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**Prevalence of silver resistance genes in bacteria isolated from human and horse wounds**

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**Keywords: silver resistance, *sil* genes, *Enterobacter cloacae*, wounds**

**Non-standard abbreviations:** Non-Silver Containing Hydrofiber<sup>®</sup> dressing = NSH

Silver Containing Hydrofiber<sup>®</sup> dressing = SCH

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## Abstract

The aim of this study was to investigate the prevalence of silver resistance genes in 172 bacterial strains which had been isolated from both human and equine wounds. PCR screening for 8 currently named genes in 3 silver resistance transcriptional units, *si/E*, *si/RS* and *si/P*, *si/CBA* and *si/F* was performed on total DNA extracted from all clinical isolates. Plasmids were isolated from *sil*-positive strains to determine if the genes were present on the chromosome. MICs and zone of inhibition assays were utilised to examine phenotypic resistance to silver nitrate and ionic silver. Evidence of silver resistance genes was demonstrated in six strains of *Enterobacter cloacae*, an organism rarely implicated as a primary pathogen in chronic wounds. MIC data showed that all strains were inhibited at silver nitrate concentrations  $\geq 5\text{mg/L}$ . When tested against a silver-containing absorbent wound dressing all strains showed inhibition of growth after 24 hours. In MIC and zone of inhibition studies, inhibition was evident but reduced in strains which contained *sil* genes. Although *sil* genes were found in six of the wound isolates studied, the genes were consistently associated with a non-pathogenic bacterium. Furthermore, investigation of phenotypic resistance in *sil*-positive isolates showed that silver continued to be effective.

## Introduction

Silver resistance has been reported in bacteria isolated from both clinical (Carr and Rosenkranz 1975; Annear et al., 1976; Hendry and Stewart, 1979; Markowitz et al., 1983; Slots et al., 1990; Klasen, 2000) and environmental (Belly and Kydd, 1982; Haefeli et al., 1984; Grewal and Tiwari, 1990; Choudhury and Kumar, 1998) settings. The genetic basis for silver resistance was first reported by McHugh *et al* in 1975

demonstrating that silver resistance was plasmid encoded and has been confirmed by others (Haefeli et al., 1984; Grewal and Tiwari, 1990; Gupta et al., 1998; Davis et al., 2005). The physiological, biochemical, genetic and structural studies of the silver resistance determinant plasmid pMG101 established the molecular basis of silver resistance (Gupta et al., 1999, Silver, 1999; Gupta et al., 2001; Silver 2003). Plasmid pMG101 is a 182 kb, transferable plasmid encoding resistance to silver (nine Open Reading frames [ORFs] in three transcriptional units), mercury, tellurite, ampicillin, chloramphenicol, tetracycline, streptomycin, and sulphonamide (McHugh *et al* in 1975; Gupta et al., 1999). The presence of this plasmid has been shown to confer resistance to silver at concentrations six or more times that of a sensitive *E. coli* can tolerate (Gupta et al., 1998). *Sil* gene functions have been assigned based on homologous genes that encode resistance to other genes. The silver resistance cassette of genes encodes two silver efflux pumps (one an ATPase and the other chemiosmotic) and two periplasmic Ag<sup>+</sup>-binding proteins (Silver 2003). The aim of this investigation was to determine the prevalence of silver resistance genes in clinical wound isolates and to determine the prevalence of silver resistance in equine and human wound care. Human wound isolates previously investigated for the presence of *silE*, *silP* and *silRS* using crude DNA preparations were further investigated using separate total and plasmid DNA isolation protocols and improved primers for the 3 *sil* genes together with additional primers for *silCBA* and *silF* in the present study. The aim of this study was to investigate the presence and prevalence of silver resistance genes in human and horse wound isolates, to confirm that genes existed on plasmids and to determine if a silver-containing wound dressing was effective against wound isolates possessing silver resistance genes.

## Materials and Methods

### Organisms

Sixty bacterial strains isolated from horse wounds at Leahurst School of Veterinary Medicine, University of Liverpool, UK were screened for silver resistance genes. In addition one hundred and twelve strains isolated from human diabetic foot ulcers (Percival et al., 2008) which had previously been investigated for the presence of 3 *sil* genes were also screened further for additional evidence of *sil* genes. All bacteria isolated from horse wounds were characterised using standard techniques and rapid identification methods (API and Vitek, BioMérieux Inc., France). The horse wound isolates which had been previously identified included; *Enterococcus faecalis* (1), *Bacillus cereus* (4), *Bacillus* spp (2), *Enterobacter hirae* (4), *Streptococcus viberus* (1), *Streptococcus* sp (1), *Streptococcus dysgalactiae* (1), *Enterococcus avium* (1), *Brevibacillus laterosporus* (1), *Staphylococcus lentus* (2), *Staphylococcus auricularis* (1), *Staphylococcus aureus* (2), *Staphylococcus haemolyticus* (2), *Enterococcus faecium* (2), *Enterococcus faecalis* (4), *Enterobacter cloacae* (6), *Enterobacter arburiae* (1), *Enterobacter amnigenus* (1), *E. coli* (11), *Acinetobacter calcoaceticus baumanii* complex (3), *Pasteurella haemolytica* (1), *Proteus vulgaris* (2), *Pseudomonas aeruginosa* (1), *Acinetobacter baumannii* (1), *Serratia marcescens* (1), *Serratia plymuthica* (1), *Morganella morganii* (2), *Providencia rettgeri* (1), *Citrobacter freundii* (1), *Providencia alcalifaciens* (1). Details of the human diabetic foot isolates can be found in Percival et al., 2008.

### DNA extraction

DNA was extracted from samples of pelleted control organisms (*E. coli* J53 and *E. coli* J53-1 pMG101) and all clinical isolates using a GenElute bacterial DNA isolation

kit (Sigma, Dorset, UK) following the manufacturers' instructions. DNA was extracted from all isolates using this method and provided high quality, clean total DNA (genomic and plasmid) for use in PCR amplification. All bacterial strains were grown on tryptone soy agar (TSA; LabM, Bury, UK) plates for 24-48 hours (37°C). Following incubation a single colony from each bacterium was used to inoculate 10 mls of tryptone soy broth (TSB; LabM, Bury, UK) and grown up overnight before carrying out DNA extraction.

### **Plasmid extraction**

Plasmid isolation was performed on control (*E. coli* J53 and *E. coli* J53-1 pMG101) and clinical test isolates using a Plasmid Miniprep kit (Qiagen, Crawley, UK) following the manufacturers' instructions. DNA was extracted from all isolates using this method and provided high quality, clean plasmid DNA for use in PCR amplification. Bacterial inocula were prepared as described above with the exception that plasmid cultures were made in Luria-Bertani (LB; LabM, Bury, UK) broth. All Plasmids were stored at -20°C until required.

### **PCR primers and protocol**

Samples were prepared for PCR by adding 3 µl template (total or plasmid DNA) to 25µl REDtaq ReadyMix PCR mix (Sigma, Dorset, UK) 0.3-0.6 µM forward primer and reverse primers. The reaction volume was made up to 50 µl with nuclease-free water. Primers were designed using Primer3 software (Rozen and Skaletsky, 2000) using the *Salmonella typhimurium* plasmid pMG101 sequence (accession number AF067954 (Gupta et al., 1999) and purchased from Thermo Electron GmbH (Ulm, Germany). *Sil* gene primer sequences were as follows: *si/E* forward

124 aggggaaacggtctgacttc, *silE* reverse atatccatgagcgggtcaac, *silRS* forward  
 125 ggcaatcgcaatcagatttt, *silRS* reverse gtggaggatactgcgagagc, *silCBA* forward  
 126 cgggaaacgctgaaaaatta, *silCBA* reverse gtacgttcccagcaccagtt, *silF* forward  
 127 cgatatgaatgctgccagt, *silF* reverse attgccctgctgaataaacg, *silB* forward  
 128 caaagaacagcgcgtgatta, *silB* reverse gtcagacattgctggcata, *silA* forward  
 129 cttgagcatgccaacaagaa, *silA* reverse cctgccagtacaggaacat, *silP* forward  
 130 cctgggtttacagcgtcatt, and *silP* reverse atggcacctgaggtttgttc. PCRs were carried out  
 131 using the following PCR protocol: 3 minutes at 95°C followed by 45 cycles of 95°C  
 132 for 40 seconds, 55-57°C for 40 seconds and 72°C for 40 seconds. A final elongation  
 133 step was performed at 72°C for 10 minutes. 20 µl of each sample was applied to a 2%  
 134 agarose Tris acetate-EDTA (TAE) gel containing 0.5 µg/ml ethidium bromide (Sigma,  
 135 Dorset, UK) and electrophoresed for 1 hour at 70 volts/cm. A 50 bp DNA ladder  
 136 (Invitrogen, Paisley, UK) was also applied to the gel to define PCR fragment sizes.  
 137 UV gel images were obtained using an Alpha Innotech Fluorchem SP system (CA,  
 138 USA).

139

#### 140 **Minimum inhibitory concentration (MIC)**

141 MICs for silver nitrate were investigated for all *sil* positive and a selection of *sil*  
 142 negative isolates following British Society for Antimicrobial Chemotherapy  
 143 guidelines (Andrews, 2001). Briefly, 150 µl of an overnight culture in cation-adjusted  
 144 Mueller Hinton Broth (MHB, LabM, Bury, UK) was mixed with an equal volume of  
 145 silver nitrate solution to give final concentrations ranging from 500 mg/L to 1 mg/L in  
 146 microtitre plates (n=3). After a 20 hour incubation at 37°C, plates were examined to  
 147 determine the lowest concentration at which microbial growth was inhibited.

148



## Corrected zone of inhibition (CZOI)

Growth inhibition was measured to test the efficacy of a silver-containing wound dressing against *sil*-positive bacteria. CZOIs for silver were determined for all bacterial strains shown to be positive for *sil* genes plus additional clinical and laboratory bacteria from both horse and human origin for comparison. Briefly, TSA plates were inoculated in duplicate with each test strain using a sterile swab and incubated for 4 hours at 37°C. Two pieces of silver Hydrofiber® dressing (SCH; ConvaTec, Deeside, UK) measuring 2.5 cm<sup>2</sup> were placed on opposite sides of the plate and hydrated with approximately 700 µl minimal recovery diluent (MRD; LabM, Bury, UK). Additional plates were prepared using a non-silver containing Hydrofiber® dressing (NSH; ConvaTec, Deeside, UK) as controls. After 30 minutes incubation, one of the two dressings was removed from the plate surface to monitor speed of kill. After 24 hours, the total width of clear zones around the perimeter of the remaining dressings were measured (horizontally and vertically). Hydration of the Hydrofiber® samples caused the dressings to contract inwards whilst increasing in height, therefore corrected zones of inhibition were calculated by subtracting the dimensions of the dressing from these zones and obtaining a mean. Data was analysed (unpaired *t*-test) and presented using GraphPad Prism 4 software.

## Results

Overall 172 bacteria isolated from chronic wounds in humans and horses were screened for the presence of the silver resistance gene cassette. A total of 6 (2 from human, 4 from horse) bacteria were shown to contain the genes; all of which were strains of *Enterobacter cloacae*. A total of 10 *E. cloacae* strains were investigated,

therefore over half of these were found to contain silver resistance genes. Initial PCR results for all *sil*-positive *Enterobacter cloacae* produced positive results for all 7 primer pairs, covering 8 silver resistance genes (Figure 1). Isolation of plasmids from all 6 *sil*-positive strains provided evidence that these genes were present extrachromosomally.

MIC results for silver nitrate demonstrated that strains possessing the *sil* gene cassette had a MIC value of  $\geq 5\text{mg/L}$ . *Sil*-negative strains, which included *E. cloacae* and *E. coli* isolates, had MIC values in the range of 1-2.5mg/L. Further experiments to determine the efficacy of ionic silver presented in a wound dressing on all *sil*-positive strains of *E. cloacae* and *sil*-negative strains of *Enterobacter* spp demonstrated that the SCH was able to kill all strains of bacteria after an initial 30 minute contact time (Figure 2A). Zones of inhibition were clearly apparent around the SCH for all strains tested (Figure 2A). Results showed a significant difference ( $P = 0.0003$ ) between the CZOI for the *sil*-positive and the *sil*-negative strains tested (Figure 2B). The zones of inhibition for *sil*-positive strains were smaller than those generated by the SCH in the presence of *sil*-negative strains. No growth inhibition was seen around the perimeter of the NSH control dressing with any strain.

## Discussion

Data from this study has shown that 3.5% of a total of 172 bacterial wound isolates possess silver resistance genes. The prevalence of *sil*-positive strains in horse wounds was 6.7% compared to 1.8% in human wounds. All 6 isolates were strains of *E. cloacae*. This suggests that silver resistant *E. cloacae* affects both human and veterinary medicine and implies the possibility that the presence of *sil* genes in *E.*

*cloacae* is inherent. The scientific basis for the strong correlation between the presence of *sil* genes and *E. cloacae* is not yet known.

Enterobacteriaceae are responsible for a variety of nosocomial infections including bacteraemia, lower respiratory tract infections, skin and soft tissue infections, urinary tract infections (UTIs), intra-abdominal infections (Paterson, 2006) and advanced periodontitis (Davis et al., 2005). They have also been shown to exhibit resistance to a number of antibiotics (Paterson, 2006). All *sil* gene positive bacteria identified in this study were strains of *E. cloacae* which are frequently isolated from both chronic and acute wounds (Cason et al., 1966; Fox, 1968). Resistance to silver was first described in *Enterobacter* spp isolated from burn wounds (Bowler and Davies, 1999 a and b). Further evidence on silver resistance transpired from studies using *Salmonella typhimurium* which had also been isolated from burn wounds (McHugh et al., 1975). In this study the presence of the *sil* gene cassette was confirmed on plasmid pMG101.

Investigation of the prevalence of silver resistance is important because of the possibility of plasmid transfer and hence cross-resistance in other bacteria. Chronic wounds are colonised and infected with an array of different bacteria. The wound therefore provides an ideal environment for the transfer of plasmids, which may contain *sil* genes, between strains particularly as biofilms are becoming significant in this area (Percival et al., 2005, 2007, 2008; Davis et al., 2008). Conversely, the situation of silver resistance may be similar to that associated with *S. aureus* methicillin resistance in that silver resistance is restricted to a particular species of bacteria and not easily transferable which will make it relatively manageable as compared to a host of organisms acquiring resistance.

223

224 Ionic silver in the form of a silver-containing wound dressing was shown to be  
225 efficacious on both the *sil*-negative and positive strains of bacteria. However, it was  
226 evident that zones of inhibition associated with the *sil*-negative isolates were greater  
227 than those associated with the *sil*-positive isolates. MIC values showed that the  
228 inhibitory concentrations of silver nitrate were only marginally higher in *sil*-positive  
229 compared to *sil*-negative strains. Since other silver resistant strains have shown MIC  
230 values of up to 5440mg/L (Ip et al., 2006; Lansdown and Williams 2007), the isolates  
231 from the current study appear only to have low level phenotypic resistance. In  
232 addition, as discussed by Chopra in 2007 there are no recognised breakpoints for  
233 determining true silver resistance, as with other antimicrobial therapies. Evidence  
234 from this study implies that the presence of *sil* genes alone, does not transcribe into  
235 phenotypic silver resistance and is not sufficient to offer complete protection from the  
236 antimicrobial activity of Ag<sup>+</sup>.

237

238 Considering the length of time that silver formulations have been used in clinical and  
239 domestic settings, it might have been expected that a higher percentage of the isolates  
240 would have tested positive for genetic Ag<sup>+</sup> resistance. The low rate of silver resistance  
241 in bacteria isolated in the present study underlines the fact that in our opinion, the  
242 danger posed by silver resistant strains in the wound care setting is low and that silver  
243 resistance genes appear to be confined to *E. cloacae* and not pathogenic bacteria  
244 associated with infected wounds. Evidence here and in the literature (Hendry and  
245 Stewart, 1979; Chopra, 2007) indicates that the organisms found to have silver  
246 resistance are rare, suggesting that silver resistance genes are difficult to transfer and  
247 be maintained by other bacteria. Hence the threat to wound care at this present time is

248 small and the positive clinical outcomes associated with silver usage far out weigh the  
249 risks of bacterial resistance.  
250

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255

256     **Transparency declaration**

257     None to declare.

258

259

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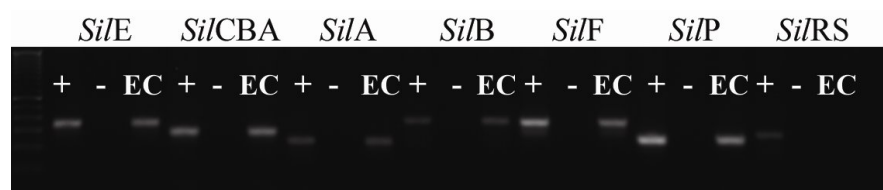
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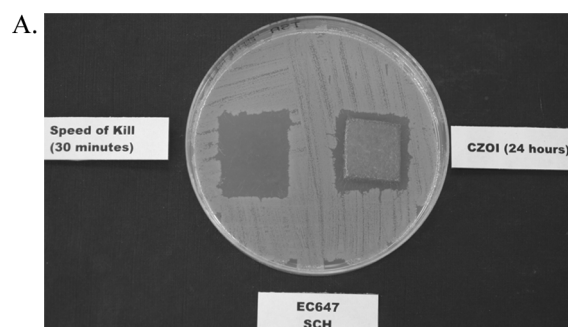
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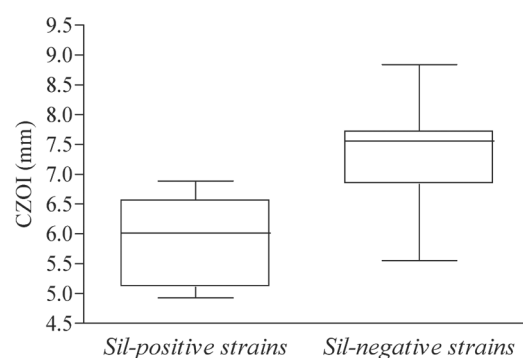


**Figure 1**

Agarose gel electrophoresis of PCR products from Plasmid DNA extracted from human *E. cloacae* strain Z951124 was one of 6 strains shown to contain the complete *sil* cassette. Positive control = plasmid DNA extracted from *E.coli* J53-1 pMG101 (+); negative control = plasmid DNA extracted from *E. coli* J53 (-) and clinical strain (Z951124) from a human diabetic foot ulcer (EC).



B. Corrected zones of inhibition of *sil* positive versus *sil* negative bacteria treated with SCH



## Figure 2

A TSA plate inoculated with horse strain EC647 (*sil*-positive) was treated with a SCH. Application of the SCH dressing for 30 minutes demonstrated that bactericidal activity occurred within that time (A, left hand side). After 24 hours a clear zone of inhibition (CZOI) was visible around the perimeter of the dressing (A, right hand side). Comparison of the width of CZOI produced by *sil*-positive versus *sil*-negative showed that zones were significantly smaller in *sil*-positive strains (B).