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In silico drug repurposing via chemical-protein interaction analysis, a proof-of-concept study: targeting pyocyanin based virulence of antibiotic resistant *Pseudomonas aeruginosa*

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Abstract

World Health Organization has classified *Pseudomonas aeruginosa* as one of top priority threat in terms of the prevailing pandemic scenario of antibiotic-resistant superbugs. This pathogen is widespread in healthcare settings and is listed as one of the top three nosocomial infectious agents. A susceptible population is vulnerable due to lack of vaccine availability to combat this opportunistic pathogen. Pyocyanin (PCN) is considered as a prime virulence factor of *P. aeruginosa* among many others. A wide range of bioactivities have been attributed to this compound primarily based on is redox active nature. We attempted to exploit structural information of PCN in order to screen available drug pool for disrupting or reducing PCN production. In this proof of concept study, PCN molecule's structure was studied for a potential drug hit via studying chemical-protein interaction (CPI). The CPI data was used to identify query-drug interactions. A screening was performed and high probability hits were selected. The predicted targets were tested for inhibition of PCN production. Piperaquine showed remarkable inhibition of PCN biosynthesis. This predicted reported target may provide a basis for the development of a reliable anti-virulence drug against acute and urinary tract infections by *P. aeruginosa*. The approach adopted here could be extended to other bacterial pathogens for potential immunogenic target predictions and ultimately successful drug development.

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Introduction

Pseudomonas aeruginosa is a gram negative ubiquitous environmental bacterium (Hardalo and Edberg, 1997). It is among identified as one of the top three nosocomial infectious agents causing complicated and persistent infections. It has the notorious capabilities such as biofilm formation and antibiotic resistance which made it a significant healthcare challenge (Costerton *et al.*, 1999, Ahearn *et al.*, 1999; Kahrstrom, 2013). Infections associated with *P. aeruginosa* may include bacteremia in burn victims, urinary tract infections, hospital-acquired pneumonia in patients on respirators and opportunistic infections in cystic fibrotic patients are now associated with this pathogen (Bodey *et al.*, 1983, Priebe and Goldberg, 2014). The pathogen has the natural tendency for drug resistance (Breidenstein *et al.*, 2011; Poole, 2011), at the same time we left with a short of conventional effective antibiotic therapeutic options (Livermore, 2009). Emergence of *P. aeruginosa* strains showing greater number of persister cells as sole survivor approach has render the quest of anti-pseudomonas biocidal agents pointless (Mulcahy *et al.*, 2010). Alternative possibilities are also limited including which may silver nanoparticles (Poon and Burd, 2004; Morones *et al.*, 2005; Percival *et al.*, 2005; Atiyeh *et al.*, 2007), along with other metallic nanoparticles (Teitzel and Parsek, 2003), colistin (Denton *et al.*, 2002; Linden *et al.*, 2003; Johansen *et al.*, 2008). However, there are still issues with these approaches. Despite all the clinical significance there is no vaccine readily available in markets against *P. aeruginosa* for preemptive protection (Priebe and Goldberg, 2014).

Pyocyanin (PCN) is considered as most vital of all virulence factors produced by *P. aeruginosa*. It has two benzene rings and a heterocycle in the middle (Byng *et al.*, 1979; Watson *et al.*, 1986; Cole and Taylor, 1986; Gardner, 1996;). It serves as virulence factor during infections; it is toxic to variety of cells ranging from prokaryotic bacteria to eukaryotic fugal, plant animal and mammalian cells even at minute concentrations (Wilson *et al.*, 1988). PCN induces apoptosis in Neutrophils, prevent the phagocytosis of these apoptotic neutrophils by Macrophages, increases the expression of inflammatory cytokines, inhibits ciliary beating of tracheal epithelial lining, depletes NAD (P)H stocks and induces apoptosis in the same. With all these actions this blue pigment tends to increase the survival chances and creates microenvironment inside lungs more favorable for *P. aeruginosa* growth (Denning *et al.*, 1998; Britigan *et al.*, 1999; Muller, 2002; Muller, 2006; Cheluvappa *et al.*, 2008; Winstanley and Fothergill, 2009).

Virulence factors and drug resistance mechanisms cover ultimate research interest in *P. aeruginosa*, the factors and mechanisms by which this bacterium damages human body and how does it manage to escape therapeutic agents. With highest frequency of nosocomial infections *P. aeruginosa* needs to be studied in detail for obtaining knowledge and information for application in medical and pharmaceutical sciences (Behzadnia *et al.*, 2014). Development of anti-virulence or anti-infective drugs would not only help in infection management but will also reduce the evolutionary selective pressure which otherwise has driven antibiotic resistance to current pandemic state (Rasko and Sperandio, 2010).

In this proof of concept study, we adopted a comprehensive computational framework for screening available drug pool for inhibition of PCN production depicted in Fig. 1. This study is focused on the structure-functional relationship of targets and drugs and drugs interacting with drugs within biological systems. The probability of drugs interacting with proteins other than their intended targets creates a window of opportunity to reevaluate and redesign the drug for other additional uses. We subjected PCN to our approach in order to screen out an anti-virulence drug which can be more effective against PCN mediated *P. aeruginosa* pathogenicity. Molecular structure of PCN was studied for potential drug hits via studying chemical-protein interactome (CPI). The CPI data was used to identify query-drug interactions. Screening was performed and high probability hits were selected. The predicted targets were tested for inhibition of PCN production. Piperaquine is reported to exhibit noteworthy inhibition of PCN production.

Materials and methods

PCN chemical and structural information

Chemico-structural information of PCN was obtained from Pubchem (CID:6817) and ChEMBL (CHEMBL2289232) databases (Wang *et al.*, 2009; Bento *et al.*, 2014). PCN structure was built using Canonical SMILES (CN_1C_2=CC=CC=C_2N=C_3C_1=CC=CC_3=O) obtained from Pubchem.

Virtual screening via DDI-CPI Server

PCN structural information was submitted to Drug-drug Interaction via Chemical Protein Interaction (DDI CPI) server in order to identify potential drug targets which may interfere with PCN biosynthesis (Luo *et al.*, 2014). DDI CPI server depending upon query (chemical) probable interaction with the respective protein targets screens potential drug interactions.

These interacting drugs are considered as good hits with possibility of drug repurposing. The server contains a pool of ~12000 drugs and their respective protein targets. Default confidence values (> 0.85) were used for the initial screening step. For screening out less probable hits the confidence threshold was set at > 0.98. Since *P. aeruginosa* has tendency of acquiring antibiotic resistance, it was appropriate to exclude any antibiotics from the screened prioritized hits.

Assessment of anti-PCN activity of the prioritized hits

High scoring drug hits were tested against PCN producing clinical strains of *P. aeruginosa* strains. Dilutions were made in sterile distilled water for each of the drug hits to be tested (Table 1). Spot assay was performed using Cetrimide agar media used for enhanced PCN pigmentation (Brown & Lowbury, 1965). Bacterial lawn were made on media plates using overnight culture. In order to validate the potential anti-PCN activity Acetylsalicylic acid (Prithiviraj *et al.*, 2005) and Ibuperfen were used as positive and negative controls respectively. Dilutions of the drugs were spotted on media plates and incubated overnight at 37 °C.

Post incubation media plates were observed for zones of inhibition under visible and ultra violet lights.

Comparative H_2O_2 anti-PCN activity assessment

Piperaquine indices oxidative stress for exhibiting its antimalarial activity. In order to enquire whether the similar mode of action was also involved in its anti-PCN activity, Hydrogen peroxide (H_2O_2) was used to exhibit similar discolouration. H_2O_2 (30%, 50µL) was applied to bacterial lawn developed on Cetrimide agar media and incubated overnight at 37^0 C. The bacterial growth and pigmentation patterns were compared to pepraquine.

Results and discussion

In this study potential anti-PCN drugs were virtually screened based on the CPI information and subsequent PCN-drug interaction of the respective drug targets used. Studying Drug-drug interactions is vital for the aim of drug repurposing. DDI and CPI has been demonstrated to be very effective in predicting drug side effects, but these side effects hold the potential for the drug to be repurposed for another clinical challenge (Luo *et al.*, 2014).

Virtual screening for anti-PCN activity

DDI-CPI server at default cut off score (> 0.85) screened 287 drug hits out of its drug and target pool against PCN. This number was reduced to 39 when cut off confidence score was raised to > 0.98. Anticancer, antiviral, antibiotics and cardiac related drugs were excluded from the high probability screened hits thus resulted in removal of 14 hits from the prioritized hits' list with final number of hits to 25 (Table 1, Fig. 1). Screening steps were performed for reducing the number of random hits by raising probability score to > 0.98. Exclusion was implemented with keeping risk-to-benefit ratio in mind and drugs with severe side effects i.e. anticancer and antiviral chemotherapy agents (Carr and Cooper, 2000, Montessori *et al.*, 2004, van Leeuwen *et al.*, 2012).

Table 1. List of high priority screened drug hits tested for anti-PCN bioactivity against *P. aeruginosa*.

Sr. No.	Drug hit	Drug info
1	Buprenorphine hydrochloride	Opioid alkaloid
2	Chlorotrianisene	Synthetic, nonsteroidal estrogen
3	Dihydroergotamine	Alkaloid used to treat migraines
4	Galantamine	Alzheimer's disease
5	Haloperidol	Antipsychotic
6	Levorphanol	Opioid analgesic
7	Piperaquine	Antimalaeria
8	Propoxyphene	Narcotic pain relievers
9	Pyronaridine	Antimalarial
10	Tadalafil	erectile dysfunction
11	Telmisartan	Angiotensin II receptor antagonist
12	Tolterodine	Antimuscarinic drug
13	Toremifene	selective estrogen receptor modulator
14	Treprostinil	Vasodilator that is used for the treatment of pulmonary arterial hypertension
15	Clomipramine	Tricyclic antidepressant
16	Dipyridamole	Vasodilator
17	Dorzolamide	Carbonic anhydrase inhibitor. It is an anti-glaucoma agent, and acts by decreasing the production of aqueous humour.
18	Estrone	Steroid, a weak estrogen, and a minor female sex hormone
19	Icosapent	Icosapent ethyl is a type of omega-3 fatty acid, a fat found in fish oil. It is used along with a proper diet to help lower fats (triglycerides) in the blood.
20	Risperidone	Antipsychotic medication
21	Sertindole	Antipsychotic medication
22	Tramadol	Narcotic-like pain reliever
23	Treprostinil	Vasodilator
24	Trimipramine	Tricyclic antidepressant
25	Oxycodone	Treat moderate to severe pain

Antibiotics were also removed due to the fact that most of the clinical pathogens harbor antibiotic resistance mechanisms the factor which compelled the current study (Laxminarayan *et al.*, 2013).

Cardiac related drugs were also for the reason that *P. aeruginosa* is associated with hospital acquired infections with higher vulnerability in patients under intensive care (Vincent *et al.*, 1995, Venier *et al.*, 2014).

Anti-PCN activity assessment

Of all drug hits tested, Piperaquine exhibited anti-PCN activity in contrast to all the hits tested and positive control Fig. 2 a, b c and d. Largest zone of inhibition was observed for dilution (4.5 M) 40.25±1.71 mm and 25.75±4.74 mm for piperaquine and acetylsalicylic acid respectively (Fig. 3). Piperaquine holds structural and chemical similarities with PCN. Interestingly Piperaquine is used as antimalarial drug in combination with artemisnin derivatives, while PCN had also been used for similar objectives during early twentieth century (Davis *et al.*, 2005, Meissner *et al.*, 2011). Both the molecules could potentially share similar mode of action as both induce oxidative stress for killing of malarial parasite. Due to its' virulence, PCN has been termed as an important virulence factor of *P. aeruginosa*.

Animal model studies have demonstrated the high virulence and cytotoxicity of PCN making it the primary cause of mortality (Wilson *et al.*, 1988). Inhibiting PCN biosynthesis will affect the pathogen's ability to cause infection as PCN is responsible for variety of duties for infection establishment due to its production at early stages of infection.

Reported anti-PCN activity could be exploited for acute *P. aeruginosa* infections i.e. Urinary Tract Infections where high amount of PCN is one of the main reason of virulence.

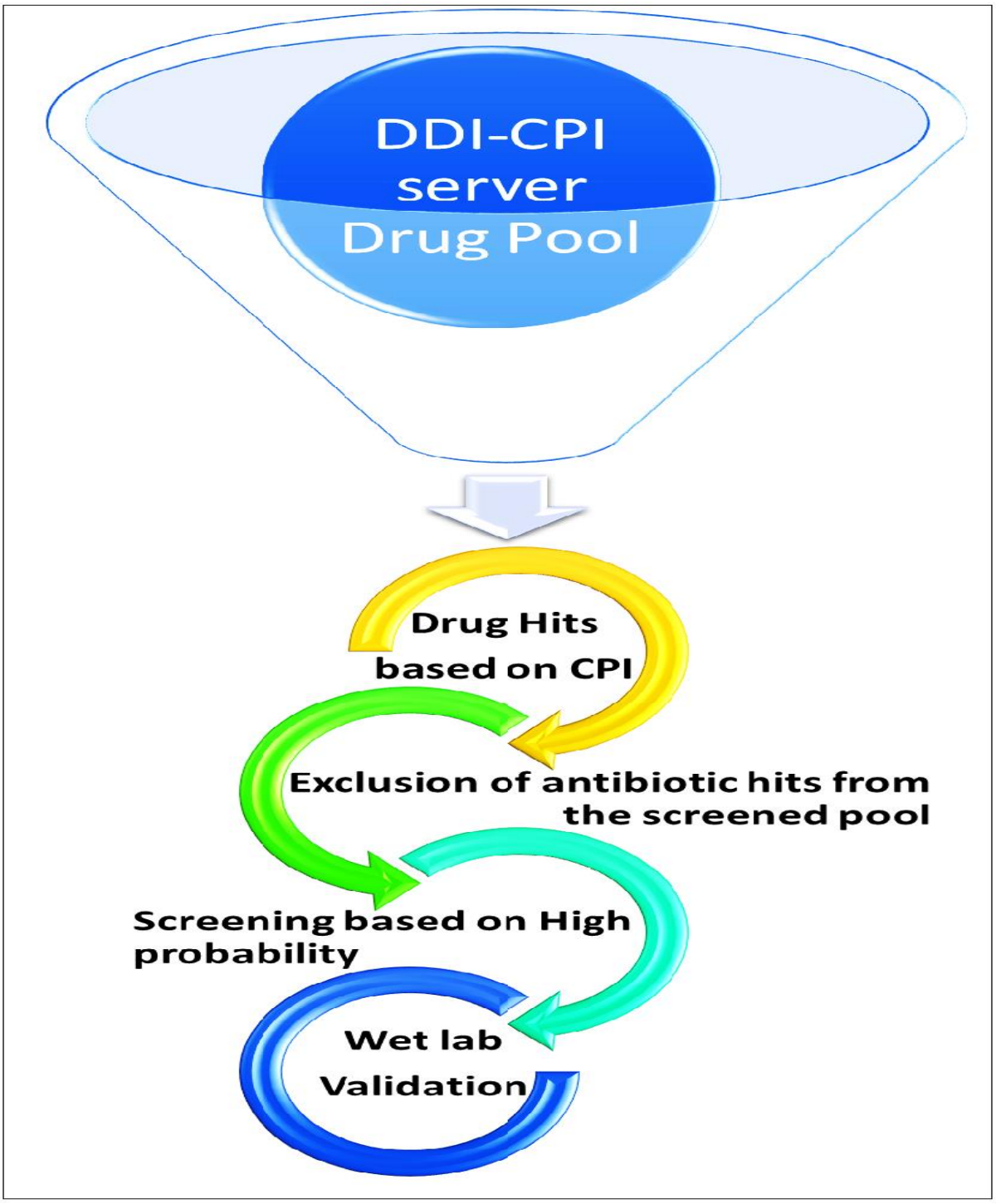

Fig. 1. Schematic workflow. DDI-CPI server pool was screened against PCN structure. Hits were screened based on probability score and cut off was set at >0.98 instead of default (>0.85). Antibiotics were removed from the screened hits. Resulting hits were tested in wet lab against *P. aeruginosa* for evaluating their potential anti-PCN activity.

There have been few drug repurposing studies focusing on anti-PCN activity with yielding products ranging from salicylic acid to raloxifene with varying degree of PCN biosynthesis inhibition (Prithiviraj *et al.*, 2005; Sui *et al.*, 2012).

Salicylic acid and its derivatives share structural similarity with Chorismate, the starting substrate of PCN biosynthetic pathway. But Chorismate is an important metabolite and is involved in multiple different pathways thus affecting the pathogen's metabolism beyond PCN biosynthesis.

Although Salicylic acid inhibits PCN but it serves as precursor for siderophore Pyochelin, an iron scavenging virulence factor (Audenaert *et al.*, 2002). Pyochelin is reported to augment oxidative injury in upper respiratory infections and effects human pulmonary epithelial cells (Britigan *et al.*, 1997, Braud *et al.*, 2010).

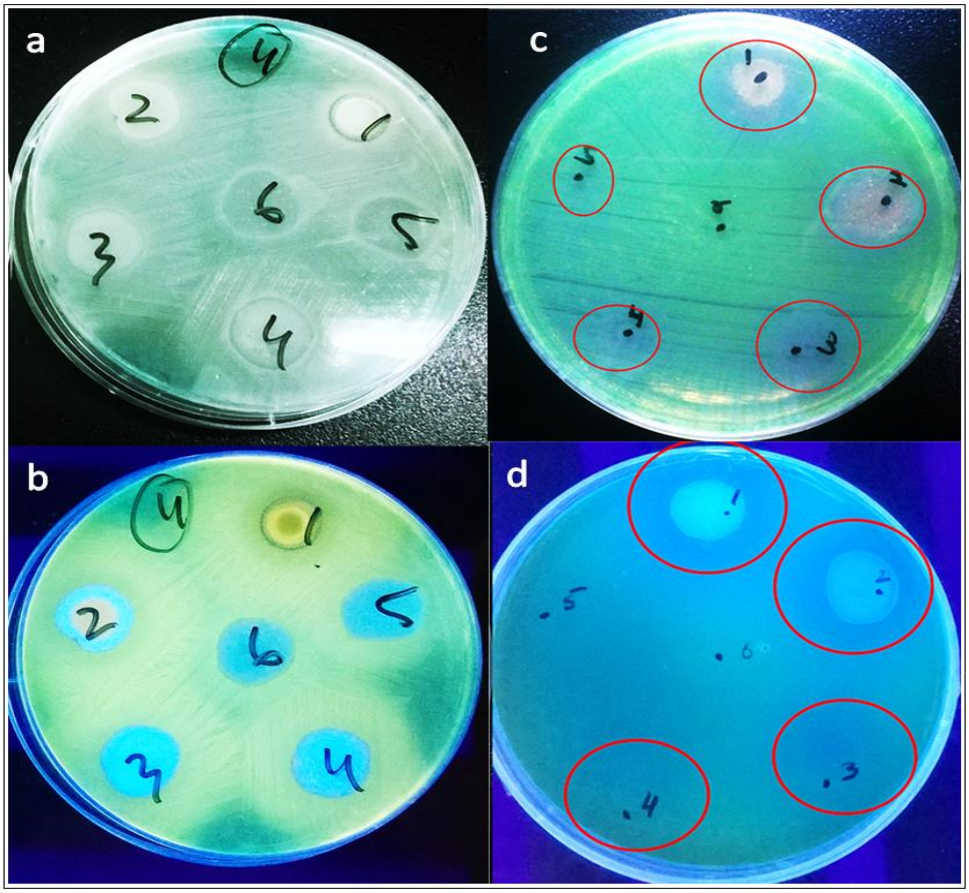

Fig. 2. Comparative anti-PCN activity of Piperaquine and Acetyalsalicylic acid. (a) & (b) Piperaquine activity in visible light and UV light and (c) & (d) Acetylsalicylic acid activity respectively.

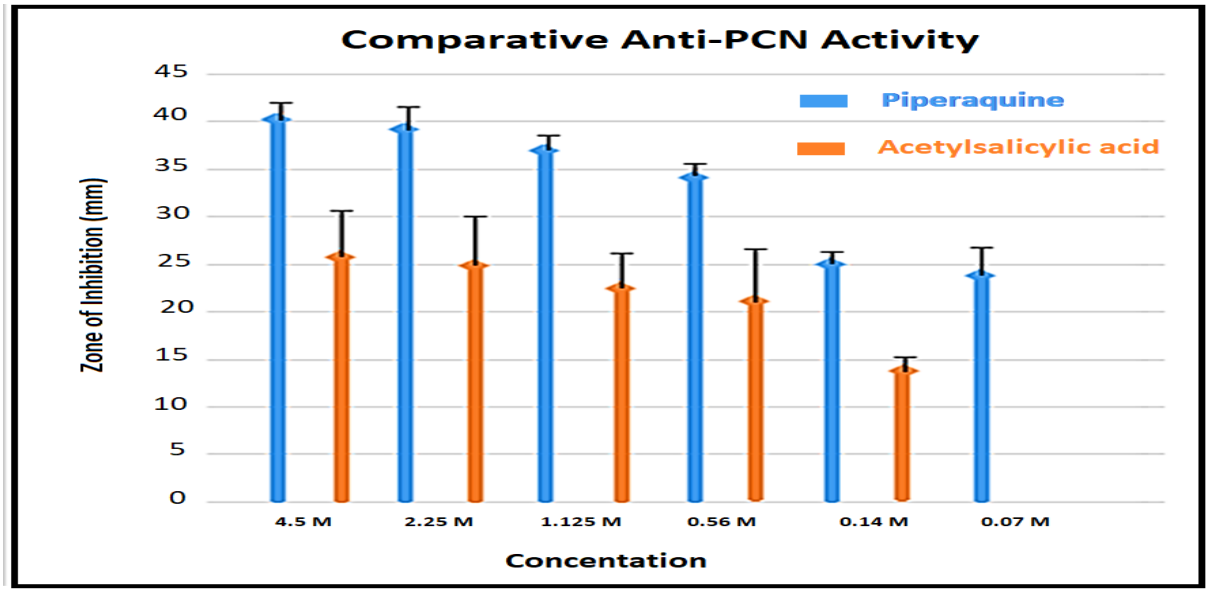

Fig. 3. Comparative anti-PCN activity of Piperaquine and Acetyalsalicylic acid.

Raloxifene on the other hand is a selective estrogen receptor modulator which mimics the effects of estrogens along with anti-cancerous activity used for preventing osteoporosis and invasive breast cancer in post-menopausal women (Gizzo *et al.*, 2013).

Raloxifene inhibits PCN by targeting Phenazine biosynthetic enzyme (PhzB2) in dose dependent manner (Sui *et al.*, 2012). But its cytotoxic effects (i.e. venous thromboembolism and stroke etc.) overshadow the potential benefits of anti-PCN activity (Adomaityte *et al.*, 2008; Lemmo, 2016).

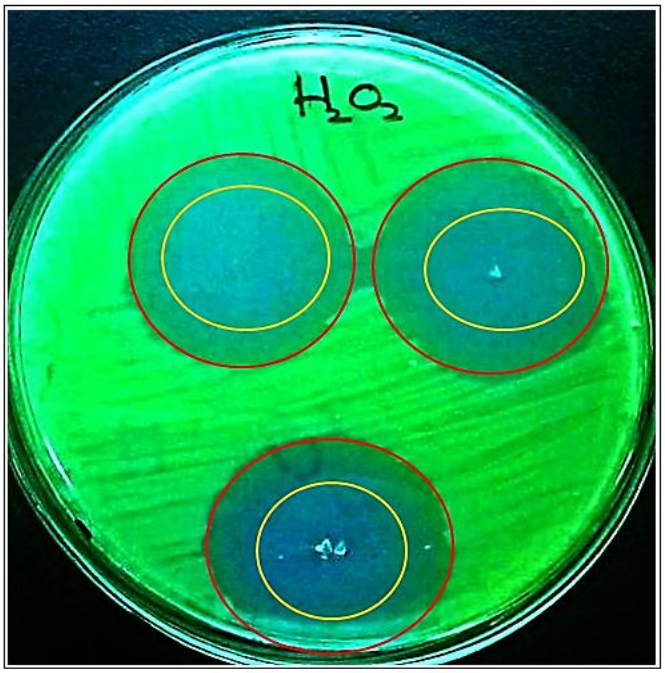

Fig. 4. Comparative bactericidal and anti-PCN activity of Hydrogen peroxide. The red circles demarcate bactericidal zones (3.46 ± 0.2mm) and decoloration is marked by yellow boundary (2.26 ± 0.18 mm) indicating diffusion of PCN produced beyond the red circle regardless of H_2O_2.

Mode of action assessment

When challenged with oxidative stress of H_2O_2, *P. aeruginosa* didn't impacted PCN production as exhibited in Fig. 4.

Two distinct zones were observed bactericidal activity was observerd as larger zone of growth inhibition (3.46 ± 0.2mm) while a smaller decoloration zone was also present within the larger zone (2.26 ± 0.18 mm). Regarding the mode of action of the new activity evaluated using drug repurposing via DDI CPI approach may not be the one conceived at the time of drug development. As indicated, when compared with piperaquine and H_2O_2, oxidative stress did not had any impact on PCN biosynthesis other than it whipped out the bacterial cells secreting it. The two zones of inhibitions indicated the bactericidal zone had PCN diffused in it from the viable cells growing beyond the active reach of H_2O_2.

Conclusion

Development of anti-infective drugs could help in managing the pandemic situation of antibiotic resistance. Using drug repurposing strategy we demonstrated that Piperaquine can inhibit PCN biosynthesis based on virtual screening and *in silico* chemical-protein interaction and drug-drug interaction assessments.

Although the mode of action could vary from the actual designed or intended one which was conceived at the time of drug development yet it may not pose a significant problem. This approach could be used for other pathogens by targeting their non-proteinaceous virulence factors.

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