ISSN: 0067-2904

Synergistic Effect of Linezolid, Tigecycline, and Vancomycin on *Staphylococcus Aureus* Isolated From Iraqi Patients with Diabetic Foot Ulcers

Hayder H. Hasan Al-hamedawy*, Suhad S. Mahmoud
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

Abstract

Ninety nine swabs were collected from patients with diabetic foot ulcers (DFU), all swabs were cultured on different selective media for screening, 46 isolates confirmed as *S. aureus* by API staph. The results of antibiotic susceptibility test revealed that all isolates were resistant to metronidazole, 34 isolates were resistant to cefoxitin, ceftriaxone, and meropenim, 23 isolates were resistant to ciprofloxacin and norfloxacin, 17 and 16 isolates were resistant to tetracycline and trimethoprim, respectively; while all isolates were sensitive to tigecycline. The results of minimum inhibitory concentration (MIC) that carried out by using vancomycin, tigecycline and linezolid for 8 isolates, MIC results were1-2 µg /ml, 0.25-0.5 µg /ml, 4 µg /ml, respectively; 4 isolates were selected according to their aggressive antibiotic resistance to test the antibiotics` combinations effects, the combination of vancomycin/ tigecycline presented promising results against *S. aureus* infections at low concentrations.

Keywords: *Staphylococcus aureus*, diabetic foot ulcer DFU, Synergistic effect, MDR.

التأثير التأزري للينزوليد ، تجيساكلين، و الفانكومايسين على البكتيريا العنقودية الذهبية المعزولة من مرضى تقرح القدم السكري

حيدر حمود حسن الحميداوي*،سهاد سعد محمود
قسم التقنيات الاحيائية ، كلية العلوم ، جامعة بغداد، بغداد، العراق

الخلاصة

تم جمع تسعة وتسعون مسحة من المرضى المصابين بتقرح قدم السكري. كل المسحاة تم تنميتها على مختلف الاوساط الانتقائية لا جل التحري عن الانواع البكتيرية الموجودة.ستة واربعون عزلة بكتيرية شخصت على انها بكتريا *S. aureus* باستخدام عدة Api staph .اظهرت نتائج اختبار الحساسية للمضادات الحياتية بأن كال العزلات كان مقاومة للمترندزول ،34 كانت مقاومة للسيفوكستين , سيفوترياكسون وميروبينيم, ،23عزلة كانت مقاومة لل سبروفلاكساسين ونورفلوكساسين ,17و 16 عزلة كانت مقاومة للتتراسايكلين و ترايمثبريم على توالي .بينما كانت جميع العزلات حساسة للتيجيساكلين. كذلك اظهرت نتائج اختبار الاحد الادنى للتركيز المثبط الذي نفذ باستخدام المضادات فانكومايسين ،تيجيساكلين، ولانزوليد على ثمان عزلات،وكانت النتائج ،µg /ml 1−2 ،µg /ml 0.25−0.5 ،µg /ml 4 على التوالي،(اربعة

*Email: haydarhmood@yahoo.com

عزلات تم اختيارها بسبب مقاومتها العالية للمضادات لاختبار التأثيرالمشترك للمضادات، الحمع بين الفانكومايسين والتيجيساكلين قدم نتائج واعدة ضد البكتيريا العنقودية الذهبية بتراكيز منخفضة.

Introduction

Staphylococcus aureus is a commonly reported pathological bacterium among diabetic foot ulcer (DFU) infections, approximately 40–50% of all *S. aureus* isolates resistance to beta-lactam and wide range of antimicrobial drugs, so that there are persistent need to develop new effective drug against such resistant bacterial infections. This pathogen presents many treatment difficulties, particularly in the provision of appropriate empiric antimicrobial therapy. [1]

Treatment guidelines have recommended empiric anti-Staphylococcal coverage for all patients with a DFI. [1, 2].

There are many complications with diabetes, Over time diabetes that can damage the heart, blood vessels, eyes, kidneys and nerves, and increase the risk of heart disease and stroke [3].Such damage can result in reduced blood flow, which – combined with nerve damage (neuropathy) in the feet – increases the chance of foot ulcers, infection, and the eventual need for limb amputation [4]. According to the previous reports there is a relationship between the Diabetes mellitus and both postoperative infections and nosocomial infections, 2.7-fold increase in nosocomial infection rate when blood glucose levels reach to more than 220 mg/dL.[5] Endogenous glycation (is the result of the covalent bonding of a sugar molecule, such as glucose or fructose, to a protein or lipid molecule, without the controlling action of an enzyme. Glycation may occur either inside the body (endogenous glycation) or outside the body (exogenous glycation))[6]. Endogenous glycation from elevated blood glucose leads to cellular dysfunction in soft tissues that must heal after surgery and decreased immune response, both cellular and humeral, thus compromising a host's ability to prevent intraoperative contamination from developing a surgical site infection [4].

Combined antimicrobial therapy may be prescribed for certain indications including: giving a broad spectrum of activity in empirical therapy, especially in high-risk situations such as neutropenic sepsis, [7] ,To treat mixed infection if one drug does not cover all possible pathogens, To achieve a synergistic effect, thus increase efficacy but decrease the dose required of each drug (and thus decrease the risk of side effects), To decrease the probability of the emergence of drug resistance and to restore or extend the spectrum of activity by including an enzyme inhibitor. [7].

Previous studies found synergistic effect when they used combination of triterpenoids with antibiotics, reduction in MIC of cefradine with oleanolic acid indicates their potential use against MRSA. [8]

Another study found synergistic effect when rutin, morin and quercetin were used in combination. Test bacteria responded to ampicillin, amoxicillin, cephradine, methicillin and ceftriaxone when these antibiotics were mixed with flavonoids. Similarly, imipenem activity was further increased against test MRSA strains when combined with flavonoids [9].

So this study aimed to develop new antibiotics combinations to treat multidrug resistant *staph aureus* bacteria to avoid side effects of high-dosage and ensure no resistance development in future usage.

Methodology

1.specimen Collection and bacterial identification:

Ninety-nine swabs were taken -by transport media- from diabetic foot patients, eighty nine of them were hospitalized; 50 patients in Al-Imam Ali general hospital, 25 in Al Kindi General Teaching Hospital, 14 in Al-shaheed alsader hospital, and 10 were out-patients (non-hospitalized). (From October 2016- April 2017)

The collected swabs were cultured on Brain Heart agar (BHA) and mannitol salt agar (MSA) for 24 hr. Among 99 swabs only 89 isolates were grown on BHA.at same time growth was appear in (84) plates on MSA (53were able to ferment mannitol aerobically while 31 were mannitol non-fermented), 15 plates recorded without growth on (MSA) because MSA is selective media for *Staphylococcus* spp. The result of biochemical test confirmed by API staph kit.

2.Antibiotic susceptibility test:

This test was performed according to Kirby-Bauer method [10] using a group of antibiotics (Cefotaxim 30 µg / Disc, Ceftriaxone 30 µg / Disc, Cefoxitin 30 µg / Disc, Ciprofloxacin 5 µg / Disc, Erythromycin 15 µg / Disc, Meropenim 10 µg / Disc, Metronidazole 5 µg / Disc, Norfloxacin 10 µg /

Disc, Tetracycline 30 µg / Disc, Tigecycline 15 µg / Disc, Trimethoprim 5 µg / Disc, Vancomycin 30 µg / Disc) (manufactured by mast group UK).

3. Determination of minimum inhibitory concentrations (MICs) methods:[11]

3.1. Preparation of inoculum:

Inoculum concentration (5 $*10^5$cfu/ml) was prepared by mixing it with an equal volume of antimicrobial solution(1*10 6 cfu/ml) in wells .

3.2 Preparation of antibiotics stock solutions and culture:

Stock solutions were prepared by dissolving the antibiotic powders (Vancomycin and Tigecycline) in distilled water or DMSO solution for linezolid.Working solutions were also prepared according to dilution law (C1V1=C2V2).100 µl from the working solution were Placed into each microtiter plate well in first column (column 1) which already contains 100 µl of sterile Mueller Hinton broth.(Here the wells in column 1 contained 200 µl of Mueller Hinton broth with double concentration antibiotic).100 µl from each well in first column (column 1) were taken by multi-channel pipette ,and placed into the wells of the second column (column 2) which already contained 100 µl sterile Mueller Hinton broth .then 100 µl from each well in second column (column 2) were taken by a multi-channel pipette ,and placed into the wells of the third column (column 3) Which already contained 100 µl sterile Mueller Hinton broth, and so on to the ninth column. (Serial dilution method). The tenth column didn't contain antibiotic to be considered as positive control , while the eleventh column contained only sterile Mueller Hinton broth consider as negative control, Column 12 contained 100µl (DMSO) with 100µl bacterial inoculum to demonstrate that the solvent did not have anti-bacterial efficacy. The last step was adding 100 µl of bacterial inoculum with concentration (1*10 ^{6} cfu/ml) to all wells .At the end, we obtained concentration (5*10^5 cfu/ml) bacterial inoculum, the required antibiotic concentration in nine columns, and positive, negative controls in addition to the solvent control columns.

3.4. Incubation and results reading:

All plates were incubated at 37 °c for 18-24 hr. in aerobic conditions.

After incubation, the plates were read by the ELASA reader, on wavelength 630 nm** to Investigate growth in wells , The least concentration of antibiotic that able to inhibit bacterial growth its considered MIC

4. Determination of combination effect of antibiotics (checkerboard titration technique): [12]

4.1. Preparation of microdilution plates:

In checkerboard (combination experiments) technique two micro titer plates were used to prepare the serial dilutions of antibiotics.

The first plate was loaded with 100 µl of sterile Mueller Hinton broth in all wells,100 µl from the first antibiotic with eight times concentration (the highest concentration required) were added to all wells in the first column (column 1) and mixed well,(e.g. the highest concentration of vancomycin was 8 µg/ml should be added 64 µg/ml , the highest concentration of Tigecycline was 2 µg/ml shuold be added16 µg/ml , the highest concentration of linezolid was 16 µg/ml should be added 128 µg/ml) ,100 µl from (column 1 wells) were taken by a multi-channel pipette and transferred into(column 2 wells) , then 100 µl from (column 2 wells)were transferred into(column 3wells), so on to the (column 8).

The second plate was loaded with 50 µl sterile Mueller Hinton broth in all wells,50µl from the second antibiotic with eight times concentration(the highest concentration required) were added to all wells in first eight wells from the first raw (raw A) and mixed well . 50 µl from (raw A wells) were taken by a multi-channel pipette and transferred horizontally into (raw B wells), then 50 µl from (raw B wells) were transferred into (raw C wells), and so on to the last raw (raw H). Here we would obtain serial dilution for antibiotic solution, horizontally and not, as usual, vertically, after preparation of serial dilutions for both antibiotics in both plates, 50 µl from wells in first plate were transferred into the same wells in the second plate. This meant 50 µl had been taken from A1 well in first plate and put into A1 well in second plate. Column 9 remained empty. The column 10 contained just broth and bacterial inoculum as positive control. Column 11 contained DMSO, the column 12 contained just media as negative control. 100µl Bacterial inoculum with concentration (1*10^6 cfu/ml) was added to all wells except (columns 9, 12) ,and mixed well. All plates were incubated over night at 37 °c in aerobic conditions after incubation the plates were read by the ELASA reader, on wavelength 630 nm to observe growth in wells.

4.2. Calculations: [12]

1. Calculate the FIC for each antibiotic as follows:

$$\text{FIC for antibiotic A} = \frac{\text{MIC of antibiotic A in combination}}{\text{MIC of antibiotic A alone}}$$

$$\text{FIC for antibiotic B} = \frac{\text{MIC of antibiotic B in combination}}{\text{MIC of antibiotic B alone}}$$

2. Calculate the summation of FIC (ΣFIC) index for each combination as follows:

$$\sum \text{FIC} = \text{FIC for antibiotic A} + \text{FIC for antibiotic B}$$

4.3. Interpretation:

Interpretation of the summation is as follows:

If ΣFIC is ≤0.5 then the relationship between antibiotics is "Synergism"

If ΣFIC is (>0.5 and≤4) then the relationship between antibiotics is "Indifference"

If ΣFIC is >4 then the relationship between antibiotics is "Antagonism"

Results and Discussion:

-Sample collection and identification

The results showed that among 99 swabs only 89 isolates were grown BHA. Growth was appeared in 84 plates on MSA (53were able to ferment mannitol aerobically while 31 were mannitol non-fermenter), 15 plates recorded without growth on MSA because it is selective media for *Staphylococcus* spp., To ensure biochemical identification further characterization preformed using API staph kit , 46 isolates confirmed as *Staphylococcus aureus*, 2 isolates were *S. xylosus*, while 5 isolates were non-differentiated which could be *Enterococcus* or *Micrococcus* that share many biochemical characters.

-Antibiotics susceptibility results:

All results of Antibiotics susceptibility test were listed in Table-1

Table 1-Antibiotics susceptibility test results:

Antibiotic	Resistant (%)	Sensitive (%)	Intermediate (%)
Cefoxitin (30 µg)	34 (*MRSA)(73.9%)	12 (MSSA)(26.1%)	-----
Ciprofloxacin (5 µg)	23(50%)	21(45.6%)	2(4.4%)
Norfloxacin (10 µg)	23(50%)	23(50%)	------
Trimethoprim (5 µg)	16(34.8%)	25(54.3%)	5(10.9%)
Tetracycline (30 µg)	17(36.9%)	25(54.3%)	4(8.8%)
Vancomycin (30 µg)	3(6.5%)	43(93.5%)	--------
Tigecycline (15 µg)	----------	46(100%)	---------
Erythromycin (15 µg)	15(32.6%)	13(28.3%)	18(39.1%)
Ceftriaxone (30 µg)	34(73.9%)	12 (26.1%)	-----
Cefotaxim (30 µg)	34 (73.9%)	12(26.1%)	-----
Metronidazole (5 µg)	46(100%)	------	------
Meropenim (10 µg)	34(73.9%)	12 (26.1%)	-----

-Minimum inhibition concentration (MIC) results:

All results of minimum inhibitory concentration test were listed in Table-2

Table 2-Antibiotics Minimum inhibition concentration (MIC)

Isolate No.	Tigecycline MIC (µg /ml)	Vancomycin MIC (µg /ml)	Linezolid MIC (µg /ml)
2	0.5	2	4
7	0.25	2	4
8	0.25	2	4
25	0.25	2	4
31	0.25	1	4
62	0.25	1	4
64	0.25	1	4
88	0.25	1	4

- Antibiotic combination effect result:

Antibiotics combination effect was tested by microtiter plate assay for 4 isolates (isolate number 2, 7, 8 and 31) selected according to their multidrug resistance patterns, each isolates were run as triplicate (each concentration run 3 times to take the average result).The combination of vancomycin and tigecycline results for isolate No.2 showed in Table-3:

*****(The result of isolate number 2 will show only a brief summary of the place and the rest of the results will be similar)

Table 3-Combination effect of Tigecycline & Vancomycin on isolate No.2. (-) = no growth, (-) = the lowest concentrations inhabit growth, (+) = growth,*s= Synergism, I = Indifference

2	Tigecycline							
Vancomycin	Antibiotics Concentration	2	1	0.5(MIC)	0.25	0.125	0.065	0.032	0.16
	8	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	2(MIC)	-	-	-	-	-	-	-	-I
	1	-	-	-	-	-	-	-I	+
	0.5	-	-	-	-	-	-	- *s	+
	0.25	-	-	-	-	-	-*s	+	+
	0.125	-	-	-	-I	-*s	+	+	+
	0.065	-	-	- I	+	+	+	+	+

Synergism illustrated in Table-3 in isolate No.2 documented 3 combinations after reading by ELISA reader device at concentration (VA/TGC) were: 0.125/0.125, 0.25/0.065 and 0.5/0.032 µg/ml, this concentration considered as synergism according to combination equation as follows: FIC (VA) = $\frac{0.125}{2}$ = 0.0625 fractional inhibitory concentration of vancomycin.

FIC (TGC) = $\frac{0.125}{0.5}$ = 0.25 fractional inhibitory concentration of tigecycline.

Σ FIC =0.0625+0.25, FIC= 0.312, by applying combination standard value of checker board titration [7] as follows:

Interpretation of the summation is as follows:
Synergism =ΣFIC is ≤0.5, Indifference =ΣFIC is >0.5 and≤4, Antagonism =ΣFIC is >4.
On mathematical logic: 0.312 <0.5 so it considers synergism, on biological and experimental concept: wells contained synergistic concentration confirmed by transporting liquid culture to BHB to ensure week growth level. Other concentration applied by the same method.

The result of third combination included linezolid and tigecycline represented in Table-4 shows that there are 2 synergism detected based upon combination standard value of checker board titration [7].

Table 4- combination effect of Tigecycline & linezolid on isolate No.2

2	Tigecycline							
Linezolid	Antibiotics Concentration	2	1	0.5(MIC)	0.25	0.125	0.062	0.031	0.016
	16	–	–	–	–	–	–	–	–
	8	–	–	–	–	–	–	–	–
	4(MIC)	–	–	–	–	–	–	–	- I
	2	–	–	–	–	–	–	- I	+
	1	–	–	–	–	–	-*s	+	+
	0.5	–	–	–	–	-*s	+	+	+
	0.25	–	–	–	- I	+	+	+	+
	0.125	–	–	- I	+	+	+	+	+

Combination results of vancomycin and linezolid showed in Table-5:

Table 5-combination effect of Vancomycin& linezolid on isolate No.2, (-) = no growth, (-) = the lowest concentrations inhabit growth, (+)= growth, *s= Synergism, I=Indifference

2	Vancomycin							
Linezolid	Antibiotics Concentration	8	4	2(MIC)	1	0.5	0.25	0.125	0.062
	16	–	–	–	–	–	–	–	–
	8	–	–	–	–	–	–	–	–
	4(MIC)	–	–	–	–	–	–	–	- I
	2	–	–	–	–	- I	- I	- I	+
	1	–	–	–	- I	+	+	+	+
	0.5	–	–	–	- I	+	+	+	+
	0.25	–	–	- I	+	+	+	+	+
	0.125	–	–	- I	+	+	+	+	+

In applying combination equation, there are no significance value <0.5 in all used concentration (FIC (VA) +FIC(LIN)), which explained there are no synergistic effect between both antibiotics.

Discussion:

The best combination procedure was the combination of vancomycin and tigecycline at the 3 concentration on all 4 isolates (each isolate is triplicate), the second combination work efficiently linezolid and tigecycline at two concentration. The first combination target to sites (vancomycin target cell wall synthesis and tigecycline targets 30s ribosomal subunit) while the second successful combination (linezolid/tigecycline) targets (linezolid target protein translation while tigecycline 30s

ribosomal subunit). In addition to that's tigecycline, linezolid and vancomycin their working point decreased after combination which give advantage to avoid side effect of high dose in addition to avoid selective pressure made by high concentration of used antimicrobial agents.

Hamza and his coworker in 2016 found synergistic effect when they used combination of triterpenoids with antibiotics, reduction in MIC of cefradine with oleanolic acid indicates their potential use against MRSA. [8]

Muhammad and his coworker in 2015 found synergistic effect when rutin, morin and quercetin were used in combination. Test bacteria responded to ampicillin, amoxicillin, cephradine, methicillin and ceftriaxone when these antibiotics were mixed with flavonoids. Similarly, imipenem activity was further increased against test MRSA strains when combined with flavonoids [9].

Conclusions:

In this study the combination of Vancomycin with Tigecycline had clearly a synergistic effect against *S. aureus,* and to a lesser extent linezolid with Tigecycline. While the combination of vancomycin with linezolid have not synergistic effect against *S. aureus*

Acknowledgements:

Special thanks to the staff of Al-imam Ali, Al Kindi, Al-shaheed alsader, and alrashad hospitals, for their help and cooperation.

References

1. Lipsky, B.A., Berendt, A.R., Cornia, P.B., Pile, J.C., Peters, E.J., Armstrong, D.G. **2012.** infectious diseases society of america clinical practice guideline for the diagnosis and treatment of diabetic foot infections. *J Am Podiatr Med Assoc*., **103**(1): 2–7. PMID: 23328846

2. Reveles, R, Bryson M., Robert J., and Elizabeth O. **2016.** "Epidemiology of Methicillin-Resistant Staphylococcus Aureus Diabetic Foot Infections in a Large Academic Hospital : Implications for Antimicrobial Stewardship"1–8.

3. Sarwar, N, Gao, P., Seshasai, S.R., Gobin, R., Kaptoge, S., Di Angelantonio, E., Ingelsson, E., Lawlor, D.A., Selvin, E., Stampfer, M., Stehouwer, CD., Lewington, S., Pennells, L., Thompson, A., Sattar, N., White, I.R., Ray, K.K. and Danesh, J. **2010.** "Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies". *Lancet.* **375**(9733): 2215–22. doi: 10.1016/S0140-6736(10)60484-9. PMC 2904878. PMID 20609967.

4. Colombier, S., Kessler, U., Ferrari, E., von Segesser, L.K., Berdajs, DA. **2013.** Influence of deep sternal wound infection on long-term survival after cardiac surgery. *Med Sci Monit*., **19**: 668–673.

5. Waldrop**,** R., McLaren, A., Calara, F. and McLemore, R. **2014.** Biofilm Growth Has a Threshold Response to Glucose in Vitro. *Clinical Orthopaedics and Related Research*, **472**(11): 3305–3310. http://doi.org/10.1007/s11999-014-3538-5

6. Ahmed, N. and Furth, A.J. **1992.** "Failure of common glycation assays to detect glycation by fructose". *Clin. Chem*. **38**(7): 1301–3. PMID 1623595

7. Wiffen, et. al. **2012.** *Oxford Handbook of Clinical Pharmacy*. 2ed Ed., Oxford University Press page 420.

8. Hamza, M., Nadir, M., Mehmood, N. and Farooq, A. **2016.** In vitro effectiveness of triterpenoids and their synergistic effect with antibiotics against Staphylococcus aureus strains. *Indian Journal of Pharmacology*, **48**(6): 710–714. http://doi.org/10.4103/0253-7613.194851

9. Muhammad Usman Amin, Muhammad Khurram2, Baharullah Khattak and Jafar Khan **2015.** Antibiotic additive and synergistic action of rutin, morin and quercetin against methicillin resistant Staphylococcus aureus. *BMC Complementary and Alternative Medicine***,** **15**: 59 DOI 10.1186/s12906-015-0580-0.

10. Morello, J., Mizer, H. and Granato, P. **2006.** *Laboratory Manual and Work Book in Microbiology*. 8th ed. McGraw Hill, London. **37**: 632-687.

11. Hasselmann, C. **2003**. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution, 1–7.

12. Verma, P. **2007**. *Methods for Determining Bactericidal Activity and Antimicrobial Interactions: Synergy Testing, Time-Kill Curves, and Population Analysis*, CRC Press : 275–298.