Transfer of Multidrug Resistance among Bacteria Isolated from Industrial Wastes

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Abstract

One hundred and twenty two (122) bacterial isolates belonging to the genera Micrococcus, Streptococcus, Pseudomonas, Actinomyces, Bacillus, Corynebacterium, Brucella, Shigella, Hafnia, Proteus and Salmonella were isolated from four different industrial waste sites. Thirty five (28.68%) of these were resistant to two or more antibiotics. Of these, multiple drug resistant species of Pseudomonas and Proteus were chosen as donors in resistance transfer studies with selected susceptible environmental and clinical isolates as recipients. Results showed that the drug resistance is transferable among environmental isolates and from environmental to clinical isolates. Following treatment with sodium dodecyl sulphate in a resistance curing protocol, thirty one (88. 57%) of the resistant isolates lost resistance to all the antimicrobial drugs to which they were previously resistant. These findings suggest that the resistance may be plasmid-mediated and promiscuous. The possible public health implication of this is discussed.

Keywords: Industrial wastes, Bacterial isolates, Antimicrobial resistance, Resistance transfer, Curing

Introduction

Current thinking on the reason for the prevalence of antibiotic resistance emphasizes the presence of antibiotics or related products as the selective pressure, inducing and sustaining resistance among bacteria (Wilkins, 1996; Waters, 2000; Neu, 1992, and Powell, 2000). It was once though that over prescription of antibiotics was the main route for development of multidrug resistant bacteria (Reilly, 2005). Thus there is a general notion that bacteria with the greatest levels of resistance come from environments with the greatest potential for significant contamination by antimicrobial agents e.g. hospitals and hospital sewage effluents (Frontaine and Hoadley, 1976), commercial fisheries (Watanabe *et al*, 1971), and abattoirs (Goyal and Hoadley, 1979). This assumes that the principal mechanisms by which humans enhance the spread of antibiotic resistance among environmental bacteria is by the introduction of antibiotic resistant bacteria/ genes into the environment (Guardabassi and Dalsgaaard, 2002). Epidemiological studies (Cohen, 1992) have, in line with this, repeatedly demonstrated the influence of antimicrobial use on the emergence, persistence, and transmission of antimicrobial resistant bacteria.

Knowledge of the incidence of antibiotic resistant bacteria in the environment is fragmentary. Their presence in lake waters (Linton, 1986), animal slurry (Hinton and Linton, 1982), and their survival on slurry treated pasture (Linton and Hinton, 1984) has been reported. Studies of resistance gene transfer elements in soil and marine bacteria (Salyers and Amabile – Cuevas, 1997) have shown that resistant and 'mobilizable' plasmids are obtainable in environmental settings. This suggests that there are, besides antibiotics, other selective pressures in nature that favour the selection and up-regulation of (resistance) plasmids and other gene transfer agents (McKeon *et al*, 1995).

The dearth of ecological studies on the dissemination of resistant bacteria in the environment has public health consequences (Guardabassi and Dalsgaaard, 2002). The main risk for public health is that resistance genes can transfer from environmental bacteria to human pathogens. This ability of resistance genes to move from one ecosystem to another is documented (Wegener *et al*, 1999; Kruse, 1999). This presumed public health significance of the occurrence of multidrug resistant bacteria in the environment necessitated this work aimed at evaluating the antibiotic resistance pattern of bacteria isolated from industrial wastes. This work also studied the transfer of such resistance from environmental to clinical isolates; and attempted to determine whether such resistance is plasmid or chromosomally borne.

Materials and Methods

Isolation of bacteria: Both solid and liquid industrial wastes were collected randomly from Charlou Industries Limited, Nsukka (manufactures of Beauty Queen® relaxers and body creams), Hardis and Dromedas Company Enugu (manufacturers of Royallux, and other facial astringents), King-size Pharmaceuticals (KP) Ogidi (alcoholic beverage section), and Nigerian Mineral water and Bottling Company Nkpor (manufactures of Limca range of soft drinks). One gram (or 1 ml) of the waste was inoculated into 1 ml of nutrient broth, shaken for 1 h and further diluted two fold. From these, nutrient agar (NA) and MacConkey agar (MA) plates were streak-inoculated and incubated for 24 h at 35^0C. After incubation, representative colonies were picked and further purified on MA plates. Purified colonies were subsequently stored on NA slants in the refrigerator as stock cultures. Isolates were identified on the basis of morphological and biochemical attributes according

to standard procedures' (Krieg and Holt, 1984; Cowan and Steel, 1965; Crabtree and Hinsdill, 1974).

Antibiotic susceptibility testing: Isolates were assayed for their sensitivities to the following: augmentin (30 μg), gentamycin (10 μg) erythromycin (30 μg), chloramphenicol (20 μg), ampiclox (30 μg), nalidixic acid (30 μg) ,streptomycin (30 μg), rifampin (10 μg), lincocin (30 μg), ofloxacin (10 μg), co-trimoxazole (30 μg) and ampicillin (30 μg) antibiotic sensitivity discs. (Optun Nig. Ltd). Overnight cultures of isolates were grown in Mueller-Hinton broth (LAB M). The cultures were standardized by diluting to O. 5 McFarland turbidity standard to produce approximately 1.5 x 10^8 colony forming units ml^{-1}. Colony counts were also performed to verify the inoculum size according to the methods of Lambert *et al* (2001). Mueller – Hinton agar (LAB M) plates were swabbed with a suspension of the inoculum and left to dry. Antibiotic discs were subsequently placed on the plates ensuring that they made good contact with the agar surfaces. Inhibition zone diameters were measured after 24 h incubation at 35^0C. Susceptibility ranges were scored according to the methods of Anon (1988), De La Rosa *et al* (1993) and Prescott *et al* (1999). As controls, selected inoculated MA plates were incubated without antibiotic discs.

Resistance curing: Isolates resistant to two or more antibiotics were selected for resistance curing using sodium dodecyl sulphate (SDS) (Tomoeda *et al*, 1968; Salisbury *et al*, 1972; Mach and Grimes, 1982, and Bhalakia, 2005). Twenty four-hour nutrient broth (LAB M) cultures of chosen isolates were standardized as above and 0.5 ml each of these was pipetted into separate sterile 100 ml broth, each containing 1 g of SDS (pH 7.6). Control broth without SDS was subjected to similar treatments. Cultures were incubated with aeration at 35^0C for 24 h. Isolates were recovered from these, purified and tested again for antibiotic susceptibility as described above.

Resistance transfer experiment: Species of *Pseudomonas* (resistant to augmentin, ceporex nalidixic acid, gentamicin, cotrimoxazole, erythromycin and ampicillin) and *Proteus* (resistant to all the test antibiotics) were chosen as donors while species of *Micrococcus* and *Salmonella* (susceptible toa all test antibiotics) and clinical isolates of *Escherichia* spp (n=5) *Salmonell*a spp (n=5) and *Enterobacter* spp (n=5) susceptible to test antibiotics were chosen as recipients. The method followed those of Bell *et al.,* (1980); Watanabe and Fukasawa (1961) as modified by Sturtevant and Feary (1969). Overnight cultures of the donors and recipients were grown at 35^0C in nutrient broth. A 0.1 ml sample of donor was added to 8 ml fresh nutrient broth with 0.1 ml of recipient: This mixture was incubated for 24 h at 35^0C. A 1:10 dilution of the culture was then used to flood MA plates incorporated with 50 μg/ml of nalidixic acid. Colonies recovered were separated on the basis of shape, glucose and lactose utilization, motility, urease production, maltose utilization, indole production, and catalase production. Recipient isolates were further subjected to standard antimicrobial sensitivity testing as described above.

Results

Antibiotic resistance: Percentage resistance of the one hundred and twenty two (122) isolates belonging to the genera *Brucella*, *Actinomyces*, *Micrococcus, Pseudomonas, Corynebacterium, Shigella, Hafnia, Streptococcus, Salmonella, Bacillus* and *Proteus* to eleven antimicrobials are shown in Table 1. Species of *Pseudomonas* showed the highest overall percentage resistances to the test antimicrobial agents and were selected on this basis as donors in the resistance transfer experiments. None of the species of *Brucella, Actinomyces, Micrococcus, Shigella, Hafnia*, *Streptococcus* and *Salmonella* showed any demonstrable resistance to lincocin, cotrimoxazole and ofloxacin. As a result, twelve (12) isolates of *Micrococcus* spp and four (4) of *Salmonella* spp, in addition to five (5) clinical isolates each of *Escherichia* spp, *Salmonella* spp and *Enterobacter* spp were used as recipients for the *in vitro* resistance transfer studies. Maximum resistance was seen towards gentamycin and ampicillin and minimum on lincocin and cotrimoxazole.

Resistance curing: On the basis of resistance to two or more antimicrobial agents, a total of thirty five (35) of the one hundred and twenty two (122) bacterial isolates of various genera (Table 2) were subjected to resistance curing protocols using 1% SDS. Total (100%) resistance elimination was achieved in all species of *Brucell*a, *Actinomyces*, *Micrococcus*, *Corynebacterium, Shigella, Hafnia, Streptococcus* and *Bacillus* tested. Various percentage resistance elimination were achieved among species of *Pseudomonas* (33.3 – 66.7%) *Salmonella* (50 – 66. 7%) and *Proteus* (50 – 80%). Overall 88.57% of the multidrug resistant isolates tested lost demonstrable resistance by exposure to the curing agent.

Resistance transfer: Results of resistance acquisition among the recipient bacteria after mating with the multidrug resistant donor species of *Pseudomonas* and *Proteus* are shown in Table 3. Resistance acquisition was notably and comparatively high among both clinical (20 – 60%) and environmental (25 – 50%) isolates of *Salmonella* spp. Eighty percent (80%) of clinical isolates of *Escherichia* spp acquired resistance to augmentin when mated with *Proteus* sp and sixty percent (60%) after mating with *Pseudomonas* sp.

Discussion

This work set out to investigate the antibiotic resistance pattern of bacteria isolated from non-clinical (environmental) sources and to determine if resistance, when present, is transferable among such isolates and from them to clinical isolates. This is necessitated by the fact that over the past two decades, understanding the dynamics of multidrug

Table 1*:* Percentage resistance of isolates to antimicrobial agents

Bacteria Genus	Number isolated and tested	% Isolates Resistant										
		Gm	E	Na	Ax	S	A m	L,	Tx s	Ta	Cx	Au
***Brucella* spp**	9	33.3	22.2	11.1	11.1	22.2	33.3	00	00	00	00	11.1
***Actinomyces* spp.**	10	10	10	00	00	00	10	00	00	00	00	00
***Micrococcus* spp.**	16	25	18.8	12.5	12.5	12.5	25	00	00	00	6.3	18.8
***Pseudomonas* spp**	8	75	75	50	75	75	75	37.5	37.5	50	62.5	50
***Corynebacterium* spp**	11	36.4	36.4	18.2	18.2	27.3	36.4	9.1	9.1	9.1	18.2	18.2
***Shigella* spp**	8	25	12.5	12.5	12.5	12.5	25	00	00	00	00	12.5
***Hafnia* spp**	12	8.33	00	00	00	8.33	3.33	00	00	00	00	00
***Streptococcus* spp**	9	22.2	11.1	00	00	11.1	22.2	00	00	00	00	11.1
***Salmonella* spp**	6	50	33.3	33.3	33.3	50	16.7	00	00	00	00	16.7
***Bacillus* spp**	20	20	15	10	15	20	10	00	00	50	00	5.0
***Protems* spp**	13	38.5	23.1	23.1	30.8	30.8	38.5	15.4	15.4	15.4	23.1	30.8

Key 1: *Gm, gentamicin; E, erythromycin; Na, nalidixic acid S, streptomycin; Am, ampicillin; L, lincocin; Txs, cotrimoxazole; Ta, ofloxacin, Cx, ceporex; Au, augmentin; Ax, ampiclox;*

Table 2: Percentage loss of resistance after SDS treatment*

Bacteria Genus	Number isolated and tested	% Loss of Resistance										
		Gm	E	Na	Ax	S	A m	L,	Tx s	Ta	Cx	Au
***Brucella* spp**	3	100	100	100	100	100	100	ND	ND	ND	ND	100
***Actinomyces* spp.**	1	100	100	ND	ND	ND	100	ND	ND	ND	ND	ND
***Micrococcus* spp.**	4	100	100	100	100	100	100	ND	ND	ND	100	100
***Pseudomonas* spp**	6	66.7	66.7	50	66.7	66.7	66.7	33.3	33.3	50	40	50
***Corynebacterium* spp**	4	100	100	100	100	100	100	100	100	100	100	100
***Shigella* spp**	2	100	100	100	100	100	100	ND	ND	ND	ND	100
***Hafnia* spp**	1	100	ND	ND	ND	100	100	ND	ND	ND	ND	ND
***Streptococcus* spp**	2	100	100	ND	ND	100	100	ND	ND	ND	ND	100
***Salmonella* spp**	3	66.7	50	50	50	66.7	00	ND	ND	ND	ND	00
***Bacillus* spp**	4	100	100	100	100	100	100	ND	ND	100	ND	100
***Proteus* spp**	5	80	66.7	66.7	75	75	80	50	50	50	66.7	75

** 1. Values were obtained in relation to numbers originally resistant to each drug (Table 1). 2. ND: Not determined (isolates were not originally resistant to the corresponding drugs.) (Table 1). Key 2: Gm, gentamicin; E, erythromycin; Na, nalidixic acid S, streptomycin; Am, ampicillin; L, lincocin; Txs, cotrimoxazole; Ta, ofloxacin, Cx, ceporex; Au, augmentin, Ax, ampiclox;*

Table 3: Pattern of resistance transfer and acquisition

Donors	Recipients Bacteria Genus	Number Treated	Percentage Acquired Resistance						
			Au	Cx	Na	Gm	Txs	E	Am
Pseudomonas sp	***Micrococcus* spp**	12	25	16.7	16.7	25	16.7	8.3	16.7
	***Salmonella* spp**	4	50	50	25	50	25	25	25
	***Escherichia* spp (Clin.)**	5	60	40	60	40	40	40	40
	***Salmonella* spp (Clin)**	5	40	40	20	60	40	20	20
	***Enterobacter* spp (Clin)**	5	60	40	40	60	60	40	60
Proteus sp	***Micrococcus* spp**	12	16.7	8.3	16.7	16.7	16.7	8.3	25
	***Salmonella* spp (Clin)**	4	50	50	25	50	25	50	25
	Escherichia Spp	5	80	40	60	60	40	20	40
	***Enterobacter* Spp (Clin)**	5	60	40	40	40	20	60	40
	Salmonella Spp (Clin)	5	40	20	40	60	40	60	60

Clin: - Indicates Clinical Isolates. Key3: Gm, gentamicin; E, erythromycin; Na, nalidixic acid; Am, ampicillin; Txs, cotrimoxazole;, Cx, ceporex; Au, augmentin;

resistance among bacteria and factors selecting for and sustaining them have become central issues in medicine and public health (Livermore, 2003). Results obtained show that bacteria isolated from industrial wastes exhibit some measure of resistance to antimicrobial agents commonly used in our society. This is in variance with the general perception (Powell, 2000; Waters, 2000, and Wilkins, 1996) that drug resistance is an exclusive self-defeating consequence of the use, misuse and overuse of antibiotics. The finding is, however in consonance with the opinion of other authors (Levy, 1998; Hart, 1998; Osterblad *et al*, 1995) who believe that drug resistance among bacteria is more widespread than can be accounted for as being a consequence of the selection pressure caused by the use of antibiotics alone.

The drug resistance transfer experiments revealed that resistance is transferable among environmental isolates and from environmental to clinical isolates. This demonstrable resistance transfer explains; in part why on a large scale antibiotic resistance in one place often spread far and wide (Levy, 1998). In addition to this our data on resistance curing (Table 2) and acquisition (Table 3) suggest strongly that the observed resistance are plasmid borne thus promoting the speculation (Mach and Grimes, 1982) about the possibility of in situ resistance transfer and its effect on public health. Plasmids, from which ever bacterial source, are the ideal vehicles for the recruitment and dissemination of resistance genes. This dissemination of plasmids (with transposons and integrons) among bacteria gives rise to gene epidemics (Livermore, 2003) which create epidemics of resistance of local, national and even international dimensions (Levy, 1998; Livermore, 2003).

Our suggestion here therefore is that the occurrence of transferable multidrug resistance traits among bacteria isolated from industrial wastes should generate concern among stake holders in medicine and public health. This should be such that appropriate containment procedures to block the transmission of resistant bacteria or of the plasmids that bear the drug resistance will be mapped and followed up to forestall the obvious public health consequences. It should be seen as an additional frontier in the widespread discourse and fight against multidrug resistant bacteria selected and maintained in the environment by the use, misuse and over use of antibacterial agents.

References

Anon, (1988) National Committee for Clinical Laboratory Stand-ards. NCCLS Supplement M 100-S2. *The Anti-microbic News-letter*; *5:* 9 -15.

Bell, J.B; Macrae, W.R. and Euiott, G.E. (1980). Incidence of R Factors in Coliforms, Fecal Coliform, and Salmonella Populations in the Red River in Canada. *Appl. Environ. Microbiol; 40:* 486-491.

Bhalakia, N. (2005). Isolation and Plasmid Analysis of Vancomycin Resistant *Staphylococcus aureus. Journal of Young Investigations*; *13 (4):* 1-6.

Cohen, M. L (1992). Epidemiology of Drug Resistance: Implications for a Post-Antimicrobial Era. *Science, 257*. 1050-5.

Cowan, S.T. and Steel, K.J. (1965). Manual for the Identification of Medical Bacteria. University Press, Cambridge.

Crabtree, K.T. and Hinsdill, R.D. (1974). Fundamental Experiments in Microbiology. W.B. Saunders Company. Toronto. Pp 153-179.

De La Rosa, M.C; Mosso, M.A; Garcia, M.L. and Plaza, C. (1993). Resistance to Antimicrobial Agents of Bacteria isolated from Non-Sterile Pharmaceuticals. *Journal of Applied Bacteriology*; *74:* 570- 577.

Frontaine, T.D. and Hoadley, A. W. (1976). Transferable drug resistance associated with coliforms isolated from hospital and domestic sewage. *Health Lab. Sci. 13.*238-245.

Goyal, S.M. and Hoadley, A.W. (1979) Salmonellae and their associated R-Plasmids in Poultry Processing Wastes. *Rev. Microbiol.; 10 (2):* 50-58.

Guardabassi, L. and Dalsgaaard, A. (2002) Occurrence and Fate of Antibiotic Resistant Bacteria in Sewage. Paper Presented to Danish Environmental Protection Agency NO. 722; 1-59.

Hart, C. A. (1998). Antibiotic Resistance: An Increasing Problem?. *BMJ. 316* 1255-6.

Hinton, M and Linton, A. H. (1982).The survival of multi- antibacterial drug-resistant *Escherichia coli* and *Salmomella typhimurium* in stored static slurry from a veal calf unit. *Journal of Hygiene*; *88:* 557-65.

Krieg, N.R and Holt, J.G. (1984). *Bergey's Manual of Systematic Bacteriology 1*. William and Wilkins, Baltimore.

Kruse, H. (1999).Indirect Transfer of Antibiotic Resistance Genes to Man. *Acta. Vet. Scand. 92 (Suppl.).* 59-65.

Lambert, R. J. W., Joynson, J., and Forbes, B. (2001).The relationships and susceptibilities of some industrial, laboratory, and clinical isolates of *Pseudomonas aeruginosa* to some antibiotics and biocides. *Journal of Appl. Microbio.; 91:* 972 – 84.

Levy, S. B. (1998). Antimicrobial Resistance: Bacteria on the Defence. *BMJ. 317* 612 – 3.

Linton, A. H. (1986). Flow of Resistance Genes in the Environment and from Animals to Man. *Journal of Antimicrobial Chemotherapy*. *18 (Suppl. C)* 189 – 197.

Linton, A. H and Hinton, M. (1984). The Ecology of Antibiotic Resistant Bacteria in Animals and their Environment. in Antimicrobials and Agriculture (Woodbone, M., Ed.), Butterworths' London, Pp 533 – 49.

Livermore, D. M (2003). Bacterial Resistance: Origins, Epidemiology and Impact. *Clinical Infectious Diseases*. *36 (Suppl. 1)* S11 – 23.

Mach, P. A. and Grimes, D. J. R (1982). – Plasmid Transfer in a Wastewater Treatment Plant. *Applied and Environ Microbio.; 44 (6):* 1395 – 1403.

McKeon, D. C., Calabrese, J. P., and Bissonette, G. K. (1995) Antibiotic – resistant Gram – negative bacteria in Rural Ground Water Supplies. *Water Resource*. *29* 1909 – 18.

Neu, H. C. *(1992)*. The Crisis in Antibiotic Resistance. *Science. 257* 1064 – 1073.

Osterblad, M., Leistevuo, J., Leistevuo, T., Jarvinen, H., Pyy, L., Tenovuo, J. and Huovinen, P. (1995). Antimicrobial and Mercury Resistance in Aerobic Gram – negative Bacilli in Fecal Flora among persons with and without Dental Amalgam Fillings. *Antimicrob. Agents Chemother. 39 (11)* 2499-2502.

Powell, W. J. (2000). Molecular Mechanisms of Antimicrobial Resistance. Technical Report # 14. *Antimicrobial Agents and Chemotherapy.* 37:414 – 418.

Prescott, L. M., Harley, J. P., and Klein, D. A. (1999) Microbiology (4th Ed.) McGraw – Hill. Boston Rurr Bridge New York.. Pp. 536, 681 – 3; 807-9.

Reilly, M. Sludge (2005) Watch – Multiresistant bacteria develop in Sewage Treatment Plants. maureen. reilly at sympatico. Ca..

Salisbury, V., Hedges, R. W., and Datta, N. (1972). Two Modes of 'Curing' Transmissible Bacterial Plasmids. *J. of Gen. Microoio. 70* 443 – 452.

Salyers, A. A. and Amabile – Cuevas, C. F. **(**1997). Why are Antibiotic Resistance Genes so Resistant to Elimination. *Antimicrobial Agents and Chemotherapy 41* 2321 – 2325.

Sturtevant, A. B., Jr. and Feary, T. W. (1969). Incidence of Infections Drug Resistance among Lactose – Fermenting Bacteria Isolated from law and treated Sewage. *Appl. Microbio. 18* 918-924.

Tomoeda, M., Inuzuka, M., Kubo, N., and Nakamura, S. (1968) Effective Elimination of Drug Resistance and Sex Factors in Escherichia coli, by Sodium Dodecyl Sulphate. *Journal of Bacteriology*. *95 (3)* 1078 – 1089.

Watanabe, T. and Fukasawa, T. (1961). Episome – mediated transfer of Drug Resistance in Enterobacteriaceae. J*. of Bacteriol. 81* 669-678.

Watanabe, T., Aoki, T., Ogata, Y., And Egusa, S. R (1971) – Factors related to fish culturing. *Ann. N. Y. Acad. Sci.*. *182*. 383-410.

Waters, V. L *(2000)*. Conjugative transfer in the dissemination of Beta-lactam and Aminoglycoside resistance. *Applied and Environ. Microbio. 66 (12)* 5406 – 5409.

Wegener, H., Aarestrup, F. Gerner-Smidt, P., and Bager, F. (1999).Transfer of Resistant Bacteria from Animals to Man. *Acta. Vet. Scand. 92 (Suppl.)* 51-58.

Wilkins, A.(1996) Antibiotic Resistance: Origin, Evolution, and Spread. *Bioassays; 40:* 847-848.