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Environmental Contamination and Toxicology

Effects of Some Metallic Compounds on *Klebsiella*

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Many industrial and waste disposal practices unconsciously pollute our environment by adding excess heavy metals to it. Although reports show an inconsistency in the toxic levels of heavy metals such as zinc, nickel, cadmium, mercury and silver between microbial groups, the toxic effects of the metals on microorganisms have been well documented (Carr et al. 1973; Ditri 1972; Forstner and Wittman 1979; Golubovoch 1974; Mascaskie and Dean 1982; Robinson and Tuovinen 1984). Little is known of the differential effects these metals have on coliform K. pneumoniae and K. oxytoca. These bacteria are widely recognized as antibiotic resistant opportunistic pathogens (Martin, et al. 1971; Eickhoff, 1971; Crouch et al. 1972; Orskov, 1974; Fox, and Rohosvsky, 1975; Cruickshank, et al. 1975) ubiquitously distributed in environments (Wong et al. 1985). Besides, they are able to fix dinitrogen. In this study, these metals were found to affect these organisms in a variety of concentrations. Such effect could affect the total coliform count in water, dinitrogen fixation, and removable of nitrate in soil and water.

MATERIALS AND METHODS

Three hundred and ninety-eight strains of Klebsiella (146 K. oxytoca, 252 K. pneumoniae including ATCC 29665 and 13030) were used in the study. All except the ATCC strains, were isolated from clinical, alfalfa sprouts and soil samples. These cultures were maintained on nutrient agar (Difco) slants at 20 °C. The cultures were subcultured every 30 days. Purity was checked by streaking plates before each subculture was performed.

All cultures were routinely grown on nutrient agar plates incubated at 37 °C for 18 hours. Cells were harvested by gently scraping colonies off agar surfaces. The cells were washed twice with sterile phosphate buffer (0.01 M). Immediately after washing, the cells were resuspended in the buffer. Each cell suspension was further diluted to give each inoculum with final concentrations approximately 10 v.c./ml. The maximium holding time was 6 hours before initiation of the experiment.

The chemically defined nitrogen-free medium (NFB) used for the tests had the following composition (gram per litre of triple-distilled deionized water): Na_2HPO_4, 0.7; NaH_2PO_4,0.3; $MgSO_4$.H 0, 0.2; $MnSO_4$, 0.1; $CoCl_2$,0.005; $FeCl_2$, 0.005; and MoO_3, 0.005. To prepare the medium, all of the chemicals were dissolved in hot water. The pH was adjusted to 6.8. A slight precipitate formed on cooling and was filtered out with a Whatman No.2 filter paper. Sterilization was by membrane filtration (with 0.22 micron milipore filter). Nitrate medium (NNB) was prepared by adding 1 gram of of potassium nitrate to a liter of NFB. Both NNB and NFB contained 1 gram of glucose per liter. Nitrate Broth (Difco) was used for nitrate reduction test.

One gram of one of the following compounds was added to a litre of nutrient agar: mercuric chloride ($HgCl_2$), uranyl nitrtate ($UO_2(NO_3)_2.6H_2O$), thorium chloride ($ThCl_4.4H_2O$), zinc chloride ($ZnCl_2$), nickel chloride ($NiCl_2.6H_2O$), cadmium acetate ($(CH_3COO)_2Cd.2H_2O$), and silver acetate (CH_3COOAg). To get maximum solubility of these compounds without hydrolyzing agar, the pH was adjusted to 6. Each litre of nutrient agar was then sterilized by autoclaving at 121°C for 15 minutes and allowed to cool to 50 °C. Each plate was arranged with one side being lifted up to a height of 5 mm. The molten agar was poured carefully into the dishes that the agar would barely touch the bottom edge of the lifted side. This process gave a gradient of agar ranging from 5 mm to zero mm in height. The plate was left in this position till the agar was set. The high and low sides of each plate were clearly marked. Then the plates were placed on a flat surface. Sterile nutrient agar without any added compound was poured onto the low side until the agar just reached the top of the high side, forming a level surface. When the agar was set, the surface moisture was aseptically air dried. Sterile swabs (cotton wool), wetted with each of the inocula prepared, were used to make a lawn on each plate containing one heavy metal. The inoculated plates were incubated at 37 °C for 24 hours. The plates were then examined.

Eighteen strains were randomly selected from 398 obtained from clinical sources, 24 from alfalfa sprouts and 8 from soils to give a total of 50 isolates. These were tested on nickel chloride, cadmium acetate, mercuric chloride, and silver acetate. The media used were: nutrient broth (Difco), NNB with 0.1% (w/v) glucose, and NFB with 0.1% (w/v) glucose.

Each of the four listed compounds was added to the media in a series of concentrations. For the more toxic silver, mercury and cadmium based compounds, the concentrations used were (mg/l): 0, 0.1, 1.0, 5.0, 10 and 20 while for the less toxic nickel chloride, the concentrations used were (mg/l): 0, 10, 50, 100, 200, 300. Two ml of each medium was carefully dispensed into one well in a multiwell repli-dish (Qualicum Ltd., Ottawa, Ont.) in ascending order of the concentrations of the metal compound. Each well was inoculated with 0.01 ml of inoculum prepared using a microliter pipette. The plates were incubated at 35 °C for 48 hours. Turbidity generation was used as the index of growth, and confirmed by cross-checking using streaking on nut-

rient agar and subsequent colonial growth in abundance after overnight incubation.

RESULTS AND DISCUSSION

Results in Table 1 show that neither K. oxytoca nor K.pneumoniae had any preferential resistance to any particular heavy metallic compounds. The percentage of survivals of each species to each compound tested was extremely close. The degree of inhibition on the gradient plates was related to each individual strain rather than the whole species. The more resistant strains appeared to be the more slimy. This indicated that the slime build-up of these organisms provided them with a defense mechanisms against the toxic metallic ions.

Table 1. Effect of compounds of heavy metals on the ability of Klebsiella spp. to grow on gradient plates

	Surviving Strains (%)	
Chemical Compound	K. oxytoca	K. pneumoniae
Cadmium acetate	22.3	23.2
Mercuric chloride	20.0	20.3
Nickel chloride	100.0	100.0
Silver acetate	23.7	22.2
Thorium chloride	100.0	100.0
Uranyl nitrate	100.0	100.0

Not all the heavy metals are toxic to these organisms. Uranyl nitrate and thorium chloride had no effect on them. At a concentration of approximately 1g/l (w/v) on them, there was no sign of any inhibition. The cause(s) of this is/are currently under investigation.

Results in Table 2 showed that cadmium acetate exerted no significant inhibitory effects at 1.0 mg/l. At this concentration,

Table 2. Effect of cadmium acetate on the survival ability of strains of Klebsiella spp.

	Surviving Strains (%)								
Concentration	Nutrient Broth			NNB			NFB		
(mgCd/l)	C	A	S	C	A	S	C	A	S
0	100	100	100	100	100	100	100	100	100
0.1	100	100	100	100	100	100	100	100	100
1.0	100	100	100	100	100	100	100	100	100
5.0	100	100	100	6	13	50	0	0	38
10.0	67	67	50	6	0	50	0	0	0
20.0	67	46	50	6	0	13	0	0	0

C, clinical isolates; A, alfalfa isolates; S, soil isolates.

Klebsiella was inhibited in NNB and NF media, but not in nutrient broth. As far as soil isolates are concerned, 50% grew in nitrate medium while only 37% grew in NFB. Higher concentrations than 10 mg/l caused inhibition. However, two strains, one from clinical and one from soil isolates, were grown in NNB. In nutrient broth, the toxicity of cadmium acetate was diminished since all strains grew well at 5 mg/l concentrations. Even when the concentration was increased to 20 mg/l, 67% of clinical isolates, 50% of isolates from legume sprouts and 50% of soil isolates were still able to grow.

The response of Klebsiella to mercuric chloride varied considerably for different growth conditions. Inhibition was distinctly shown in glucose NFB, particularly on clinical isolates (Table 3). At 0.1 mg/l there were 17% and 11 % survival rates respectively in NNB and NFB. Both alfalfa sprouts and soil isolates were more resistant. But in the nutrient broth the inhibition was only found when the concentration of mercuric chloride was raised to 5.0 mg/l. At this concentration, there was no growth in NNB and NFB. At concentration of 10 mg/l of the same compound, 5 clinical, 5 botanical and 4 soil isolates survived.

Table 3. Effect of mercuric chloride on the ability of strains of Klebsiella spp. to grow.

	Strains exhibiting growth (%)								
Concentration	Nutrient Broth			NNB			NFB		
(mg/l)	C	A	S	C	A	S	C	A	S
0	100	100	100	100	100	100	100	100	100
0.1	100	100	100	17	67	88	11	71	75
1.0	94	100	100	0	0	25	0	0	13
5.0	39	100	100	0	0	0	0	0	0
10.0	28	21	50	0	0	0	0	0	0
20.0	28	4	0	0	0	0	0	0	0

C, clinical isolates; A, alfalfa isolates; S, soil isolates.

Table 4. Effect of silver acetate on the ability of strains of Klebsiella spp. to grow.

	Strains Exhibiting Growth (%)								
Concentration	Nutrient Broth			NNB			NFB		
(mg/l)	C	A	S	C	A	S	C	A	S
0	100	100	100	100	100	100	100	100	100
0.1	39	38	63	0	0	0	0	0	0
1.0	27	21	50	0	0	0	0	0	0
5.0	27	21	50	0	0	0	0	0	0
10.0	27	21	50	0	0	0	0	0	0
20.0	22	17	37	0	0	0	0	0	0

C, clinical isolates; A, alfalfa isolates; S, soil isolates.

Of the four metallic compounds tested, silver acetate proved to be the most lethal to Klebsiella, particularly when the organic nitrogen was not available in the media (Table 4). At a 0.1 mg/l concentration of silver acetate, there was no growth of any isolates in either NNB or NFB. However, when organic nitrogen was available, i.e. nutrient broth, 38 % of legume, 63% of soil and 39% of clinical isolates were not inhibited. Even when the concentration of the compound was raised to 20 mg/l the survival rates of these isolates was still above 20%.

Nickel chloride appeared to be the least toxic amongst the four compounds (Table 5). However, the isolates from alfalfa sprouts were more tolerant to this metal than were the other isolates. Amongst the three groups of isolates, the soil isolate were the most susceptible to nickel chloride. At 10mg/l concentration, no growth of soil isolates occurred in either NNB or NFB. But at this concentration, 22% of clinical and 29% of alfalfa sprout isolates survived in NNB, and 25% of clinical and alfalfa sprout isolates grew in NFB. When the concentration was quintupled, only alfalfa sprout isolates grew in NNB and NFB (8% in NNB, 13% in NFB). In nutrient broth, more than 50% of the isolates from alfalfa sprouts remained unaffected by the presence of nickel chloride at 50 mg/l level. When this level was raised to 300 mg/l, 3% of the 50 strains tested still grew (6% of the clinical and 8% of the alfalfa sprout isolates).

Table 5. Effect of nickel chloride on the ability of strains of Klebsiella spp. to grow

	Strains Exhibiting Growth (%)								
Concentration	Nutrient Broth			NNB			NFB		
(mg/l)	C	A	S	C	A	S	C	A	S
0	100	100	100	100	100	100	100	100	100
10	78	83	100	22	29	0	6	25	0
50	22	54	0	0	0	0	0	13	0
100	17	42	0	0	0	0	0	4	0
200	17	38	0	0	0	0	0	0	0
300	6	8	0	0	0	0	0	0	0

C, clinical isolates; A, alfalfa isolates; S, Soil isolates.

Solubilities of these compounds decrease as the pH increases. At the same time, the toxic effects of these compounds on Klebsiella diminished. This was particularly distinct when mercuric chloride, silver acetate, nickel chloride or cadmium chloride was added to the media. This suggested that by lowering the pH, these compounds become solubilized and more toxic to these organisms. Some of these such as compounds of nickel and cadmium are commonly found in the soil. At low pH these compounds could be released into water system to kill these organisms or prevent then to assimilate nitrate and to fix dinitrogen. The toxic effects of these compounds on coliform Klebsiella could also affect the total coliform count in water.

Apart from those in the nutrient broth, strains surviving in NNB and NFB appeared to be light pink in color. This coloration was not observed in the presence of other heavy metal compounds tested, except nickel chloride. In this respect no distinct differences were observed between K pneumoniae and K. oxytoca.

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REFERENCES

Carr HS Wlodowski TJ Rosenkranz HS (1973) Silver sulfadiazine:In vitro antibacterial activity. Antimicrob. Agents Chemother. 4:585-587.

Crouch JRF, Atherto JG, Platt H (1972) Venereal transmission of Klebsiella aerogenes in a thorough bred stud from a persistently infected stallion. Vet. Rec. 90:21-24.

Cruickshank R, Duguid JP, Marmion BP, Swain RHA (1975). Medical Microbiology. 12th Ed. Vol. 2. Churchill Livingstone, Edinburgh, London.

Difco Manual (1984) 10th ed. Difco Laboratories, Detroit.

Ditri FM (1972) The environmental mercury problem. CRC Press Inc. Boca Raton, Fla.

Eickhoff TC (1971) Nosocomial infections due to Klebsiella pneumoniae: Mechanisms of intra-hospital spread. In: Proceedings of the International Conference on Nosocomial Infections. (Brachman PS, Eickhoff TC eds.). Am. Hospit. Asoc. Chicago. PP. 117-125.

Forstner U Wittman GTW (1979) Metal Pollution in Aquatic Environment. Spring Verlag, New York.

Fox JG, Rohosvsky MV (1975) Meningitis causes by Klebsiella spp. in two Rhesus monkeys. J. Am. Vet. Med. Assoc. 67:634-636.

Golubovoch VN (1974) Toxic effect of silver ions on various groups of microorganisms. Mikrobiol. 43:922-924.

Martin WJ, Yu PKW, Washington JA II (1971) Epidemiological significance of Klebsiella pneumoniae. A 3 months study. Mayo Clinic Proceed. 46:786-793.

Mascaskie LE Dean ACR (1982) Cadmium accumulation by microorganisms. Environ. Technol. Lett. 3:49-56.

Orskov I (1974) Klebsiella In Bergey's Manual of Determinative Bacteriology. 8th ed. (Buchanan RE, Gibbons NE eds.) The Williams & Wilkins Company, Baltimore. pp. 321-324.

Robinson JB Tuovinen OH (1984) Mechanisms of microbial resistance and detoxification of mercury and organomercury compounds: physiological, biochemical and genetic analyses. Microbiol. Rev. 48:95-124.

Rother JA Millbank JW Thornton I (1982) Effects of heavy metal additions on ammonification and nitrification in soils contaminated with cadmium, lead and zinc. Plant Soil 69:239-258.

Sakaguchi T Sakaguchi S (1982) Uptake of metals into the cadmium and zinc resistant Shigella flexneri. Jpn. J. Ind. Health. 24:245-252.

Williams SE Wollum AG (1981) Effect of cadmium on soil bacteria and actinomycetes. J. Environ. Qual. 10:142-144.

Wong SH, Cullimore DR, Bruce LR (1985) Selective medium for isolation and enumeration of Klebsiella spp. Appl. Environ. Microbiol. 49:1022-1024.

Wysor MS Zollinhofer RE (1972) On the mode of action of silver suladiazine. Patho. Microbiol. 38:296-308.

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