INTRODUCTION

Quorum sensing (QS) systems, which are intra- and inter-species' signaling systems present in both gram negative and gram positive bacteria ***(Sturme et al., 2002)***, have been discovered by which they regulate expression of several genes, many of these coding for virulence factors ***(Li and Tian, 2012).***

This revealed that each bacterial species secretes one or more chemical signal molecule(s) and has receptors for the same ones; these can either diffuse freely into & out of the cell, or some are partly effluxed by efflux pumps; whenever the local concentration reaches a threshold level (which corresponds to a certain bacterial density- *quorum*), a set of genes' inductions as well as repressions start to be activated via an activated transcription factor; this in turn allows for community-based decisions among bacteria ***(De Kievit and Iglewski, 2000)***.

Many bacterial species were found out to be living in a complex structure known as biofilm which is firmly attached to biotic and abiotic surfaces. It consists of many microcolonies (bacteria living in close relation), existing in a matrix of extracellular polymeric substance with channels for free diffusion of nutrients, waste products & autoinducers ***(Sanchez et al., 2013).***

Biofilm community forms the ideal environment for QS since it allows local elevation of signal molecules' concentrations ***(Li and Tian, 2012)*** and it is also under QS

system control ***(Davies et al., 1998).*** Biofilm production has been accused for emergence of Multi drug resistant (MDR) strains among many organisms by allowing selection for highly resistant ones ***(Sanchez et al., 2013)***.

Fortunately, *Pseudomonas aeruginosa* has the most accurately studied bacterial QS behavior; It is a gram negative opportunistic bacillus that primarily infects individuals who are immunocompromised or having breaches in normal barriers such as in cases of burns, using indwelling medical devices, or prolonged use of broad-spectrum antibiotics ***(De Kievit and Iglewski, 2000).*** Thus, it is responsible for several nosocomial infections, on top of these comes ventilation associated pneumonia, urinary tract infections (especially true in catheterised patients) and gram negative septicemia ***(Van Delden and Iglewski, 1998).***

Quorum- controlled pseudomonal genes include those essential for elastase-enzyme production, biofilm formation, as well as production of rhamnolipids , pyocyanin, proteases, and hemolysin ***(Ishida et al., 2007).***

Many factors account for pseudomonal strong resistance to antibiotics like the low outer membrane permeability, and the presence of several active multidrug efflux pumps, as well as their existence in biofilm. MDR pseudomonal strains, that resist most of the present antibiotics, increased morbidity and mortality among hospitalised patients ***(Aloush et al., 2006).***

The growing fear of heading into a postantibiotic era permitted thinking of novel strategies to combat infections other than chemicals that affect bacterial growth and allow natural selection of resistant strains; instead, focusing on chemicals that decrease bacterial *virulence*, which facilitates clearance of the bacterial load by the host's self ***(K Bhardwaj et al., 2013).***

Quorum sensing inhibitors (QSIs) are chemicals that interfere with QS-controlled gene regulation; these include naturally existing molecules & new synthetic homologues, many of which are still under trials and observation; however, it has been observed that many of previously used drugs that were already in clinical use but for completely different purposes, showed QS inhibitory effects ***(Yang et al., 2009).***

Acetylsalicylic acid (aspirin) has been widely studied for this purpose and proved to be a potent QSI ***(Prithiviraj et al., 2005)***; the mechanism of inhibition remained unrevealed for several years, but it was finally found out to be due to inhibition of prostaglandin synthesis in the fungal cell through COX-like enzyme inhibitory effects ***(Alem and Douglas, 2004).***

Unlike Aspirin, which is a nonselective cycloxygenase (COX) inhibitor, a selective COX2 inhibitor is expected to have the same effects on quorum sensing inhibition without having toxic effects on gastric mucosa ***(Umaru et al., 2009)***; moreover, COX2-inhibitors have been demonstrated to be accountable for inhibition of COX-dependable multidrug efflux pumps in

prokaryotic cells, possibly leading to premature intracellular increase of autoinducer and several antibiotics concentrations, thus leading to premature expression of quorum-controlled genes facilitating host clearance. It could be responsible for decreased resistance to antibiotics as well ***(Kalle and Rizvi, 2011)***, which means that along with the QSI effects, it is a promising solution for the MDR organisms problem.

AIM OF THE WORK

The aim of this work is to compare the in vitro effects of Aspirin (acetylsalicylic acid) with Etodolac (a selective COX-2 inhibitor) on *Pseudomonas aeruginosa* clinical isolates obtained from patients with hospital acquired infections, as well as to their untreated isolate counterparts. This comparison was done in terms of QS inhibition through focusing on inhibition of biofilm formation, elastase enzyme production and pyocyanin production as QS-controlled virulence factors, which is the ultimate goal of therapy.

PSEUDOMONAS AERUGINOSA

Pseudomonas aeruginosa (*P. aeruginosa*) is a member of the Gamma Proteobacteria class and is the prototype of genus Pseudomonas group which contains 12 other members ***(Todar, 2008-2012; Jayaseelan et al., 2013)***.

It is a non-fermentative gram negative bacillus, measuring 0.5–0.8 μm wide and 1.5–3.0 μm long. Almost all strains are motile by means of a single polar flagellum ***(Jayaseelan et al., 2013)***, and it is classified as a facultative anaerobe due to its ability to use nitrogen as the terminal electron accepter in absence of oxygen ***(Mesquita et al., 2013)***.

It is known to be highly ubiquitous in nature, living in a wide variety of habitats, with preference of moist conditions like soil and water sources, as well as, decaying organic matter ***(Osman et al., 2010, Todar, 2008-2012)*** and other terrestrial environments. It can also colonize living eukaryotic organisms ***(Fourie et al., 2016)***, this occurs commonly on plants but only rarely does it colonize surfaces of animals or human beings ***(Todar, 2008-2012)***. Nevertheless, it has the ability to infect an extensive range of hosts from yeasts, plants, invertebrate and vertebrate animal hosts, as well as human beings ***(Lau et al., 2004).***

Probable habitats of *Pseudomonas aeruginosa* in various human- related community settings include swimming pools, whirlpools, hot tubs, contact lens' solutions, home humidifiers,

both bulk soil and rhizosphere as well as on vegetables and in sinks, water pipes and tap water ***(Mesquita et al., 2013).***

As mentioned before, it is only seldomly a member of the normal bacterial flora, with normal colonization rates of only 0-2% on skin, and highest rates of 2.6-24% in the gastrointestinal tract which is still relatively low. However, these rates are dramatically increased on hospitalization, especially in presence of breaches to cutaneous or mucosal barriers, with decreased immunity or with disruption in normal microbial flora due to prolonged antimicrobial uptake; Colonization rates may exceed 50% in these conditions ***(Lister et al., 2009).***

Normally, there's a selective pressure of nutrient limitation and competition between different microorganisms thus regulating colonization by this potentially pathogenic bacterium. However, when this equilibrium is disrupted in any way, infection by this opportunistic bacterium occurs ***(Fourie et al., 2016)***. Thus, it primarily infects individuals who are immunocompromised like elderly patients ***(Fernandez et al., 2011)***, patients suffering from cancer or acquired immunodeficiency syndrome (AIDS), or those having breaches in normal barriers, such as in cases of burns or use of indwelling medical devices. It also commonly occurs with prolonged use of broad-spectrum antibiotics ***(De Kievit and Iglewski, 2000).*** Infection by this species is additionally popular among cystic fibrosis (CF) patients, and is very difficult to treat; In fact, it's identified as its

prime lung pathogen, where the lungs of nearly all CF patients are chronically colonized by *P.aeruginosa*; thus, it actively contributes to the progression and death rates of this disease, being the leading cause of death in these patients with decreased lifespan of over 80% of the cases ***(Finnan et al., 2004; Lau et al., 2004; Jayaseelan et al., 2013 and Mesquita et al., 2013)***. This is also true with other forms of bronchiectasis [non-cystic fibrosis], and among patients with chronic obstructive pulmonary disease ***(Kuang et al., 2011)***.

As a result, Intensive care units' (ICU) patients are particularly susceptible, this is because they have significant underlying medical conditions and are malnourished, both of which lead to impaired immune responses, along with extensive use of invasive devices. Moreover, circumstances necessitating immediate life saving measures, commonly occurring in ICUs, often do not allow thorough infection control measures to be undertaken, which increases the risk of infection.

Besides, ICU patients often have history of frequent hospital admissions which increases the risk of colonization by multidrug resistant organisms. These patients also commonly receive multiple courses of broad-spectrum antibiotics, owing to the complexity and severity of their illnesses, this is habitually given empirically in cases of severe sepsis when the focus of infection could not be identified promptly, further increasing the risk of emergence of resistant strains ***(Hatcher et al., 2012).***

The clinical importance of such organism has arisen from the facts that it's becoming increasingly responsible for an ever-widening spectrum of nosocomial infections and encountering increasing antimicrobial resistance among clinical isolates ***(Todar, 2008-2012; Mesquita et al., 2013)***. With the increase in the immunocompromised population globally, resurgence of many infectious diseases including those caused by *P.aeruginosa* has occurred in the past three decades ***(Lister et al., 2009).***

Epidemiology

Serious *P.aeruginosa* infections are predominantly hospital acquired infections (HAI) ***(Lister et al., 2009)***. It comes as a second leading cause of gram negative nosocomial infections ***(Lutz and Lee, 2011)***; In a study done in Greece *P.aeruginosa* was related to 8% of infections and colonizations during a study period of two years ***(Kotsogiannou, 2013)***. It is responsible for a wide variety of HAI, on top of these comes ventilation associated pneumonia (VAP), urinary tract infections (UTI) (especially true in catheterized patients) and gram negative septicemia ***(Van Delden and Iglewski, 1998).***

However, it is also involved in a variety of other infections such as osteomyelitis, endocarditis, meningitis and skin & soft tissue infections ***(Hatcher et al., 2012)***; It can also ultimately lead to multiorgan system failure ***(Gad et al., 2007)***.

Diseases caused by it are often more serious than other nosocomial bacterial infections. ***Ashour and El-Sharif (2009)***, in a study conducted on hospitalized cancer patients, stated that mortality rates were higher in cases infected with *P. aeruginosa* than any other nosocomial infection.

In hospital settings, it can be found in a large variety of moist environments which act as reservoirs for infection; these include sinks and taps, mops, soaps, some disinfectants, medicines as well as medical devices like ventilators and other respiratory equipment, and hot tubs, physiotherapy and hydrotherapy pools. Accordingly, we are facing a very critical problem when trying to apply infection control measures ***(Giamerellou, 2002; Lister et al., 2009 and Hatcher et al., 2012)***. IV lines have also been commonly reported as a cause of infection outbreaks ***(Mesquita et al., 2013)***. In hospitals, they tend to persist, since there is constant exchange between patients, hospital and environmental habitats ***(Osman et al., 2012).***

In a study by ***Kotsogiannou (2013)***, Data indicated clonal spread among ICU and normal ward patients; this supports the postulation that colonization during ICU hospitalization contributes to infection and spread to other wards.

It is the commonest cause of VAP ***(Hatcher et al., 2012)***, and the second most common bacterium causing sepsis in ICUs ***(Gad et al., 2007)***. Its' ability to live on minimal nutritional requirements and in diverse environmental conditions (from

4°C to 42°C) ***(Lutz and Lee, 2011)*** in addition to its' innate and acquired resistance to many antibiotics helps in persistence of *P.aeruginosa* in both community and hospital settings ***(Lister et al., 2009).***

Genome:

Several *P.aeruginosa* strains' genomes have been fully sequenced over the past decade. *Pseudomonas aeruginosa PAO1* was the first, it was isolated from a human wound and sequenced by ***Stover et al. (2000)***; Seven more different clinical isolates had been also sequenced by 2013 ***(Mesquita et al., 2013)***.

P.aeruginosa has the largest sequenced bacterial genomes, ranging from 6.26 million base pairs (Mbp) to 6.8 Mbp of *P.aeruginosa PAO1* and *P.aeruginosa* NCGM2.S1 respectively ***(Mesquita et al., 2013).*** *P.aeruginosa PAO1* genome, had 5567 predicted genes or open reading frames, compared to only 4.64 Mbp (4279 genes) in *Eschericia coli K12*, 2.81 Mbp (2594 genes) in *Staphylococcus aureus N315* and 1.83 Mbp (1714 genes) in *Haemophilus influenzae Rd* ***(Stover et al., 2000 and Lambert, 2002)***. It also has the highest known GC content among bacteria of around 66.6 % ***(Mesquita et al., 2013)***.

Comparative genomic analysis of the different sequenced *P.aeruginosa* strains revealed that its genome is a mosaic consisting of two components, the core and accessory genomes. The core genome is the part shared by all strains and comprises

about 90% of the total genome content, it includes many common metabolic and pathogenic factors. On the other hand, accessory genome constitutes genomic segments that are varying between different strains; it is composed of genetic elements like transposons, insertion sequences, plasmids, prophages and genomic islands acquired by one of the horizontal gene transfer processes like conjugation, transduction or transformation.

Four genomic islands have been identified in *P.aeruginosa*, PAG-1, PAG-2, PAG-3 and the flagellum islands; these have the same characteristics as the rest of accessory genome mentioned in the paragraph below. However their role in pathogenicity is yet to be determined ***(Finnan et al., 2004)***.

The accessory genome is widely dispersed within the core genome, but tends to cluster in certain loci named Regions of Genomic Plasticity (RGP). RGPs can be predicted by having different characteristics from rest of genomic DNA like unusual GC content or codon usage; they also tend to be associated with transfer ribonucleic acid (tRNA) genes that apparently act as hotspots for foreign deoxyribonucliec acid (DNA) insertion ***(Mesquita et al.,2013).***

An approximate calculation of the number of genes needed for cell growth and division in a minimal salts medium, including all enzymes needed for metabolism and structural proteins, is around 1500 genes only. *P.aeruginosa*, therefore,

possesses considerable additional genetic capacity compared to other organisms ***(Lambert, 2002)***.

The complexity of this genome comes from the presence of many regulatory genes; In fact, it contains the highest proportion of regulatory genes than in any other observed bacterial genome. In addition, the number of paralogus gene groups is 50% higher than expected for this genome size. This means that it has a greater number of distinct gene families, and that it's larger size is due to genetic diversity rather than mere gene duplication events; all of which is ultimately leading to functional diversity ***(Stover et al., 2000)***.

Furthermore, upon determining some gene functions, a large number of genes were found to be involved in catabolism, transport and efflux of many different organic compounds; as well as four different chemotaxis systems ***(Stover et al., 2000).***

All of the above explain *P.aeruginosa*'s highly adaptable nature, making it able to live on a wide variety of substrates and tolerating diverse environmental stresses. It can also alter its properties in response to changes in the environment; this includes the ability to develop resistance where antibiotics are used extensively ***(Lambert, 2002).***

It is noteworthy however to mention that some genetic regions which may encode virulence factors may not be conserved among some clinical isolates ***(Finnan et al., 2004).***

Antimicrobial Resistance:

P.aeruginosa is inherently resistant to a wide range of antimicrobials and antiseptics, with only few classes of drugs being effective in treatment of its infections; all of these have to cross the cell wall to reach their targets.

Despite being able to overcome these inherent defense mechanisms, all of the following drug categories without exception are unfortunately prone to be rendered ineffective by mutational or acquired resistance ***(Livermore, 2002).***

Effective Classes of Drugs:

The aminoglycosides (including gentamicin, tobramycin, amikacin, Netilmicin) inhibit protein synthesis by binding to the 30S subunit of the ribosome. Quinolones (ciprofloxacin, Levofloxacin) bind to the A subunit of DNA gyrase, which is normally essential for maintaining ordered structure of the chromosome inside the cells. The Antipseudomonal β-lactams include four different antimicrobial categories, Antipseudomonal Penicillins (e.g. piperacillin- tazobactam, Ticarcillin- clavulanic acid), Antipseudomonal Cephalosporins (e.g. Ceftazidime, Cefepime) Carbapenems (e.g. imipenem, meropenem, Doripenem) and Monobactams (e.g. aztreonam), all of which inhibit the peptidoglycan-assembling transpeptidases located on the outer face of the cytoplasmic membrane. Finally, the polymyxins / cationic peptides (colomycin, colistin) bind to phospholipids in the

cytoplasmic membrane, destroying its barrier function ***(Lambert, 2002; Hatcher et al., 2012 and Magiorakos et al., 2012).***

The above categories, with all the drugs stated under them, constitute the full antimicrobial panel that should be used for complete antimicrobial susceptibility testing of a *P.aeruginosa* isolate **(Table 1)**, as is proposed by joint expert groups from European Centre for Disease Prevention and Control (ECDC) and Center for Disease Control and Prevention (CDC) based on documents and breakpoints from the Clinical Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the United States Food and Drug Administration (FDA) ***(Magiorakos et al., 2012).***