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Short Communication

Isolation of silver- and antibiotic-resistant *Enterobacter cloacae* from teeth

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Antibiotic-resistant bacteria pose a serious threat to human health; hence the mechanisms that lead to their selection need to be investigated. One possible mechanism is that the silver and mercury in amalgam dental restorations may select for bacteria that contain heavy metal and antibiotic-resistance determinants, leading to the spread of these resistances, particularly if they are contained on the same mobile genetic element. The incidence of silver-resistant bacteria on teeth is investigated in this work. Two silver-resistant *Enterobacter cloacae* isolates were isolated from infected teeth containing dental restorations. Both isolates were also resistant to ampicillin, erythromycin, and clindamycin. The *silE* gene, which is encoded on the silver resistance operon, has been sequenced from both isolates. Results suggest that the silver resistance operon is encoded on plasmid DNA.

Key words: antibiotic resistance; dental amalgam; *Enterobacter cloacae*; *sil*; silver resistance

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The control of antibiotic-resistant bacteria is a major problem for the medical community. A key issue is the potential increase of selective pressure on antibiotic-resistant bacteria. A current concern is that the presence of mercury in amalgam dental restorations may provide an indirect selective pressure for antibiotic resistance in oral bacteria (24). This is because mobile elements encoding mercury- and silver-resistance genes often also encode antibiotic resistance (8, 12, 21–23, 26, 27). Should mercury select for such resistant strains it would also provide an indirect selection for antibiotic-resistant bacteria. Numerous studies have attempted to resolve this question (10, 19, 24, 30). What has not been addressed is whether the silver contained in amalgam fillings may also select for oral bacteria, which encode genetically linked silver and antibiotic resistance.

In the UK as of 1997, approximately 22 million amalgam fillings were placed every year by the National Health Service (9). Amalgam dental restorations are composed of 50% Hg, 35% Ag, 13% Sn, and 2% Cu (18). Should a link between amalgam dental restorations and silver and antibiotic resistance be confirmed, this would pose a substantial selective pressure for antibiotic-resistant bacteria.

Numerous environmental silver-resistant isolates have been documented (4, 8, 11, 14). The majority of silver-resistant clinical isolates are from burns wards, where silver sulfadiazine or silver nitrate was used in a topical cream, and also from patients with silver tracheotomy tubes (3, 7, 15, 20, 21).

Silver- and antibiotic-resistant clinical bacterial isolates have been documented (7, 20, 21). These studies were carried out prior to the characterization of the silver-resistance determinant and therefore the majority of isolates have not been subjected to molecular analysis and the silver- and antibiotic-resistance determinants have not been conclusively linked to the same mobile genetic elements. However, the *Salmonella typhimurium* isolate from McHugh *et al.*'s study (21) has been characterised. This isolate has been shown to contain a 180 kb *Inc*H1 plasmid, pMG101, which encodes transferable resistance to silver nitrate, mercuric chloride, ampicillin, chloramphenicol, tetracycline, streptomycin, and sulphonamide (12, 21). Interestingly, further study of plasmids encoding silver resistance showed that of those tested, all belonged to the *Inc*H incompatibility group (13).

From study of the *S. typhimurium* pMG101 plasmid, a model for the mechanism of silver resistance has been proposed and reviewed in depth (12, 28). Briefly, the silver-resistance (*sil*) operon encodes two parallel silver efflux pumps. The expression of these proteins is regulated at the transcriptional level by the *silE, silS*, and *silR* gene products (12). Of importance to this work, significant

conservation of the *silE* DNA sequence has been demonstrated across numerous isolates, making it an ideal target when PCR screening for the *sil* operon (13).

Whether silver-resistant bacteria can be isolated from teeth has not been investigated.

The aim of this study was to determine if silver-resistant bacteria were associated with amalgam dental restorations and antibiotic resistance. A collection of oral isolates was screened for resistance to silver, silver sulfadiazine, and antibiotics. The collection of bacteria (obtained from Dr D. Spratt, The Eastman Dental Institute, UK) was previously isolated from seven extracted teeth for a prior study (1). The teeth were removed from seven different patients, after attempts to restore previously infected dental restorations failed due to re-infection. Of the seven teeth, five contained ceramo-metal crowns, one an amalgam restoration, and one a composite resin restoration; samples from an eighth tooth lacking a dental restoration were taken as a control. None of the patients had received antibiotics in the previous 6 months. The teeth were processed according to a validated protocol to ensure there was no contamination of the internal surfaces from an external source; this included surface decontamination of the teeth with 30% (v/v) hydrogen peroxide solution (Sigma Chemical Ltd, Poole, UK) and 10% (w/v) povidine-iodine solution (Betadine, Seton Healthcare Group plc, Oldham, UK) (1).

The collection was initially screened for silver resistance on Mueller Hinton (MH) agar (Oxoid, Hampshire, UK) containing 50 μM, 200 μM, 300 μM, and 500 μM $AgNO_3$ (Sigma, Dorset, UK). The MIC of silver nitrate ($AgNO_3$) and silver sulfadiazine (AgSd) on putative silver-resistant strains was then determined using an agar dilution technique with the following concentrations of $AgNO_3$ or AgSd (Sigma): 0; 50 μM; 100 μM; 250 μM; 600 μM; and 1000 μM. Plates were incubated aerobically and anaerobically at 37°C for 18 h. Media composition has been shown to affect the MIC values of heavy metals (25). To account for this, silver-sensitive *Staphylococcus aureus* NCTC 6571 (Oxford strain), *Pseudomonas aeruginosa* NCTC 10662, and *Enterobacter cloacae* strain 05 (donated by P. Stapleton, School of Pharmacy, UCL, London) were used as negative control strains. All had an MIC of 250 μM for $AgNO_3$ and 100 μM for AgSd. *Pseudomonas stutzeri* AG259 with an MIC of 1000 μM for $AgNO_3$ was used as positive control strain (donated by J. T. Trevors, University of Guelph, Canada).

From 97 isolates screened, two were identified as silver-resistant, both having an MIC for $AgNO_3$ of 1000 μM. With an MIC of 50 μM, neither isolate was resistant to silver sulfadiazine. These isolates were identified, using an API 20 E identification system (bioMérieux SA, Lyon, France), as *E. cloacae* and were designated *E. cloacae* Ag703 and *E. cloacae* Ag1157. *E. cloacae* Ag703 was isolated from a molar tooth that had had a ceramo-metal crown. *E. cloacae* Ag1157 was isolated from a molar tooth that had had an amalgam restoration.

The identification was confirmed by sequencing of the 16S rRNA gene, which was polymerase chain reaction (PCR) amplified from genomic DNA using the following degenerate primers (Sigma-Genosys, Haverhill, UK): 27F-AGAGTTTGATCMTGGCTCAG and 1492R-TACGGYTACCTTGTTACGACTT (M = C:A; Y = C:T) (17). All genomic DNA was isolated using a PureGene kit following the supplier's instructions (Gentra Systems, Minneapolis, MN). Typical PCR reactions consisted of an initial 95°C for 4 min followed by 30 cycles of 95°C for 1 min, 50–60°C for 1 min (depending on the primer Tms [melting temperatures]) and 72°C for 1–5 min (depending on the expected product length). PCR products were purified using QIAquick PCR Purification Kit following the manufacturer's protocol (Qiagen®, West Sussex, UK). All DNA sequencing was performed using ABI BigDye terminator mix (PE Biosystems, Buckinghamshire, UK) and analysed on a Perkin Elmer 310 genetic analyser.

The *silE* gene sequence is well conserved at the DNA level (13). PCR primers internal for the *silE* gene (Forward GTACTCCCCCGGACATCAC and reverse GGCCAGACTGACCGTTATTC) were derived from those used by Gupta *et al.* (13). These were used to PCR screen genomic DNA obtained from the silver-resistant isolates. A 362 bp PCR product was amplified from both strains. The sequence from the *E. cloacae* Ag703 PCR product (Accession number AY679159) shared the greatest homology with the *silE* gene of *Escherichia coli* pTJ100 (99% homology/300 bp) (Accession number AY214164). This ~100 kb conjugative plasmid encodes resistance to tetracycline and ampicillin (16). The PCR product shared slightly lower homology (96% homology/300 bp) with *silE* from pMG101 (Accession number AF067954). The predicted pMG101 mature SilE peptide is encoded by a 368 nucleotide sequence and contains 10 conserved histidine residues (12). The SilE protein also shares significant homology with an *E. coli* copper binding protein, PcoE (Accession number X83541.1) (6). The predicted protein sequence encoded by the *E. cloacae* AG703 PCR product shared 84% identity with the pMG101 SilE and 41% identity with the *E. coli* PcoE. The *E. cloacae* PCR was predicted to encode six of the 10 histidine residues conserved in SilE. Southern Blot analysis was carried out on undigested and *Bam*HI (Promega, Southampton, UK) digested preparations of genomic DNA, and plasmid DNA from both isolates. Genomic DNA was isolated using a PureGene kit following the supplier's instructions and plasmid DNA was isolated using QIAGEN® QIAprep Spin miniprep and midiprep kits following the supplier's protocol (West Sussex, UK). Hybridization probes were derived from 16S rRNA and *silE* sequence PCR amplified from *E. cloacae* Ag703genomic DNA. Prior to labelling, probes were agarose gel purified using the QIAquick© gel extraction kit (Qiagen®) according to the manufacturer's instructions and confirmed via sequencing. Southern Transfer of DNA, labelling of probe, hybridization, signal generation, and stripping of blots between experiments were performed using the ECL™ direct nucleic acid labelling and detection system kit (Amersham Life Science, Little Chalfont, UK) following the manufacturer's instructions. DNA was autocrosslinked to Hybond N + membrane (Amersham Pharmacia Ltd, Buckinghamshire, UK) in a Stratalinker® UV crosslinker (Stratagene Ltd, Cambridge, UK). Blots were washed at high stringency (0.2% SSC in primary wash buffer) and Hyperfilm™ ECL™ film was used for signal detection.

Both isolates contain multiple plasmids and appear to have the same plasmid profile (Fig. 1), indicating they may be the same strain. The silE probe hybridised to uncut plasmid DNA running level with the 48.5 kb marker (Fig. 2) and to DNA fragments larger than 48.5 kb in uncut total DNA. This DNA band is barely visible on an agarose gel in uncut plasmid DNA (Fig. 1), suggesting this plasmid is present at a relatively low copy number. The difference in hybridization profile in uncut total genomic vs. plasmid DNA may be an artefact of the different methods by which the total genomic DNA and plasmid DNA where isolated. The total genomic DNA isolation protocol is not optimised for the isolation of large plasmids. Alternatively, in the total genomic DNA

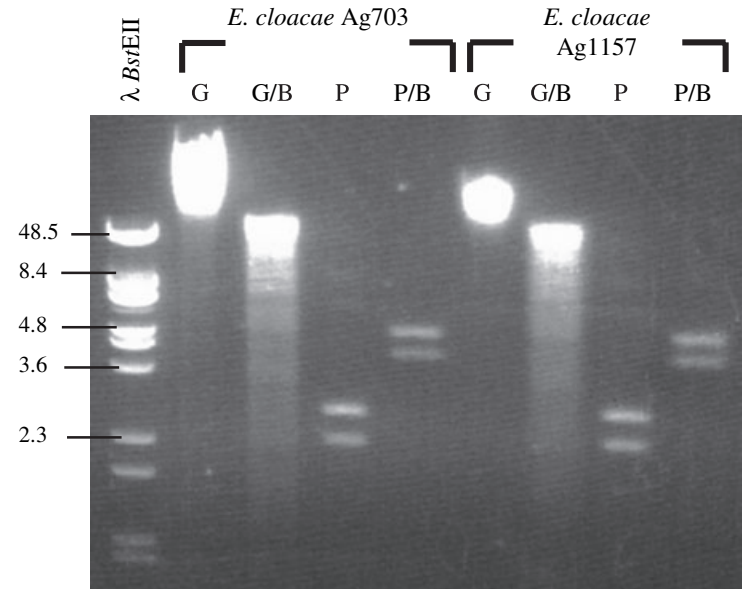

Fig. 1. Agarose gel of undigested and *Bam*HI digested genomic and plasmid DNA isolated from *E. cloacae* Ag703 and Ag1157. The same plasmid profile is seen in both isolates. Figures left of the gel refer to the size (in Kb) of the λ *Bst*EII digested molecular weight markers. Key to lanes: G = Genomic DNA; P = Plasmid DNA; B = *Bam*HI digested.

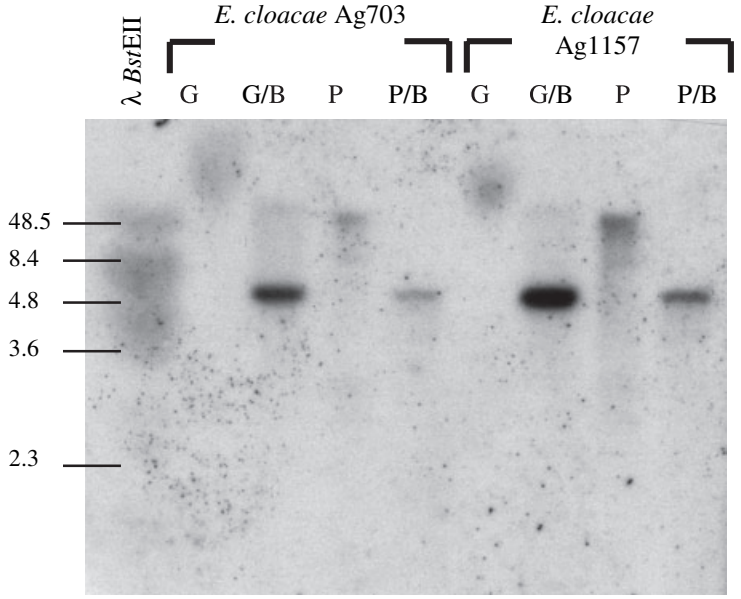

Fig. 2. Southern blot of undigested and *Bam*HI digested genomic and plasmid DNA isolated from *E. cloacae* Ag703 and Ag1157. The blot is hybridized with PCR amplified *silE* probe from *E. cloacae* Ag703 and λ probe. Subsequent hybridization of this blot with 16sRNA probe revealed no hybridization to plasmid DNA (results not shown). Figures left of the blot refer to the size (in Kb) of the λ *Bst*EII digested molecular weight markers.

lanes the plasmid DNA may be complexed with chromosomal DNA within the agarose gel. In both silver-resistant isolates the same hybridization pattern was observed in the *Bam*HI digested total genomic and plasmid DNA (Fig. 2). To confirm plasmid DNA preparations were not contaminated with chromosomal DNA, the same blot was probed with the 16S rRNA probe. No hybridization was seen in plasmid DNA lanes (results not shown). Together these results strongly suggest the *silE* gene is encoded on extrachromosomal DNA in both isolates.

The antibiotic resistance profile of the silver-resistant isolates was screened using antibiotic disk diffusion (Oxoid) on bacterial lawns grown on MH agar. Zones of inhibition were judged to be resistant according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines (2). Antibiotic-sensitive *E. coli* NCTC 10418 was used as a negative control. The antibiotics screened and the concentration of antibiotic per disk were as follows: piperacillin 100 μg; trimethoprim 2.5 μg; gentamicin 10 μg; ciprofloxacin 5 μg; ceftazidine 30 μg; ampicillin 10 μg; erythromycin 5 μg; clindamycin 2 μg; cephaloridine 5 μg; tetracycline 10 μg; and penicillin G 1 μg. Resistance to $HgCl_2$ was ascertained by streaking onto MH $HgCl_2$ 40 μM (Sigma). Mercury-resistant *S. aureus* NCTC 50581 and *S. aureus* NCTC 6571 (Oxford strain) were used as positive and negative controls, respectively. All media were incubated aerobically at 37°C for 18 h. Both isolates were resistant to ampicillin, penicillin G, erythromycin, and clindamycin.

This is the first time silver-resistant bacteria have been isolated from teeth. Silver-resistant clinical isolates have been documented from numerous burns wards where silver sulfadiazine or silver nitrate was used as a topical antimicrobial (3, 15, 20, 21). It should be noted that the silver-resistant isolates identified in this study, *E. cloacae* Ag703 and Ag1157, were not resistant to silver sulfadiazine.

Although *E. cloacae* Ag703 and Ag1157 were obtained from the site of an infection, we cannot say if they were the causative agent. However, silver-resistant *E. cloacae* have been isolated in outbreaks in various burns units (15, 20). Interestingly many of these isolates, like *E. cloacae* Ag703 and Ag1157, were also resistant to multiple antibiotics. *E. cloacae* Ag703 and Ag1157 were resistant to antibiotics which are recommended and commonly used for the treatment of dental infections, i.e. ampicillin and erythromycin (5, 31). This may have hindered effective treatment.

E. cloacae do not normally colonise a healthy mouth. However, a study of 427 adults with advanced periodontitis recovered nonoral gram-negative facultative rods from 43% of the subjects, with *E. cloacae* being the most commonly isolated species (29). The same is true with respect to re-infected endodontic treatments, where nonoral isolates are found at the site of the infection (1, 31). Such isolates may have been introduced during re-installation of the filling. In this

case it may be that silver resistance has allowed the *E. cloacae* isolates to fill a niche they would not normally inhabit. The collection records state that two different patients harbour what appears to be the same strain. It is possible the patients came into contact with it via the same source, for example the same dentist. However, the collection records do not state where previous dental care was given.

The *sil* operons characterised to date have all been located on *Inc*H plasmids (12, 13). In this study hybridization of *silE* probe to what appears to be a large plasmid is consistent with these findings. Whether the observed antibiotic resistances are encoded on this plasmid remains to be investigated.

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