RESEARCH ARTICLE

Genetic identification of methicillin resistant Staphylococcus aureus (MRSA) isolated from diabetic foot ulcers and evaluating the inhibition activity of reuterin against this bacteria

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Abstract

Objective: To genetically diagnose the isolates of methicillin-resistant staphylococcus aureus taken from patients with severe diabetic foot infections, and to test the inhibitory effect of reuterin on the growth of these bacteria.

Method: The experimental study was conducted from June to November 2021 at the Diabetes Unit of Al-Faihaa General Hospital, Basrah, Iraq, and comprised 62 foot ulcer swabs from the necrotic lesions of patients with type 2 diabetes. The swabs were cultivated first in brain heart infusion broth media, and then streaked on Mannitol salt agar and staphylococcus chromogeneic agar media for phenotypic and genetic analysis. The genetic identification of bacteria was confirmed by deoxyribonucleic acid extraction, and methicillin-resistant staphylococcus aureus was confirmed by the presence of plasmid mecA gene. The inhibition activity of reuterin towards methicillin-resistant staphylococcus aureus was determined using the minimum inhibitory concentration test. All data was analyzed with SPSS version 23.

Results: A total of 62 swabs were taken from the necrotic lesions of type 2 diabetes mellitus (T2DM) patients with diabetic foot ulcers. Of the total isolates, 9(14.5%) gave mauve to purple colour on staphylococcus chromogeneic agar, which was then genetically confirmed as methicillin-resistant staphylococcus aureus. The minimum inhibitory concentration value of these bacteria was 10µl/ml at 16mm inhibition zone diameter. There was no cytotoxicity of reuterin to human red blood cells.

Conclusion: Reuterin was found to be a natural antimicrobial substance suitable for use to disinfect diabetic foot wounds from bacterial contamination and infection, especially those caused by methicillin-resistant staphylococcus aureus.

Key Words: Methicillin, Staphylococcus aureus, Agar, Diabetic, hydroxypropionaldehyde, Microbial Sensitivity, Mannitol, DNA, Erythrocytes, Plasmids, Brain

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Introduction

Diabetic foot infections (DFIs) are inflammation of the thin tissues or bones located beneath the malleoli. They usually start with skin ulcers in about 25% of cases, spreading contiguously from the skin to the deep subcutaneous tissue and/or bone. These infections are often complicated by contagion microbial pathogens, and staphylococcus (S.) aureus is the most causative factor which is associated with previous antibiotic treatments and a long recovery time[1]. The need for amputation and surgical debridement increases in patients infected with this bacteria which may be minor or significant. The prevalence of methicillin-resistant S. aureus (MRSA) strain in DFIs is 15-30% and there is a worrying trend of increase in several countries, with some studies identifying new strains of MRSA distinctive by the presence of mecA gene that confers resistance to methicillin antibiotic. These strain also have mobile genetic elements (MGEs) that are composed of 15-25% of the genome, including plasmids, transposons, pathogenicity islands, prophages and integrons. An undestanding of MGEs is essential to increase human knowledge on the genetic drivers of antibiotic resistance (AR)[2].

S. aureus is a gram-positive bacteria and facultative anaerobe occuring naturally on the human skin and in the nasal passages. This bacterium is toxic, and one of the effects of its toxin is to reduce the effectiveness of antibiotics. Besides, exfoliative toxins facilitate bacterial invasion of the host skin[3]. Methicillin, the first synthetic penicillin in 1960, was used to treat S. aureus infections, but soon many methicillin-resistant strains appeared in hospitals[2]. Two main classes of MRSA strain have been found. The first is known as nosocomial infections that are often transmitted in healthcare settings and are also called hospital-acquired MRSA (HA-MRSA). The second

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class is known as community-acquired MRSA (CA-MRSA). Diabetic patients may get both kinds of infections due to frequent sores and ulceration[4]. The moderate-severity CA-MRSA can be treated with co-trimoxazole (trimethoprim/sulfamethoxazole), doxycycline or clindamycin after susceptibility results are available, while both severe CA-MRSA and HA-MRSA infections must be treated with glycopeptides, linezolid or daptomycin[2].

Reuterin, a compound derived from lactobacillus (L.) reuteri, is a useful material in sterilising biological tissues in healthcare or pharmaceutical applications[5]. It has a broad inhibitory spectrum towards most bacteria, including S. aureus. The inhibitory effect of reuterin comes from its ability to inactivate the bacterial proteins as well as small molecules that contain thiol groups and the primary amines[6]. Second, the dimmer shape of reuterin structure, which is similar to the structure of ribose sugar, can specifically inhibit the reductive ribonucleotide enzyme which is often required for the generation of deoxynucleotide that is necessary for synthesis of deoxyribonucleic acid (DNA) in bacteria[7].

The increasing resistance to antibiotics, in particular methicillin and vancomycin, need to be further studied to assess the requirment for new antibiotics in the treatment of DFIs, and to evaluate the therapeutic approach to this problem. The current study was planned to genetically diagnose MRSA isolates taken from patients with severe DFIs, and to test the inhibitory effect of reuterin on the growth of these bacteria.

Materials and Methods

The experimental study was conducted from June to November 2021 at the Diabetes Unit of Al-Faihaa General Hospital, Basrah, Iraq. After approval from the institutional ethics review committee, the sample was raised using convenience sampling technique. Those included were patients type 2 diabetes mellitus (T2DM) with diabetic foot ulcers (DFUs). Swabs were taken from the necrotic lesions after taking informed consent from the patients. Those not willing to participate were excluded. .

The swab samples were placed first in sterile tubes containing brain heart infusion broth (BHIB) (HiMedia, India), and then transported to the laboratory where they were incubated for 24h at 37°C. After the broths gave positive growth, they were inoculated by streaking method on Manitol salt agar (HiMedia, India) and MRSA staphylococcus chromogeneic agar media (Conda/Pronadisa, Spain) and incubated for 24h at 37°C. The grown colonies were observed and microscopically examined after gram staining for the shape and assemblies of a cell, and then cultured using nutrient agar (HiMedia, India) for another testing[8].

DNA extraction was obtained using a genomic DNA mini-kit (Geneaid, Taiwan), according to the manufacturer's instructions.

The primers used for staphylococcus genus-specific included Staph 756F (5-AACTCTGTTATTAGGGAAGAACA-3) and Staph 750R

(5-CCACCTTCCTCCGGTTTGTCACC-3)[9]. The mecA gene primers used to determine plasmid gene included MecA1 (5'-GTAGAAATGACTGAACGTCCGATAA-3') and MecA2 (5'-CCAATTCCACATTGT TTCGGTCTAA-3')[10].

Thermo-cycling conditions (TechneTC-300G,UK) for Staph 16S rRNA gene were set at 95°C for 5 min, followed by 35 cycles of 95°C for 30s, 55°C for 30s, 72°C for 1 min, and 72°C for 5 min. For mecA gene, the conditions were set at 3 min for 95°C followed by 30 cycles for 1 min at 94°C, 53°C for 30s, 72°C for 1 min, and 72°C for 6 min.

The reuterin activity was quantified by the determination of the minimum inhibitory concentration (MIC) towards MRSA using the well-diffusion method on Mueller-Hinton agar (MHA)[11,12]. Concentrations of standard reuterin included 1µl, 5µl, 10µl, 20µl, 30µl, 40µl, 50 µl, 60µl, 70µl, 80µl, 90µl, 100µl, 200µl in distilled water. Further, 0.1ml from MRSA physiological broth saline of turbidity 0.5 McFarland standard measured at 600nm, which gives an approximate number of cells 1.5×108CFU/ml, was diffused by a sterile diffuser on MHA plates. Pores of 6mm diameter were then made in each plate with a sterile cork perforator. The pores were then filled with the addition of 50µl of each concentration prepared from standard reuterin with distilled water as control. Petri-dishs were then incubated at 37°C for 24h. The diameters of bacterial inhibitory zones around pores were measured in millimetre units.

The cytotoxicity of the prepared reuterin dilutions to human red blood cells (RBCs) was tested in line with literature.[1] All data was analyzed with SPSS version 23.

Results

A total of 62 swabs were taken from the necrotic lesions of type 2 diabetes mellitus (T2DM) patients with diabetic foot ulcers (DFUs) under the supervision of a physician. The mean age of the patients was 46±3 years and mean duration of DFIs 14±1.5 years. Of the total isolates, 13(21%) were found to have S. aureus infections and of these, 9(69.2%) gave mauve to purple colour on staphylococcus chromogeneic agar, which was then genetically confirmed as MRSA.

Table-1: Minimum inhibitory concentration (MIC) test of reuterin against methicillin-resistant staphylococcus aureus (MRSA).

Conc. µl/ml	Inhibition zone diameter (mm)	MIC value
control	0	
1	0	
5	0	
10	16	
20	21	
30	24	
40	27	10 µl/ml
50	28	
60	28	
70	29	
80	29	
90	30	
100	30	
200	31	

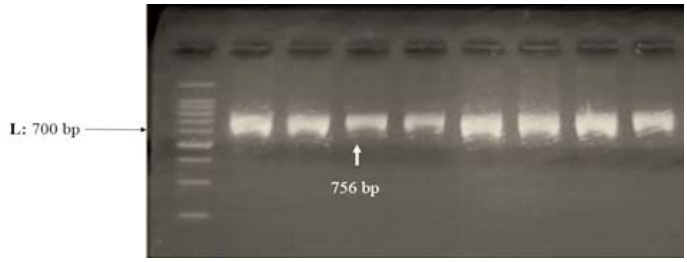

Figure-1: Agarose 1% gel electrophoresis for Staph 16SrDNA PCR products at position756bp (Ladder: 100-1000bp).
DNA: Deoxyribonucelic acid, PCR: Polymerase chain reaction.

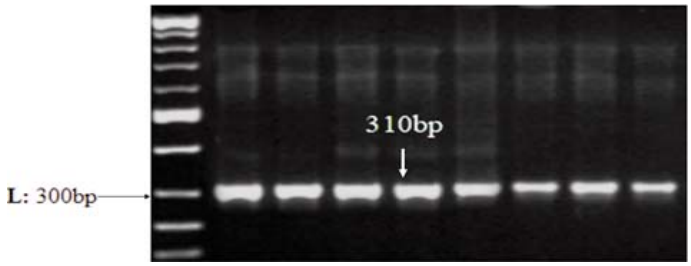

Figure-2: Agarose 1% gel electrophoresis for specific mecA gene products at position 310bp (Ladder: 100-1000bp).

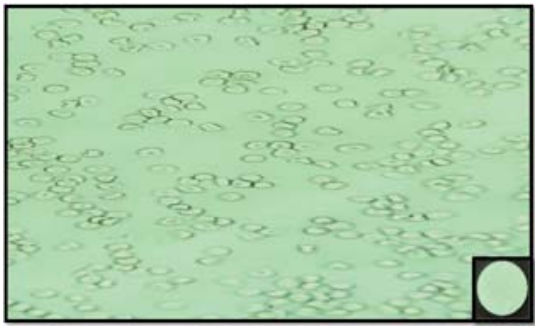

Figure-3: Red blood cells (RBCs) under microscope (40X) after cytotoxicity test.

Electrophoresis showed that DNA gave single bands for S. aureus visualised under ultraviolet (UV) transilluminator .

Polymerase chain reaction (PCR) products for the amplification of Staph 16SrDNA under the UV transilluminator display all 13(21%) to be S. aureus, and gave a single band at position 756bp (Figure 1).Furthermore, 9(69.2%) isolates showed MRSA, and, as confirmed by the amplification of mecA genes, gave bands at 310bp (Figure 2).

The lowest reuterin concentration required to inhibit the growth of MRSA was 10µl/ml with inhibition zone diameter of 16mm, while inhibition of the zone increased with the increasing of concentration, and 10µl/ml was considered the MIC (Table).

Cytotoxicity test showed that the compound was not cytotoxic, and the RBCs settled at the bottom of the tubes, leaving a clear solution (Figure 3). They were intact and did not suffer from lysis during the 8-hour observation period.

Discussion

The current findings agreed with those of Gadban et al.[13] who isolated S. aureus from DFUs at two local hospitals in Al-Basrah governorate of Iraq, but different from Osariemen et al. in Nigeria[14]. Perim et al. reported that S. aureus was predominant among bacteria isolated from DFUs in a Brazilian hospital[15]. This variation can be attributed partially to differences in associated microorganisms over time and geographic variation or type and severity of infection included in the studies[16].

The current results related to agricultural phenotypic and microscopic diagnosis were in agreement with Merlino et al. who described the growth and identification of colonies[17]. Gaillot et al. reported that S. aureus isolates produced purple (positive) colonies usually 2-3mm in diameter after 24h of incubation with an halo mat surrounding the colonies of S. aureus. [18].

Based on Staph 16SrDNA gene, staphylococcus spp. was the most common and abundant bacterial isolate, which could indicate virulence factors for staphylococcus spp., particularly S. aureus, as a pathogenic bacteria in DFUs, as has been reported in other studies[19].

The amplification of the genomic Staph 16SrDNA and mecA genes in all of the extracted DNA bacterial isolates confirmed that all of them were MRSA, while the PCR technique confirmed identification in addition to preventing bacterial loss[20].

MIC is the lowest concentration of an antimicrobial

metabolite that inhibits the visible development of a microorganism after an overnight incubation period[21]..Generally, as the reuterin concentrations increased, there was an increase in the diameters of the inhibitory zones, demonstrating the inhibitory effect of reuterin on S aureus. This finding was consistent with earlier studies[21, 22].

In the current study, reuterin was significantly nontoxic. Sung et al. reported that the median lethal dose of reuterin in mice was estimated to be 7.55, while Chen et al. reported that the estimated median lethal dose of reuterin in mice ranged 260-320mg/kg[23]. Also, 0.4-1.2mM of reuterin was enough to sterilise biological tissues contaminated by pseudomonas (P.) aeruginosa, escherichia (E.) coli, S. aureus, and bacillus (B.) subtilis within 4 hours[5]. Chen et al. reported that mammalian cells were less sensitive to reuterin exposure when cytotoxicity was assessed in vitro using a mouse-derived established cell line of 3T3 fibroblasts, and a 7-day treatment with reuterin of rats infected with trypanosoma (T.) brucei resulted in 68% reduction in parasitemia, and an increase in rat survival[23].

The long-term effects of reuterin on human health may occur after prolonged exposure to lower concentrations, while the metabolic fate of reuterin in humans is not completely known[24]. Reuterin has been reported to exhibit moderate cytotoxicity in the human hepatoma cell line HepG2, and was generally recognised as a safe flavouring compound[25]. More in-depth toxicological studies are needed to establish the safety profile before the compound can be recommended for use in various fields.

Limitation: The current study has limitations as the sample size was not calculated which could have affected the power of the study.

Conclusion

Reuterin was found to be a natural antimicrobial substance suitable for use to disinfect diabetic foot wounds from bacterial contamination and infection, especially those casued by MRSA.

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