IJMS
Vol 30, No 2, June 2005

Original Article

In vitro Susceptibility of *Pseudomonas aeruginosa* Isolated from a Burn Center to Silver Sulfadiazine and Silver Nitrate in Shiraz, South of Iran

A. Japoni, M. Hayati, A. Alborzi, Sh. Farshad, S.A. Abbasian

Abstract

Background: *Pseudomonas aeruginosa*, as an etiological agent, has a prominent infection role in serious burned patients. Burned patients usually treated with antiseptic ointments such as silver sulfadiazine (SSD). This study evaluated the effectiveness of 1% SSD and different concentrations of silver nitrate solution (SNS) on resistant isolates of *P. aeruginosa*.

Methods: Three groups of *P. aeruginosa* isolates were collected consisting of 63 strains from burned patients (group I), 15 strains from burn-hospital environment (group II) and 70 strains from non-burn patients as control group. The Minimum Inhibitory Concentrations (MICs) of SSD and SNS were determined by agar dilution method and their susceptibility to SSD was evaluated by agar well diffusion method.

Results: In group I, 60 (95%) strains were resistant to SSD, whereas only 5 of them were resistant to SNS. In group II, eight out of 15 strains were resistant to SSD with MICs similar to group I while they did not show any resistance to SNS ($P<0.001$). In control group, all strains were sensitive to SSD and SNS ($P<0.001$).

Conclusion: Most of burned patient isolates were resistant to SSD while most of them were sensitive to SNS. In contrast, all the control isolates were sensitive to SSD and SNS. Frequent administrations of SSD ointment in burned patients surely have caused resistant strains to emerge. Cessation of SNS application in clinic or less administration of SSD in non-burn patients did not induce resistance strains.

Iran J Med Sci 2005; 30(2): 63-67.

Keywords ● *Pseudomonas aeruginosa* ● silver sulfadiazine ● silver nitrate

Professor Alborzi Clinical Microbiology, Research Center, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, Iran.

Correspondence:
Aziz Japoni PhD,
Prof Alborzi Clinical Microbiology, Research Center, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, Iran.
Tel: +98 711 6262225
Fax: +98 711 6287071
E-mail: Japonia@hotmail.com

Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogenic bacterium. This bacterium in hostile conditions such as colonization in burned skin surface produces large amounts of exopolysac charide that bind with water and form gels. The production of such slime material plus iron binding siderophores, pyochelin and pyroverdin

results in the extraction of iron and other nutrients from its environment and thrives in microcolonies.[1] The existence of such array of the exoenzymes and gels with cell surface enzyme penicillinases makes the organism very difficult to treat or eradicate from burn wound and medical equipment and devices in burn units.[1,2] Therefore, serious infections caused by *P. aeruginosa* remain a common complication in burn patients, contributing substantially to burn morbidity and mortality. Unfortunately, burned patients routinely are exposed to water either for hydrotherapy or washing of the wounds and cross-transmission of resistant strains from small tube or large Hubbard tank to another patient has been proven.[2]

Silver compounds are widely used as effective antimicrobial agents to combat pathogens both in clinics and for public health purpose.[3] Silver cations (Ag^+) are microbiocidal at low concentration and have been administrated to treat burn lesion, wound and ulcer.[3] Application of silver sulfadiazine (SSD) for burn infection was introduced by Fox.[4] However, concern about its efficacy arose when resistant bacteria reported in a burn unit of Birmingham hospital in UK following SSD treatment of patients with extensive burns.[5] Silver nitrate was administrated previously in clinic as a topical antiseptic. However, the advent of more effective silver compounds and new antiseptics decreased or in some clinics ceased its application.

This study was, therefore, conducted to evaluate the current level of *in vitro* susceptibility of *P. aeruginosa* to silver based creams which are broadly administrated as a topical anti-pseudomonal agent in curing burned patients. In addition the level of susceptibility of *P. aeruginosa* to SNS which is not used anymore as a topical antiseptic agent in our hospitals was determined to assess the relationship between application of drug and emergence of resistant strains.

Material and methods

Pseudomonas aeruginosa *strains*

Sixty three, 15 and 70 strains of *P. aeruginosa* were isolated from burned patients (group I), environment of Ghotbedin Burn Hospital (group II) and non-burned patients admitted to Nemazee Hospital as control (control group), all affiliated with Shiraz University of Medical Sciences, Shiraz Iran, respectively from January to April 2002. Control group had various pseudomonal infections but was not exposed to silver compounds.

A sterile cotton swab was used for sampling from patients or the environment. Samples were quickly cultured on nutrient agar medium (Oxoid, UK) and incubated overnight at 37 ^oC. Any suspicious colony was then subcultured and purified. The isolates were identified as a *P. aeruginosa* based on oxidase test, triple sugar iron (TSI) fermentation, color, pyocyanin pigment production, odour and or ability to grow at 42 ^oC. The isolates were preserved at -70 ^oC in Luria-Bertani broth (LB) containing 50% (v/v) glycerol.

Antiseptics supplies

Pure silver nitrate powder was purchased from BDH Laboratory Supplies Poole (Anala R, England). Pure SSD powder and ointment (1%) were supplied by Sobhan Pharmacy Company (Tehran, Iran).

Susceptibility tests

Minimum inhibitory concentrations (MICs) for SSD and SNS were determined using agar dilution and agar well diffusion method.

Agar dilution method

There is not a consistent guide to differentiate silver resistant isolates from sensitive ones. Hendry and his colleagues used 0.5 mM of SNS as a cutoff point for differentiation of resistant strains from sensitive ones.[6] In this study we used Hendry's procedure with the same media and conditions to determine MICs for SSD and SNS in *P. aeruginosa* isolates. Therefore bacteria with MIC>0.5 mM were considered as a resistant isolate and <0.5 mM as a sensitive one.

Agar well diffusion method

Muller-Hinton agar with 5 mm well containing holes and 6 mm diameter was prepared. The surface of the agar was swabbed with suspension of each isolate adjusted to 0.5 Mac-Farland (approximately10^8 CFU/ml). An antibiotic sensitive American Typing Culture Collection (ATCC) 27853 was used as a control strain in antibacterial susceptibility determination. The well was filled under aseptic condition with 1% SSD ointment using sterile syringe and needle. The plates were incubated aerobically in darkness for 24 hrs at 37 ^oC. Resistance and susceptibility was defined as no zone of inhibition or significant inhibition zone, respectively.[7]

Statistical analysis

Data are presented as mean±SD. The difference between MIC values for SSD and SNS in groups I and control was analyzed separately by paired Student's *t* test. The difference between MIC values for SSD and SNS in group II was obtained by non-parametric test (Willcoxon). The correlation between MIC val-

Table 1: Mean±SD of MIC for SSD & SNS and frequency of resistant (R) and sensitive (S) strains in *P. aeruginosa* isolated from patients of groups I, II and control.

Groups	SSD(mM)	R (%)	S (%)	SNS (mM)	R (%)	S (%)
I	7.8±3.5*#	60 (95)	3 (5)	0.49±0.16*#	5 (8)	58 (92)
II	2.06±3.3	8 (53)	7 (47)	0.49±0.16	0 (0)	15 (100)
Control	0.2±.052	0 (0)	70 (100)	0.29±0.12	0 (0)	70 (100)

* Difference between Means of MICs for SSD or SNS in groups I and II, (P<0.001).
# Difference between Means MICs for SSD and SNS in groups I and control (P<0.001).

Table 2: MIC ranges and the lowest concentration of SSD (mM) or SNS (mM) which prevent growth of 50%, (MIC_{50}) or 90% (MIC_{90}) of *P. aeruginosa* isolates.

	SDS			SNS		
Groups	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
I	0.25–>10	>10	>10	0.25–1	0.5	0.5
II	0.12–10	0.75	2	0.12–0.5	0.25	0.5
Control	0.12–0.25	0.25	0.25	0.12–0.38	0.25	0.25

ues and inhibition zone for each group was calculated by correlation coefficient test. The difference between means for MIC groups I and II was estimated by non-parametric test (Mann-Whitney) while this difference between group I and control group was obtained by Student's *t* test and the significant level was defined as *P*<0.05.

Results

The results of susceptibility tests showed that 60 (95%) of isolates in group I were resistant to SSD, whereas 5 (8%) of the isolates were resistant to SNS (Table 1). MIC ranges of these isolates to SSD were between 0.25–>10 mM and to SNS between 0.25–1 mM (Table2). The lowest concentration of antibacterial agent which prevented growth of 50% and 90% of bacteria (MIC_{50} and MIC_{90}) to SSD and SNS were >10, 0.5 and >10, 0.5 mM, respectively (Tables 2). The results of agar well diffusion method revealed that only three strains had inhibition zone with diameter of 10, 11, and 11 mm and with MIC values of 0.25, 0.38, and 0.38 mM, respectively. Nevertheless, the remainder of the isolates (60 strains) in this group had no inhibition zone. As shown in Table 1 the difference between MIC values for SSD and SNS was highly significant (P<0.001). In group I the correlation between MIC values and the diameter of inhibition zone for SSD was observed (P<0.001).

Group II included 15 strains isolated from the environment of burn center. In this group eight strains (53%) were resistant to SSD. MIC ranges of these isolates to SSD, MIC_{50} and MIC_{90} were, 0.12–10 mM.0.75and 2 mM, respectively, while MIC ranges of these isolates to SNS was 0.12–0.5 mM. None of these isolates showed any resistance to SNS (Table 2). The result of agar well diffusion method showed that eight strains had no zone of inhibition while seven strains showed inhibition zones between 10–11 mm. In group II correlation between the MIC values and the diameter of inhibition zone for SSD was observed (P<0.04).

Control group consisted of 70 strains isolated from non-burned patients in Nemazee hospital. In this group all the strains were sensitive to SSD and SNS with the MIC ranges between 0.12–0.25 mM and 0.12–0.38 mM, respectively (Tables 2). In control group all seventy isolates showed zone of inhibition between 10-24 mm. In control group also the correlation between the MIC values and the diameter of inhibition zone for SSD existed (P=0.007).

Analysis of MIC distribution to SSD and SNS in all the three groups showed that in burned-patients group 40 isolates (63.3%) were highly resistant to SSD (MICs ranges between 0.25–>10 mM), while in groups II and control the intermediate and lower range of MIC values to SSD between 0.12–10 and 0.12–0.25 mM were obtained, respectively (Table 2).

Discussion

Although resistance to SSD in *P. aeruginosa was* reported, its resistance mechanism has not been determined.[8] It is suggested that resistance of *Pseudomonas* to silver based topical antimicrobials in part is based on the mutation of outer membrane proteins that transport ions including silver across bacterial membrane.[9,10] Gentamicin-resistant strains of *P. aeruginosa* which were isolated from burned-patients have been reported.[11] These stains showed cross-resistance to SSD but their resistance was unstable and did not persist on subculture media. According to the report in USA, an epidemic sepsis of *Enterobacter cloacae* in burned patients occurred which led to 13 deaths.[12] The MIC values of SSD for these

strains were 3200 µg/ml whilst the strains isolated from non-burned patients were all sensitive to SSD. Similarly, Rosenkranz et al isolated two SSD resistant strains of En*trobacter cloace* in a burn unit where SSD was in use. These strains showed high resistance to SSD (MIC= 400 µg/ml) and were cross-resistant to silver benzoate but not to silver nitrate.[13]

Analysis of our data indicated that immergence of resistant strains of *P. aeruginosa* with high MIC (>10 mM) in burned patients occurred due to extensive administration of SSD. Similar to Rosenkranz et al. our strains did not show any cross-resistance to SNS. One possibility for this phenomenon is that different mechanisms may contribute to the resistance to SSD and SNS.[8,13] MIC distribution for SSD revealed that most of burned patient strains (63.4%) had this value at the highest level (>10 mM). This type of resistance can occur by transferable elements such as plasmid, phage or transposon, etc.[14-16] Nevertheless, resistance genes can be originated from membrane genes, the function of which is transporting ions and organic materials (including silver compounds) across bacterial membrane.[8,9] However, mutations in these genes may alter their proteins function by decreasing of bacterial membrane permeability.[17] Alternatively, the synergies between low permeability, due to mutation, and efflux pumps could be responsible for resistance.[18,19] Several efflux operons whose responsibility is to execrate solutes and antibiotics out of *P. aeruginosa* have been discovered.[20,21] Nevertheless, to date resistance mechanisms of *P. aeruginosa* to silver compounds and SSD have not been known precisely. Therefore, further molecular genetic basic researches are needed to initiate and clarify the basis resistance of *P. aeruginosa* to SSD.

Our data also showed that the difference between MIC means values of SSD in groups I and control or group I and II was highly significant (Table 1, P<0.001). This mean frequent application of SSD in burn patients could facilitate resistant strains to be selected while the difference between burn patients and environmental isolates could reflect less contact of these strains to SSD. It is clear that continuous administration of SSD may emerge resistant strains. Regarding of this problem periodical application of this drug or administration of combination of SSD with other anti-pseudomonal antiseptics can promote wound healing,[22] or improve SSD efficacy.[23]

In recent years other alternative silver compounds such as nanocrystal silver delivery system has been introduced.[24] The antimicrobial efficacy of nanocrystal silver delivery system has been proven. Surprisingly, as compared to SSD, administration of this new drug in burn patients has markedly reduced the cost of nursing, supplies and painkiller.[24,25] This finding warns us to regularly survey the efficacy of all administrated silver compounds in clinics.

Conclusion

Due to frequent administration of SSD ointment in burned patients majority of the isolate were resistant to this antiseptic ointment. In contrast, all the control isolates were sensitive to SSD and SNS. This could happen due to the cease of application of SNS in clinic or less administration of SSD in the control group.

Acknowledgment

The project was financially support by budget provided by Professor Alborzi Clinical Microbiology Research Center.

References

1 Vasil ML, Ochsner UA. The response of Pseudomonas aeruginosa to iron: genetics, biochemistry and virulence. *Mol Microbiol* 1999; 34: 399-413.

2 Tredget EE, Shankowsky HA, Joffe AM, et al. Epidemiology of infections with Pseudomonas aeruginosa in burn patients: the role of hydrotherapy. *Clin Infect Dis* 1992; 15: 941-9.

3 Silver S. Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. *FEMS Microbiol Rev* 2003; 27: 341-53.

4 Fox CL. Silver sulfadiazine--a new topical therapy for Pseudomonas in burns. Therapy of Pseudomonas infection in burns. *Arch Surg* 1968; 96: 184-8.

5 Lowbury EJ, Babb JR, Bridges K, Jackson DM. Topical chemoprophylaxis with silver sulphadiazine and silver nitrate chlorhexidine creams: emergence of sulphonamide-resistant Gram-negative bacilli. *Br Med J* 1976; 1: 493-6.

6 Hendry AT, Stewart IO. Silver-resistant Enterobacteriaceae from hospital patients. *Can J Microbiol* 1979; 25: 915-21.

7 Starodub ME, Trevors JT. Silver resistance in Escherichia coli R1. *J Med Microbiol* 1989; 29: 101-10.

8 Pirnay JP, De Vos D, Cochez C, et al. Molecular epidemiology of Pseudomonas aeruginosa colonization in a burn unit: persistence of a multi drug-resistant clone and a silver sulfadiazine-resistant clone. *J Clin Microbiol* 2003; 41: 1192-202.

9 Vasishta R, Saxena M, Chhibber S. Contribution of silver ion resistance to the pathogenicity of Pseudomonas aeruginosa with special reference to burn wound sepsis. *Folia Microbiol* 1991; 36: 498-501.

10 Daikos GL, Lolans VT, Jackson GG. Alterations in outer membrane proteins of Pseudomonas aeruginosa associated with selective resistance to quinolones. *Antimicrob Agents Chemother* 1988; 32: 785-7.

11 Bridges K, Kidson A, Lowbury EJ, Wilkins MD. Gentamicin and silver-resistant pseudomonas in a burns unit. *Br Med J* 1979; 1: 446-9.

12 Hendry AT, Stewart IO. Silver-resistant Enterobacteriaceae from hospital patients. *Can J Microbiol* 1979; 25: 915-21.

13 Rosenkranz HS, Coward JE, Wlodkowski TJ, Carr HS. Properties of silver sulfadiazine-resistant Enterobacter *cloacae*. *Antimicrob Agents Chemother* 1974; 5: 199-201.

14 Gupta A, Maynes M, Silver S. Effects of halides on plasmid-mediated silver resistance in *Escherichia coli*. *Appl Environ Microbiol* 1998; 64: 5042-5.

15 Nakayama K, Kanaya S, Ohnishi M, et al. The complete nucleotide sequence of *phi CTX*, a cytotoxin-converting phage of Pseudomonas aeruginosa: implications for phage evolution and horizontal gene transfer via bacteriophages. *Mol Microbiol* 1999; 31: 399-419.

16 Krishnapillai V, Royle P, Lehrer J. Insertions of the transposon Tn1 into the Pseudomonas aeruginosa chromosome. *Genetics* 1981; 97: 495-511.

17 Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clin Microbiol Rev* 1997; 10: 781-91.

18 Nikaido H. Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrob Agents Chemother* 1989: 33: 1831–6.

19 Nikaido H. Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* 1996; 178: 5853-9.

20 Poole K, Krebes K, McNally C, Neshat S. Multiple antibiotic resistance in *Pseudomonas* aeruginosa: evidence for involvement of an efflux operon. *J Bacteriol* 1993; 175: 7363-72.

21 Li XZ, Poole K, Nikaido H. Contributions of MexAB-OprM and an EmrE homolog to intrinsic resistance of Pseudomonas aeruginosa to aminoglycosides and dyes. *J Antimicrob Chemother* 2003; 47: 27-33.

22 Muller MJ, Hollyoak MA, Moaveni Z, et al. Retardation of wound healing by silver sulfadiazine is reversed by *Aloe vera* and nystatin. *Burns* 2003; 29: 834-6.

23 Fraser JF, Bodman J, Sturgess R, et al. An *in vitro* study of the anti-microbial efficacy of a 1% silver sulphadiazine and 0.2% chlorhexidine digluconate cream, 1% silver sulphadiazine cream and a silver coated dressing. *Burns* 2004; 30: 35-41.

24 Holder IA, Durkee P, Supp AP, Boyce ST. Assessment of a silver-coated barrier dressing for potential use with skin grafts on excised burns. *Burns* 2003; 29: 445-8.

25 Ohura T, Sanada H, Mino Y. Clinical activity based cost effectiveness of traditional versus modern wound management in patients with pressure ulcers. *Wounds* 2004; 16: 157–63.