0.1% (w/v) crystal violet for 10 min. To remove the excessive stain, the test tubes were washed with deionized water. The formation of biofilm was confirmed by

the presence of visible stained film on the walls and bottom of the tube (Jain *et al*., 2013). Two isolates were observed to be positive for biofilm formation, named as 'isolate 1' and 'isolate 2', and selected for further study.

***Characterization of selected isolates***

Colony characteristics of isolates including size, shape, surface, elevation, edge, and colour were noted. The Gram reaction was determined and the morphology of isolates studied with the help of a compound microscope (Magnaster SM 100). A set of biochemical tests were performed for catalase, bile solubility, coagulase, blood hemolysis, $H_2S$ production, oxidase, citrate, and pigment production (lghamdi, 2022). The tentative identification of the isolates was done based on the categorization described in the *Bergey's Manual of Determinative Bacteriology* (Bergey and Holt, 2000).

***Thermo-tolerance and survival assays***

Initially, the growth curve study over a broad range of temperature was carried out to assess the lethal and sub-lethal temperatures for the two isolates. Thermotolerance and survival assays were designed based on these temperatures. Five groups were made for each isolate in thermotolerance studies. The 1st group was control (incubation at 37ºC). The 2nd group was placed directly at lethal temperature (60ºC for isolate 1, and 70ºC for isolate 2). The 3rd group was pre-treated at sublethal temperature for 15 min (55ºC for isolate 1, and 65ºC for isolate 2) before subjecting to lethal temperature treatment for 20 min. The 4th group (preconditioned) was pre-treated at sublethal temperature for 15 min and allowed to recover at 37ºC for 1 h; and the 5th group was pre-treated at sublethal temperature for 15 min and allowed to recover at 37ºC for 2 h before subjecting to lethal temperature treatment for 20 min. For each group, both the isolates were grown aerobically at 37°C to an $OD_{600}$ of 0.5 in LB broth. One mL of each culture was then pipetted out in Eppendorf tubes, treated as per the group experimental design, and then placed directly at respective lethal temperatures for varied time intervals (0-20 min) as per treatment. After each time interval, the tubes were withdrawn and cfu $mL^{-1}$ determined by serial dilution and plating on LB agar plates. The survival was calculated as the ratio of cfu $mL^{-1}$ after treatment to the cfu $mL^{-1}$ at zero time (Runde *et al*., 2014).

***Protein profiling by SDS-PAGE***

For protein profiling, the soluble protein extracts were obtained from group 1 (isolates placed at 37°C), group 2 (isolates placed directly at lethal temperatures for 20 min) and group 3 (isolates pretreated at sublethal temperature for 15 min and allowed to recover at 37°C for 1 h before subjecting to lethal temperature for 20 min - preconditioned). For preparation of protein extracts, bacterial cells were harvested after heat treatment and lysed in supplemented buffer A (50 mM 150 mM NaCl, pH 8 supplemented with 0.33 mg $mL^{-1}$ lysozyme, 20 µg $mL^{-1}$ DNase I, 20 µg $mL^{-1}$ RNase I, 1 mM PMSF and 1% triton X-100) by shaking vigorously at 750 rpm, 90 min, 37°C followed by first centrifugation at 3000 rpm (2 min, 4°C) and second centrifugation at 12000 rpm (30 min, 4°C). The protein content of extract was estimated by Lowry's method (Lowry *et al.*, 1951); and 500 ng protein was loaded in each well of 12% SDS polyacrylamide gels in standard tris/ glycine tank buffer (0.025 M tris. HCl, 0.129 M glycine, 0.1% SDS). Electrophoresis was carried out at 100 V in mini-protean II apparatus (Bio-Rad) (Laemmli, 1970). Broad range molecular weight markers of 35-205 kDa (Genei Labs Pvt. Ltd.) were used as standard gels and stained with silver nitrate 12 mM (Urban-Chmiel *et al*., 2013). The protein profiles of the three groups were compared for each isolate.

***Quantitative biofilm formation assay***

For studying the inhibitory effect of HtpG on biofilm forming capacity in each isolate, the treatment leading to maximum thermotolerance (preconditioning - pretreatment at sublethal temperature followed by recovery at 37°C for 1 h) was carried out in presence of HtpG inhibitor geldanamycin [5 µM dissolved in dimethylsulphoxide (DMSO)]. This was labelled as group 3. Group 1 was control; group 2 was preconditioned and group 4 was preconditioned in presence of DMSO only. Biofilm formation was evaluated quantitatively by crystal violet staining method (Christensen *et al.*, 1982).

The cultures were grown overnight on a shaking incubator at 37°C and diluted at 1:100 in a fresh medium. The aliquots (250 µL) of cultures were dispensed into the wells of microtiter plates and incubated for 24 h under static conditions (covered and incubated at 37ºC without shaking). After incubation, the planktonic cells were discarded, and the wells were washed with PBS (pH, 7.3) and allowed to dry. Dried wells were stained with 0.1% (w/v) crystal violet for 10 min, again washed in deionized water and allowed to dry. Biofilm-bound crystal violet was then extracted by adding 20% glacial acetic acid and incubated for 10 min. The contents of wells were read at 570 nm (Jain *et al*., 2013; Subramenium *et al*., 2015).

***Antibiotic susceptibility test***

The same four groups, as taken in biofilm formation assays, were used in the antibiotic susceptibility tests. The antibiotic susceptibility of isolates to six antibiotics was determined by Kirby-Bauer disc diffusion method (Bauer *et al*., 1966). The 100 µL inoculum ($1.5 \times 10^8$ cfu $mL^{-1}$) was spread evenly on Mueller-Hinton agar plates. Antibiotic discs, placed on the plates, were incubated at 37°C for 24 h. The diameters of inhibition zones were measured after 24 h. The experiments were performed in triplicate for each antibiotic. The results were interpreted as per CLSI recommendations (Salman and Senthikumar, 2015).

***Statistical analysis***

All the above experiments were conducted in a completely randomized design. The data generated were expressed as mean ± SD of three replicates. For data analysis, GraphPad Prism 8 software (*www.graphpadprism.com*) was used. An unpaired t-test was used to assess the possible differences among the means; and p values <0.05 was considered as significant (Grudniak *et al*., 2018).

## RESULTS AND DISCUSSION

Biofilms are recognized as virulence factors and the biofilm formation progresses in three steps *viz*., attachment, biofilm maturation, and dispersal. The strategies that disrupt the any stage of biofilm formation are considered valuable in controlling the infections (Jiao *et al*., 2019). Therefore, the present study was primarily focused on assessing this capability and screen the isolates obtained from the oral cavity for biofilm formation. The colony morphology of two isolates revealed that isolate 1 was Gram-positive coccus forming yellow coloured colony of 0.1-2.0 mm size, having smooth surface, concave elevation and entire edge. The isolate 2 was Gram-positive coccus forming white coloured colony of 1.0-3.0 mm size having rough surface, concave elevation and irregular edge. The biochemical characterization revealed that the isolate 1 was pigment producing, positive for catalase, coagulase, and citrate, but negative for $H_2S$ production, oxidase and bile solubility tests; and showed β-hemolysis. The isolate 2 was non-pigmented, positive for oxidase and negative for catalase, $H_2S$ production, citrate and coagulase while bile solubility tests showed α-hemolysis. Based on the above characteristics the isolate 1 was tentatively identified as *Staphylococcus* sp. and being coagulase-positive the isolate could possibly be *Staphylococcus aureus*. The isolate 2 was tentatively identified as *Streptococcus* spp. and since it showed negative bile solubility, the isolate could probably be *Streptococcus mutans*. In oral microflora, α-hemolytic *Streptococci* are most frequently reported isolates, and among other bacteria, the coagulase-negative *Staphylococci* are commonly isolated (Sweeny *et al*., 2004).

***Thermotolerance, survival assay and protein profiling***

The percent survival after a pre-treatment at 55°C (for *Staphylococcus aureus*) and 65°C (for *Streptococcus mutans*), followed by 0, 1 and 2 h recovery at 37°C is given in Fig. 1. A 24% higher survival in *S. aureus* and 16% higher survival in *S. mutans* was observed following pre-conditioning

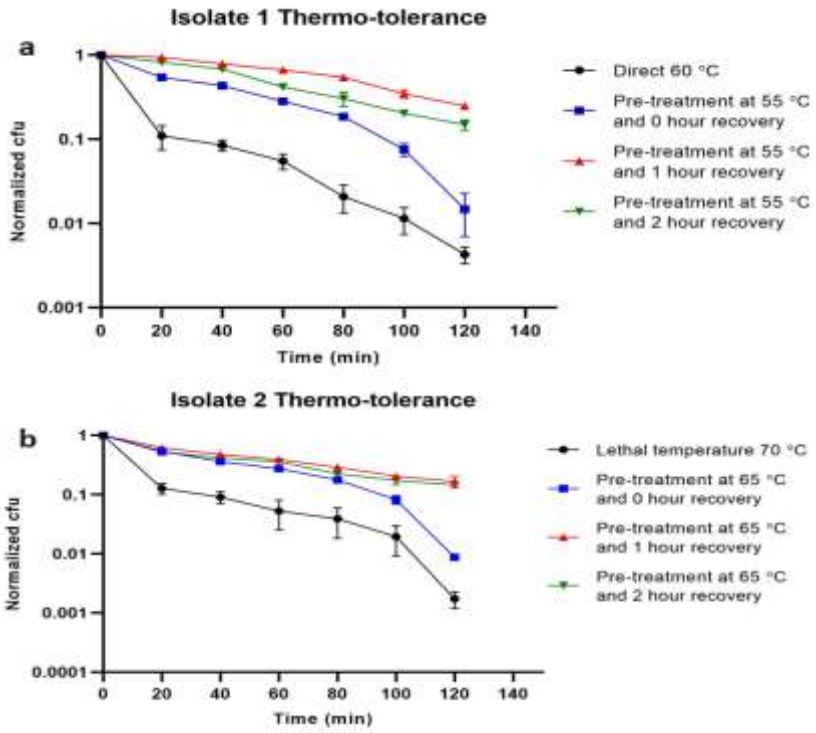


**Fig. 1: Survival of *Staphylococcus aureus*, isolate 1 (a); and *Streptococcus mutans*, isolate 2 (b) in thermo-tolerance experiments. Data expressed as normalized CFU values at each treatment. Each plotted point represents three trials expressed as mean ± SD**

at sublethal temperature and 1 h recovery at 37°C. The improved survival indicated the acquired thermotolerance. Heat-induced thermo-tolerance has been reported in many bacteria like *Bacillus cereus* (Periago *et al*., 2002) and *Lactobacillus bulgaricus* (Gouesbet *et al*., 2001). The strong experimental evidences suggest the involvement of a class of proteins called 'chaperones' in the development of acquired thermo-tolerance (Maleki *et al*., 2016). The expression of heat shock proteins (hsps) under sublethal heat stress increased heat resistance of *E. coli* and were the key contributors to the development of thermotolerance (Li and Gänzle, 2016). Earlier the over-expression of proteins with molecular masses corresponding to the major heat shock proteins in thermotolerance strains of *E. coli* have been observed by Patki and Shah (2017).

The protein profiling results in present study exhibited altered protein milieu in the soluble fraction of cell extracts (Fig. 2a,b). Bands corresponding to the molecular weights of 99, 77 and 40 kDa

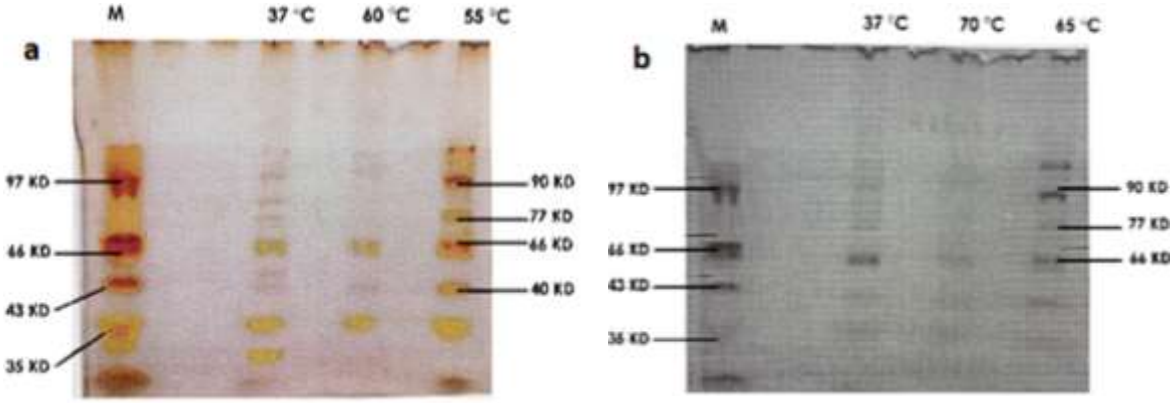


**Fig. 2: SDS-PAGE protein profiles of isolate 1 (*Staphylococcus aureus*) and isolate 2 (*Streptococcus mutans*). The 12% gels were stained with silver stain. Molecular weight marker (35-205 kDa) [M] and soluble protein fractions were loaded. a) shows protein profile of isolate 1 cultured at 37°C, treated directly to lethal temperature of 65°C and pre-treated at sublethal temperature of 55°C followed by 1 h recovery at 37°C. b) shows protein profile of isolate 2 cultured at 37°C, treated directly to lethal temperature of 70°C and pre-treated at sublethal temperature of 65°C followed by 1 h recovery at 37°C.**

showed increased intensity in pre-conditioned group for both the isolates. The bacterial homolog of Hsp90, HtpG (a molecular chaperone) is known to have a molecular weight around 77-78 kDa. SDS-PAGE profiles suggested the overexpression of HtpG among other chaperones.

***Quantitative biofilm formation***

*S. mutans* is known to colonize the oral cavity through mechanisms like adhesion to solid surfaces, acid production, and interaction with other bacterial species; thus leading to biofilm formation (Krzyściak *et al*., 2014). Similarly, *S. aureus* exhibits a wide range of virulence factors like production of degradative enzymes, antigenic toxin production, hemolysin production, and biofilm formation. These are reported to play a vital role in the development of periodontal diseases (Kim and Lee, 2015).

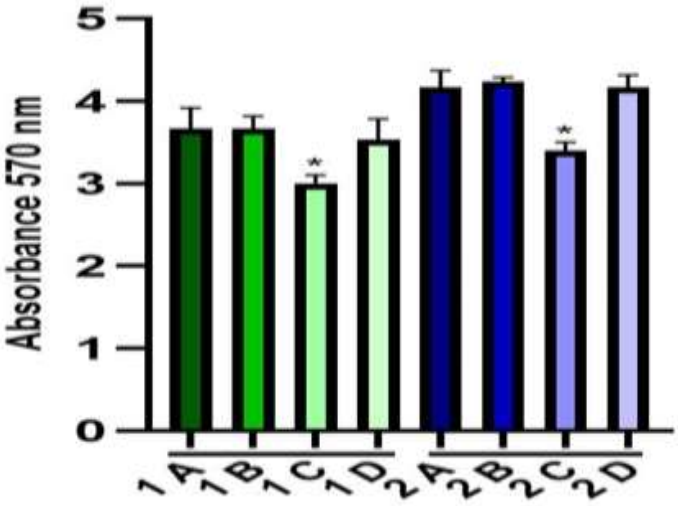


**Fig. 3: Quantification of biofilm formation by isolate 1 (*Staphylococcus aureus*) and isolate 2 (*Streptococcus mutans*) after 24 h incubation. A) Control (cultured at 37°C), B) Preconditioned (at sublethal temperatures followed by 1 h recovery at 37°C), C) Preconditioned (at sublethal temperatures followed by 1 h recovery at 37°C and geldanamycin treatment), D) Preconditioned (at sublethal temperatures followed by 1 h recovery at 37°C and DMSO treatment). Each plot point represents the mean of three independent experimental values expressed as mean ± SD; *means $p<0.05$.**

In present study, biofilm formation was observed in control group (1A and 2A) of both the isolates supporting their crude identity (Fig. 3). Pre-conditioned thermotolerant group (1B and 2B) also showed biofilm formation comparable with control. While DMSO treatment did not affect the biofilm formation (1D and 2D), a 19% (1C) and 18.26% (2C) reduction in biofilm formation in GA-treated groups was observed in isolates 1 and 2, respectively. Similar down-regulation of DnaK and GroEl, two other bacterial chaperones, caused a reduction in biofilm formation in *S. mutans* (Dong *et al*., 2021). *S. aureus* is also a major bacterium found in oral infections through biofilm formation. Arita-Morioka *et al*. (2015) have reported that the molecular chaperone Dnak is indispensable for biofilm formation in *E. coli* and *S. aureus* including the methicillin-resistant strain. HtpG has also been implicated in various studies to be involved in physiological activities related to biofilm formation (Grudniak *et al*., 2018; Dong *et al*., 2021).

Biofilm in the form of supragingival plaques is an etiologic agent in dental caries and periodontal diseases. Bacteria within biofilms exhibit a tremendous amount of drug resistance through mechanisms like reduced antibiotic penetrance, drug efflux, quorum sensing, horizontal gene transfer of antibiotic resistance genes due to genetic diversity and the oral cavity provides an excellent environment for microbial colonization (Rath *et al*., 2021). *S. mutans* is widely recognized as the main cariogenic bacterium in oral biofilms. Antimicrobials aimed at interrupting EPS synthesis of *S mutans* are promising approaches in controlling oral biofilms (Kuang *et al*., 2018).

***Antibiotic susceptibility of isolates***

The disc diffusion assays showed a negligible effect of HtpG inhibition on the susceptibility of the test isolates to common antibiotics (Table 1). The pre-conditioned isolates also did not show significant decrease in susceptibility as compared to the control. Antibiotic resistance in oral microflora is recognized as a global problem that increases the cost of dental healthcare and limits

**Table 1: Antibiotic susceptibility of *Staphylococcus aureus* and *Streptococcus mutans* against six commercial antibiotics by Kirby-Bauer disc diffusion method**

| Antibiotic (concentration) | Control | Pre-conditioned | Pre-conditioned + geldanamycin (5 µM) | Pre-conditioned + DMSO |
|---|---|---|---|---|
| | Zone of inhibition (cm) | | | |
| *Staphylococcus aureus* | | | | |
| Streptomycin (10 µg) | 2.93 ± 0.15 | 3.10 ± 0.10 | 2.90 ± 0.10 | 3.46 ± 0.20 |
| Erythromycin (15 µg) | 3.03 ± 0.05 | 2.80 ± 0.10 | 3.46 ± 0.05 | 3.00 ± 0.10 |
| Ampicillin (10 µg) | 1.50 ± 0.20 | 1.43 ± 0.15 | 1.93 ± 0.05 | 1.52 ± 0.34 |
| Amoxicillin (10 µg) | 1.56 ± 0.11 | 1.53 ± 0.15 | 1.50 ± 0.10 | 1.43 ± 0.05 |
| Cefoperazone (75 µg) | 2.30 ± 0.30 | 2.13 ± 0.15 | 2.33 ± 0.15 | 2.30 ± 0.10 |
| Cefotaxime (30 µg) | 2.13 ± 0.15 | 1.86 ± 0.15 | 2.43 ± 0.25 | 2.06 ± 0.20 |
| *Streptococcus mutans* | | | | |
| Streptomycin (10 µg) | 2.93 ± 0.15 | 3.10 ± 0.10 | 2.90 ± 0.10 | 3.46 ± 0.20 |
| Erythromycin (15 µg) | 3.03 ± 0.05 | 2.80 ± 0.10 | 3.46 ± 0.05 | 3.00 ± 0.10 |
| Ampicillin (10 µg) | 1.50 ± 0.20 | 1.43 ± 0.15 | 1.93 ± 0.05 | 1.52 ± 0.34 |
| Amoxicillin (10 µg) | 1.56 ± 0.11 | 1.53 ± 0.15 | 1.50 ± 0.10 | 1.43 ± 0.05 |
| Cefoperazone (75 µg) | 2.30 ± 0.30 | 2.13 ± 0.15 | 2.33 ±0.15 | 2.30 ± 0.10 |
| Cefotaxime (30 µg) | 2.13 ± 0.15 | 1.86 ± 0.15 | 2.43 ± 0.25 | 2.06 ± 0.20 |

Each value in the table represents the mean of three different readings ± SD

the effectiveness of treatment. The α-hemolytic S*treptococci* and Gram-negative *Prevotella* reportedly show resistance to a range of antibiotics frequently prescribed in dentistry (Sweeny *et al*., 2004). Although biofilm formation contributes to antimicrobial resistance, even planktonic bacteria are known to exhibit notable resistance through mechanisms like drug target mutation, acquisition or enhanced expression of drug efflux pumps or stabilization of outer membrane, and low permeability through phospholipid or membrane protein modifications (Wang *et al*., 2018). Drug resistance in *P. aeruginosa* is related to the outer membrane stabilization through the assembly of membrane proteins and depletion of a chaperone SurA, important for outer membrane integrity, leads to increased antibiotic resistance (Klein *et al*., 2019). The GroEL/ GroES chaperonin system is responsible for the development of aminoglycoside antibiotic resistance in *E. coli* (Goltermann *et al*., 2013). Persistence is another important mechanism shared between planktonic and biofilm forms of bacteria which is a transient state of tolerance in the presence of an antibiotic (Banin *et al*., 2017). HtpG reportedly contributes to the intestinal persistence of *Salmonella typhimurium* in pigs (Verbrugghe *et al*., 2015). In present study, we tried to throw some light on the effect of HtpG inhibition on the susceptibility of test isolates to known antibiotics. A negligible effect of HtpG inhibition was observed on the susceptibility of test isolates to common antibiotics **(**Table 2). This indicates poor involvement of chaperone in the mechanisms of drug resistance by planktonic strains. Despite the conservation in structure and mechanism there are notable differences between the eukaryotic and prokaryotic orthologues (Buchner, 2010), these may assume completely different conformations during nucleotide-binding and client protein binding. Therefore, inhibitors of Hsp90 may not successfully inhibit or block all the prokaryotic chaperone interactions. The absence of any obvious effect of HtpG inhibition on antibiotic susceptibility in present study may also be the result of limited inhibitor function.

**Conclusion:** Microbial pathogens withstand stressful host cell environments by strategic use of molecular chaperones. Chaperone involvement in microbial virulence and development of antibiotic resistance have spotlighted them as novel and promising targets in infectious diseases. The study showed that inhibition of HtpG by GA drastically reduced the capacity of bacterial isolates to form biofilms. As existence in biofilms confers protection to the cells from antibiotics, it is a major contributor to the development of antibiotic resistance. At the same time, HtpG showed very little

influence on other mechanisms of drug resistance in planktonic cells of the same isolates. The combined involvement in biofilm formation and other mechanisms of bacterial virulence as reported in previous studies marks HtpG as a target worthy of consideration in antimicrobial therapeutics. The structural differences between prokaryotic and eukaryotic orthologues of Hsp90 need to be exploited to develop more specific microbial inhibitors.

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**Author's contribution:** Acquisition and recording of data, design of study, data analysis, review of the draft of manuscript and approval to the version submitted by Shruti Purohit. Conceptualization and design of study, analysis and interpretation of data, writing the manuscript and approval of the version submitted by Jyoti Patki

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**Conflict of interest:** The authors have no competing interests relevant to this manuscript.

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