ASSOCIATION OF METAL TOLERANCE WITH MULTIDRUG RESISTANCE AMONG BACTERIA ISOLATED FROM SEWAGE

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ABSTRACT

Objectives: Sewage effluent from the sewage treatment plant of the University of Nigeria, Nsukka, was analyzed for the presence of metal and non-metal ions and for the presence of metal tolerant and drug resistant bacteria. Methods: Plasmid mediation of metal tolerance and multiple drug resistance was demonstrated by sodium dodecyl sulphate (SDS) curing and direct cell transfer experiments. Results: Ions found present (in mgL^{-1}) include, among others, Mercury (50.148), Lead (41.906), Sodium (907.240), and Potassium (700.00). Bacterial populations isolated from the effluent were members of the genera *Enterobacter* (n = 15), *Escherichia* (n = 18), *Achromobacter* (n = 18), *Acinetobacter* (n = 25) *Klebsiella* (n = 12), *Pseudomonas* (n = 08), *Proteus* (n = 20) and *Serratia* (n = 10). *Enterobacter* spp showed high percentage tolerance of 73% to Lead. Species of *Acinetobacter* and *Pseudomonas* showed, to varying degrees, across – the – board tolerance to all the individual salts. Also an across-the-board resistance of between 25 – 75% and 8.3 – 41.7% to the test drugs was exhibited by *Pseudomonas* and *Klebsiella* spp respectively. Sixty per cent each of *Acinetobacter* and *Klebsiella* spp lost both metal tolerance and drug resistance attributes simultaneously following the SDS curing protocol. Overall percentage loss of both characteristics was 57.1%. Acquisition of metal tolerance and multidrug resistance by recipients was total (100%) and so was the subsequent loss of these capabilities following SDS treatment of these recipients. Discussion: The public health hazard derivable from these findings is discussed.

KEY WORDS: Sewage; Bacterial isolates; Metal tolerance; Drug resistance; Drug transfer; Drug curing.

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INTRODUCTION

Sewage is waste matter resulting from the discharge into the sewers of human excreta and wastewater originating from a community and its industries (Guardabassi and Dalsgaard, 2002). It has a high content of both organic and inorganic matter, as well as high densities of living organisms, including pathogenic, commensal and environmental bacteria. This characteristic makes sewage a particularly suitable niche for the spread of antibiotic resistance (Guardabassi and Dalsgaard, 2002). Sometimes the acquisition of antibiotic resistance is not dependent on the presence of antibiotics but on a multitude of other substances occurring in sewage. Such substances include heavy metals and biocides. Heavy metals are widespread in sewage as a consequence of industrial pollution and there has been considerable speculation about possible genetic association between bacterial tolerance for these metals and multiple antibiotic resistance (Dhakephalkar and Chopade, 1994; Roane and Kellogg, 1996; Wireman *et al*., 1997).

It has been suggested that genes encoding resistance to heavy metals (and biocides) can be located together with antibiotic resistance genes on either the same genetic structure (eg. plasmid), or different genetic structures within the same bacterial strain (Guardabassi and Dalsgaard, 2002). McArthur and Tuckfield, (2000) had suggested that metal and antibiotic resistance among bacteria are linked very closely together and that expression of antibiotic resistance may be dependent on exposure to metals. This is supported by the finding that unspecific mechanisms conferring resistance to both metals and antibiotics exist in some bacterial species (eg. Active pump – efflux system encoded by the *mar* A gene in *E. coli*) and that indirect evidence that bacteria (of the same genus) isolated from heavy-metal polluted marine sediment are significantly more resistant to antibiotics than their counterparts isolated from unpolluted sites (Rasmussen and Sorensen, 1998). Similarly metal tolerance and drug resistance among bacteria have been shown to increase proportionally along industrial contamination gradients (Osborn *et al*., 1997; Roane and Kellogg, 1996) and use of metal based antimicrobial agents. Nakahara *et al,* (1977a, 1977b, and 1977c) have suggested that the combined expression of antibiotic resistance and metal tolerance may not be a fortuitous phenomenon but rather is caused by selection resulting from metals present in an environment.

The importance of enhanced drug resistance due to metal presence or contamination stems from the abundance of metal – contaminated locations in the environment (McArthur and Tuckfield, 2000). The potential public health impact of this results from the ability of microbes to disperse from water, soil, and sewage through various mechanisms (Hamilton and Lenton, 1988) and thus enter the atmosphere. Once in the atmosphere, bacteria can be distributed over large geographical areas and subsequently return to earth through rain, hail, or dry fall thus spreading drug resistant genes. This should give cause for concern and impetus to studies aimed at understanding the correlation between metal tolerance and multidrug resistance among sewage borne bacteria. The work described in this paper was performed to measure the amount of some metal ions in the sewage of an academic community, check the antibiotic resistance and metal tolerance of bacteria isolated there from and verify any link between heavy metal pollution and antimicrobial resistance with any concurrent transfer and loss of metal tolerance and drug resistance on such bacteria. This work also attempts to verify relationship between loss of metal tolerance and drug resistance in such populations.

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METHODS

Determination of metal ion contents and pH of sewage sample

Raw sewage samples were collected from University of Nigeria, Nsukka sewage plant in clean grease free 250 ml flasks. The Mercury (Hg), Lead (Pb), Zinc (Zn), Aluminum (Al), Copper (Cu), Sodium (Na) and Potassium (K) ion contents of a sample was determined using a Gallenkamp flame analyzer (FGA 330c England). Chloride (Cl) ion content was determined by titration against 0.25N $AgNO_3$ using methyl orange (Merck) (0.25%) as indicator. pH was determined using pH meter (7020 Richmond Surrey, England). This metal analysis was repeated two times at intervals of 20 days and the mean values were recorded.

Isolation of bacteria

Raw sewage was collected in a sterile 250 ml beaker and filtered through Whatman No. 40 filter paper. Microbial analysis was implemented by modification of the method of Verme *et al,* (1976). One milliliter of the filtrate was inoculated into 9 ml lactose broth and incubated with shaking at 37°C for 24 hrs. From this, 0.1 ml was inoculated into nutrient agar (NA) plate and incubated for 24 hrs at 37°C. Distinct and representative colonies from the NA plates were further purified and stored on NA slants in a refrigerator. The bacteria isolates were subjected to standard morphological and biochemical tests and identified based on the criteria of Krieg and Holt (1984) and Cowan and Steel (1965). Sewage samples were collected from three different points every four days for sixty days.

Antimicrobial susceptibility tests

The bacteria isolates were inoculated into Mueller Hinton broth, incubated and the resultant growth diluted with sterile normal saline to match the turbidity of 0.5 McFarland standards (Cooksey *et al*., 1978). From these and using the methods of Bauer *et al.,* (1966) on Mueller Hinton agar plates, the isolates were assayed for their sensitivities to the following: gentamicin (10μg), lincocin (30 μg), rifampin (10 μg), erythromycin (30 μg), chloramphenicol (20 μg), streptomycin (30 μg), ampiclox (30 μg) co-trimoxazole (30 μg), ampicillin (30 μg) and ofloxacin (10 μg). Antibiotic discs were obtained from Optun (Nig). After incubation (for 24 hrs at 37°C) and measurement of inhibition zone diameters, susceptibility ranges were decided according to Anon (1988c), De La Rosa *et al.,* (1993) and Prescott *et at.,* (1999). Control plates were incubated without antibiotic discs.

Determination of metal tolerance

Molten sterile Mueller Hinton agar in 60ml bottles were supplemented with filter sterilized (Millipore III type HA) solutions (in distilled water) of the following salts, corresponding to cationic concentration (microgram per millilitre): $HgCl_2$ (10), $ZnSO_4$ (1600), K_2CrO_4 (1600), Pb $(NO_3)_2$ (3200), and Al_2 $(SO_4)_3$ (200) for Hg, Zn, K, Pb and Al respectively (Marques *et al*., 1979; Calomiris *et al*., 1984). The salt-agar media were adjusted to a final pH of 7.0 using NaOH. Plates were prepared from the molten agar and inoculated by single-streak with the isolates, incubated for 24 hrs at 37°C and results deciphered in accordance with Marques *et al.* (1979) and Nakahara *et al.* (1977b). MHA plates without salts were inoculated and incubated as controls.

Plasmid curing

Isolates that showed tolerance to two or more of the salts as well as resistance to two or more antibiotics were selected and subjected to plasmid curing treatments following the methods of Salisbury *et al,* (1972) and Bhalakia, (2005) as follows: To 100 ml of nutrient broth, 1 g of sodium dodecyl sulphate (SDS) was added. The solution was autoclaved, the pH adjusted to 7.6 and the solution steamed for 1 hr. Twenty four hour broth cultures were standardized as described above and 0.5 ml was pipetted into each 100 ml broth. Control broth without SLS was subjected to similar treatment. Cultures were incubated with aeration at 37°C for 24 hrs. The cells including those from the control tubes were then tested for antibiotic susceptibility and metal tolerance tests as described above.

Metal tolerance and multidrug resistance transfer tests

Two bacteria isolates that showed remarkable drug resistance and metal tolerance were selected as donors. These were *Enterobacter* sp (which showed tolerance to lead, aluminum, zinc and mercury and resistance to gentamicin, erythromycin, ampiclox, ampicillin and chloramphenicol) and *Pseudomonas* sp (which showed tolerance to all the test metal salts and resistance to all the drugs tested). One *Escherichia* sp (susceptible to potassium, zinc and aluminum ions and lincocin, streptomycin, cotrimazole and ofloxacin) and one *Proteus* sp (susceptible to zinc and potassium; and lincocin, cotrimoxazole, ofloxacin, chloromphenical and rifampicin) were chosen as recipients. The transfer experiment was done according to the methods of Watanabe and Fukasawa, (1961) as modified by Sturtevant and Feary (1969). Nutrient broth culture tubes were prepared and inoculated with chosen isolates. After incubation for 24 hrs at 37°C, 0.1 ml of donor culture was mixed with 0.2 ml of recipient culture in 2 ml sterile nutrient broth. The mixtures were incubated at 37°C for 24 hrs. After mixed growth, a loopful of each mixture was smeared onto MacConkey agar plates with appropriate concentrations of individual metals and antibiotics as shown under determination of metal tolerance above. Colonies recovered were separated on the basis of indole production, glucose and lactose utilization with the production of acid and/or gas: Recipient isolates were further subjected to drug resistance, metal tolerance and plasmid curing tests.

RESULTS

Substantial amount of metal (and non-metal) ions was shown to be present in the sewage sample with mercury being as high as 50.148mgL^{-1} and lead 41.906mgL^{-1}. Others included, (in mgL^{-1}) Aluminium (0.453), Chloride (1240.25), Sodium (907.24), Copper (7.148), Potassium (700), and Zinc (0.848). The mean pH of the sewage samples was 6.6.

A total of 126 bacteria isolates belonging to the genera *Enterobacter, Escherichia, Serratia, Achromobacter, Acinetobacter, Pseudomonas, Kiebsiella* and *Proteus* were isolated and tested for their tolerance to various metals. The highest percentage tolerance of 73% was shown by *Enterobacter* spp to lead but leading overall tolerance to all the metals was shown by *Acinetobacter* spp and *Pseudomonas* spp which showed growth (to varying degrees) on all media containing the different metallic salts (Table 1).

Of all the 126 bacteria isolates analyzed for multiple drug resistance, *Acinetobacter* spp (between 8% and 40%), *Pseudomonas* spp (25% to 75%) and *Klebsiella* spp (8.3% to 41.7%) contained species that were not inhibited by any single drug (Table 2). The highest percentage resistance (75%) was observed among species of *Pseudomonas* against ampicillin

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and ampiclox. Streptomycin and lincocin showed, among all the drugs, the highest potency against the test isolates.

A remarkable loss of both tolerance to metals and resistance to antimicrobial agents was observed in both *Escherichia* spp and *Acinetobacter* spp. The two *Escherichia* spp isolates lost both abilities after curing treatment (Table 3). Sixty per cent of the *Acinetobacter* spp and *Klebsiella* spp lost both attributes simultaneously. The overall percentage simultaneous loss of both metal tolerance and multidrug resistance – a measure of the total number of isolates that concurrently lost both capabilities in relation to the total number treated to the curing protocol, was 57.1%.

Results of the metal tolerance and drug resistance transfer tests showed that the recipient isolates acquired resistance to the drugs and tolerance to the metals to which they were susceptible and to which the donors were tolerant. The recipients lost the tolerance and resistance after they were subjected to the curing treatment.

Table 1: Percentage Tolerance of Isolates to Metals

Bacteria Genus	No of Isolates tested	% Isolates tolerant to				
		Hg	Zn	K	Pb	Al
Enterobacter spp	15	20	7	-*	73	27
Escherichia spp	18	11	-	-	33	-
Serratia spp	10	10	-	-	40	20
Achromobacter spp	18	33	-	-	33	22
Acinetobacter spp	25	48	12	8	40	24
Pseudomonas spp	8	50	1	13	36	38
Klebsiella spp	12	42	-	-	42	25
Proteus spp	20	35	-	-	30	25

*-: No tolerance.

Table 2: Percentage Resistance of Isolates to Antimicrobial Agents

Bacteria Genus	No of tested isolates	% Isolates resistant to									
		Gm*	L	Rg	E	C	S	Ax	Txs	Ta	Am
Enterobacter spp	15	26.7	-	-	20	13.3	-	26.7	-	-	33.3
Escherichia spp	18	16.7	-	11.1	11.1	11.1	-	16.7	-	-	22.2
Serratia spp	20	10	-	10	-	10	-	10	-	-	15
Achromobacter spp	18	22.2	-	-	-	16.7	-	22.2	-	-	22.2
Acinetobacter spp	25	40	4	12	12	12	-	12	8	24	40
Pseudomonas spp	8	37.5	25	37.5	37.5	37.5	25	75	37.5	37.5	75
Klebsiella spp	12	41.7	16.7	8.3	16.7	25	8.3	16.7	16.7	16.7	41.7
Proteus spp	20	40	-	10	20	15	15	15	15	10	35

*Gm, gentamicin; L, lincocin; Ra, rifampicin; E, erythromycin; C, chloramphenicol, S, Streptomycin; Ax, ampiclox; Txs, cotrimoxazole; Ta, ofloxacin; Am, amplicillin.

Table 3: Percentage Loss of Tolerance to Metals and Resistance to Antimicrobial Agents by Isolates after Curing

Bacteria Genus	No. subjected to curing	% Loss of metal tolerance (MT)	% Loss of antimicrobial agent resistance (AR)	% Simultaneous loss of both MT and AR
Enterobacter spp	4	75	75	75
Escherichia spp	2	100	100	100
Achromobacter spp	4	50	75	50
Acinetobacter spp	10	60	60	60
Pseudomonas spp	4	0	0	0
Klebsiella spp	5	80	60	60
Proteus spp	6	50	66.6	50

Overall percentage simultaneous loss of both MT and AR = 57.1%

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DISCUSSION

Results of this investigation showed the presence of metal ions in the sewage sample studied and resistance to metal ions by some bacteria isolates, suggesting a possible link. We speculate that the presence of metal ions such as mercury (50.148 mgL^{-1}), lead (41.906 mgL^{-1}), and zinc (0.848 mgL^{-1}) in sewage may have acted as the selection force inducing and maintaining metal tolerance and multidrug resistance among sewage-borne bacteria. The 57.1% concurrent loss of both capabilities in isolate bacteria after plasmid curing treatments and total transfer of those attributes to susceptible isolates suggests that the genes controlling these attributes may be located on the same plasmid and their sustenance in the host bacteria may be the result of metal or antibiotic selective pressure in the environment.

These results are in line with earlier suggestions (Fontaine and Hoadley, 2000; Nakahara *et al*., 1977a) that combined expressions of antibiotic resistance and metal tolerance may not be a chance phenomenon but rather the results of selection by metals present in an environment. It has also been asserted that heavy metals, disinfectants, antibacterials and antimicrobials – all can select for different kinds of bacteria, including those resistant to lifesaving antibiotics (Levy, 1998b; Moken *et al*., 1997).

The public health implication and hazard derivable from these findings stem from the possible (horizontal) transfer of the genetic elements (notably plasmid) responsible for these resistance abilities to pathogenic bacteria that they might come in contact with. This suggestion follows from earlier observations (Guardabassi and Dalsgaard, 2002) that sewage is a possible vehicle for the dissemination of antibiotic resistance genes in the indigenous micro flora of aquatic environments. Resistance genes can be taken up by indigenous bacteria and spread by mechanisms of genetic transfer. Examples of resistance genes originating in commensal or environmental bacteria and transferring to pathogens have previously been described (Hart, 1998) This results from the inherent genetic fluidity of bacteria (Levy, 1998a). Indeed, the exchange of genes (among bacteria) is so pervasive that the entire bacterial world can be thought of as one huge multicellular organism in which the cells interchange their genes with ease (Levy, 1998b). This point of view implies that spread can occur from one ecosystem to another. This has been documented by various cases in which transmission of resistant bacteria has been demonstrated between animals and man (Kruse, 1999; Wegener *et al* 1999).

Sewage connects antibiotic selective environments such as hospitals, chemical industries, farms and slaughter houses to natural environments (Guardabassi and Dalsgaard, 2002). When disseminated to natural environments, such microbes may create their own dispersal agents from water and soil through various mechanisms (Hamilton and Lenton, 1988) and thus enter the atmosphere. Once in the atmosphere, bacteria can be distributed over large geographical areas and subsequently return to earth through rain, snow, hail or dry fall (Yuriewa, 1997) thus aiding in the worldwide distribution of various (multidrug resistant and metal tolerant) bacteria or their genes. The occurrence, fate, and especially transfer of multidrug resistant and metal tolerant bacteria from sewage deserve further consideration and insight in view of the foregoing.

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