Journal of Antimicrobial Chemotherapy (2002) **49**, 777–783
DOI: 10.1093/jac/dkf019

JAC

Prevalence and antibiotic resistance profile of mercury-resistant oral bacteria from children with and without mercury amalgam fillings

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Received 10 July 2001; returned 10 December 2001; revised 17 December 2001; accepted 5 February 2002

Genes encoding resistance to mercury and to antibiotics are often carried on the same mobile genetic element and so it is possible that mercury-containing dental materials may select for bacteria resistant to mercury and to antibiotics. The main aim of this study was to determine whether the prevalence of Hg-resistant oral bacteria was greater in children with mercury amalgam fillings than in those without. A secondary aim was to determine whether the Hg-resistant isolates were also antibiotic resistant. Bacteria in dental plaque and saliva from 41 children with amalgam fillings and 42 children without such fillings were screened for mercury resistance by cultivation on a $HgCl_2$-containing medium. Surviving organisms were identified and their susceptibility to mercury and to several antibiotics was determined. Seventy-eight per cent and 74% of children in the amalgam group and amalgam-free group, respectively, harboured Hg-resistant bacteria; this difference was not statistically significant. Nor was there any significant difference between the groups in terms of the proportions of Hg-resistant bacteria in the oral microflora of the children. Of Hg-resistant bacteria, 88% and 92% from the amalgam group and the amalgam-free group, respectively, were streptococci; 41% and 33% were resistant to at least one antibiotic, most frequently tetracycline. The results of this study show that there was no significant difference between children with amalgam fillings and those without such fillings with regard to the prevalence, or the proportion, of Hg-resistant bacteria in their oral microflora. The study also found that Hg-resistant bacteria were common in children regardless of whether or not they had amalgam fillings and that many of these organisms were also resistant to antibiotics.

Introduction

For many years, mercury amalgam has been one of the most widely used materials for the restoration of carious lesions. The typical filling in a human molar tooth contains *c.* 750–1000 mg of mercury and studies have shown that the body retention of mercury from dental amalgam fillings is between 3 and 17 μg/day.[1] A major concern in the use of mercury amalgams, therefore, has been related to its toxicity towards both the patient and the dental practitioner.[2,3] A less-considered side effect of the use of dental amalgams is the promotion of mercury resistance in oral bacteria. Mercury is known to be released from dental amalgams and a study in Canada has shown that the level of mercury in plaque taken from amalgam surfaces ranged from 0.5 to 1.3 μg/mg dry weight of plaque.[4] A further study by the same authors subsequently confirmed that oral bacteria such as streptococci and actinomycetes were resistant to mercury concentrations of between 5 and 40 mg/L.[5]

Genes encoding resistance to antimicrobial agents are often linked genetically and can be transferred together,[6,7] and there are many reports of antibiotic-resistant bacteria also being resistant to mercury.[8–11] Hence, it is possible that the constant exposure of oral bacteria to the mercury released

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from amalgam fillings could promote the development and maintenance of mercury- and antibiotic-resistant bacteria in the oral cavity. Only a few studies have looked at the combined prevalence of mercury and antibiotic resistance in oral bacteria and most of these have been carried out with primates. Summers *et al.*[11] have demonstrated a relationship between the presence of amalgam fillings and the occurrence of mercury- and antibiotic-resistant bacteria in monkeys. In their longitudinal study they found that placement of amalgam fillings resulted in a significant increase in the incidence of Hg-resistant bacteria. The Hg-resistant organisms isolated included oral streptococci, members of the Enterobacteriaceae and enterococci. Most of these Hg-resistant isolates were also resistant to one or more antibiotics. The study also revealed that, in some of the enterobacterial isolates, determinants for mercury resistance and antibiotic resistance were carried on the same plasmid and, more importantly, in the same integron. Only two studies appear to have been published concerning the prevalence of antibiotic- and mercury-resistant bacteria in human subjects,[12,13] and, of these, only one has examined the oral microflora.[13] The latter study found no association between the presence of mercury amalgam fillings and the prevalence of Hg-resistant oral bacteria.

The fact that mercury from dental amalgams may be promoting an increase in antibiotic resistance in oral bacteria is obviously of concern to dental practitioners, as antibiotics are used in the treatment of a number of oral infections e.g. periodontitis and abscesses.[14] Oral bacteria such as the streptococci are also a major cause of bacterial endocarditis and infections in immunocompromised individuals for which antibiotic therapy is essential.[15] Furthermore, the ingress of these bacteria into the gut may result in the spread of antibiotic resistance to the normal gut microflora. The aims of this study were to determine whether the presence of mercury amalgam results in an increase in the prevalence, or oral load, of mercury-resistant oral bacteria and to investigate whether such bacteria also exhibit resistance to antibiotics.

Materials and methods

Patient selection and sampling

Healthy children aged between 5 and 16 years attending the Department of Paediatric Dentistry at the Eastman Dental Hospital were recruited. These included one group of children without amalgam restorations and a second group of children who had at least two tooth surfaces restored with amalgam. In each child, the number of tooth surfaces restored with amalgam was counted. Children with chronic medical disorders, known viral carriage or who had been treated with antibiotics during the preceding 3 months were excluded.

Plaque was collected from around the gingival margins and the surfaces of all the teeth using an alginate swab. The swab was then placed into a sterile bijou bottle containing 4 mL of Calgon–Ringer's solution (Oxoid Ltd, Basingstoke, UK) and five glass beads. One millilitre of saliva was collected from each child by gently dribbling into a sterile universal container. The samples were taken to the laboratory for processing within 30 min of collection.

Sample processing

On arrival in the laboratory, the swab from each patient was vortexed for 30 s and then added to the saliva sample from the same patient and vortexed for a further 30 s. Serial 1 in 10 dilutions (up to 10^{-7}) of the sample were then prepared in Tryptone Soy broth (Oxoid). Preliminary screening of the samples for the presence of Hg-resistant microbes was carried out as follows. Duplicate 100 μL aliquots of the undiluted sample and of dilutions (10^{-1}, 10^{-2}, 10^{-3}) of the sample were spread over the surfaces of Mueller–Hinton (MH) agar (Oxoid) containing 40 μM $HgCl_2$. These plates were prepared on the same day that the specimen was collected and were kept out of direct sunlight before use. Four plates were used for each dilution: two were incubated at 37°C anaerobically for 48 h and two aerobically for 48 h. Duplicate 100 μL aliquots of dilutions (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) of each sample were also inoculated on to Hg-free MH plates and these were also incubated under aerobic and anaerobic conditions to determine, respectively, the total aerobic and anaerobic viable counts. Anaerobic incubation was carried out in an anaerobic chamber (Fred Baker Scientific, Runcorn, UK). Following incubation, colonies growing on the Hg-containing and Hg-free media were enumerated. Representative colonies (at least four from both the aerobically and anaerobically incubated plates) from the mercury-containing plates were subcultured on to Hg-containing MH and Hg-free MH plates to obtain pure cultures. The MIC of mercury for each of the isolates was determined using an agar dilution technique employing MH medium containing a range of $HgCl_2$ concentrations (1.0–1024 μM). Mercury-resistant strains of *Staphylococcus aureus* NCTC 50581 and *Enterococcus faecium* 6641H1 (kindly supplied by Professor Anne Summers, University of Georgia, USA) were used as positive controls. The MICs of a range of antibiotics (penicillin, ampicillin, erythromycin, vancomycin and tetracycline) for each isolate were then determined using an agar dilution technique employing Isosensitest agar containing 5% horse blood. The concentrations of antibiotics used were: pencillin, ampicillin and erythromycin, 0.008–8.0 mg/L; vancomycin, 0.0625–16 mg/L; tetracycline, 0.016–128 mg/L. The MIC of $HgCl_2$ for each of the control Hg-resistant strains under the test conditions described above was 32 μM. Oral isolates able to grow on the MH agar containing 16 μM $HgCl_2$ or greater were regarded as being mercury resistant and these were identified to genus level on the basis of Gram's stain, morphology, atmospheric requirements and standard biochemical tests. The vast majority of mercury-resistant isolates were found to be streptococci

Table 1. Identity of mercury-resistant bacteria from the two patient groups

Organism	No. (%) of isolates from children: with amalgam fillings	without amalgam fillings
Streptococcus sanguis	3 (4)	1 (2)
S. mitis	7 (9)	9 (14)
S. oralis	36 (48)	17 (27)
Streptococcus salivarius	5 (7)	8 (13)
Streptococcus parasanguis	2 (3)	6 (9)
Streptococcus vestibularis	0 (0)	1 (2)
Streptococcus anginosus	0 (0)	2(3)
Other streptococci	13 (17)	15 (23)
S. aureus	1 (1)	1 (2)
Coagulase-negative staphylococci	3 (4)	4 (6)
Stomatococcus sp.	4 (5)	0 (0)
Pseudomonas sp.	1 (1)	0 (0)
Total	75	64

and these were further identified to species level on the basis of their carbohydrate-fermenting profiles.[16]

Statistical analysis

The outcome measure to be analysed statistically was the proportion of Hg-resistant microbes in the oral microflora expressed as a percentage of both the aerobic and anaerobic counts. Both outcomes were severely positively skewed and could not be subjected to parametric statistical tests. Initially, therefore, data were analysed using the non-parametric Mann–Whitney test using SPSS software[17] and checks were therefore made that the overall shape and distribution of data from each group did not significantly differ from each other using the Kolmogorov–Smirnov test. In order to optimize statistical power (i.e. to increase the likelihood of correctly identifying a significant result), the alternative approach of transforming the data into a near-normal distribution and undertaking multivariate multilevel regression[18] was also implemented using MLwiN software.[19] Any differences in the proportion of Hg-resistant bacteria between the two groups was assessed using the χ^2 test. All statistical tests were two-tailed, with the 5% level of statistical significance adopted throughout.

Results

Forty-one children were recruited into the amalgam group; mean age 10.7 years, S.D. 3.1 (range 6.3–16.8 years). The minimal number of tooth surfaces restored with amalgam for each child was two (mean number of surfaces 4.7, range 2–12). A further 42 children without amalgam restorations, mean age 10.2 years, S.D. 2.9 (range 5.9–16.3 years), were included in the control group.

Bacteria able to grow on MH medium containing 16 μM $HgCl_2$ were considered to be mercury resistant, as the MIC of $HgCl_2$ for the two control mercury-resistant bacteria (*S. aureus* and *E. faecium*) was 32 μM.

Of those children who had amalgam fillings, 32 (78%) harboured mercury-resistant bacteria. A similar number (30) and proportion (71%) of children without amalgam fillings also harboured bacteria resistant to mercury. There was no significant difference between the two groups in terms of the number of individuals harbouring Hg-resistant bacteria.

Of the Hg-resistant bacteria isolated, 88% of those obtained from the group with amalgam fillings were streptococci (Table 1). Streptococci comprised a similar proportion (92%) of the Hg-resistant bacterial isolates from the group without amalgam fillings. Of those streptococci that could be identified to species level, *Streptococcus oralis* comprised the greatest proportion of Hg-resistant bacteria in both patient groups. However, Hg-resistant strains of this organism were isolated more frequently from children with amalgam fillings than from those without ($P < 0.05$). No Hg-resistant obligate anaerobes were isolated and only one Hg-resistant Gram-negative bacterium (a pseudomonad) was found.

Table 2 shows the proportions of Hg-resistant bacteria (expressed as percentages of the total viable aerobic and anaerobic counts) present in the samples. There was no significant difference between the two groups in terms of the proportions of Hg-resistant bacteria present in the saliva/plaque samples. This was the case regardless of whether the proportions were calculated on the basis of the total viable aerobic count ($P = 0.107$) or the total viable anaerobic count ($P = 0.256$).

Table 2. Proportions of mercury-resistant microbes in the oral microflora of children with and without amalgam fillings

	Proportion of aerobic count (%)		Proportion of anaerobic count (%)	
	without amalgam	with amalgam	without amalgam	with amalgam
Range	0–15.00	0–14.88	0–17.32	0–9.28
Median	0.0083	0.085	0.0140	0.0550
Inter-quartile range	0.2848	0.7978	0.3261	0.7948
Mann–Whitney *P* value (Kolmogorov–Smirnov *P* value)	0.101 (0.225)	0.218 (0.174)		
Multivariate regression *P* value[a]	0.107	0.256		

[a]Multivariate regression in this instance uses multilevel modelling to analyse the two outcomes (with respect to the amalgam groups) simultaneously in order to optimize statistical power.

Table 3. Resistance to antibiotics of mercury-resistant bacterial isolates from children

	No. (%) of Hg-resistant isolates exhibiting resistance	
Antibiotic	without amalgam fillings	with amalgam fillings
Penicillin	5 (8)	5 (7)
Ampicillin	0 (0)	0 (0)
Erythromycin	9 (14)	14 (19)
Vancomycin	0 (0)	1 (1)
Tetracycline	11 (17)	19 (25)
Any antibiotic	21 (33)	31 (41)

Thirty-one (41%) and 21 (33%) of the Hg-resistant bacterial isolates from the group with and without amalgam fillings, respectively, were also resistant to at least one antibiotic; some of these isolates were resistant to more than one antibiotic (Table 3). None of the Hg-resistant bacteria exhibited resistance to ampicillin and only one (a *Pseudomonas* sp.) was resistant to vancomycin. The Hg-resistant bacteria were most often resistant to tetracycline and, to a lesser extent, to erythromycin. Resistance to penicillin was not encountered frequently among these bacteria. Table 4 shows that of the Hg-resistant isolates from children with amalgam fillings, the majority of isolates that were also resistant to one or more antibiotics were strains of *S. oralis*. This was in marked contrast with those isolates displaying resistance to both Hg and an antibiotic from children without amalgam fillings (Table 4). In this group, the antibiotic-resistant isolates showed a greater species diversity with no particular species being dominant in terms of frequency of isolation.

Discussion

In this study we have compared the prevalence of Hg-resistant bacteria in the oral cavity of children with and without amalgam fillings and also the proportions of Hg-resistant oral bacteria in these two groups. There was no significant difference between the groups in terms of either the number of children harbouring Hg-resistant bacteria or the oral load of such organisms.

There are, unfortunately, few studies with which the results of this investigation can be compared directly and, to complicate matters, there are many methodological differences between studies. One of the main problems with regard to the latter is that there is no internationally accepted criterion defining what is meant by 'mercury resistance' and a variety of $HgCl_2$ concentrations have been used in media to differentiate between 'sensitive' and 'resistant' strains. These include 7.4, 8.4, 18.4, 20, 37, 50, 59 and 100 μM.[10–13,20–26] The only study in humans that is comparable to the present one appears to be that of Edlund *et al.*,[13] who also found that there was no significant difference in the proportion of Hg-resistant bacteria in the saliva of individuals with and without amalgam fillings. However, a number of differences in experimental design between the two studies make direct comparison difficult. First, the present study used children rather than adults and four times as many individuals were included in each group; this increases the reliability of the data analysis. Secondly, the present study involved the processing of saliva plus dental plaque rather than just saliva: the former approach

Table 4. Antibiotic resistance profiles of mercury-resistant bacteria from children with (+Am) and without (–Am) amalgam fillings

	Number of isolates displaying resistance							
	tetracycline		erythromycin		penicillin		vancomycin	
Hg-resistant isolate	+Am	–Am	+Am	–Am	+Am	–Am	+Am	–Am
S. oralis	12	1	9	2	0	1	0	0
S. mitis	2	4	1	0	1	0	0	0
S. sanguis	0	0	1	0	0	0	0	0
S. parasanguis	0	3	0	2	0	2	0	0
S. salivarius	0	0	0	2	0	0	0	0
Other streptococci	4	3	2	2	1	1	0	0
Stomatococcus spp.	0	0	0	0	2	0	0	0
Pseudomonas sp.	0	0	1	0	1	0	1	0
S. aureus	1	0	0	0	0	0	0	0
Coagulase-negative staphylococci	0	0	0	0	0	1	0	0

provides a more representative sample of the oral microflora. Thirdly, in the study by Edlund *et al.*[13] the medium used for isolation of Hg-resistant bacteria contained blood (unlike the medium used in the present study), which is known to neutralize the antibacterial activity of mercury compounds.[5,21,27] Finally, unlike the present study, Edlund *et al.*[13] did not determine the MICs of mercury for the putative Hg-resistant organisms detected on the primary plates, nor did they use Hg-resistant organisms as controls to ensure that the oral isolates were truly Hg resistant. Interestingly, the proportions of 'Hg-resistant' bacteria in the saliva samples reported by Edlund *et al.*[13] were extremely high (of the order of *c.* 50%) regardless of whether or not the individual had amalgam fillings. This is in marked contrast with the proportions of Hg-resistant bacteria found in the present study, which were generally <1% of the total viable count, raising the possibility that many of the organisms classified as Hg resistant in the study of Edlund *et al.*[13] were not truly Hg resistant. In the present study, however, all of the putative Hg-resistant organisms isolated on the primary plates were retested and designated as Hg resistant only if their MIC on retesting in pure culture was identical to, or greater than, that of control, Hg-resistant, strains of *S. aureus* and *E. faecium*. While there are no other comparable studies in humans, Summers *et al.*[11] carried out a longitudinal study of Hg-resistant oral bacteria in monkeys before and after placement of mercury amalgam fillings and found that the proportion of Hg-resistant oral streptococci (i.e. strains with an MIC > 50 μM $HgCl_2$) increased following amalgam installation.

A few studies have also reported on the effect of amalgam fillings on mercury resistance in the intestinal microflora, although the results are conflicting. Osterblad *et al.*[12] found that there was no difference between individuals with and without amalgam fillings with respect to the proportion of Hg-resistant Gram-negative aerobic bacilli, whereas Wireman *et al.*[10] reported that installation of amalgam fillings in monkeys increased the number of faecal bacteria (including aerobic Gram-negative bacilli) resistant to mercury. Edlund *et al.*[13] found a significant increase in the proportion of Hg-resistant *Bacteroides* spp. (but not in the proportion of Hg-resistant *Escherichia coli* or enterococci) in the intestinal microflora of adults with amalgam fillings compared with those without.

In the present study the majority of Hg-resistant bacteria isolated from both subject groups were streptococci. Furthermore, in the case of the amalgam group, most of these were *S. oralis*. Even in the amalgam-free group, *S. oralis* comprised a high proportion of the Hg-resistant isolates. *S. oralis*, together with *Streptococcus mitis*, are common members of the microflora of several oral habitats including supragingival plaque, saliva and mucosal surfaces.[28] Unfortunately, very little is known concerning the susceptibility of oral streptococci (or, indeed, any oral bacteria) to mercury. In a study of the susceptibility of oral streptococci and *Actinomyces* spp. to mercuric chloride, Lyttle & Bowden[5] found that the former were more resistant to mercury than the latter. All 10 of the streptococci tested were able to grow in a medium containing 5 mg/L (18.4 μM) $HgCl_2$ but only eight and five strains were able to grow at 36.8 and 110.4 μM $HgCl_2$, respectively. Surprisingly, in the present study, no Hg-resistant species from other genera encountered frequently in the oral microflora of children were isolated. Although the MH medium used for isolation of Hg-resistant organisms was not supplemented with blood (because of its neutralizing effect on the $HgCl_2$) it is able to support the growth of the predominant organisms present in plaque and saliva, i.e. *Streptococcus*

spp., *Actinomyces* spp., *Haemophilus* spp. and *Veillonella* spp.[28,29]

Many of the Hg-resistant isolates (41% and 33%, respectively, in the amalgam-containing and amalgam-free groups) were also resistant to one or more antibiotics. While no similar study on human subjects has been carried out, Summers *et al.*,[11] in an analysis of antibiotic resistance in Hg-resistant oral streptococci from monkeys, also found that a high proportion (59%) was resistant to one or more antibiotics; resistance to streptomycin was encountered most frequently. In both groups of children in the present study, resistance to tetracycline and erythromycin was encountered most frequently. Resistance to these two antibiotics appears to be common, and occurs at high frequencies, in oral streptococci. For example, Ioannidou *et al.*[30] reported that 38.5% and 23% of 200 oral viridans group streptococci isolated from the oropharynx of healthy children were resistant to erythromycin and tetracycline, respectively. Resistance to tetracycline and erythromycin was found to be common among viridans group streptococci responsible for a variety of infections in a survey carried out by Teng *et al.*[31] Of the 207 isolates tested, 53% were resistant to tetracycline while 40% were resistant to erythromycin. A number of other studies have reported similar high frequencies of resistance to erythromycin (38–53.3%),[32–34] and to tetracycline (12–41%);[32,35] in viridans group streptococci isolated from patients with serious infections. Of particular interest, and concern, was the finding that most of the Hg- and antibiotic-resistant isolates from the amalgam group were identified as being strains of *S. oralis*, a bacterium that is very closely related to *Streptococcus pneumoniae*, with which it shares over 99% sequence homology.[36] This bacterium, as well as being associated with bacterial endocarditis, has been shown to be the causative agent of a variety of infections in immunocompromised patients (particularly neutropenic patients and individuals undergoing transplants) and so may be regarded as a significant human pathogen.[16,37–39] In keeping with the results of this study, other investigations have shown that this bacterium is frequently resistant to tetracycline and erythromycin. Hence, Teng *et al.*[31] found that 60% of 40 isolates were resistant to tetracycline while 55% were resistant to erythromycin. Wisplinghoff *et al.*[40] found that 26% of 19 strains of *S. oralis* isolated from blood samples from neutropenic patients were resistant to tetracycline.

The results of this study have shown that there was no statistically significant difference in the prevalence, or the proportion, of Hg-resistant bacteria in the oral microflora of children with and without amalgam fillings. Surprisingly, mercury-resistant oral bacteria were isolated frequently from children regardless of their exposure to amalgam fillings and many of these bacteria were found to be resistant to antibiotics, principally tetracycline and erythromycin. We are currently undertaking studies to investigate the nature of the genes encoding Hg resistance and antibiotic resistance in these bacteria and possible linkage between them.

Acknowledgements

We would like to thank Professor Anne Summers for providing bacterial strains and for helpful discussions. This work was supported by project grant 9810729 from the Medical Research Council, UK.

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