BioMetals 1998, **11**, 27–32

Multiple parameters for the comprehensive evaluation of the susceptibility of *Escherichia coli* to the silver ion

Guojing Zhao & S. Edward Stevens, Jr

Department of Microbiology and Molecular Cell Sciences, The University of Memphis, Memphis, TN 38152, USA

Received 15 July 1997; accepted for publication 17 September 1997

The susceptibility of *Escherichia coli* B to the antibacterial activity of silver ions was measured in terms of the initial inhibitory concentration, complete inhibitory concentration, postagent effect for bacteriostatic susceptibility, minimum bactericidal concentration, maximum tolerant concentration, and log killing time for bactericidal activity. At a concentration of 9.45 μM and an inoculum size of 10^{4-5} CFU ml^{-1}, silver caused growth delay of *E. coli*; at a concentration of 18.90 μM, silver completely inhibited bacterial growth. Prolonged postagent effects ranged between 1.5 and 12 h at 0.75 × the initial inhibitory concentration, 1.0 × the initial inhibitory concentration, and 1.5 × the initial inhibitory concentration of the silver ion. One log-unit of viable bacterial population size was lost every 30 min at the minimum bactericidal concentration of the silver ion. Silver tolerance was determined as 20 times the initial inhibitory concentration with 48 h of exposure. This study presents an evaluative model as a reference for the quantitative analysis of the susceptibility of bacteria to silver ions.

Keywords: antibacterial activity, *E. coli* B, parameters, silver ion

Introduction

Silver has been known to be a disinfectant for about 1200 years and has been widely used during this century in the treatment of clinical diseases, including newborn eye prophylaxis, topical burn wounds, orthopaedic infections, and so on. Silver serves as a potent antibacterial agent acting against an exceptionally broad spectrum of bacteria while exhibiting low toxicity to mammalian cells. Since silver therapy is of significant clinical benefit in the control of bacterial infections, various forms of new agents containing the silver ion, such as creams, solutions, electrodes, ligatures, foils, nylons, biological skin and catheters, have been developed over the past decades in medical, biological and pharmaceutical preparations (Fox 1968, Long 1972, Grier 1977, Becker & Spadaro 1978, Thibodeau *et al.* 1978, Gilman *et al.* 1980, Deitch *et al.* 1983, Ersek & Denton 1988, Moing *et al.* 1990, Bhagava *et al.* 1992). Accordingly, the bioavailability and antimicrobial properties (oligodynamic action) of the silver ion have been extensively investigated. Berger *et al.* (1976a and 1976b) first measured the minimum inhibitory concentrations (MICs) of electrically generated silver for 16 clinical isolates of bacteria and 7 species of fungi. By comparing the bactericidal effect of silver nitrate with that of silver-coated nylon fiber, MacKeen *et al.* (1987) reported the latter may be an effective antibacterial agent. Recently, Hamilton-Miller *et al.* (1993) reassessed the antimicrobial activity of silver sulphadiazine against 409 strains *in vitro*. No resistant strains were found including methicillin-resistant *Staphylococcus aureus* and *Acinetobacter* spp.

Address for correspondence: Dr. S. Edward Stevens, Jr, Department of Microbiology and Molecular Cell Sciences, 509 Life Science Building, The University of Memphis, Memphis, TN 38152, USA. Tel: (901) 678 4182; Fax: (901) 678 4457; E-mail: EStevens@admin1.Memphis.edu

Silver is the most toxic element to microorganisms in the following sequence: Ag > Hg > Cu > Cd > Cr > Pb > Co > Au > Zn > Fe > Mn > Mo > Sn (Golubovich & Rabatnova 1974, Berger *et al.* 1976b). With the rise of antibiotic-resistant bacteria, silver is re-emerging as a modern medicine because all pathogenic organisms have failed to develop an immunity to it. However, the mechanism for such bacterial sensitivity to silver is poorly understood. Our primary study has been designed to establish an evaluative model as a reference for the quantitative analysis of bacterial susceptibility to the silver ion. The parameters we measured were the initial inhibitory concentration (IIC), the complete inhibitory concentration (CIC), the postagent effect (PAE), the minimum bactericidal concentration (MBC), the maximum tolerant concentration (MTC), and the log killing time (LKT) (Table 1) for antibacterial activity of standard silver nitrate solution against our model strain, *Escherichia coli* B. *Escherichia coli* is the most extensively studied and best understood bacterium (Gottschalk 1988). A large data base exists to which comparisons may be readily made. Moreover, recently *E. coli* has become a threat to human health because of publicized outbreaks of *E. coli* 0157:H7 (Griffin and Tauxe 1991). Silver nitrate is the most common silver compound used as an effective medicine in clinical practice. There is strong evidence in the literature that the active component of this silver salt is the silver itself (Berger *et al.* 1976b). Silver nitrate is a substance that releases silver ions rapidly.

In this report, the analysis lends itself to an understanding of the response behavior of bacteria to the silver ion. The information would shed light on the relationship among agent dose, bacterial density, exposure time, and toxic features in the interaction of *E. coli* and silver. Various concentrations of silver exert bacteriostatic or bactericidal action to a different degree. The IIC of silver causes growth delay or slow growth of bacteria, while the CIC of silver results in no growth of bacteria at all. Moreover, the MBC of silver induces irrevocable cell death. The PAE indicates bacteriostatic persistence, while the MTC or LKT indicates bactericidal effectiveness. Together these parameters may have important pharmacodynamic implications for therapeutic intervention in human infectious diseases.

Materials and methods

Bacterial strain and culture condition

Escherichia coli B, an ATCC 23848 wild type strain, was stored on Luria broth (LB, containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl in 1 liter of deionized water) agar at 4°C. In preparation for experiments, a stored culture of *E. coli* B was routinely grown overnight in LB at 37°C on a shaker at 100 r.p.m. The culture turbidity was measured with a Bausch & Lomb Spectronic 20 (MacKeen *et al.* 1987) and colony forming units (CFU) were determined by viable plate counts. The overnight cultures served as the inocula for experiments. Unless otherwise stated, the standard growth medium and conditions for experimental cultures were as described above for the overnight culture.

Silver nitrate

A standard solution of $AgNO_3$ (Fisher Scientific, Fair Lawn, NJ, USA) was prepared employing the Mohr method (Fifield & Kealey 1975). $AgNO_3$ was suspended in deionized water and titrated against NaCl (which was first dried in an oven at 500–600°C), until cutoff was reached as indicated by K_2CrO_4 (Fisher Scientific, Fair Lawn, NJ, USA). The test concentrations of $AgNO_3$ were obtained by dilution. Silver ion concentrations were calculated relative to $AgNO_3$.

Table 1. Abbreviations of the parameters used in the present study.

Abbreviation	Parameter name	Reference
IIC	Initial Inhibitory Concentration	This study.
CIC	Complete Inhibitory Concentration	This study.
CAE	Continuous Agent Effect	Odenholt-Tornqvist *et al.* 1992,
CA SIE	Continuous Agent Sub-IIC Effect	Zhanel *et al.* 1992.
CA IE	Continuous Agent IIC Effect	
CA SPIE	Continuous Agent Supra-IIC Effect	
PAE	Postagent Effect	Odenholt-Tornqvist *et al.* 1992,
PA SIE	Postagent Sub-IIC Effect	Zhanel *et al.* 1992.
PA IE	Postagent IIC Effect	
PA SPIE	Postagent Supra-IIC Effect	
MBC	Minimum Bactericidal Concentration	Bailey and Scott 1974.
MTC	Maximum Tolerant Concentration	Sabath *et al.* 1977.
LKT	Log Killing Time	Joklik *et al.* 1988.

Assays for bacteriostatic susceptibility

1. Determination of the initial inhibitory concentration (IIC) and the complete inhibitory concentration (CIC). The IIC of the silver ion was read as the lowest concentration in which no visible turbidity change was observed when the control tube showed significant turbid growth between 1.5 and 6 h incubation. The CIC of the silver ion was read as the lowest concentration which completely prevented visible growth of bacteria after 24 h of incubation. Twofold serial dilutions of the silver solution were used to determine the IIC and CIC of the silver ion in reference to *E. coli* B. Briefly, eight series of twofold dilutions in broth were set up with the desired concentrations of $AgNO_3$. Four different concentrations of cells at *ca.* 5×10^3, 5×10^4, 5×10^5, 5×10^7 cells per ml, were added to these dilutions of $AgNO_3$ in a volume of 0.5 ml. After mixing, the tubes were incubated for 24 h at standard conditions. During testing, progressive increases in a series of test cultures showing visible growth were noted every 3 h.

2. Determination of the postagent effect (PAE) and the continuous agent effect (CAE). The PAE is defined as the difference in time required for test cultures (those exposed to the silver ion for a fixed time with subsequent removal of the silver ion) and control cultures (no silver) to grow (increase in cell number) by the equivalent of 1 $\log_{10}$ unit. The CAE is defined as the difference in time required for test cultures (those exposed continuously to the silver ion) and control cultures to increase in cell number by the equivalent of 1 $\log_{10}$ unit. The PAE and CAE determinations were performed after a 1 h exposure to silver nitrate, based on the kinetics of silver ion uptake by *E. coli* reported by Modak & Fox (1973) and MacKeen *et al.* (1987). The agent-bacterium combination (silver nitrate and *E coli* B in LB), was studied on different occasions in terms of continuous agent sub-IIC effect (CA SIE), continuous agent IIC effect (CA IE), continuous agent supra-IIC effect (CA SPIE), postagent sub-IIC effect (PA SIE), postagent IIC effect (PA IE) and postagent supra-IIC effect (PA SIE) (Table 1). Briefly, the test culture was diluted to obtain a starting inoculum of *ca.* 5×10^4 CFU ml^{-1}, and then exposed to sub-IIC ($0.75 \times$ the IIC), IIC, and supra-IIC ($1.5 \times$ the IIC) of silver nitrate for 1 h at standard conditions. After incubation, one set of tubes was kept for culture while the other set of tubes was centrifuged at 8000 **g** for 15 min and washed three times to remove or eliminate silver ions. The cells were resuspended in fresh medium and reincubated for another 24 h. The control culture was treated similarly. During the incubation, samples were withdrawn every hour and if necessary, diluted in physiological saline. The dilutions of each sample were seeded on LB agar plates and colonies were counted to determine the number of CFU. Only plates with 20 to 200 colonies were used in calculations.

The PAEs, including PA SIE, PA IE and PA SPIE were calculated according to the following formula: PAE = T_{PA}–C, where T_{PA} is the time required for the viable counts of the cultures exposed to the sub-IIC, IIC, or supra-IIC of silver to increase by 1 $\log_{10}$ unit above the counts observed immediately after removal of the silver ion and C is the corresponding time for the unexposed control (Odenholt-Tornqvist *et al.* 1992, Zhanel *et al.* 1992). The CAEs involving CA SIE, CA IE or CA SPIE were defined as: CAE = $T_{CA} - C$, where T_{CA} is the time for cultures exposed to the sub-IIC, IIC, or supra-IIC of silver to increase by 1 $\log_{10}$ unit above the counts observed immediately after 1 h initial incubation with silver nitrate and C is the corresponding time for the unexposed control (Odenholt-Tornqvist *et al.* 1992, Zhanel *et al.* 1992).

Assays for bactericidal activities

1. Determination of the minimum bactericidal concentration (MBC). The MBC is defined as the lowest concentration of the silver ion which reduced the originally measured inoculum by > 99.9% within 48 h (Bailey and Scott 1974). A 0.5 ml aliquot was removed from the nonturbid tubes of the tube dilution set after 24 h of incubation, mixed with 12 ml of the same medium solidified with 1.5% agar and made into a pourplate. The cells were incubated at 37°C for another 48 h, and the number of surviving bacteria on the plate was then determined (Bailey and Scott 1974).

2. Determination of the maximum tolerant concentration (MTC). The MTC was defined as the lack of bacterial colonies observed in silver-free medium beyond a dose relative to the IIC of the silver ion. In experiments where silver was removed, two sets of 0.5 ml aliquots of the cultures containing 10, 20, 40 and 60 times the IIC of the silver ion, taken after 24 h and 48 h of incubation respectively, were transferred into 30 ml silver-free medium (to eliminate the silver ion), and incubated at standard conditions for another 48 h. The MBC was determined as described above (Sabath *et al.* 1977).

3. Determination of log killing time (LKT). The LKT is defined as the mean time for a 1 $\log_{10}$ unit reduction in the viability of bacterial cells (Joklik *et al.* 1988). Time-kill studies with the silver ion were performed by using a final bacterial concentration of 1×10^6 CFU ml^{-1} with various silver ion concentrations in each tube. All test tubes were then placed in a shaking incubator at 37°C. Samples (100 µl) were withdrawn at designated intervals then taken through a serial dilution and spread onto LB agar plates for use in counting viable cells at a level of detection of 20 to 200 CFU per plate, after incubating for 24 h.

All microbiological assays were independently performed a minimum of three times.

Results

To determine the bacteriostatic effect of silver on *E. coli* the progressive changes in visual turbidity of cultures containing various concentrations of silver were examined. After 6 h, the control cultures

appeared turbid (with the exception of those with a bacterial population size of 10^{7-8} CFU ml^{-1} which showed visible growth after 1.5 h). Progressive increases in turbidity were observed every 3 h in those test tubes with the silver ion from lower to higher concentrations. After 18 h, no further turbidity change was observed during an additional 48 h of incubation. At a bacterial population size of 10^{4-5} cells ml^{-1}, the inhibitory range of silver was between 9.45 and 18.90 μM. At an initial inoculum of 10^{4-5} cells ml^{-1}, and a silver concentration of 18.90 μM, growth did not occur. An increase in the initial population density of an order of magnitude (10^{5-6} CFU ml^{-1}) at the same concentration of the silver ion resulted in a 50% level of inhibition, whereas no bacteriostatic action was detectable with an initial population density of 10^{7-8} CFU ml^{-1}. The IIC, CIC and MBC varied depending on the starting concentration (population density) of *E. coli* B (Figure 1).

Results of the PAE determinations for the silver ion are displayed in Table 2. As shown, the growth of the control increased 1 $\log_{10}$ unit in approximately 2 h; with a concentration equal to or greater than the IIC of the silver ion under continuous or postagent treatment, the cultures took roughly 7 h longer to achieve a similar increase. The PAE and CAE were similar.

After 48 h of cell exposure to a silver ion concentration 20 times the IIC, all cells were irreversibly damaged (effectively killed) as shown in Table 3. Thus, the MTC can be determined as 20 times the IIC after 48 h exposure, or 60 times greater after 24 h exposure.

The killing pattern of the silver ion shown in Figure 2 was determined by examining the $\log_{10}$ CFU of viable bacteria per ml versus time of exposure to the silver ion. The killing curves indicated that the silver ion produced immediate killing and a rapid rate of loss in viability. At a concentration of 40 times the IIC, the population of bacteria decreased one order of magnitude every 7.5 min, while at the MBC, 30 min was required to exert the same efficacy, as seen in the log-kill curve in Figure 3.

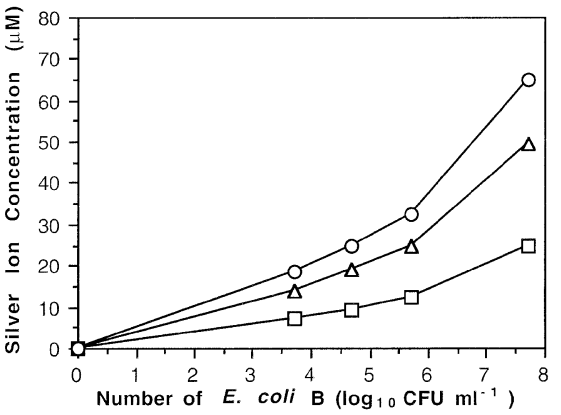

Figure 1. Influence of inoculum size of *E. coli* B on the MBC (○), the CIC (△), and the IIC (□) of the silver ion.

Discussion

We present a set of parameters (Table 1) which are important to a definition of antibacterial activity of the silver ion in the form of silver nitrate. The initial inhibitory (IIC) and complete inhibitory (CIC) concentrations are explicit criteria for the quantitative and qualitative analysis of the silver effect. Most antibacterial agents, including the silver ion, exhibit two distinct bacteriostatic properties. These result in a growth delay with later regrowth and a growth shutdown in bacteria. A growth delay involves a short-term cell killing, prolonged lag phase and

Table 2. The continuous agent (CAE) and postagent (PAE) effect[a] for the silver ion on *E. coli* B.

Variable	Control culture	Test culture					
		0.75 × the IIC (sub-IIC)		IIC[b]		1.5 × the IIC (supra-IIC)	
		CA SIE	PA SIE	CA IE	PA IE	CA SPIE	PA SPIE
C[c]	2	–	–	–	–	–	–
T[d]	–	4	3.5	10	9	14	13
T-C[e]	–	2	1.5	8	7	12	11

[a]The following formula was used to quantify the CAE and PAE: CAE or PAE = *T–C*; [b]The IIC was 9.45 μM for 5×10^4 CFU ml^{-1} of bacteria; [c]*C* = time (h) required for the count of CFU in the control culture (no silver) to increase by 1 log unit above the count immediately after 1 h incubation; [d]*T* = time (h) required for the count of CFU in the test culture (with sub-IIC, IIC, or supra-IIC of silver) to increase 1 log unit above the count immediately after 1 h incubation. [e]*T–C* = the difference in time required for test cultures and control culture to grow (increase in cell number) by the equivalent of 1 $\log_{10}$ unit.

Table 3. Determination of maximum tolerant concentration (MTC) of *E. coli*[a] B to the silver ion.

Silver-treatment	Times (×) the IIC[b] of the silver ion				
time (h)	0	10[c]	20	40	60
24	+[d]	+	+	+	–
48	+	+	–[e]	–	–

[a]Initial inoculum concentration was 5×10^5 CFU ml^{-1}; [b] The IIC was 12.05 μM; [c] 10 times the IIC was 120.5 μM; [d]d +: The test tubes exhibited bacterial growth after additional 48 h incubation following the removal of silver; [e] –: The test tubes had no bacterial growth after additional 48 h incubation following the removal of silver.

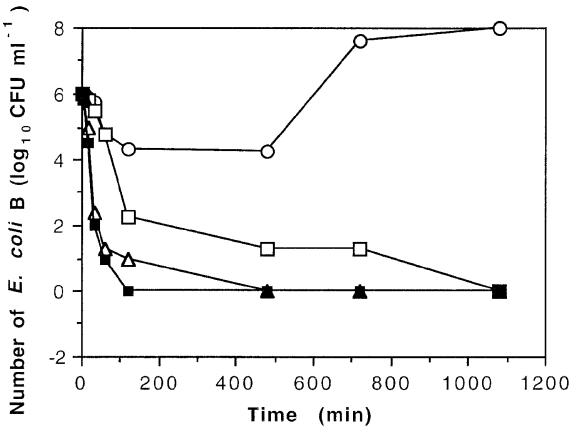

Figure 2. The killing curves of the silver ion against *E. coli* B. The inoculum was 10^6 CFU ml^{-1} and the silver concentrations were the IIC (○), the MBC (□), 10 times the IIC (△) and 40 times the IIC (■).

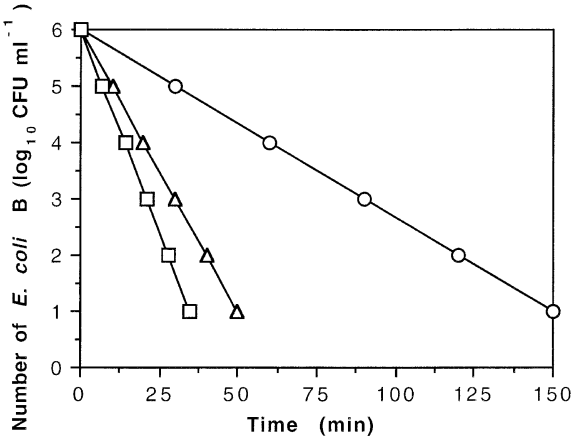

Figure 3. Log killing time (LKT) of *E. coli* B at silver ion concentrations of the MBC (○), 10 times the IIC (△), and 40 times the IIC (□).

decreased specific growth rate in log phase in the inhibitory behavior (unpublished data). Obviously, the IIC serves as an index of the growth delay and the CIC becomes a marker for growth shutdown in bacteria.

The data demonstrate that microbial population size was a limiting factor in the inhibition activity by the silver ion. However, with the increase of bacterial population size, the ratio of silver ions per cell is decreased. It is postulated that the antimicrobial properties depend upon specific sites for silver binding within the cell. Slawson *et al.* (1990) have reported that silver binding may occur in two stages: first, a rapid, reversible, metabolically independent surface binding; second, a metabolically dependent irreversible, intracellular accumulation. This could be a possible mechanism for bacterial sensitivity to silver. The lack of significant difference between the postagent (PAE) and continuous agent (CAE) effects would also support the proposed mechanism. PAE could thus serve as a practical parameter in the dosing regimes of silver used in a clinical application.

The tolerance property illustrated by the maximum tolerant concentration (MTC) implies that a correspondingly small proportion of the organisms could escape cell damage by silver. Originally, tolerance was defined as a decreased rate of killing or slow loss of viability upon exposure to bactericidal agents (Handwerger and Tomasz 1985). However, tolerance is currently poorly defined. The MTC could be used as a simple means of determining tolerance. The results show that the MTC of *E. coli* B was 20 times the IIC after 48 h exposure.

The time-kill study (LKT) indicated a close relationship between the silver concentration and the killing time. The killing action of the silver ion is characterized by the onset of immediate killing, a short killing time, and a rapid rate of kill. This action provides a possible explanation for why bacteria have so far failed to develop strong resistance to silver.

In conclusion, multiple parameters were clearly defined to evaluate the dose response relationship between the silver ion and *E. coli* B. At relatively low concentrations, the silver ion was inhibitory to the growth of *E. coli* B, showing a rapid killing activity and a prolonged post-agent effect. The parameters described in this model are beneficial in establishing both bacteriostatic and bactericidal properties of the silver ion. This model develops an evaluative reference for comparative analyses of a variety of silver agents and other antibacterial agents. Under this framework, our data facilitate further study and insight into the biological effects of silver agents.

Acknowledgment

This research was supported by the W. Harry Feinstone Chair of Excellence in Molecular Biology (held by S.E.S.)

References

Bailey WR, Scott EG. 1974 Determination of susceptibility of bacteria to antimicrobial agents. In Bailey WR, Scott EG, eds. *Diagnostic Microbiology*. St Louis: The C.V. Mosby Co; 316–317.

Becker RO, Spadaro JA. 1978 Treatment of orthopaedic infections with electrically generated silver ions. *J Bone and Joint Surg* **60(A)**, 871–881.

Berger TJ, Spadaro JA, Chapin SE, Becker RO. 1976a Electrically generated silver ions: quantitative effects on bacterial and mammalian cells. *Antimicrob Agents Chemother* **9**, 357–358.

Berger TJ, Spadaro JA, Bierman R, Chapin SE, Becker RO. 1976b. Antifungal properties of electrically generated metallic ions. *Antimicrob Agents Chemother* **10**, 856–860.

Bhagava KB, Shirali GN, Abhyankar VS, Garde KC. 1992 Treatment of allergic and vasomotor rhinitis by the local application of different concentrations of silver nitrate. *J Laryngol Otol* **106**, 699–701.

Deitch ED, Marino AA, Gillespie TE, Albright JA. 1983 Silver-nylon: a new antimicrobial agent. *Antimicrob Agents Chemother* **23**, 356–359.

Ersek RA, Denton DR. 1988 Cross-linked silver-impregnated skin for burn wound management. *J Burn Care Rehabil* **9**, 476–481.

Fifield FW, Kealey D. 1975 *Principle and Practice of Analytical Chemistry*. London: International Textbook Company Limited.

Fox CL, Jr 1968 Silver sulphadiazine: a new topical therapy for *Pseudomonas* in burns. *Arch Surg* **96**, 184–188.

Gilman AG, Goodman LS, Gilman A. 1980 *Goodman and Gilman's Pharmacological Basis of Therapeutics*, 6th ed. New York: Macmillan Publishing Co.

Golubovich VN, Rabotnova IL. 1974 Kinetics of growth inhibition in *Candida utilis* by silver ions. *Microbiol* **43**, 948–950.

Gottschalk G. 1988 *Bacterial Metabolism*. New York: Springer-Verlag.

Grier N. 1977 Silver and its compounds. In N. Grier, ed. *Disinfection, Sterilization, and Preservation.* Philadelphia: Lea & Febiger; 376–387.

Griffin PM, Tauxe RV. 1991 The epidemiology of infections caused by *Escherichia coli* 0157:H7, other enterohemorrhagic *Escherichia coli* and associated hemolytic uremic syndrome. *Epidemiological Review* **13**, 60–98.

Hamilton-Miller JM, Shah S, Smith C. 1993 Silver sulphadiazine: a comprehensive in vitro reassessment. *Chemotherapy* **39**, 405–409.

Handwerger S, Tomasz A. 1985 Antibiotic to tolerance among clinical isolates of bacteria. *Rev Infect Dis* **7**, 368–386.

Joklik WK, Willett HP, Amos DB, Wilfert CM. 1988 *Zinsser Microbiology*, 19th edn. Connecticut: Appleton & Lange.

Long DM. 1972. Silver nitrate therapy as an adjunct in the treatment of myelomeningocele. *J Neurosurg* **36**, 769–772.

MacKeen PC, Person S, Warner SC, Snipes W, Stevens SE, Jr 1987 Silver-coated nylon fiber as an antibacterial agent. *Antimicrob Agents Chemother* **31**, 93–99.

Modak SM, Fox CL, Jr 1973 Binding of silver sulfadiazine to the cellular components of *Pseudomonas aeruginosa. Biochem Pharmacol* **22**, 2391–2404.

Moing G, Thunus L, Dauphin JF, Depy-Dupont G, Deby C. 1990 Anti-inflammatory properties of a preparation containing low doses of copper, gold and silver. *Int J Tissue React* **12**, 251–262.

Odenholt-Tornqvist I.E, Lowdin E, Cars O. 1992 Post-antibiotic sub-MIC effects of vancomycin, roxithromycin, sparfloxacin, and amikacin *Antimicrob Agents Chemother* **36**, 1852–1858.

Sabath LD, Wheeler N, Laverdiere M, Blazevic D, Wilkinson BJ. 1977 A new type of penicillin resistance of *Staphylococcus aureus. Lancet* **1**, 443–447.

Slawson RM, Lee H, Trevors JT. 1990. Bacterial interactions with silver. *Biol Metals* **3**, 151–154.

Thibodeau EA, Handelman SL, Marquis RE. 1978 Inhibition and killing of oral bacteria by silver ions generated with low density direct current. *J Dent Res* **57**, 922–926.

Zhanel GG, Karlowsky JA, Davidson RJ, Hoban DJ. 1992 Effect of pooled human cerebrospinal fluid on the postantibiotic effects of cefotaxime, ciprofloxacin, and gentamicin against *Escherichia coli. Antimicrob Agents Chemother* **36**, 1136–1139.