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Original Article

Effect of the combined use of Esomeprazole and Imipenem against *Pseudomonas aeruginosa*

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Antimicrobial activity of esomeprazole (0.25 mM) and imipenem (30 µg/ml) was tested independently and in combination against planktonic and biofilm associated cells of *Pseudomonas aeruginosa*. Esomeprazole (0.25 mM) and imipenem (30 µg/ml) were added to broth media in test tubes to determine their effect on growth of *Pseudomonas aeruginosa* at 2, 4, 6, 24 and 72 hours after inoculation at 37°C. Viable bacterial counts (cfu/ml) were measured by dilution and plating on nutrient agar medium and the biomass was measured at 600 nm using a spectrophotometer. Biomasses of bacterial biofilms were determined by tube adherence assay and measuring the absorbance at 580 nm wavelength. *Pseudomonas aeruginosa* showed decreased growth and biomass of sessile bacteria ($p<0.05$) after exposure to imipenem and esomeprazole compared to untreated controls after 48 and 72 hours of exposureat 37°C. However, the reduction in viable cells after exposure to esomeprazole was not significant after 48 and 72 hours of incubation at 37°C ($p>0.05$). Biomass of bacterial biofilms were significantly reduced after exposure to esomeprazole and imipenem compared to untreated controls ($p<0.05$) as determined by tube adherence assay. Esomeprazole and imipenem demonstrated antibacterial and anti-biofilm property against planktonic and biofilm embedded cells of *P. aeruginosa* which may be useful for treatment of bacterial infections.

Key words: Antimicrobial activity, Biofilm, Biomass, Bacterial infection

Introduction

Biofilms are commonly found in natural, industrial and clinical environments and demonstrate higher level of antimicrobial resistance which contributes mostly to the etiology of infectious diseases[1, 2]. Initially bacteria grow freely as planktonic cells which subsequently attach to biotic and abiotic surfaces. Persistence of bacteria within biofilms has been shown to be critical in many chronic infections like cystic fibrosis[3]. Growth of pathogenic bacteria within biofilms is now reported to cause more than 60% of all human bacterial infections[4].

Pseudomonas aeruginosa has been considered as one of the most common pathogens in microbiology. It is more prevalent in burn patients, patients with otitis externa and in immunocompromised patients. One of the most important characteristics of this organism is it can produce biofilm on catheter and other medical devices. Thus it can cause nosocomial and community acquired infections which are important in internal medicine as well as in public health.

Proton pump inhibitors (PPIs) have been shown to have antimicrobial properties against *Helicobacter pylori* and *Streptococcus mutans* in previous studies[5, 6]. In a previous *in vitro* study, addition of omeprazole and lansoprazole showed antimicrobial activity against *Streptococcus mutans* biofilms, a common pathogen of human oral flora. However, antibiofilm and antimicrobial effect of PPI on other pathogens need to be studied.

Imipenem is considered as a standard antibiotic against *P. aeruginosa* which is used in intravenous route[7]. As the number of peptic ulcer patients is on the rise, some researchers are thinking how proton pump inhibitors can also be used as an antimicrobial agent against pathogens. Thus the treatment cost of the patients could be lower if PPIs and antibiotics are used in combination rather than if new treatment agents need to be discovered. In a previous study PPIs showed *in vitro* antimicrobial activity of Tigecycline against some common clinical pathogen like *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecalis*, and three species of *Enterobacteriaceae* (*Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae*[8]. Combination therapy of rifampin with piperacillin was reported effective against biofilm embedded *P. aeruginosa* infection[9]. The objective of this study was to investigate the effect of esomeprazole in preventing the formation of biofilm in pathogenic bacteria. Whether the addition of esomeprazole could enhance the *in vitro* activity of imipenem against planktonic cells and biofilm-embeded *P. aeruginos* was also investigated.

Materials and Methods

Bacterial strains and culture conditions

Laboratory isolated strain of *Pseudomonas aeruginosa* was used in this study. The isolate was stored at -20°C in Trypticase Soy Broth containing 20% glycerol. Fresh isolates were subcultured twice in Brain heart infusion broth (BHIB, Himedia Laboratories Ltd., India) for 24 hours at 35°C prior to each investigation. An

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inoculum was prepared from an overnight culture and diluted accordingly based on the absorbance at 600 nm wavelength and quantitated subsequently by dilution plate method on Mueller Hinton agar (Himedia Laboratories Ltd., India) plates. Colony counts were obtained from plates showing countable number of colonies (30-300 per plate).

Antimicrobial agents and proton pump inhibitors

A stock solution of esomeprazole (1.0 M) was prepared in Normal saline (0.9%) solution and added to Mueller Hinton Broth (MHB) media accordingly to have a final concentration of 0.25 mM. A stock solution of imipenem (3.0 mg/ml) was prepared in sterile distilled water and diluted in broth media to constitute a final concentration of 30.0µg/ml.

Tube adherence assay for determination of antimicrobial activity

Biofilms were prepared using commercially available borosilicate test tubes containing Mueller Hinton broth and 10^6 cfu/ml *P. aeruginosa*. Inoculated tubes were incubated at 37°C for 72 hours to determine antimicrobial and anti-biofilm activity of esomeprazole and imipenem on sessile and biofilm associated cells. Test tubes were inoculated with esomeprazole (E), imipenem (I), esomeprazole+imipenem (E+I). Tube containing only *P. aeruginosa* without any inhibitory compound was considered as control (C).

Inhibition of planktonic cells by esomeprazole and imipenem

Test tube containing broth media and inhibitors were incubated at 37°C for 72 hours. Broth samples were taken out at 2, 4, 6, 24, 48 and 72 hours interval and diluted (1:10) in sterile normal saline and drop plated on Mueller Hinton agar plates. Plates were incubated at 37°C for 18-24 hours. Plates showing 30-300 colonies were counted to calculate the number of viable bacteria per ml at different time interval in different interventions. Optical density of the cell growth was measured at 600 nm wavelength to determine the change in the biomass.

Inhibition of biofilms by PPI and imipenem

Tubes containing broth media with esomeprazole (E), imipenem (I), esomeprazole+imipenem (E+I) were incubated at 37°C for 48 hours. A tube containing only *P. aeruginosa* was used as control (C). Broth media was decanted from the tube after 48 hours of incubation. Biofilm formed interior of the test tubes were washed 3 times using 2 ml of phosphate buffered saline. The layer of biofilm lining the test tube was stained using 2ml crystal violet solution (0.10%, w/v) for 30 minutes. Crystal violet solution was poured out of the tube and washed for 3 times using 2 ml normal saline (0.90% NaCl, w/v). Stained cells in the biofilms formed in test tubes were destained with 95% ethanol (v/v). The absorbance of the destained ethanol was measured at 580 nm wavelength to determine the amounts of biomass in biofilms[10].

Microscopic analysis

Pseudomonas aeruginosa was grown in a beaker containing 20ml of MHB with different inhibitors and a coverslip was submerged in the broth to provide an artificial surface for the formation of biofilm. After 48 hours the glass cover slip was removed and the biofilm layer was washed 3 times with phosphate buffered saline and stained with 0.1% (w/v) crystal violet solution of 30 minutes. Excess stain was removed and washed with PBS. Cover slips at different interventions were then visualized at 40× magnification under bright field microscope.

Statistical analysis

Statistical analysis was carried out using online software (www.socialscistatistics.com). The growth of bacterial isolates (cfu/ml) and spectrophotometer results (OD_{600}) over time were compared between isolates exposed or not exposed to interventions using two tailed t-test for 2 dependent means. A p value <0.05 was considered significant.

Results

Esomeprazole-treated bacteria demonstrated decreased sessile bacterial growth and biomass

The biomass of *P. aeruginosa* was significantly reduced after exposure to esomeprazole compared to the untreated control after 48 and 72 hours of incubation at 37°C ($p<0.05$) (Fig 1A). *Pseudomonas aeruginosa* showed decreased sessile bacterial growth after exposed to esomeprazole as compared to the untreated control (Fig 1B). After 72 hours of exposure, the mean value of $\log_{10}$CFU/ml in control tube was 7.85 CFU/ml, compared to the 7.27 CFU/ml in esomeprazole-exposed strains. However,

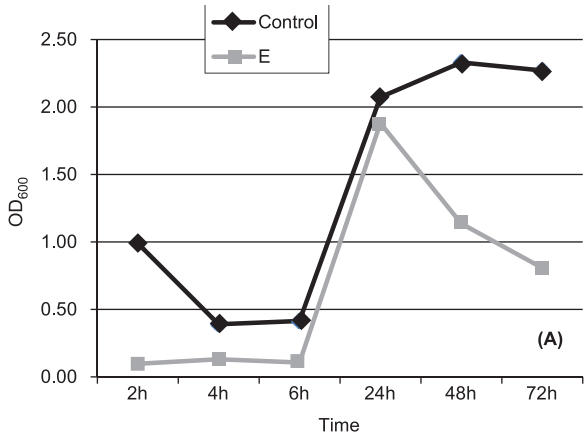

Figure 1A. *Effect of Esomeprazole (E) on biomass of Pseudomonas aeruginosa*

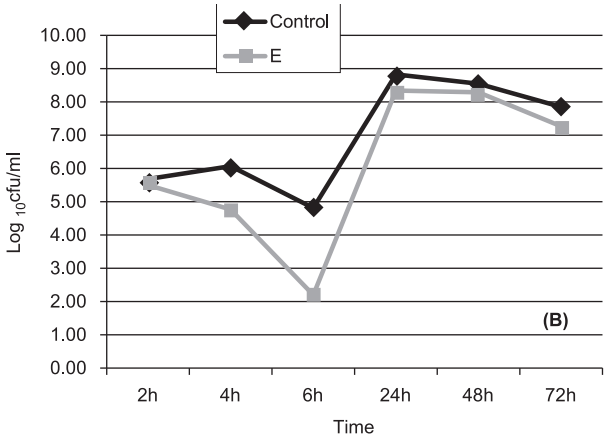

Figure 1B. *Effect of Esomeprazole (E) on growth of Pseudomonas aeruginosa*

the reduction in CFU was not significant compared to the control after 48 h and 72 h of incubation at 37°C ($p>0.05$).

Increased killing of sessile bacteria and decreased biomass were observed in bacteria given imipenem alone and with esomeprazole.

Decreased biomass formation were observed for esomeprazole-treated bacteria with imipenem and antibiotic alone compared to untreated controls (Figure 2A, 3A) ($p<0.05$). There was a significant decrease in sessile bacterial growth when they were exposed to standard antibiotic and adjunctive therapy (Fig 2B, 3B) ($p<0.05$).

Decreased biofilms were observed in esomeprazole-treated bacteria with or without antibiotics.

After 48 hours of exposure to standard antibiotic and adjuvant therapy, the amount of biofilms formed by *P. aeruginosa* in tube adherence assay was found to be significantly lower than the untreated control ($p<0.05$). Absorbance of P. aeruginosa biofilm decreased from 1.54±0.18 in control to 0.53±0.35 in imipenem exposed isolates and from 0.57±0.29 in esomeprazole-treated isolates to 0.34±0.23 in isolates exposed to imipenem and esomeprazole.

Light micrographs of P. aeruginosa *exposed to esomeprazole and imipenem*

Light micrographs of P. aeruginosa cells exposed to esomeprazole and impenem individually and in combination showed reduced biofilm growth compared to the untreated controls (Figure 4).

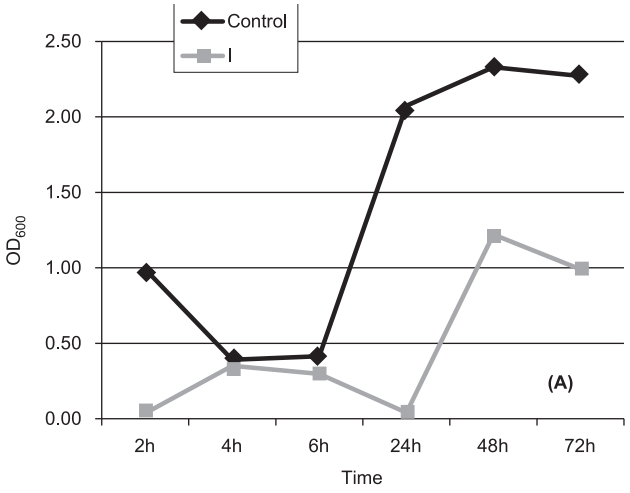

Figure 2A. *Effect of Imipenem (I) on biomass of P. aeruginosa*

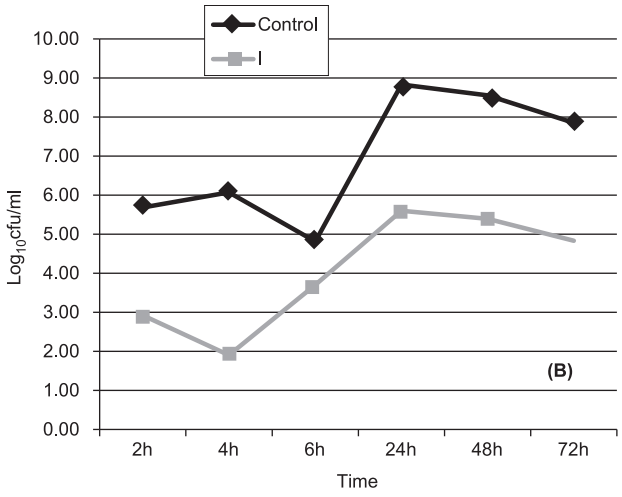

Figure 2B. *Effect of Imipenem (I) on growth of Pseudomonas aeruginosa*

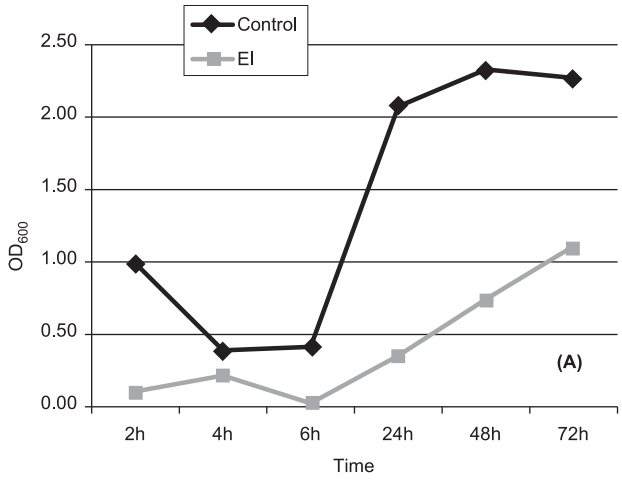

Figure 3A. *Effects of esomeprazole, as adjunctive therapy with standard antibiotics, on biomass for Pseudomonas aeruginosa*

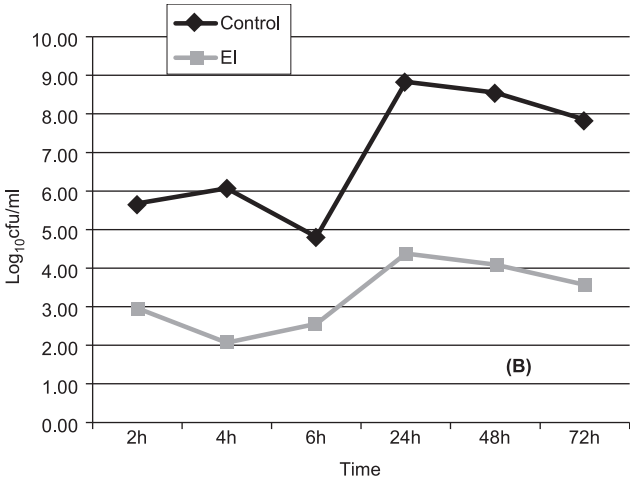

Figure 3B. *Effects of esomeprazole, as adjunctive therapy with standard antibiotics, on viable cells ofPseudomonas aeruginosa*

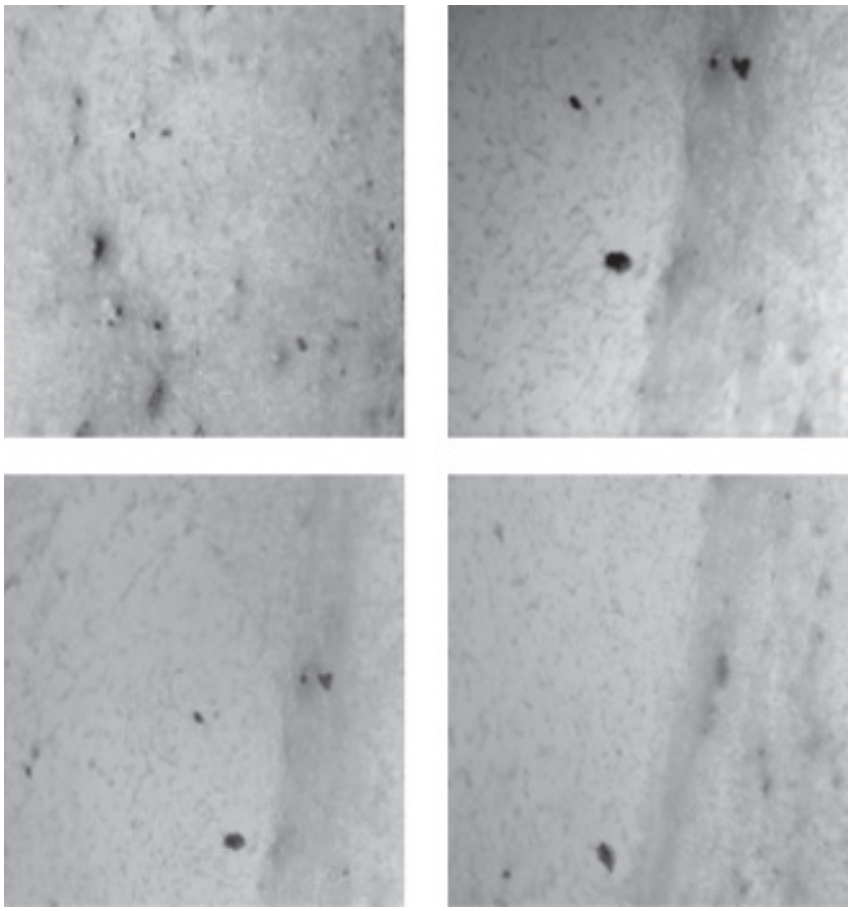

Figure 4. *Light microscopic image of P. aeruginosa biofilm on glass surface*

Discussion

Biofilm producing capacity of pathogenic bacteria is an important threat to health-care associated infections. With the development of modern technology, a number of artificial devices have been introduced in medical treatment such as, antibiotic-impregnated catheters, coated catheters, heart valves which provide artificial surface for the formation of biofilms. Biofilms act as a barrier and make it difficult to treat biofilm-embedded bacteria, as such they display tolerance to antibiotic killing activity and the host immune system[11, 12]. Traditional antibiotics were developed to kill planktonic bacteria and often have a limited effect on the killing of sessile bacteria entrapped within a biofilm. Development of antibiotic resistance in sessile bacteria present in the environment make the situation even more complicated when they form biofilms and complicate treatment procedure. Hence, it is necessary to develop alternative treatment options for treatment and prevention of biofilm-associated infections[13].

Nosocomial infections are very common in hospitals especially with catheters due to production of biofilms by *P. aeruginosa*. Imipenem is a conventional and standard antibiotic which acts directly against *P. aeruginosa*. So, it is expected that the application of this antibiotic will also demonstrate anti-biofilm activity against pathogenic bacteria[10]. Proton pump inhibitor (PPI) such as, esomeprazole will further enhance the efficacy of antibiotics. Esomeprazole is a new drug available in the market which belongs to the Proton Pump Inhibitors. In a previous *in vitro* study, anti biofilm activity of lansoprazole and omeprazole was tested against oral biofilm producing bacteria, *S. mutans*. It was found that lansoprazole at 0.01 mM concentration markedly inhibited biofilm glycolysis[6]. A novel PPI (benzimidazole) showed anti-biofilm activity against multiple pathogens including *S. aureus* and *P. aeruginosa*. Application of benzimidazole as a coating on the surface of catheters significantly reduced biofilm formation in a catheter infection model[6]. Although it is now possible to coat intravascular catheters with antibiotics, there are a number of reports on catheter-associated bloodstream infections[14]. Inhibition of biofilms formation by the commensal gut flora may assist *Clostridium difficile* to penetrate colonic epithelial cell and cause disease. However, this field requires further studies[15]. In another study esomeprazole showed significant reduction ($p<0.05$) in viable cell count and biomass in Calgary biofilm device in microwell plates for *P. aeruginosa* but not for *S. aureus*. In this study growth of planktonic and biofilm associated *P. aeruginsa* was significantly reduced in presence of esomeprazole and imipenem in tube adherence assay. In spite

of the novelty of this study, there are some limitations of this study. Physiologically relevant concentrations of imipenem and esomeprazole were only used in this study. Future dose-response studies need to be conducted for greater killing or a decrease in biomass with different dosing procedures. Further *in vitro/in vivo* studies will be required to confirm these results. This research would be more useful if we could examine on some of the special characteristics of *P. aeruginosa* and see the effects of esomeprazole on quorum sensing and colony formation in adverse environmental condition.

This study has public health importance for control and prevention of nosocomial and community acquired infections by *Pseudomonas aeruginosa*. A follow up research should focus on the novel benefits and toxicities associated with these findings.

Acknowledgement

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Conflict of interest

Authors declare no conflict of interest.

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