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ORIGINAL ARTICLE

Invitro Study of Effect of *Lactobacillus Plantarum* on *Pseudomonas aeruginosa* Isolated from Wound Infection

[1]Ayat S. Elnahal*, [2]Maii A. Shams Eldeen, [3]Mohamed I. El Sawaf, [2]Abd-ElRehem G. Ads
[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Kafr-Elsheik University, Egypt
[2] Department of Medical Microbiology and Immunology, Faculty of Medicine,Tanta University, Egypt
[3]Department of General Surgery, Faculty of Medicine, Tanta University, Egypt

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****Corresponding Author:***
Ayat Shaaban Mousa Elnahal
Department of Medical Microbiology and Immunology, Faculty of Medicine, Kafr-Elsheik University, Egypt
Tel:01016882446
dr_ayat2020@yahoo.com

ABSTRACT

***Background**: Pseudomonas aeruginosa (P. aeruginosa) is one of the most common causative bacteria in chronic wound infection. It is increasingly becoming resistant to many anti-pseudomonal agents. So, it is necessary to advance the field of alternative treatment. Probiotics are viable, non-pathogenic microorganisms that alter the micro-flora of the gastro intestinal tract of the host, yielding a positive influence on health and body physiology. **Objective**: To investigate the in-vitro effect exerted by Lactobacillus plantarum (L. plantarum) on the antibiotic sensitivity of P. aeruginosa. **Methodology:** This study was carried out in Medical Microbiology & Immunology Department, Faculty of Medicine, Tanta University on forty patients admitted, during the period of research (February 2017 to August 2017). P. aeruginosa isolates were identified by results of culture and conventional biochemical reactions. The effect of L.plantarum on antibiotic sensitivity of P. aeruginosa was detected by measuring the antibiotic sensitivity of the isolated strains of P. aeruginosa before and after addition of L.plantarum. **Results**: Among the forty wound samples investigated, the most predominant bacterial isolate was P. aeruginosa (50%). P. aeruginosa infection was more common in males than females with predominance in old age. P.aeruginosa susceptibility pattern to different antimicrobial agents significantly increased after addition of L. plantarum. 100% of isolates became sensitive to colistin sulphate followed by imipenem (70%), ciprofloxacin (65%), pipracillin–tazobactam (60%), gentamicin (35%), aztreonam (30%) and pipracillin (20%). **Conclusion**: P. aeruginosa was the predominant bacterial isolate in the studied wound infections. P. aeruginosa susceptibility pattern to different antimicrobial agents significantly increased after addition of L. plantarum.*

INTRODUCTION

Chronic wound is a worldwide problem for health systems because chronic wounds produce large expenditures for hospitalization and treatments[1]. The deleterious effect of microbial infection on wound healing has been recognized for decades and the control of bio-burden is accepted as an important aspect of wound management[2]. Wounds are complex micro-environments where infections by bacterial pathogens such as *Pseudomonas aeruginosa* or *Staphylococcus aureus* represent major concerns in patient treatment[3]. The presence of dead, denatured tissues and moist environment makes the wound vulnerable to infection by *P. aeruginosa*. Patients and their relatives are important sources of *P. aeruginosa* in intensive care units (ICU) or other critical care units and have become a potential source of healthcare-associated infections (HAIs)[4]. *Pseudomonas aeruginosa* virulence determinants include cell-associated factors e.g (lipopolysaccharide endotoxin flagellum, pili) and extracellular factors e.g (alginate, exotoxin A, exoenzyme S, pyocyanin, elastase, etc.)[5]. In burn infections, *P. aeruginosa* damages epithelial tissues through the secretion of proteases, such as elastase, encoded by the quorum sensing (QS) -regulated gene[6]. The cytotoxic blue pigment pyocyanin blocks wound healing by promoting the establishment of oxidative stress conditions and p38 mitogen-activated protein kinase (MAPK) pathway activation in infected tissues[7]. *Pseudomonas aeruginosa* is naturally resistant to many antibiotics and it is increasingly becoming resistant to many anti-pseudomonal agents. Therefore, treatment of wounds or burn patients infected with *P. aeruginosa* became very difficult due to limited treatment options[4]. So it is necessary to advance the field of alternative treatments that are financially accessible to the least economically developed countries[8]. To address the difficulties in treating *P. aeruginosa* infections, several approaches were undertaken including intensifying research to develop new antibiotics, use of different antibiotic combinations and identification of alternative treatment methods using non-antibiotic means. However, the scope for the development of new antibiotics that will be more effective than the existing antibiotics and against which the frequency of resistance development will be lower than the current antibiotics is very limited. Therefore, a lot of researchers focused on developing new antibiotic combinations to treat multi drug resistant (MDR) *P. aeruginosa* infections[9]. In addition to this a lot of researches are being carried out to develop non-antibiotic therapeutics against this pathogen using probiotics, phages and phytomedicines. Other investigations suggest that some of these non-antibiotic therapeutic agents alone or in combination with antibiotics are highly effective against MDR *P. aeruginosa* strains, indicating that non-antibiotic antimicrobial agents in future may play a significant role in the management of *P. aeruginosa* infections[10]. Probiotics are defined by world health organization (WHO) as "living micro-organisms which, when administered in adequate amounts confer ahealth benefit to the host"[11]. They are viable, nonpathogenic microorganisms that alter the micro-flora of the gastrointestinal tract (GIT) of the host, yielding a positive influence on health and body physiology[12]. Lactobacilli are well studied gut propiotic as they can provide several health promoting effects as they enhance host immune functions and provide protection against enteric pathogens through secreted acids, bacteriocins and other by-products that exert anti-microbial effects. They can also inhibit the growth of enteric pathogens such as *Eschericcvhia coli, Salmonella typhi* and *Shigella* species[13]. *Lactobacillus plantarum* has an important antipathogenic capacity on *P.aeruginosa*. It is able to reduce the viability of *P. aeruginosa* in both planktonic and biofilm forms. It also interferes with normal functioning of *P. aeruginosa* quorum sensing system that inhibits both biofilm and virulence factor (elastase, pyocyanin, and rhamnolipids) formation. Anti-pathogenic properties mentioned above, together with the immunomodulatory, tissue repair, and angiogenic properties of *L. plantarum*; show that it's use in the treatment of infected chronic wounds is highly feasible.[14]

The aim of this work is to study the effect of *L. plantarum* on the antibiotic sensitivity of isolated *P. aeruginosa* strains.

METHODOLOGY

This study was carried out in Medical Microbiology and Immunology Department, Faculty of Medicine, Tanta University on 40 patients admitted, during the period of research (between February 2017 and August 2017), to the Inpatient Surgical Units. 20 cases from Burn Unit ,10 cases from Vascular Unit, 3 cases from GIT Unit, 2 cases from I.C.U and 5 cases from Outpatient Surgical Units of Tanta University Hospitals. All specimens were pus that was collected from surgical site infection and out patients different wound infection.

Two swabs were introduced deeply into depth of the wound and soiled by wound disharge. All samples were cultured on ordinary media (Nutrient agar), Differential media (MacConkey's agar) then blood agar and incubated aerobically at 37^0c for 24 hours.

- **Isolation and identification of *Pseudomonas aeruginosa* strains**:

Suspected colonies were identified as *P. aeruginosa* according to the standard microbiological techniques which included:[15] microscopic appearance of Gram negative bacilli, pigment production on nutrient agar ,characteristic fruity aroma, growth as non-lactose fermenting pale yellow colonies on MacConkey's agar and oxidase test positive.

- **Preparation of Lactobacillus plantarum strain:**

L. plantarum was grown on blood agar at 37°C in candle jar over night. The *L.plantarum* colonies were very minute alpha haemolytic colonies, gram positive bacilli and catalase test negative.

All isolated *Pseudomonas aeruginosa* strains were subjected to:

- Testing antibiotic sensitivity of *Pseudomonas aeruginosa* strains before and after addition of *L.plantarum* using Modified Kirby-Bauer disk diffusion method[16]

Direct colony suspension method

- A single colony from the identified *P. aeruginosa* isolates was added to two test tubes containing 1ml BHI broth with and with out addition of 4 colonies of *L. plantarum*. All The tubes were incubated overnight at 37˚c aerobically.
- After incubation for 24 hours, each mixture was spread on MacConkey agar plates by using sterile inoculating loop, then the plates were incubated over night at 37˚c aerobically.
- From a fresh pure (24 hours culture), a direct colony suspension equivalent to 0.5 McFarland standard was made from all plates.

Disc diffusion method:

- A sterile swab was immersed in tested organism's suspension (*P. aeruginosa* alone and *P. aeruginosa* after addition of *L . plantarum*) and excess fluid was removed by pressing and rotating the swab against the side of the tube above the level of the suspension.
- The surface of Muller-Hinton agar plate was swabbed three times; rotating the plate approximately 60 degrees each time to ensure an even distribution of inoculums.
- The selected antibiotic discs were evenly distributed on the inoculated plate.
- Within 30 minutes of applying the discs, the plate was incubated aerobically at 37˚c. After 24 hours incubation, each plate was examined.
- The diameters of zones of inhibition were measured, including the diameter of the disc, zones were measured to the nearest whole millimeter,

using a ruler which is placed on the back of the inverted Petri dish.

Interpretation of results:

Using the (CLSI., 2016) interpretative criteria, the zone diameters of each antimicrobial were interpreted as susceptible (s), Intermediate (I), and resistant (R) categories for selected antimicrobial discs. (Aztreoneme (30µg), Colistin sulphate (10µg), Ciprofloxacin (5µg), Sulpha methoxazole trimethoprim (25µg), Piperacillen (100µg), Piperacillen tazobactam (100µg/10µg), Gentamycin (10µg), Imipeneme (10µg).

RESULTS

Pseudomonas aeuroginosa was the commonest organism isolated (50%) as shown in (table 1). Males were more common than females and age group from 40-60 years are the predominant age group as shown in (table 2). The majority of cases (100%) were sensitive to colistin sulphate followed by imipenem (70%) followed by ciprofloxacin (65%). *P. aeuroginosa* susceptibility pattern to different antimicrobial agents significantly increased after addition of *L.plantarum* (P value <0.01*). *P. aeuroginosa* susceptibility to pipracillin highly increased after addition of *L. plantarum* (100% of cases were sensitive to pipracillin after addition of *L. plantarum* compared to 20% of cases before it' saddition*)* followed by pipracillin-tazobactam which increased (from 60% to 100%) as shown in (table3 and Fig 1).

Table 1: Causative organisms isolated from 40 patients included in this study

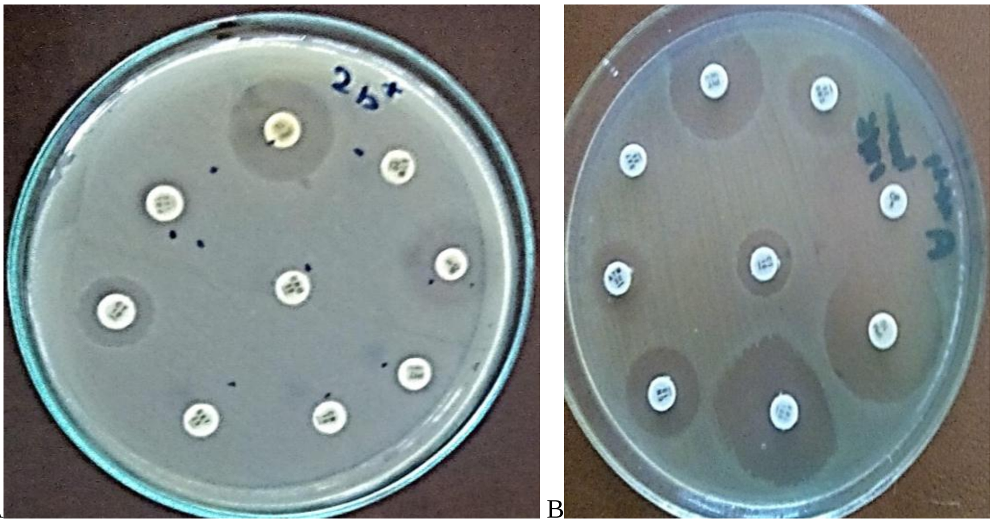
Causative organism	No. Of cases	%
Pseudomonas aeruginosa	15	37.5%
Klebsiella pneomonae	8	20%
Escherichia coli	6	15%
Mixed:	**(5)**	**(12.5%)**
P.aeruginosa & K. pneomonae	2	5%
P. aeruginosa & E. coli	2	5%
P.aeruginosa & Acinetobacter	1	2.5%
Saphylococcus aureus	4	10%
Acinetobacter	2	5%

Table 2: The relation between age, sex and *Pseudomonas aeuroginosa* infection

Sex	Age Range	No	%	Mean ± SD	P value	
Male 15 (75%)	3-20	4	20	8.3 ± 6.57	P1	0.00023
	20-40	3	15	40 ± 0		
	40-60	8	40	54 ± 8.24	P^2	0.00965
Female 5 (25%)	3-20	2	10	9.5 ±.77	P1	-
	20-40	0	0	0		
	40-60	3	15	64 ± 1.73	P^2	0.0028
Total		20	100			

Table 3: Comparison between antimicrobial susceptibility pattern of *P.aeuroginosa* isolates to different antimicrobial agents before and after addition of *L.plantarum*

Antimicobials	Sensitive cases				Intermediate cases				Resistant cases				P value
	Before		After		Before		After		Before		After		
	No	%	No	%	No	%	No	%	No	%	No	%	
Aztreonam	6	30	10	50	1	5	5	25	13	65	5	25	0.0010* (<0.01*)
Colistin sulphate	20	100	20	100	-	-	-	-	-	-	-	-	-
Ciprofloxacin	13	65	18	90	5	25	2	10	2	10	-	-	0.002* (<0.01*)
Pipracillin	4	20	20	100	9	45	-	-	7	35	-	-	0.0000007 *(<0.0٠1*)
Pipracillin-tazobactam	12	60	20	100	7	35	-	-	1	5	-	-	0.0017* (<0.01*)
Gentamicin	7	35	12	60	3	15	2	10	10	50	6	30	0.004* (<0.01*)
Imipenem	14	70	19	95	4	20	1	5	2	10	-	-	0.007* (<0.01*)
Sulphamethoxazole/Tri methoprim	-	-	-	-	-	-	-	-	20	100	20	100	-



A B

Fig. 1: Antibiotic sensitivity of *P .aeuroginosa* before and after addition of *L. plantarum.* (A) Antibiotic sensitivity of *P .aeuroginosa* before addition of *L. plantarum*. (B) Antibiotic sensitivity of *P .aeuroginosa* after addition of *L. plantarum*

DISCUSSION

In this study a total of 40 wound swabs were investigated . The predominance of *P. aeruginosa* in isolates recovered from wounds of patients seeking care in Out Patient and In Patients Surgical Units of Tanta University Hospitals was reported. An overall incidence of *P. aeruginosa* was 20 cases (50%) followed by *K. pneumoniae* (8 cases, 20%), *E. coli* (6 cases,15%) , *S. aureus* (4 cases, 10%), *Acinetobacter* (2 cases , 5%) . This incidence is more or less similar to that reported in an Egyptian study reported that the most common organism isolated from wounds was *P.aureginosa* (49%) followed by *S. aureus* (21%) and *Klebsiella* (15%).[17]On the other hand , an egyptian study reported that the commonest isolated organism from wounds was *S.aureus* (50%), followed by Enterobacteriaceae (37.5%) and *P.aeruginosa* (25%) came in the third place.[18]

Also, two Indian studies reported that *S. aureus* was the most predominant organism isolated from wounds representing (29%) and (50.4%) respectively.[19] This discrepancy in results may be explained by difference in the period of time in which the different studies were done , difference in the transport of samples and number of cases and to the difference between countries . When factors such as age and sex of the patient were considered, it was found that out of the 20 clinical cases infected with *P. aeuroginosa* , 15 cases were males (75%) and 5 cases were females (25%)and the patients age ranged from 3 to 60 years with predominance of old age; patients in the age group 40-60 years represent 55% of study group and this was statistically significant (p<0.01*) .

Mahmoud et al[20] reported similar results showing that *P. aeruginosa* infections were common in males (66.7%) than female (33.3%) and the mean age was 25.8 years. This may be attributed to that the males are more exposed to burns and wounds due to their nature of work . On the other hand an europian study reported that *P. aeruginosa* infections were more common in female than in male and the mean age was 32 years.[21] This discrepancy may be explained by difference in the place where samples taken and type of patient predominant in this place. Concerning the results of antimicrobial susceptibility of *P. aeruginosa* isolates it was found that colistin sulphate was effective in all isolates(100%) sensitivity followed by imipenem (70%) ,ciprofloxacin (65%) , pipracillin –tazobactam (60%) , aztreonam(30%), gentamicin (35%) and pipracillin (20%) while all isolates were resistant to sulphamethoxazole /trimethoprim as *P.auroeginosa* is entrinsically resistant to sulphamethoxazole/ trimethoprim. The results of this study agree more or less with the study by Mohamed[17] reported that *P. aeruginosa* isolates were most sensitive to Colistin sulphate (84%), followed by amikacin (39%) and imipenem (35%).[17]

While an Egyptian study found that *P. aeruginosa* isolates were most sensitive to Imipenem (100%), Meropenem (100%) ,Gentamicin (100%) and Amikacin (100%) followed by Ceftazidime (95%), Levofloxacin (90%), Piperacillin (90%), Amoxy/ clavulanic acid (20%), Cephalexin (10 %) and Sulfa/trimethoprim (5 %).[22]

Additionally, an Indian study reported that, *P. aeruginosa* was most sensitive to pipracillin – tazobactam (89.71%), followed by imipenem (88.24%), pipracillin (69.12%), and gentamicin (30.88%)[19.] All these studies showed different sensitivity pattern compared to our study and this may be attributed to many factors like, different antibiotic policies , different infection control measures or the presence of other underlying diseases that may be associated with the isolation of different antibiotic resistant bacteria and the emergence of resistant strains due to emprical use of antibiotics.

Concerning the invitro effect of *L. plantarum* on *P. aeuroginosa* isolates, our study showed that *P. aeuroginosa* susceptibility pattern to different antimicrobial agents significantly increased after addition of *L. plantarum* (P value <0.01*). *P. aeuroginosa* susceptibility to pipracillin highly increased after addition of *L. plantarum* (100% of cases were sensitive to pipracillin after addition of *L. plantarum* compared to 20% of cases before addition of *L. plantarum*) followed by pipracillin-tazobactam which increased from 60% to 100%followed by imipenem increased from 70% to 95% followed by ciprofloxacin increased from 65% to 90% followed by gentamicin which increased from 35% to 60% followed by aztreonam which increased from 30% to 50%. The results of our study agree with a chinese study reported that the antibiotic susceptibility zone of multiple drug resistant *P. aeruginosa* had increased after *L. plantarum* addition such as meropenem, pipracillin-tazobactam, aztreonam, and ceftazidime , but it wasn't statistically significant[23] , this may be related to many factors such as environmental factors (flora quantity, PH, temperature, in vivo and in vitro, etc.), length of time of probiotics intervention, and the concentration of probiotics used. As different doses of *L. plantarum* may contribute to different effects on the bacterial resistance pattern; however, further investigation is needed to clarify this issue as there is ashortage of research concerning this part.

Additionally an Indian study found that susceptibility pattern to different antimicrobial agents like amikacin, meropenem, aztreonam and ciprofloxacin increased after addition of probiotic like Lactobacillus rhamnosus and *Lactobacillus acidophilus* [24]

CONCLUSION

P. aeruginosa was the most predominant bacterial isolate in the studied wound infections with a higher incidence in males than females and apredominance in old age. *P. aeruginosa* susceptibility pattern to different antimicrobial agents significantly increased after addition o f *L. plantarum.* It is possible that in near future many more alternative medicines will reach into the clinics and will be used for the treatment of *P. aeruginosa* infections.

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