ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1995, p. 2499–2502 Vol. 39, No. 11
0066-4804/95/$04.00+0

Antimicrobial and Mercury Resistance in Aerobic Gram-Negative Bacilli in Fecal Flora among Persons with and without Dental Amalgam Fillings

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Received 13 June 1995/Returned for modification 31 July 1995/Accepted 7 September 1995

Antimicrobial resistance is more widespread than can be accounted for as being a consequence of the selection pressure caused by the use of antibiotics alone. In this study, we tested the hypothesis that a high mercury content in feces might select for mercury-resistant bacteria and thus for antimicrobial resistance linked to mercury resistance. Three subject groups with different exposures to dental amalgam fillings were compared. None of the subjects had taken antimicrobial agents during the three preceding months or longer. The group exposed to dental amalgam ($n = 92$) had 13 times more mercury in feces than the group that had never been exposed to amalgam ($n = 43$) and the group whose amalgam fillings had been removed ($n = 56$). No significant differences in either mercury resistance or antibiotic resistance in the fecal aerobic gram-negative flora of these subject groups were seen. The following antimicrobial resistance frequencies were detected with a replica plating method: ≥1% resistance was seen in 40% of the subjects for ampicillin, 14% of the subjects for cefuroxime, 6% of the subjects for nalidixic acid, 14% of the subjects for trimethoprim, 19% of the subjects for sulfamethoxazole, and 25% of the subjects for tetracycline. The amount of mercury in feces derived from amalgam was not selective for any resistance factors in aerobic gram-negative bacteria, but antimicrobial resistance was widespread even among healthy subjects with no recent exposure to antibiotics.

The growing incidence of pathogens with antimicrobial resistance is being recognized as a serious problem in the treatment of bacterial infectious diseases. If antimicrobial drugs are to remain effective in the future, ways of limiting the spread of resistance must be found. For this to be possible, all factors promoting this spread must be considered. The use of antimicrobial drugs has been shown to be one such factor (5, 11, 14, 17, 18, 20). The use of mercury as a disinfectant has been suggested as a factor promoting the spread of mercury resistance in hospital settings (24). Different genes for antimicrobial resistance are often spread together in cassettes on transposons. Heavy metal ion resistance, for example, against mercury (Hg), cadmium, and silver is found together with antimicrobial resistance determinants (7, 12, 22, 26).

It has been suggested that the environmental load of mercury, including that released from dental amalgam, could promote and maintain antimicrobial resistance together with mercury resistance in human normal flora (25). It was argued that the mercury concentrations found in saliva (derived mainly from dental amalgam fillings, which consist of approximately 50% inorganic mercury) and intestinal tissue (derived from food and water as well as dental amalgam) might be high enough to select for mercury-resistant bacterial strains living in these microenvironments.

We decided to explore the possible differences in antimicrobial and mercury resistance between three human test groups having different histories of dental amalgam fillings: a group who had never been exposed to dental amalgam fillings (nonamalgam [NA] group), one who had had all amalgam fillings removed (AR group), and one having various numbers of amalgam fillings (A group). The total concentrations of Hg in feces were determined for all subjects to find out the actual amount of environmental stress that mercury was causing the intestinal bacteria. In the gram-negative population, the spread of resistance factors is a frequent event; thus, we chose to study this part of the fecal flora. In addition to exploring the possible connection between mercury load and antimicrobial resistance, this study also presents data on the occurrence of antimicrobial resistance in the fecal bacteria of healthy Finnish adults.

MATERIALS AND METHODS

Subjects. The subjects examined were divided into three groups: those who had never had any dental amalgam fillings (NA; $n = 43$; mean age, 22 years; range, 18 to 26 years; female/male ratio, 3.00:1), those who had had their amalgam fillings removed (AR; $n = 56$; mean age, 50 years; range, 31 to 72 years; female/male ratio, 2.29:1), and those who still had amalgam fillings (A; $n = 92$; mean age, 48; range, 19 to 83 years; female/male ratio, 1.01:1). According to their statements, none of the subjects had been taking any antibiotics during the preceding 3 months. All subjects volunteered for this study, which was approved by the Ethical Committee of the Turku University.

Fecal samples. Fecal samples were collected from November 1993 to December 1994. The subjects were provided beforehand with sterile containers for stool collection and were told to bring a fresh sample, preferably collected in the morning on the same day. All samples were cultured before 4 p.m. on the same day. Four 10-fold dilutions of the stool samples were made in physiological saline, plated onto plates containing MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, England), a medium which is selective for aerobic gram-negative enteric bacilli, and incubated aerobically overnight at 35°C. Plates with 100 to 1,000 separate colonies (mean of 302) were chosen for determination of resistance.

Determination of antibiotic resistance frequencies. The plates were replicated with a replica plating method (16) onto a series of Iso-Sensitest agar plates (Oxoid Ltd.) with fixed amounts of the following antimicrobial agents: ampicillin (AMP), 32 μg/ml; cefuroxime (CXM), 16 μg/ml; nalidixic acid (NAL), 32 μg/ml;

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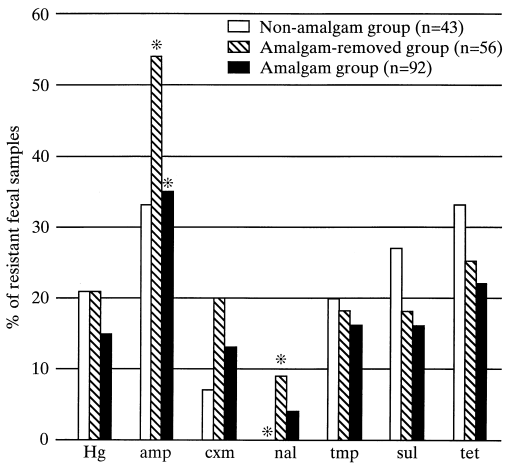

FIG. 1. Percentage of subjects having ≥1% resistant bacteria on antibiotic- and Hg-containing replica plates. Abbreviations: Hg; $HgCl_2$; amp, AMP; cxm, CXM; nal, NAL; tmp, TMP; sul, SUL; tet, TET. Asterisks note significant difference ($P < 0.05$).

trimethoprim (TMP), 8 μg/ml; sulfamethoxazole (SUL), 512 μg/ml; and tetracycline (TET), 4 μg/ml. Control plates with pure agar were included at the beginning and end of each series. Each new batch of plates was checked with appropriate control strains. The numbers of colonies growing on the plates containing antibiotics were counted after overnight incubation at 35°C. If ≥1% of the colonies transferred from the master plate grew on an antibiotic-containing plate, the subject was considered to have bacteria resistant to the agent.

Determination of mercury resistance. Preliminary tests showed that the very prominent inoculation effect of Hg made it impossible to include a $HgCl_2$-containing plate in the replica series mentioned above without modifications. This has also been noted by other laboratories (25). Therefore, an MH II (Mueller-Hinton II [BBL, Becton Dickinson and Company, Cockeysville, Md.]) agar plate with 10 μg of $HgCl_2$ (Merck Oy, Espoo, Finland) per ml was included last in the replica plate series, and the velvet pad carrying the sample colonies was blotted with another sterile velvet cloth before the sample was transferred to the Hg plate. This was followed by a control plate to confirm that all colonies had been transferred. The colonies growing on the Hg plate still had to be checked by a more reliable method, since even our susceptible control strains occasionally gave colonies on the Hg plate, but this method served well as a first selection step. Colonies growing after overnight incubation at 35°C were counted, and one to five colonies, of different morphologies if possible, were picked onto lactose agar (MacConkey broth [Oxoid] plus Bacto-agar [Difco Laboratories, Detroit, Mich.]). Less than five colonies were tested only when fewer than this number grew on the replica plate. These colonies were then tested for mercury resistance with a bacterial dilution method as follows.

Several colonies on one lactose agar plate were touched with a plastic inoculation needle and transferred to a tube containing 5 ml of physiological saline. From this, four 15-fold dilutions were made on a microtiter plate, and 1 μl was transferred to an agar plate 140 mm in diameter. The dilution series was done to ensure that the inoculum was not too heavy by confirming that only 1 to 10 colonies grew from the last dilution. The same $HgCl_2$ concentration (10 μg/ml) and agar (MH II) as for the replica plates were used. The dilutions of bacteria were also transferred to a control plate without $HgCl_2$. ATCC 35218 (*Escherichia coli*) and ATCC 27853 (*Pseudomonas aeruginosa*) (both Hg resistant), as well as *E. coli* C600, carrying the plasmid ColE1::Tn7 (13), and *E. coli* ATCC 25922 (both Hg susceptible), were used as controls. Strains showing growth in any of the last three dilutions after overnight incubation at 35°C were counted as mercury resistant.

Bacterial conjugation experiments. Bacterial conjugation experiments were done with brain heart infusion broth (Difco laboratories) by standard methods (21). A NAL-resistant strain of *E. coli* C600 was used as the recipient. MH II agar plates containing 10 μg of $HgCl_2$ per ml and 64 μg of NAL per ml were used for the selection of transconjugants. Colonies growing on these plates were further checked by the dilution method described above. Those proving to be mercury resistant by this method were confirmed to be transconjugants by showing that they had the same metabolic profile as the recipient strain by the API-20E system (BioMérieux, Lyon, France).

Antibiotic resistance profiles of the Hg-resistant strains. MICs were determined according to the guidelines set up by the National Committee for Clinical Laboratory Standards (23). The following antibiotics were tested (breakpoints for resistance are given in parentheses): AMP (≥16 μg/ml), CXM (≥16 μg/ml), NAL (≥32 μg/ml), TMP (≥16 μg/ml), TET (≥8 μg/ml), SUL (≥512 μg/ml), ciprofloxacin (CIP) (≥2 μg/ml), spectinomycin (SPE) (≥64 μg/ml), streptomycin (STR) (≥64 μg/ml), chloramphenicol (CHL; ≥16 μg/ml), and kanamycin (KAN; ≥32 μg/ml). All of the antimicrobial preparations used were from Sigma Chemical Co., St. Louis, Mo.

Determination of total mercury content in feces. The fecal samples were freeze-dried in mercury-free containers and sent for analysis to the Oulu Regional Institute of Occupational Health. Special emphasis was put on the avoidance of contamination by testing of all of the materials used for mercury traces. The samples were dissolved in 5 ml of 65% nitric acid in the CEM MDS-2000 microwave sample preparation system (CEM Corporation, Matthews, N.C.). Total mercury content was determined by cold-vapor atomic absorption spectrometry (Varian Techtron, SpectrAA-400; Varian Associates, Sunnyvale, Calif.) with a modification (15) of the method of Magos and Cernik (19). This method detects both inorganic (Hg^0 and Hg^{2+}) and organic Hg, which added together give the total Hg content in feces in concentrations down to 0.01 ng/mg.

Statistical methods. The chi-square analysis-of-contingency table test and Fisher's exact test were used to examine the significance of the differences between groups. Spearman correlation coefficients were calculated to assess possible connections between Hg concentrations in feces and the incidence of resistance.

RESULTS

Concentration of Hg in feces. The mean concentrations of total Hg in dry feces were 0.061 ng/mg for the NA group (standard deviation [SD], 0.038), 0.096 ng/mg for the AR group (SD, 0.145), and 1.044 ng/mg for the A group (SD, 2.853). Thus, in the A group, there were more than 17 times more Hg in feces than in the NA group and 11 times more than in the AR group.

Antimicrobial resistance. The frequency of antibiotic-resistant fecal samples did not differ between any of the three subject groups (Fig. 1), either counted separately or with the NA and AR groups (both groups consisting of people not having dental amalgam fillings) combined and compared with the A group. There was only a marginally significant difference between the AR and A groups for AMP resistance ($P = 0.0379$) and between the NA and AR groups for NAL resistance ($P = 0.0350$). There were no significant differences in dominantly (≥50%) antibiotic-resistant flora between the three groups. Disregarding groups and plotting resistance to each agent against Hg concentrations in feces produced no correlation; samples with a high concentration of Hg did not have a higher incidence of Hg resistance (Spearman correlation coefficient, 0.083) or any other resistance. The mean percentages of resistant fecal samples for each agent are presented in Table 1. Since Hg resistance could not be determined by the same method as antibiotic resistance, it has not been grouped into ≥1% and ≥50% levels; all samples with any level of resistance have been placed in one group. The mean Hg resistance was 19%.

Multiple antimicrobial resistance. Hg resistance was common among multiply resistant strains, occurring in 36% of all strains that were resistant to two or more antibiotics (Fig. 2).

TABLE 1. Percentage of antibiotic-resistant bacteria in fecal samples

Antimicrobial agent (breakpoint concn [μg/ml])	% of samples[a]	
	≥1% resistant	≥50% resistant
AMP (32)	40	19
CXM (16)	14	0.5
NAL (32)	6	0.5
TMP (8)	14	7
SUL (512)	19	12
TET (4)	25	12

[a] ≥1% and ≥50% resistant colonies per test subject, with the NA, AR, and A groups combined ($n = 191$).

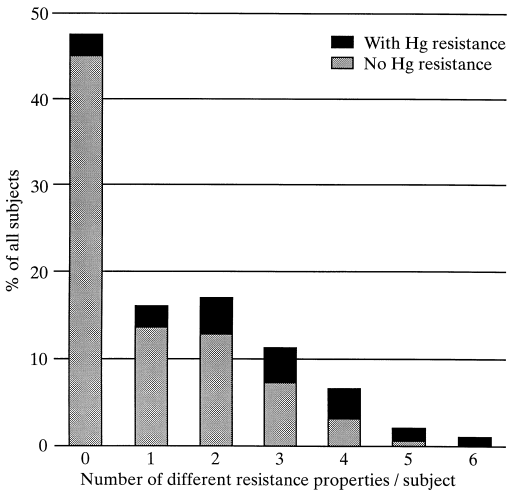

FIG. 2. Subjects (n = 191) having multiple antimicrobial resistance (≥1%) in their population of fecal gram-negative bacilli. Seventy-four percent of the subjects having Hg resistance have two or more other resistance properties.

However, again there were no significant differences between the three groups.

Antibiotic resistance profiles. Antibiotic resistance profiles of the Hg-resistant strains that were found by the bacterial dilution method are presented in Table 2. Nine subjects had two or more strains, as determined from the MIC profiles. The most common resistance pattern was Hg resistance combined with AMP resistance (17 strains), with Hg resistance alone coming second (4 strains). Altogether 36 of the 46 different strains found had AMP resistance in any combination. Twelve strains had Hg resistance combined with AMP, TMP, SUL, and TET resistance plus various other resistance determinants. Thirteen strains had Hg resistance together with SUL, TET, and STR resistance, as well as other resistance. None of the strains were resistant to CIP. No significant differences in profile distribution were seen between the NA, AR, and A groups.

Antibiotic resistance linked to Hg resistance. The bacterial conjugation was successful for the isolates from 22 of 35 subjects or 50 of 105 isolates tested. Eighty-two percent of the strains transferring resistance were *E. coli*; the rest were representatives of *Enterobacter*, *Klebsiella*, and *Citrobacter* species. All resistance factors found in the Hg-resistant strains, except CXM and NAL, were transferred on some occasion. SUL together with TET was the most frequently transferred resistance (from 18 different donor strains), followed by STR (14 strains), and AMP (13 strains). No significant difference in transfer frequency between the three subject groups was seen.

DISCUSSION

Despite the 17-fold difference in mercury concentration in feces between subjects not having and subjects having dental amalgam fillings, no differences in Hg resistance between these groups were seen. Thus, the phenomenon of increased resistance to Hg seen in Hg-contaminated natural soils and waters (2, 27) does not have a counterpart in the human intestine, at least as far as gram-negative aerobic bacteria are concerned, despite our observation that the total Hg concentration in human feces often reaches the concentrations used for detection of resistance in the laboratory. The Hg^{2+} ion is very reactive and lipid soluble and binds readily to the thiol groups of proteins (10), and the total concentration seems to be less important for the biological activity than is the chemical form of the metal (8). Ca^{2+} and Mg^{2+} ions can also directly protect bacteria from the toxic effects of Hg (8). Thus, it could be that the Hg in feces exists in nontoxic forms and is unable to harm living bacteria to any great extent. Even in the more simple environment of an agar plate, $HgCl_2$ can be seen to be very reactive; $HgCl_2$ plates have a shelf life of only about 5 days at 4°C (unpublished observations). Our results clearly show that Hg resistance is a common component of a multiresistant profile, as was also seen on the basis of population studies. However, Hg from dental amalgam does not appear to be a major selective agent for the spread of such linked resistance.

To our knowledge, frequencies of Hg resistance in the fecal normal flora has previously been reported only by Summers et al. (25). They found that a major number—63%—of their samples carried Hg resistance on some level. This is more than three times the amount found in our study. This difference may be a consequence of the different detection methods used. Hg resistance levels among isolated gram-negative bacilli range from 9% in clinical isolates of *E. coli* in St. Louis, Mo. (24), to 61.5% in *E. coli* strains isolated from foodstuffs in India (9). Since resistance levels are usually higher in developing countries (1), the 19% found in this study is within the range expected in an industrialized country.

The differences in the occurrence of resistance between the AR group and the other two groups for NAL and AMP cannot be explained by the differences in Hg concentrations now observed. The groups differ in many respects, since it is in practice impossible to find two large-enough age-matched populations that fit the criteria for this study. Only eight subjects in the A group were younger than 30 years, while all of the subjects in the NA group were under 27; consequently, a comparison of subjects of similar ages is statistically impossible. Past antibiotic use might play a role, and the mercury released

TABLE 2. Antimicrobial resistance found in isolated Hg-resistant strains

Resistance profile of Hg-resistant strain	No. of strains	Conjugation positive
Hg	4	+
Hg AMP	17	+
Hg AMP TMP SUL TET	2	+
Hg AMP TMP SUL TET STR	2	+
Hg AMP TMP SUL TET CHL	3	+
Hg AMP TMP SUL TET STR CHL	2	+
Hg AMP TMP SUL TET STR CHL KAN	1	+
Hg AMP TMP SUL TET STR CHL KAN SPE	1	
Hg AMP TMP SUL TET STR SPE	1	+
Hg AMP TMP SUL STR SPE	1	+
Hg AMP TMP TET CHL KAN SPE CXM NAL	1	
Hg AMP TMP CXM NAL	1	
Hg AMP SUL TET STR CHL KAN	1	+
Hg AMP SUL TET STR CHL	1	
Hg AMP SUL TET CHL SPE	1	
Hg AMP CHL	1	
Hg TMP SUL TET STR SPE	1	+
Hg TMP SUL TET SPE	1	+
Hg SUL TET STR KAN	3	+
Hg CHL	1	
Σ[a] 36 17 21 21 14 13 7 7 2 2	46[b]	

[a] Σ, column total.

[b] Total number of different strains; isolates from one patient that have different MIC profiles (differing by more than 2 one-step dilutions) and isolates from different patients are counted as different strains.

at the removal of the old amalgam fillings in the AR group should not be forgotten. This might have had a lasting impact on the bacterial flora.

In line with the hypothesis that mercury promotes the spread of resistance, there could be many agents harmful to bacteria that might act as confounding variables in this study, selecting for resistance and covering the possible effect of mercury. These could be components of food or drugs other than antibiotics. Traces of antibiotics in food, derived from meat or milk, might have some marginal impact. In this study, that possibility can be disregarded, since the use of antibiotics as animal feed additives is prohibited in Finland, and there is little import of these products. Different responses might be seen in other members of the intestinal ecosystem; enterococci and anaerobes are probably affected in other ways and by other agents than enterobacteria, and we did not look at these in this study.

A number of groups have reported on the frequency of antimicrobial resistance in the normal flora of various human populations, but comparison of the results is hampered by the use of different methods and breakpoints. Some of the more recent studies, however, have used some kind of quantitative method, and all of these give ≥50% (or dominant flora) as one level of resistance (3, 4, 6, 17). Our results on the ≥50% level correspond fairly well to the results from these studies; for AMP, TET, and SUL, the resistance frequencies lie around 10 to 20%, and Bonten et al. (3) report a TMP resistance of 7 to 10%, which is similar to our 7%. Levy et al. (17) found a NAL resistance of 0.7%, also very close to our 0.5%. The frequencies of antimicrobial resistance in the normal fecal flora of our subject groups from a town in southern Finland are thus very similar to those found in other parts of northern Europe and the northern United States. Seen on the ≥1% level, the resistance frequencies we have found are strikingly high, considering the fact that the subjects had not recently been given any antibiotics. Two of five people had resistance to AMP and one of four had resistance to TET, and even CXM resistance was at 14%. This is a worrying aspect for physicians who are daily confronted with urinary tract infections and other infections caused by gram-negative bacteria. Unfortunately, there is no other solution to the problem than a more sparing use of the prescription pad.

In conclusion, antimicrobial resistance of fecal gram-negative bacilli is widely distributed among healthy adults, but dental amalgam alone is not a major factor in promoting its spread.

ACKNOWLEDGMENTS

We thank Rolf Österblad for language revision and Tarja Boman and Anna-Liisa Lumiaho for skillful technical assistance.

This work was partly supported by grants from the Finnish Dental Association, the Sigrid Juselius Foundation, the Oskar Öflund Foundation, and the Academy of Finland.

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