Isolation and identification of burn wound superbugs by molecular technique and their susceptibility to silver nanoparticles

R Mala and A S Ruby Celsia
Department of Biotechnology, Mepco Schlenk Engineering College, Sivakasi-626005, Tamil Nadu, India

E-mail: maalsindia@gmail.com

Abstract. Burn wound is a global problem affecting millions of people. It is the major cause of mortality and morbidity. This study was aimed to isolate and identify the wound isolates by 16S rRNA and to assess their susceptibility to antibiotics and silver nanoparticles. Silver nanoparticles were synthesized using aqueous extract of A.indica. The silver nanoparticles were characterized by FESEM, XRD, FTIR and DSC. Antibacterial susceptibility of the isolates was assessed by well diffusion method. The wound isolates were identified as S.aureus and E.coli. Both isolates were resistant to β lactum antibiotics, aminoglycoside, quinolones and macrolides. The inhibition zone exhibited by all antibiotics against both organisms was less than 5 mm. The size of silver nanoparticles were recorded as 55 nm. XRD confirmed the crystalline nature of the nanoparticles. TGA and DSC of silver nanoparticles showed the loss of weight and the melting point of silver nanoparticles was recorded at 871.3°C. Silver nanoparticles inhibited S.aureus and E.coli with an inhibition zone of 27 mm and 32 mm respectively. Therefore the study demonstrated that only silver containing dressings can be used in burn wounds infected by multi drug resistant super bugs.

1. Introduction

Globally death due to burns is 265,000/year [1]. In United States alone, approximately 1.1 million people suffer from burn wounds. Majority of incidences are occupational hazards. Among this, 4500 people die and 20,000 people suffer from the loss of 25% of their body surface area [2]. It exposes the sterile internal environment to the pathogens. Full thickness wound extends beneath the dermis and muscles. It severely affects the vascular system and neuronal communication. Pathogens complicate the healing and delay the healing cascade. Identification of pathogen infecting and colonizing the wound and its susceptibility pattern to antibiotics is essential to adopt the treatment strategy. Traditionally, pathogens were identified by culture morphology and biochemical characteristics. But identification of pathogens by molecular techniques is highly advanced and accurate. Due to the inappropriate use of antibiotics, emergence of multidrug resistance among pathogens is becoming a common problem. It is a challenge to treat infections by superbugs which are resistant to multiple classes of antibiotics simultaneously. It is an alarming threat to mankind. Pathogens are developing resistance rapidly to those antibiotics which are reserved exclusively for ICU.

maalsindia@gmail.com
Infection of wound by super bug predisposes the patient to bacteraemia, sepsis and multi organ failure [3-5]. Efficient wound healing treatment must ensure the clearance of bacteria before causing extensive damage to the host. Sensitivity of burn isolates to antibiotic was investigated by Chauhan et al [6]. After the discovery of penicillin, the use of antibiotics becomes enormous and now pathogens have evolved strategies to resist them. New antibiotics in clinical trial are also less. So there is an urgent need to find alternative solution to antibiotic resistance. Advent of nano materials paved an attractive solution to many bottle necks in therapy and diagnosis. Silver was used before the era of antibiotics and it is revived at a nano scale to combat infectious agents. Silver nanoparticles posses many advantages. The properties of materials are different at nano scale which is successfully used to kill pathogens. Silver nanoparticles are lethal to pathogens in multiple ways. Hence, the present work was aimed to identify pathogens from burn wound and to reveal the resistance of the pathogens to antibiotics. To conquer infectious agents and to improve the rate of wound healing, silver nanoparticles were synthesized using Azadarachta indica (neem) extract and its potency to be lethal to the pathogens were evaluated.

2. Materials and Methods
All media constituents, chemicals and antibiotics used in the study were purchased from Himedia, Mumbai, India.

2.1 Isolation of pathogens from wound
Pathogens were isolated from the wound using sterile swab stick. The swab stick was streaked onto blood agar medium and incubated at 37ºC. The isolated colonies were selected and used for identification.

2.2. Identification of pathogens
Individual colonies isolated from the wound were further used for identification by 16S rRNA sequencing. The isolates were grown to log phase in nutrient agar medium and DNA was extracted using alkali lysis method [8]. PCR was done with 8F (AGAGTTTGATCCTGGCTCAG) and 1541R primers (AAGGAGGTGATCCAGCCGCA). The PCR product was purified using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 27F/1492R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase (Applied Biosystems). Single-pass sequencing was performed on each template using below 16S rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The 16S rRNA sequence was blast using NCBI blast similarity search tool. The phylogenetic analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences [8].The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminate poorly aligned positions and divergent regions that removes alignment noise [9]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering [10].

2.3 Synthesis and characterization of silver nanoparticles
1.0 g of tender A.indica leaves were surface sterilized with 70% of ethanol and extracted with 50ml of double distilled water. The sample was centrifuged at 10,000 rpm for 15 minutes. The supernatant was used for the synthesis of nanoparticles. To 50 ml of the aqueous solution of silver nitrate (1mM), 500µl plant extract was added and kept at room temperature till color changes to reddish pink color.
The UV-Vis absorption spectrum of the silver nanoparticles was analyzed by UV-Visible Spectrophotometer. The morphology of silver nanoparticle was recorded in FESEM. The dried powder was used for FTIR, DSC and XRD analysis.

2.4 Susceptibility of isolates to antibiotics and silver nanoparticles by agar well diffusion method
Antibacterial activity of silver nano particles was evaluated by well diffusion method [11]. The isolates were grown to log phase and the OD was adjusted to 10^8 CFU/ml. 100 µl of sample was swabbed over Mueller and Hinton agar medium. A well was punctured in the plate and 100µl of silver nanoparticle was loaded on to it. The plates were incubated at 37º C for 24 hours. The inhibition zone was measured after 24 hours. The susceptibility of organisms was inferred as per CLSI standards [12].

3. Results and Discussion

3.1 Isolation and identification of wound pathogens
Burn wound are one of the occupational hazards that affects many people. Burn wounds are caused by heat or by chemicals. Fire accidents are responsible for burn wounds in fire crackers and match work industries. Burn wounds are most commonly infected by pathogens. In the present study, pathogens were isolated from burn wound (figure 1). It was partial thickness burn wound of a child. Burn wounds are difficult to heal as the damage to the skin penetrates into the dermis. The pathogens were identified by 16S rRNA as S.aureus (Accession: KY770792.1; GI:1176922624) and E.coli (Accession: KY770797.1; GI:1176922629).

The phylogenetic tree constructed with the Bioinformatics tool is shown in figure 2 and figure 3. Thus the isolates were identified using molecular technique as S.aureus and E.coli. Colonization of skin by S.aureus was supported in earlier studies [13]. Presence of S.aureus in wound if untreated may lead to sepsis. Rai et.al. [14] investigated the bacteriological profile and the susceptibility of isolates from wound. The study supported the present observation that 13% of Gram negative organisms in wound sample were E.coli. Biological system provides all essential nutrients for the luxurious growth of pathogens. They readily form biofilm on the wound and they are 1000 fold resistant to antibiotics.
Biofilm architecture prevents the entry of any chemotherapeutic agents into the EPS layer as shown in figure 4.

3.2. Susceptibility of wound isolates to antibiotics
The susceptibility of the wound isolates to antibiotics is shown in figure 5 and 6.
Antibiotics like azithromycin (macrolide), amikacin/salbactum, ciprofloxacin (quinolones), doxycycline (tetracyclins), gentamicin (aminoglycoside) and sulfadiazine, (sulphonamide) showed no inhibition zone against S.aureus and E.coli. Gentamicin showed a maximum of 3 mm zone of inhibition against S.aureus. The results clearly confirmed that the isolates were superbugs. Resistant genes were transferred both horizontally and vertically between organisms. This is a threat to treatment strategy. Delay in clearing the bioburden delays the next stages of healing phenomenon. Resistance of pathogens to azithromycin is caused by methylation of target site or preventing the binding of antibiotic to the ribosomal subunit. Resistance was also developed through the expression of efflux pump and by inactivation of the drug [15]. Amikacin is an aminoglycoside resisted by pathogens by reducing their permeability and modification of the drug through enzymes [16]. Tanaka [17] investigated the genes responsible for resistance towards ciprofloxacin and quinolones and confirmed that numerous and multiple complicated mutations are responsible for resistance. Resistance to β-lactum antibiotics is due to the synthesis of β-lactamase and with the assistance of altered penicillin binding proteins. All the changes are heritable and transferred between organisms. As wound site is a niche for consortium of pathogens, transfer of resistant genes between organisms is easy. Organisms within the biofilm are 1000 fold resistant to antibiotics. Hence, in clinical unit the elimination of pathogen becomes complicated. It forces scientists to identify novel agents to kill pathogens without any harmful effect on the host system.

3.3 Synthesis and characterization of silver nanoparticles

The morphological characteristics of silver nanoparticles are shown in figure 7. Figure 8 shows the EDX spectrum of silver nano particles. The particles were homogeneous. EDX spectrum attests that the particles were silver nanoparticles. The particle size distribution of nano materials is shown in figure 9. This shows that particles with narrow size can be synthesized using phytochemicals. UV-Visible absorption spectrum of silver nanoparticles is shown in figure 10. Surface Plasmon Resonance (SPR) of silver nanoparticles was observed at 420 nm. The presence of bioactive compounds in A.indica is responsible for the reduction of silver into silver nanoparticles. Particles with less size have absorption characteristics at lower wavelength. Increase in particle size shifts the SPR to longer wavelength [18]. So the data supports the small size of silver nanoparticles.
Figure 9. Size distribution of silver nano particles

Figure 10. UV-Visible absorption spectrum of silver nano particles

FTIR spectrum of silver nanoparticles and the A.indica extract are shown in figure 11 and 12. Silver nano particles showed peaks at 446 cm\(^{-1}\), 517 cm\(^{-1}\), 635 cm\(^{-1}\), 691 cm\(^{-1}\), 781 cm\(^{-1}\), 815 cm\(^{-1}\), 173 cm\(^{-1}\), 1217 cm\(^{-1}\), 1316 cm\(^{-1}\), 1339 cm\(^{-1}\), 1362 cm\(^{-1}\), 1394 cm\(^{-1}\), 1454 cm\(^{-1}\), 1510 cm\(^{-1}\), 1624 cm\(^{-1}\), 1747 cm\(^{-1}\), 2312 cm\(^{-1}\), 2883 cm\(^{-1}\), 3444 cm\(^{-1}\), 3737 cm\(^{-1}\) and 3899 cm\(^{-1}\). Aqueous extract of A.indica revealed peaks at 419.49 cm\(^{-1}\), 486.99 cm\(^{-1}\), 518 cm\(^{-1}\), 590.18 cm\(^{-1}\), 780 cm\(^{-1}\), 819 cm\(^{-1}\), 864 cm\(^{-1}\), 901.60 cm\(^{-1}\), 1074.28 cm\(^{-1}\), 1137.17 cm\(^{-1}\), 1215.07 cm\(^{-1}\), 1264 cm\(^{-1}\), 1309 cm\(^{-1}\), 1427.6 cm\(^{-1}\), 1639.38 cm\(^{-1}\), 2390.71 cm\(^{-1}\), 2933.5 cm\(^{-1}\) and 3354.94 cm\(^{-1}\).
Prabhu et al. [19] reported the role of flavanoids in reducing silver to silver nanoparticles. Peaks from 1315 cm\(^{-1}\) to 1037 cm\(^{-1}\) and from 1456 cm\(^{-1}\) to 1600 cm\(^{-1}\) are attributed to the presence of phenolic groups [20]. Thus the results confirmed the presence of bioactive compounds bound to the silver nanoparticles which are abstracted from the plant extract. In addition to this A. indica extract is well known for its antibacterial activity. Therefore biological method of synthesis not only stabilizes the nanoparticles but also strengthens the activity of silver nano particles against pathogens. XRD spectrum of silver nanoparticles is shown in figure13. The image confirms the crystalline nature of silver nanoparticles. [21].

Thermal properties of silver nanoparticle are represented in figure 14 and 15. TGA shows that 44.58% of weight was lost ultimately up to 1200 °C. This weight loss is due to the loss of organic groups attached to the silver nanoparticles obtained from A. indica. No significant moisture was lost around 100 °C indicating that the silver nanoparticles was dry and devoid of water. In DSC, an endothermic peak was observed at 871.3 °C. It is the melting point of silver nanoparticles. This is less than the melting point of silver which melts at 962 °C [22].
3.4 Susceptibility of wound isolates to silver nanoparticles
The isolates *S.aureus* and *E.coli* were susceptible to silver nanoparticles and the results are represented in figure 16. Silver nanoparticle exhibited 27 mm and 32 mm zone of inhibition against *S.aureus* and *E.coli* respectively. The antibacterial activity of silver nanoparticles is well established and it is depicted in figure 17 to 19. Different mechanisms were proposed for the antibacterial activity of silver nanoparticles. It is hypothesized by Durán et al.[23] that silver nanoparticle disrupts the outer membrane of bacteria, impairs replication and interacts with thiol groups of proteins to cause lethal effect. Another hypothesis proposed at the lethal effect is due to the generation of reactive oxygen species which damages the membrane architecture, biomolecules and inhibition of enzyme activities [24].

![Figure 16. Susceptibility of wound isolates to silver nanoparticles](image1)

![Figure 17. Mechanism of antibacterial activity of silver nanoparticles](image2)

![Figure 18. Susceptibility of *S.aureus* to silver nanoparticles](image3)

![Figure 19. Susceptibility of *E.coli* to silver nanoparticles](image4)
From the above observations, we conclude that the silver nanoparticles synthesized act against the multi-drug resistant bacteria in multiple ways. Our earlier studies with silver nanoparticle also demonstrated its broad spectrum anti bacterial [25].

4. Conclusion

Generally breached skin surface is a well nourished source for the growth of pathogens. Severity of the burn wounds depend upon the thickness of the wound and the area of skin lost by the injury. The present study proved that wound site was colonized by pathogens that were simultaneously resistant to many classes of antibiotics. The wound isolates were superbugs, resistant to β lactum antibiotics, macrolides, aminoglycosides and quionolones. But the isolates were susceptible to the silver nano particles synthesized using A.indica. Thus the study revealed the rapid development of resistance among pathogens and confirmed their susceptibility to silver nano particles.

5. Acknowledgement.

We sincerely thank DST Nano Mission, Delhi, India for providing financial assistance. We thank Dr. S. Arivazhagan, Principal and Dr. M. L. Stephen Raj, Senior Professor and HOD, Department of Biotechnology, Mepco Schlenk Engineering College for being instrumental in the project. We sincerely thank Government Hospital, Sivakasi, Tamil Nadu, India and Mrs A. Annie Aglin, Assistant Professor, Department of Biotechnology for their assistance in collecting wound samples. We express our immense thanks to Dr. K.Parthiban, Yaazh Xenomics, Coimbatore for his efforts in identification of pathogens. We express our thanks to Mr. G. Selvakumar, Assistant Professor, PSG TECHS COE Indu Tech, Coimbatore for his assistance in recording FESEM. We thank, Dr. Bhuvaneswari, HOD and Mr. Ponnudi, Assistant Professor, Department of Physics, Alagappa Chettiar College of Engineering and Technology, Karaikudi, Tamil Nadu, India for recording XRD.

6. References

[1]. World Health Organization. Fact sheet on burns. www.who.int/mediacentre/factsheets/fs365/en/
[2]. American Burn Associations 2002 Burn Incidence Fact Sheet
[3]. Ramey P I, Barret J P, Herndon D N 1999 Crit Care Clin 15 333-52
[4]. Cumming J, Purdue G F, Hunt J L and O’Keefe G E 2001 J Trauma 50 510-5
[5]. Hettiaratchy S and Dziewulski P 2004 BMJ 328 1427-9
[6]. Chauhan J R, Khare S, Lal P, Kunhikatta V, Thunga G, Nair S and Sreekumar N C 2016 Ind J Burns 24 (1) 69-73
[7]. Birnboim H C 1983. Methods Enzymol 100 243-55
[8]. Edgar R C 2004 Nucleic Acids Res 32(5) 1792-1797
[9]. Talavera G and Castresana J 2007 Systematic Biology 56 564-77
[10]. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard J F, Guindon S, Lefort V, Lescot M, Claverie J M and Gascuel O 2008 Nucleic Acids Res 136 W 465-9
[11]. Bauer A W, Kirby W M, Sherris, J C and Tuck M 1966 Am J of clin pathol 45 (4) 493–6
[12]. Clinical and Laboratory Standards Institute 2016 CLSI document M100-S26 Wayne, PA
[13]. Otto M 2010 Expert Rev Dermatol 5(2) 183–95
[14]. Rai S, Narayan Yadav U, Pant N D, KrishnaYakha J, Tripathi, P P , Poudel A and Lekhak B 2017 Int. J. Microbiology. Article ID 2529085, 5 pages, doi:10.1155/2017/2529085.
[15]. Leclercq R 2002 Clin Infect Dis 482-92
[16]. Chow J W 2000 Clin Infect Dis 31 586-9
[17]. Tanaka M, Wang T, Onodera Y, Uchida Y and Sato K 2000 J Infect Chemother 6(3) 131-9
[18]. Anandalakshmi K, Venugobal J and Ramasamy V 2016 Applied Nanoscience 6 (3) 399–408
[19]. Prabhu N, Raj D T, Yamuna G K, Ayisha S S and Joseph Puspha I D  2010  *Dig J Nanomater Bios* 5:185–89
[20]. Jeeva K, Thiyagarajan M, Elangovan V, Geetha N and Venkatachalam P 2014  *Ind Crops Prod* 527:14–20
[21]. Shameli, K, Ahmad M, Bin, Zargar M , Yunus W M Z W and Ibrahim N A 2011  *Int. J. Nanomedicine* 6, 331–41
[22]. Silver (Ag) - US Research Nanomaterials  www.us-nano.com/inc/sdetail/126.
[23]. Durán N, Marcato P D, Conti R De Alves O L, Costa FTM and Brocchi M 2010  *J. Braz. Chem. Soc.* 21, 949–59
[24]. Grigor’eva A, Saranina I, Tikunova N, Safonov A, Timoshenko N, Rebrov A, Ryabchikova E, 2013.  *BioMetals* 26, 479–88
[25]. VaralakshmiV, Suganiya A S, Mala R  2015  *Recent Patents on Nanotecnol*  9 (3) 212-21