Silver-based dressings have been used extensively in wound management in recent years, but data on their antimicrobial activity in the clinical setting are limited. In order to explore their effects on chronic leg ulcer flora, 14 ulcers were cultured after at least 3 weeks treatment with Aquacel Ag® or Acticoat®. Phenotypic and genetic silver resistance were investigated in a total of 56 isolates. Silver-based dressings had a limited effect on primary wound pathogens, which were present in 79% of the cultures before, and 71% after, treatment. One silver-resistant Enterobacter cloacae strain was identified (silver nitrate minimal inhibitory concentration (MIC) > 512 mg/l, positive for silE, silS and silP). Further studies in vitro showed that inducible silver-resistance was more frequent in Enterobacteriaceae with cephalosporin-resistance and that silver nitrate had mainly a bacteriostatic effect on Staphylococcus aureus. Monitoring of silver resistance should be considered in areas where silver is used extensively. Key words: silver; silver-resistance; wound dressing; ESBL; derepressed mutant.

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Silver is a toxic heavy metal with antimicrobial activity. In its ionized form, it has been used in wound care since at least the 19th century (1). Its use fell into oblivion at the beginning of the antibiotic era, but limited usage of silver recommenced in the 1960s in the management of severe burn wounds (2). With the emergence of multi-resistant bacteria, interest in alternatives to antibiotics has increased. Since the turn of the century a wide variety of silver-based dressings has become commercially available for different wound types, and the clinical use of silver has consequently increased. In England, the prescribed quantity of one of the leading silver-based dressings increased by 500% between 2003 and 2004 and by 130% between 2004 and 2005 (3).

Every time a new antimicrobial substance has been introduced, it has been a matter of time before bacterial resistance has emerged (4). Silver has been shown to induce resistance in a range of bacterial species (1, 5, 6). Several of the silver-resistant strains have been collected from silver-treated patients at burn centres, where these strains have sometimes caused outbreaks (7, 8).

An important mechanism of bacterial resistance to silver is encoded by the sil gene complex, which leads to reduced permeability through efflux pumps (9). This gene complex has so far been identified only on plasmids carrying genes encoding resistance to antibiotics (10, 11). Silver may thereby exert an indirect selective pressure, but there are also concerns regarding cross-resistance to antibiotics (12–14). The clinical implication of silver-resistance is, however, not yet clear.

The antimicrobial effects of silver are unspecific and are mediated by the formation of complexes with proteins, nucleic acids and biologically important anions, which impairs their function (1). The high reactivity of silver ions and their tendency to form complexes with proteins can make it difficult to show a bactericidal silver effect in vitro, since it is largely dependent on the medium used (15, 16). An infected chronic leg ulcer represents an even more complex environment, which could significantly decrease the bioavailability of silver and thereby its bactericidal toxicity. Silver concentrations within the wound environment can thereby be sub-optimal. Although silver-based dressings are marketed as alternatives to antibiotics, few randomized controlled studies on chronic leg ulcers have investigated the direct antimicrobial effects of silver-based dressings (17). To further complicate the situation, there are no standardized methods to determine bacterial susceptibility to silver and no accepted breakpoints (18). Thus, it is a challenge to establish routines for following the prevalence of silver-resistance at a clinical microbiology laboratory or to interpret published data on the efficacy of silver as an antimicrobial substance or as a selector of resistance.

The aims of this study were to explore the effects of silver-based dressings on the quality of the bacterial flora in chronic leg ulcers, and on primary wound pathogens in particular (Staphylococcus aureus, betahemolytic streptococci and Pseudomonas aeruginosa). In addition, the frequency of silver resistance, the presence of three sil genes, and the ability to develop silver-resistance during silver exposure in vitro were...
investigated. In the last case, Gram-negative bacteria frequently found in chronic wounds and with different degrees of susceptibility to antibiotics, beta-lactams in particular, were used.

MATERIALS AND METHODS

Bacterial isolates

A total of 56 isolates were included in the study (for distribution, see Tables I and II). They were routinely collected during the period November 2006 to September 2007 from chronic leg ulcers exhibiting signs of infection or delayed healing. The patients attended the wound treatment centre at Uppsala University Hospital, Sweden. Depending on the length of the treatment, the patients were categorized into two groups.

Group 1 (n = 7)

This group was treated with Aquacel Ag® (ConvaTec, NJ, USA) for 3–5 weeks. The wound dressings were changed three times a week. Bacterial samples were collected before and after silver treatment, and other antibacterial therapies, topical or systemic, were registered. None of the patients had been treated with topical silver in the last month prior to the first culture.

Group 2 (n = 7)

This group was treated with silver-based dressings for at least 2 months, and only the local treatment was recorded. Aquacel Ag® or Acticoat® (Smith & Nephew, Mölndal, Sweden) were used, and the dressing were changed with the same intervals as in group 1. Bacterial samples were collected only at the end of the observation period.

Bacterial cultures

After washing the ulcers with tap water according to local recommendations, samples were collected from the edge of the ulcers, using swabs. The swabs were thereafter placed in transport medium with charcoal (Copan, Corona, CA, USA). The samples were cultured on blood agar (Oxoid Ltd Base, Hampshire, UK), chocolate agar (Acumedia Manufacturers, Lancing, MI, USA), cystine lactose electrolyte deficient (CLED) agar (Becton-Dickinson, Franklin Lakes, NJ, USA), and anaerobic agar (Acumedia Manufacturers, Lancing, MI, USA). The agar plates were incubated for 48 h at 37ºC in appropriate atmospheres. Bacteria were identified using standard laboratory procedures and the Vitek 2® AutoMicrobiotic System (bioMerieux, Hazelwood, MI, USA). Carriage of genes encoding Panton-Valentine leukocidin or other staphylococcal exotoxins were not investigated. All isolates were frozen at −70ºC until analysed.

Antibiotic susceptibility

For primary wound pathogens and any silver-resistant isolate, the susceptibility to appropriate antibiotics was tested on IsoSensitest broth (Oxoid Ltd) containing silver nitrate at concentrations ranging from 4 to 512 mg/l. The final bacterial concentration was 10⁵ colony-forming units (cfu)/ml, controlled by viable count. After 24 h incubation, MIC was recorded as the lowest concentration yielding no visible growth. A silver nitrate MIC>512 mg/l classified the bacterium as silver-resistant.

Screening for silver resistance

The MIC of silver nitrate was determined by suspending bacteria in IsoSensitest broth (Oxoid Ltd) containing silver nitrate at concentrations ranging from 4 to 512 mg/l. The final bacterial concentration was 10⁵ colony-forming units (cfu)/ml, controlled by viable count. After 24 h incubation, MIC was recorded as the lowest concentration yielding no visible growth. A silver nitrate MIC>512 mg/l classified the bacterium as silver-resistant.

Minimum bactericidal concentration of silver

Minimum bactericidal concentration (MBC) was determined for all isolates of S. aureus and three randomly chosen Gram-negative rods (Enterobacter cloacae SM0700909 II, Proteus mirabilis SM0604597 II, and P. aeruginosa SM0700863 II) collected from patient group 2. The MIC was determined according to the SRGA guidelines and corresponded to the lowest silver concentration that killed 99.9% of a bacterial inoculum.

Exposure of bacteria to silver in vitro

Due to the results of the clinical investigation, the ability to develop silver-resistance during silver exposure in vitro was explored in 14 strains with different degrees of susceptibility to antibiotics. Two were culture collection strains and 12 were clinical strains isolated at the Departments of Clinical Microbiology, Uppsala or Malmö University Hospital: Escherichia coli S0506373 (multiresistant, extended spectrum beta-lactamase (ESBL) positive), ATCC 25922, B0709322, and B0801711, E. cloacae S0707396, S4279/06, A13210/03 (all three derepressed mutants (resistant to cefotaxime and ceftazidime), the first strain in addition colistin-resistant), B0708431, and S0801360, Klebsiella pneumoniae CCUG 54718 (multi-resistant, ESBL positive) and B0716185, and P. aeruginosa 20080 (multi-resistant, metallo-beta-lactamase positive), A36634 (carbapenem-resistant), and A12884.

To induce silver-resistance, 1 cfu of each strain was inoculated into IsoSensitest broth. After an overnight incubation at 37ºC, 10 μl of the bacterial suspension was inoculated into a series of tubes, each containing 1 ml IsoSensitest broth with increasing concentrations of silver nitrate (8–512 mg/l). The tubes were incubated overnight, and a new inoculum was collected from the tube with the highest silver nitrate concentration and still visible growth. The experiment was repeated until a silver nitrate MIC>512 mg/l was reached, or 10 passages had been performed. Susceptibility testing to appropriate antibiotics with the disc diffusion method was performed before and after the silver exposure. MBC-determination was performed on all strains with an inhibition zone difference of 5 mm or more.

To control how stable the resistance to silver was, all silver-resistant strains were subcultured six times on CLED-agar (Becton-Dickinson) without silver. After each passage, at least five colonies from each strain were tested to determine if they were still able to grow in IsoSensitest broth supplemented with silver nitrate (512 mg/l).

Detection of silver resistance genes

To investigate whether any of the strains carried genes encoding silver resistance (silE, silP, and silS), a polymerase chain reaction (PCR) assay was performed. Templates were prepared by heating a bacterial suspension at 95ºC for 10 min. Each PCR reaction contained 2 μl template DNA, 4 μl respective primer pair and HotStarTaq MasterMix (Qiagen, Hilden, Germany) to a total reaction volume of 25 μl. The primers had the following sequences: silE forward 5'-GTACTCCCCCGGAGCAGATCAT ATT-3' and reverse 5'-GGCCAGAGCTGACCGGTATTT-3'; silP forward 5'-CATGACATCATCCTGAAGACAGAAATGC-3' and reverse 5'-CATGACATCATCCTGAAGACAGAAATGC-3' and reverse 5'-CATGACATCATCCTGAAGACAGAAATGC-3'.
and reverse 5’-CGGGCAGACCAGCAATAACGATA-3’; silS forward 5’-GGGATGATCCGGATCGATGACAA-3’ and reverse 5’-GTTTCCTGCTGACAGCTTACAGATAGC-3’ (19). The mixtures were processed in a GeneAmp PCR system 9700 cycler (PE Applied Biosystems, Foster City, CA, USA), the programme consisting of 95°C for 2 min, followed by 40 cycles of 1 min at 95°C, 1 min at 55°C, and 3 min at 72°C. The final extension step was performed at 72°C for 5 min. As positive and negative controls, *K. pneumoniae* strain U-0608239 (carrier of the complete sil gene complex) and dh2o were included. Each DNA sample was run twice. Ten µl of each PCR reaction was analysed on a 1.2% agarose gel (Shelton Scientific Inc., Shelton, CT, USA) with ethidium bromide. The size of the PCR products was compared with a DNA molecular weight marker (o’generuler™, Fermentas). After completed electrophoresis, the gel was photographed and the bands analysed.

**Arbitrarily primed PCR**

Ten isolates from three patients from group 1 were analysed with arbitrarily-primed polymerase chain reaction (AP-PCR) in order to compare the DNA-patterns of isolates of the same species before and after treatment. Included isolates were *S. aureus* from patient 2, *Enterococcus* sp. and *Proteus* sp. from patient 4 and *S. aureus* and *P. aeruginosa* from patient 5. DNA was extracted by an M48 robot (GenoVision, West Chester, PA, USA) with the MagAttract DNA Mini M48 Kit (Qiagen, Solna, Sweden). The primers used had the following sequences: ERIC1R 5’-TGTAAGCTCTGGG-GATTACC-3’, ERIC2 5’-AGTAAATGACTGGGTGAGCG-3’, A70-10 5’-CAGACACGCC-3’, 208 5’-ACGGCCGACC-3’, and 272 5’-AGCGGCGCAA-3’ (20). The same PCR kit as above was used, and the reaction mixture contained 10×PCR buffer, 1.5 mM MgCl2, 200 µM of each dNTP, 0.5 µM primer (Eurogentec S.A., Seraing, Belgium), 0.625 U HotStarTaq DNA polymerase, and 5 µl template DNA. The total reaction volume was 25 µl. The PCR programme consisted of 95°C for 4 min, followed by 45 cycles of 1 min at 95°C, 1 min at 36°C, and 2 min at 72°C. The final extension step was performed at 72°C for 5 min. The PCR products were analysed as described above.

**RESULTS**

**Bacterial findings after silver treatment** in vivo

Five out of 7 patients in group 1 were colonized with primary wound pathogens before initiating the treatment. The species most frequently isolated was *S. aureus*, followed by *P. aeruginosa*. For details, see Table I. Three out of 5 patients were still culture-positive for *S. aureus* and two out of two for *P. aeruginosa* after 3 weeks with topical silver treatment. Continued growth of the same species in leg ulcers after treatment was in all five investigated cases due to a lack of bacterial eradication, as shown by the indistinguishable DNA patterns generated by AP-PCR (Fig. 1). The two patients in whom primary wound pathogens were successfully eradicated had received additional treatment with antibiotic drugs. No increased antibiotic resistance was observed after the silver treatment.

In group 2 patients, primary wound pathogens could be found in all chronic wounds except for one after
bacteria were thereby more than 32 times higher than the MBCs for the Gram-negative bacteria.

**Induction of silver-resistance after repeated exposure in vitro**

Five out of the 14 silver-exposed strains (36%) developed resistance to silver. Of these five strains, four were resistant to third-generation cephalosporins before the silver exposure. To induce silver-resistance, 2–8 passages were necessary (Table III).

Two out of five *E. cloacae* strains (S4279/06 and S0707396) developed resistance to silver nitrate after 2–3 passages. Both were resistant to cefotaxime and ceftazidime. In addition, resistance to silver was induced in the two included ESBL-producing strains. For *K. pneumoniae* strain CCUG 54718 two passages were required, whereas *E. coli* strain S0506373 needed as many as eight passages.

The only strain without antibiotic resistance prior to the exposure and in which silver-resistance could be induced was *E. coli* strain B0709322. For this strain, five passages were necessary, but its silver resistance was relatively unstable. When the five silver-resistant strains were subcultured on a medium without silver, only *E. coli* strain B0709322 lost its silver-resistance; this occurred after two passages.

**Sil genes**

The three *sil* genes were detected in four strains: two *E. cloacae* strains and two *K. pneumoniae* strains (Table III). Of these strains, three exhibited a silver nitrate resistance.

**Table II. Bacterial findings according to laboratory records after long-term treatment with silver-based dressings (group 2)**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Topical treatment</th>
<th>Duration of treatment (months)</th>
<th>Bacterial findings after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aquacel Ag®</td>
<td>3</td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td>2</td>
<td>Acticoat</td>
<td>5</td>
<td>Faecal flora</td>
</tr>
<tr>
<td>3</td>
<td>Acetic acid</td>
<td>3</td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td>4</td>
<td>Aquacel Ag®</td>
<td>14</td>
<td><em>E. cloacae</em></td>
</tr>
<tr>
<td>5</td>
<td>Aquacel Ag®</td>
<td>2</td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td>6</td>
<td>Aquacel Ag®</td>
<td>2</td>
<td>Nephrus sp.</td>
</tr>
<tr>
<td>7</td>
<td>Aquacel Ag®</td>
<td>3</td>
<td>Skin flora</td>
</tr>
</tbody>
</table>

**Table III. Characteristic of strains with phenotypic and/or genetic resistance to silver in the study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Silver-resistance genes</th>
<th>No. of passages</th>
<th>Other properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. cloacae</em></td>
<td>+ +  +</td>
<td>NA</td>
<td>Cefotaxime I</td>
</tr>
<tr>
<td>SM0700965 II</td>
<td>+ +  +</td>
<td>2</td>
<td>Stable</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>+ +  +</td>
<td>3</td>
<td>D mutant</td>
</tr>
<tr>
<td>S0707396</td>
<td>+ +  +</td>
<td>5</td>
<td>Carbapenem R°</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>− −  −</td>
<td>8</td>
<td>D mutant</td>
</tr>
<tr>
<td>B0709322</td>
<td>− −  −</td>
<td>Unstable</td>
<td>Colistin R</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>+ +  +</td>
<td>2</td>
<td>ESBL positive</td>
</tr>
<tr>
<td>B0716185</td>
<td>+ +  +</td>
<td>stable</td>
<td>ESBL positive,</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>+ +  +</td>
<td>8</td>
<td>Outbreak strain</td>
</tr>
<tr>
<td>CCUG 54718</td>
<td>+ +  +</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>+ +  +</td>
<td>Stable</td>
<td></td>
</tr>
</tbody>
</table>

*For induction of silver-resistance and its stability.  
*After silver exposure.

NA: not applicable; NI: no induction; I: indeterminate; R: resistant.

**Minimal inhibitory concentrations and Minimum bactericidal concentrations for silver nitrate**

The MIC values for silver nitrate were determined to 16–32 mg/l for the *S. aureus* strains and to 8–16 mg/l for the three randomly selected Gram-negative rods isolated from patient group 2. The MBCs for the Gram-negatives were close to their MICs and were in the range 16–32 mg/l. All *S. aureus* strains had a silver nitrate MBC > 512 mg/l. The MBCs for the Gram-positive
MIC > 512 mg/l after exposure. The \textit{sil}E and \textit{sil}S genes, but not the \textit{sil}P gene, were found in two strains (\textit{E. cloacae} S0707396 and \textit{P. aeruginosa} A12884), of which the \textit{E. cloacae} strain grew in a silver nitrate concentration of 512 mg/l. The silver resistance demonstrated in \textit{E. coli} strains B0709322 and S056373 could not be explained by the presence of \textit{sil} genes. Both strains were \textit{PCR}-negative for all three genes. None of the Gram-positive isolates were \textit{PCR}-positive.

\textbf{Resistance to antibiotics after silver exposure in vitro}

In one of the silver-resistant strains, there was more than a 5 mm change in the size of the inhibition zone after silver exposure. \textit{E. cloacae} strain S4279/06 showed a 32-fold increase of the MIC-value for imipenem (before: 0.125 mg/l; after: 2.0 mg/l), and a 16-fold increase for meropenem (before: 0.125 mg/l; after: 2.0 mg/l).

\textbf{DISCUSSION}

In the present study, 14 patients with chronic leg ulcers were treated with silver-based dressings for 3 weeks up to several months, and the bacterial flora was thereafter explored. Although it has been suggested that silver is ideal for topical management of wound infections and at-risk wounds and that the need for systemic antibiotics can thereby be reduced (6), it could not eradicate or prevent primary wound pathogens from colonizing the wounds. Furthermore, silver appeared to have had a limited impact on the faecal flora, and it took only 3 weeks of Aquacel Ag\textsuperscript{®} treatment to find a silver-resistant \textit{E. cloacae} strain carrying \textit{sil} genes. This is, to our knowledge, the first documented clinical isolate exhibiting silver resistance after local silver treatment in Sweden. This is also the first time the exposure time and conditions have been given for selection of silver-resistance with silver-based wound dressings \textit{in vivo}.

Several studies have reported silver resistance among Gram-negative bacteria in clinical settings. Members of the \textit{Enterobacteriaceae} family and \textit{P. aeruginosa} predominate the reports (7, 8, 21–25) and \textit{E. cloacae} seems to be one of the more prone species to carry \textit{sil} genes and develop phenotypic silver resistance according to recent studies (19, 26).

In contrast, Gram-positive bacteria are seldom implicated in phenotypic or genetic silver resistance (27). In this study, they had slightly higher silver MICs than the Gram-negative bacteria without being multi-resistant, which is in accordance with other studies (26). Most remarkable were the high silver nitrate MBCs for the \textit{S. aureus} isolates, indicating a possible tolerance to or only a bacteriostatic activity of silver nitrate. This could be part of the explanation to why the performance of silver nitrate has been poorer than nanocrystalline silver both \textit{in vitro} and \textit{in vivo} on \textit{S. aureus} (28–30).

Lack of bactericidal activity includes other silver salts and beta-haemolytic streptococci needs to be further explored, but commonly used inhibition tests or tests that cannot discover a killing rate less than 99.9% should not be applied.

For the Gram-negative bacteria the silver nitrate MICs were almost identical to the silver nitrate MBCs, and these bacteria appeared to have chosen another way to handle the silver toxicity. The most frequent resistance mechanism in this study was inducible efflux, and the pumps were encoded by \textit{sil} genes. Since these genes are often harboured by plasmids carrying antibiotic resistance genes (10, 11), use of silver can indirectly take part in the selection of multi-resistant members of the \textit{Enterobacteriaceae} family. Two ESBL-producing strains were included in the study, and both developed silver resistance after exposure. The clinical relevance of this finding is supported by the fact that the only silver-resistant bacterium found in the study of Ip et al. (26) was an ESBL producer. A silent horizontal transfer between non-primary wound pathogens may not be a clinical problem for the silver-using dermatologist but for the infection control team at the hospital.

Another effective way to block silver is by decreased permeability. The main mechanism is loss of porins (water-filled protein channels) in the outer membrane of Gram-negative bacteria (13). This can lead to a cross-resistance to hydrophilic antibiotics, such as cephalosporins and carbapenems (13). In this context the patient isolate SM0700965 II was of interest. Its reduced susceptibility to cefotaxime could not be explained by induced production of AmpC, a chromosomally mediated beta-lactamase of \textit{E. cloacae}. Further experiments were therefore carried out.

The results showed that the relationship between carriage of \textit{sil} genes and phenotypic silver resistance was not straightforward. However, stable phenotypic silver resistance seemed to be associated with reduced susceptibility to third-generation cephalosporins. A cross-resistance to carbapenems was, in addition, observed in a derepressed \textit{E. cloacae} strain after silver exposure \textit{in vitro}. A porin deficiency could be the link between resistance to silver and to beta-lactams with broader spectrum (13, 31, 32). Users of silver-based dressings, especially in areas where ESBL-producing enterobacteria or derepressed \textit{E. cloacae} strains are a clinical problem, should consider the potential risk that a combination of these bacteria with a certain silver pressure may have for the development of carbapenem-resistance and outbreaks. The \textit{sil} gene carrying \textit{K. pneumoniae} strain CCUG 54718 caused the largest Scandinavian outbreak by an ESBL producer, so far, and it originated from a patient with extensive wounds (33). Attention should therefore not only be paid to primary wound pathogens and their antibiograms when topical silver treatment is recommended.
In conclusion, silver-based wound dressings are marketed as an alternative to antibiotics. In this study they had a limited effect on the colonizing primary wound pathogens and the faecal flora of chronic leg ulcers. Apart from the fact that only 3 weeks of treatment was necessary to select for a silver-resistant bacterium, a cross-resistance to cephalosporins was identified. To be able to monitor silver resistance at clinical microbiology laboratories, it is necessary to establish an international standard with accepted breakpoints.

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