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In vitro Susceptibility of *Pseudomonas aeruginosa* Isolated from Acute and Chronic Pulmonary Infection to Antibiotics, *Lactobacillus* Competition and Metal Nanoparticles

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SR and NMA conceived and designed the study. SR acquired, performed the experiments and wrote the article. GBP and NMA analyzed the data. IQ and BM helped in reviewing manuscript.

Key words
Antibacterial activity, Antibiotic, *Lactobacilli,* Nanoparticles, *Pseudomonas aeruginosa,* Pulmonary infections.

ABSTRACT

Antibiotic resistance in *Pseudomonas aeruginosa* is a major barrier to successful treatment of infection. Additional and novel measures to control this pathogen are needed, along with contemporary information about antibiotic resistances that are present in isolates from different environments. In the present study 72 samples from blood, 43 from sputum, and 19 were obtained from tracheal aspirates patients suffering from chronic and acute lung infections admitted to a local hospital in Lahore. Susceptibility of 134 isolates of *P. aeruginosa* was tested against selected antibiotics (meropenem, imipenem, piperacillin, amoxicillin, amikacin, gentamicin, tobramycin, kanamycin, clarithromycin, clarithromycin, cefepime, cefixime, levofloxacin, and ciprofloxacin), *Lactobacillus* strains and metal nanoparticles (copper, ferric and zinc). *P. aeruginosa* isolates showed *in vitro* resistance against 11 of 14 antibodies tested. The isolates were highly susceptible to meropenem, piperacillin, and amoxicillin. It was also observed that the growth of these resistant *P. aeruginosa* strains was significantly inhibited in the presence of *Lactobacilli* spp. and nanoparticles of silver, zinc and ferric oxide at a concentration of 12, 200 and 1µg/ml, respectively. This study may help in the development of chemotherapeutic methods against multidrug resistant bacterial pathogens in chronic and acute lung infections. It provides a practical approach towards the use of nanoparticles to enhance antimicrobial activity against these pathogens.

INTRODUCTION

Bacterial infections cause morbidity and mortality in millions of people all over the world and also have a serious impact on the world economy (Oveisi *et al*., 2001; Thabit *et al*., 2015). The development of resistance against many clinically useful antibiotics has become alarming (Ferri *et al*., 2017). Antimicrobial resistance among *Pseudomonas aeruginosa* strains is a continuing and growing problem worldwide (Linden *et al*., 2003). *P. aeruginosa* has developed resistance to many antimicrobial agents including carbapenems, polymyxins, fluoroquinolones, cephalosporins, and aminoglycosides (Thabit *et al*., 2015). Newer approaches to control *P. aeruginosa* tissue colonization, spread and induction of disease are clearly needed.

One such approach might be the use of non-pathogenic bacterial strains with anti-*P. aeruginosa* activity that could be applied to sites of colonization such as burned skin, the oropharyngeal mucosa or the GI tract to inhibit *P. aeruginosa* colonization. *Lactobacillus* strains are well known for their probiotic role in medical science (Gordon *et al*., 1957). They produce bacteriocins, bioactive peptides with antimicrobial activity against Gram-positive bacteria such as *Staphylococcus aureus, Bacillus cereus*, *Listeria monocytogenes* and *Clostridium botulinum* (Nettles and Barefoot, 1993) and have been shown to protect mice against *P. aeruginosa* burn-wound infection (Argenta *et al*., 2016; Qamar *et al*., 2017).

Another approach is the use of silver ions, a practice that has had a major impact in reducing burn-wound infections by *P. aeruginosa* and other pathogens (Politano *et al*., 2013). Silver metal has a history of use in ayurvaidic medicine and other historical herbal treatments for infections (Alexander, 2009). Wilding *et al*. (2016) has reported this metal and its nanoparticles (AgNPs) efficiently inhibit the growth of Gram-positive and Gram-negative bacteria due to their antibacterial activity. Silver NPs were reported to be effective against bacterial cells by promoting cell wall lysis with release of intracellular contents, inhibiting cellular respiration and eventually damaging DNA (Li *et al*., 2011). The antibacterial activity of silver nanoparticles against pathogenic bacteria displayed the highest activity against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Klebsiella pneumoniae,* and *Proteus*

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mirabilis (Buszewski *et al*., 2018).

This *in vitro* study was designed to evaluate the impact of lactobacilli, nanoparticles and different commercial antibiotics on multiple drug resistant clinical isolates of *Pseudomonas aeruginosa* obtained from patients in Lahore, Pakistan.

MATERIALS AND METHODS

The blood, endotracheal aspirates and sputum samples were collected from patients with acute and chronic respiratory diseases (Table I) admitted in local government hospitals of Lahore, Punjab, Pakistan and the samples were transferred and processed in Department of Zoology, Government College University, Lahore. All the samples were collected using techniques as per international biomedical standards (Singh *et al*., 2015). Cetrimide agar was used for selective screening of *P. aeruginosa* strains (Fig. 1A). *P. aeruginosa* BS14 (KT721565) was used as a positive control for antibiotic testing. The agar well diffusion method was used for susceptibility testing of *P. aeruginosa* against lactobacilli strains and nanoparticles. Lactobacilli were isolated from various food sources, and those isolated from yogurt were found to produce high quantities of bacteriocin-type toxins. These toxins have the ability to inhibit the growth of other bacterial strains. Nanoparticles of silver were used in aqueous (1mM $AgNO_3$) solution.

Table I.- Clinical isolates of *Pseudomonas aeruginosa* showing age, sex and source of sampling.

Source	No. of samples	Female	Male	Age in years
Blood, sputum	34	03	31	25-35
and endotracheal	47	11	36	36-45
aspirates	53	13	40	46-55
Total	134	27	107	-

Antagonistic properties of antibiotics against P. aeruginosa

P. aeruginosa isolates were cultured in brain heart infusion (BHI) broth following inoculation from a plate culture using a sterile cotton swab and incubated for 24 h at 37°C for preparation of a turbidity suspension of 0.5 McFarland units (0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension 1.5x 10^8 colony forming units (CFU/ml)). *P. aeruginosa* cultures were spread by adding 100 µl (~1.5 107cfu) of culture media onto sterile agar plates and antibiotic disks were placed on the plates at specific intervals to observe the antagonistic properties of the drugs against the isolates. Plates were incubated at 37°C for 24h and the zones of inhibition around the wells, in mm, measured. Susceptibilities were determined using standard Kirby-Bauer criteria (Hudzicki, 2009).

Antagonistic properties of lactobacilli against P. aeruginosa

P. aeruginosa isolates were cultured and suspended in BHI broth as described and 100 µl (1.5×10^7cfu) spread uniformly on sterile agar plates into 3-4 wells with 6 mm diameters had been previously cut with 20 mm distance between them. Strains of *Lactobacillus* were identified by using a universal primer for 16s ribosomal DNA (Forward primer 5`AGAGTTTGATCMTGGCTCAG 3` and reverse primer 5`TACGGYTACCTTGTTACGACTT3`). *Lactobacilli* were inoculated into MRS broth (a selective culture media for *Lactobacilli*) and incubated for 24 h at 37°C. Next, the culture was centrifuged at 5500 rpm (9000g) for 5 -10 min and the clear supernatant was used as an unpurified bacteriocin extract. Solutions (50 µl) were added into each 6 mm diameter well and sterile distilled water was used as a control. The plates were incubated at 37°C for 24 h and the zones of inhibition around the wells in mm measured. MRS broth was further used as negative control (Sgouras, 2004).

Anti-bacterial properties of nanoparticles against P. aeruginosa

The solutions of nanoparticles of several compounds were obtained from the chemistry department at GCU Lahore courtesy of Ms. Misbah Naz (Naz *et al*., 2017). Solutions of nanoparticles of silver nitrate, zinc oxide and ferric oxide with concentration of 12 µg/ml, 200 µg/ml and 1 µg/ml, respectively, were used to check the susceptibility of *P. aeruginosa* against these nanoparticles. *P. aeruginosa* colonies were spread on sterile agar plates and 3-4 wells of 6 mm diameter were made in a single plate with 20 mm distance between them. Fiftyµl solutions of silver nitrate**,** ferric oxide, or zinc oxide orsterile distilled water as a control were added to wells. Plates were incubated at 37°C for 24 h and the zone of inhibition around the wells in mm measured (Hassan *et al*., 2014; Swaroop *et al*., 2015).

RESULTS

In the present study, a total of 134 isolates of *P. aeruginosa* were obtained from hospitalized patients and their antimicrobial susceptibility patterns, susceptibility to metal nanoparticles and inhibition by *Lactobacillus* determined. Figure 1B shows the media used for isolation and testing of the *P. aeruginosa* isolates for antibiotic sensitivity, nanoparticle sensitivity and susceptibility to *Lactobacillus* bacteriocins in culture supernates.

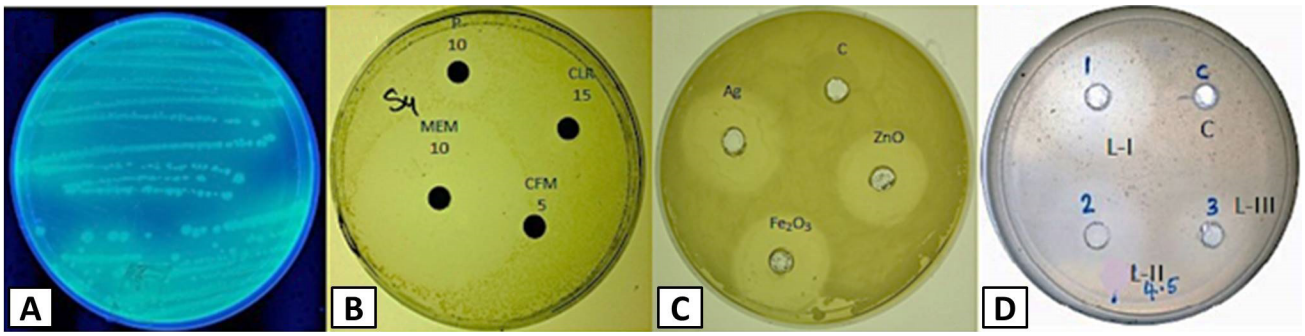

Fig. 1. Documentation of methods for testing susceptibilities of *P. aeruginosa* clinical isolates. **A**, isolated colonies of *P. aeruginosa* on cetrimide agar plate; **B**, antibiotics susceptibility test of *P. aeruginosa* (MEM, meropenem; CLA, clarithromycin; CFM, cefixime); **C**, effect of nanoparticles on *P. aeruginosa* growth (Ag, silver nitrate; ZnO, zinc oxide; Fe_2O_3, ferric oxide); **D**, effect of supernates of three *Latobacilli* spp. on *P. aeruginosa* (L-I, *Lactobacillus delbruekii* subsp. *Bulgaricus* (MH100728); L-II, *Lactobacillus curvatus* (MH107109); L-III, *Lactobacillus grminis* (MH108629).

Table II.- Susceptibility of 134 *P. aeruginosa* strains to antibiotics.

Group / Antibiotic (potency in µg)	R n (%)	I n (%)	S n (%)
Carbapenems			
Meropenem (MEM)10 µg	8 (6)	-	126 (94)
Imipenem (IRM) 10 µg	108 (81)	9 (7)	17 (13)
Penicillin			
Piperacillin (PIP) 30 µg	39 (29)	7 (5)	88 (66)
Amoxicillin (AX) 25 µg	5 (4)	17 (13)	111 (83)
Amino-glycosides			
Amikacin (AK) 30 µg	50 (37)	21 (16)	63 (47)
Gentamicin (GM) 10 µg	52 (39)	31 (23)	51 (38)
Tobramycin (TN) 10 µg	105 (78)	-	29 (22)
Kanamycin (K) 30 µg	79 (59)	14 (10)	41 (31)
Macrolides			
Clarithromycin (CLA) 2µg	121 (90)	4 (3)	9 (7)
Erythromycin (E) 15 µg	90 (67)	20 (15)	24 (18)
Cephalosporins			
Cefepime (CPM) 30 µg	102 (76)	-	32 (24)
Ceftazidime (CAZ) 10 µg	72 (54)	23 (17)	39 (29)
Cefixime (CFM) 5 µg	67(50)	59(44)	8(6)
Gyrase inhibitors			
Levofloxacin (LEV) 1 µg	98 (73)	15 (11)	21 (16)
Ciprofloxacin (CIP) 5 µg	102 (76)	11 (8)	21 (16)

R, resistant; I, intermediate; S, susceptible.

Susceptibility of P. aeruginosa *isolates to antibiotics*

The Kirby-Bauer method was used to determine the antimicrobial susceptibilities of 134 clinical isolates of *P. aeruginosa* to clinically relevant and available antibiotics. Ninety-four percent of *P. aeruginosa* isolates were sensitive to meropenem but only thirteen percent susceptible to imipenem. There was relatively high susceptibility to penicillins ranging from 66%-83% but only moderate to low susceptibility to aminoglycosides (22%-47%). There was overall low susceptibility to the macrolides (7%-18%), cephalosporins (24%-29%) and gyrase inhibitors (7%-29%; Table II). Thus 66%-94% of the strains were susceptible to only 1 of the three antibiotics: meropenem, piperacillin or amoxicillin.

Antagonistic properties of lactobacilli against P. aeruginosa

The antibacterial activities of culture supernates from *Lactobacillus delbruekii* subsp. *Bulgaricus* (MH100728), *Lactobacillus curvatus* (MH107109) and *Lactobacillus graminis* (MH108629) were tested using the well-plate method. One-hundred percent of *P. aeruginosa* clinical isolates had zones of inhibition ranging from13 mm to 24 mm in diameter indicating they all had some potential susceptibility to factors in unpurified bacterial supernate from cultures of *Lactobacilli* strains containing $1x10^7$ /mL (Fig. 1D).

Anti-bacterial properties of nanoparticles against P. aeruginosa

The well-in-plate method was used to determine the effects of nanoparticles on the growth of *P. aeruginosa* strains. The method is shown in Figure 1C. Clear zones of inhibition were obtained against the majority of the clinical isolates, indicating that nanoparticles had a strong deleterious effect on *P. aeruginosa* growth. Eighty percent of strains showed susceptibility to the nanoparticles (Table III), with clear zones of inhibition measured with average diameters of 9 mm. However, 20% of the clinical isolates

showed growth in the presence of nanoparticles (Naz *et al*., 2017).

Table III.- Effect of nanoparticles on *P. aeruginosa* growth.

Nanoparticles	Conc.	Susceptible n (%)	Resistant n (%)
Ag	12 µg/ml	111(83)	23(17)
ZnO	200µg/ml	107(80)	27(20)
Fe_2O_3	100 µg/ml	103(77)	31(23)

DISCUSSION

To address issues of antibiotic resistances and potential countermeasures, their occurrence, geographic manifestations and potential for spread needs to be continuously monitored and reported. In this study we analyzed the antibiotic resistance profiles, susceptibility to nanoparticles and killing by anti-bacterial factors in supernates of common probiotic organisms against 134 clinical isolates of *P. aeruginosa* from Lahore, Pakistan. Fifty-four percent of the *P. aeruginosa* isolates were from blood, 32% from sputum and 14% from tracheal aspirates. Similar results had been reported in different studies from other regions (Arora *et al*., 2011). We found 66-94% of isolates were susceptible to meropenem or penicillins, and but had a high level of resistance to the macrolides (82%-93%), cephalosporins (71%-76%) and gyrase inhibitors (84%). A more detailed analysis of the antibiotic-resistance findings showed strains of *P. aeruginosa* from the Lahore area hospitals are resistant to clarithromycin (90%), imipenem (81%), macrolides (84%) and tobramycin (78%) but are sensitive to meropenem (94%), amoxicillin (83%) and piperacillin (66%). In a Saudi Arabian hospital setting, Ahmad and Al-Harbi (2014) reported that there was a 70.7% susceptibility of *P. aeruginosa* isolates to meropenem but also concluded ciprofloxacin was the most active agent (85.4% susceptibility) against *P. aeruginosa* strains along with amikacin (95.1% susceptibility). They proposed that combinations of these antibiotics and β-lactams are useful and should be tested in treating multi-drug resistant strains. However, among our patients there was low susceptibility to ciprofloxacin and amikacin, indicating a distinct pattern of resistances in *P. aeruginosa* isolates from this patient population. Overall, in the Lahore hospital population, the *P. aeruginosa* isolates appear to have acquired a fairly high level of antibiotic resistances. In contrast, the majority of the isolates were killed by factors present in *Lactobacillus* probiotic supernates or by nanoparticles of either silver oxide, zinc oxide or ferric oxide, indicating two potential approaches for probiotic interventions or topical therapies to control drug-resistant *P. aeruginosa* colonization.

None of the antimicrobial agents were effective against all the multi-drug strains tested commiserates with the current worldwide problem in the treatment of multi-drug resistant nosocomial infections. In previous studies, *P. aeruginosa* isolates showed intermediate to full resistance against a variety of antimicrobial agents (Nicasio *et al*., 2008; Souli *et al*., 2008; Siegel, 2008; Giske *et al*., 2008; Slama, 2008; Chopra *et al*., 2008), encompassing clarithromycin, cefepime, ciprofloxacin, levofloxacin, imipenem tobramycin, amikacin, erythromycin, kanamycin, ceftazidime, and gentamicin. In our populations, many isolates were susceptible to meropenem, piperacillinor amoxicillin, indicating they represent the first-line drugs to consider in treating *P. aeruginosa* infections in the Lahore area hospitals.

Lactic acid bacteria are dispersed in nature and present in food items and are also found in vaginal flora where they promote resistance to urinary tract infections (UTI). Many strains of the genus *Lactobacillus* are also capable of colonizing the oral cavity and the gastrointestinal tracts and are important antagonists against certain pathogens (Antonio *et al*., 1999; Redondo-Lo´pez *et al*., 1990). The antimicrobial activity of strains of *Lactobacillus* against bacterial pathogens includes the production of bacteriocins, lactic acid and hydrogen peroxide (Servin, 2004). Bacteriocins inhibit the growth of susceptible microbial strains and can serve as signaling peptides or quorum sensing molecules (Dobson *et al*., 2012) or compete with pathogens for nutrients (Reid and Burton, 2002). Our results showed the presence of strong antibacterial activity in culture supernates of *Lactobacillus delbruekii* subsp. *Bulgaricus, Lactobacillus curvatus* and *Lactobacillus graminis* ageists a majority of the *P. aeruginosa* nosocomial isolates. This initial finding suggests a potential for these organisms to serve a probiotics to prevent *P. aeruginosa* colonization of mucosal surfaces.

We also evaluated the susceptibility of the *P. aeruginosa* clinical isolates to silver, zinc and ferric oxide nanoparticles, additional potential topical therapeutics for this pathogen. Overall we found all three NPs tested inhibited the growth of the majority of *P. aeruginosa* strains from the current study. Bayroodi and Jalal (2016) reported that the antimicrobial effects of AgNP could be enhanced by synergism with ZnO NPs when co-evaluated against resistant bacterial strains. Brayner *et al*. (2006) and Tam *et al*. (2008) reported damage to the membrane of bacterial cells by the ZnO NPs, resulting in leakage of the contents to the outside of the cell. Complexes of ciprofloxacin with copper II and zinc II showed higher antibacterial activity against *P. aeruginosa* than ciprofloxacin alone (Anacona and Toledo, 2001). Overall, NPs either alone or in

combination with antibiotics present another therapeutic option for treating multi-resistant *P. aeruginosa* infections.

In terms of demographics, the patients in this study were between the ages of 25 to 55 years, indicating a relatively young population in this cohort experiencing clinically significant *P. aeruginosa* infection. Almost 80% of our patients were male. This tends to reflect the gender demographic in many studies of *P. aeruginosa* infection outside of CF. However, Nadeem *et al*. (2009) reported in his study of a total of 1008 isolates of *P. aeruginosa* that 532 isolates were from male patients (52.7%; 504 adults and 28 children), and 476 isolates were from female patients (47.9%; 442 adults and 34 children). The gender distribution of *P. aeruginosa* reported by Ali *et al*. (2015) indicated 66.2% of the isolates of *P. aeruginosa*were from males, and the incidence of multidrug resistant *P. aeruginosa* in males was reported as 72.1%. Notably, the most susceptible age group was reported to be 10-19 years old. Although 80% of the *P. aeruginosa* isolates in this study were from patients 0-60 years old, in other studies (Ahmed and Al-Harbi, 2014), a higher rate of *P. aeruginosa* infections was reported among elderly (61-80 years old) patients. Overall, while *P. aeruginosa* nosocomial infections are often associated with males over 60 years of age *it is clear this can vary among different hospitalized populations.*

CONCLUSION

Sixty-six to >90 percent of the total *P. aeruginosa* isolates from the Lahore region showed *in vitro* resistance to many of the commercially available antibiotics tested. Meropenem, piperacillin, and amoxicillin were the drugs for which there was the greatest susceptibility and represent recommended treatments for infections due to *P. aeruginosa* in our region. A significant killing of these resistant *P. Aeruginosa* strains by factors present in supernates of *Lactobacilli* spp. was observed, suggesting that the use of *Lactobacilli* spp. as probiotics may be of value for the treatment or prevention of *P. aeruginosa* colonization. We also found strong *in vitro* anti-bacterial efficacy of Ag, Zn and Fe3 oxide NPs against the local *P. aeruginosa* isolates, suggestive of additional research into their practical application in a healthcare department.

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Statement of conflict of interest

I have declared no conflict of interest.

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